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IMPROVING OUTCOME IN MELANOMA BY EARLY DETECTION AND IDENTIFICATION OF NOVEL PROGNOSTIC MARKERS

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ABSTRACT

The incidence of cutaneous melanoma continues to rise rapidly. Problems are faced throughout its management and this thesis addresses them as follows: secondary prevention, prediction of prognosis and early detection of metastatic disease. Secondary prevention has been shown to be an effective strategy for improving survival in other cancers. A study of the rapid access, early detection, pigmented lesion clinic at Mount Vernon Hospital demonstrates for the first time that such an approach improves outcome in melanoma.

Refining current prognostic capability in melanoma is essential for improving disease management. Tissue microarray was used to investigate 480 specimens from 120 primary melanomas for novel prognostic markers. Several markers were identified, including MCAM, CD44v3, Nm23 and P-cadherin, which showed a significant bearing on melanoma patient outcome. Their identification reveals valuable markers for predicting outcome and potential targets for therapeutic manipulation.

Immunoscintigraphy is the use of radiolabelled tumour-specific antibodies to detect disease. Previously the whole monoclonal antibody LHM2, directed against the high molecular weight melanoma-associated antigen was studied. Whilst being highly melanoma-specific, its use in melanoma detection is limited. Its large size (150 kDa) results in background accumulation in normal organs, reducing image quality and hindering metastasis detection. The single chain Fv fragment (scFv) is the smallest part of the whole monoclonal antibody that maintains full antigen-binding capability. Its potential superiority over whole monoclonal antibodies has been demonstrated; however it requires further refinement before routine use in patients. Two methods of improving tumour targeting with scFvs were investigated: use of a cocktail of scFvs binding different epitopes and use of scFv multimers. The use of a scFv cocktail improved tumour targeting in the spleen and muscle. Dimeric scFvs were shown to improve tumour retention whilst maintaining rapid blood clearance, leading to overall improved tumour targeting compared with monomers.

Dedicated

to my darling Sandy

and

to my parents

ACKNOWLEDGMENTS

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CHAPTER 1

INTRODUCTION

Melanoma continues to present an increasing clinical problem. Dilemmas are faced throughout the management of melanoma: prevention of the disease, early detection of melanoma, prediction of prognosis, treatment of the primary cutaneous lesion and early detection and treatment of metastases.

1.1 THE CLINICAL PROBLEM

1.1.1 History

The earliest published case of melanoma was in 1787, when John Hunter reported a patient with a "cancerous, fungous excrescence", although Hunter never described the disease as such. René Laennec, the inventor of the stethoscope, in 1806 was the first to describe the disease entity and six years later he coined the term "melanosis", derived from the Greek word for black (Davis & McLeod, 1998).

The first case of melanoma in the English literature is that by William Norris in 1820. He went on to write up a further eight cases in 1857 and can be credited as the first to associate a number of features with the disease, such as the association with a fair complexion, a high number of moles and the recognition of various subtypes. In addition, he is probably the earliest advocate of performing wide local excision for the primary lesion. Sir James Paget (1853), in his *Lecture on Surgical Pathology*, identified melanoma as "medullary cancers modified by the formation of black pigment in their elemental structures". Sir Jonathon Hutchinson later reported on subungual melanoma (1857) and the eponymous Hutchinson's freckle (1892) (Davis & McLeod, 1998).

The basis of the management of melanoma in the whole of the first half of the last century relied on two Hunterian lectures delivered by William Handley in 1907. On the basis of a single autopsy study he advocated wide local excision or the primary lesion, regional node dissection and amputation in selected cases. Thus it can be seen here that many of the observations made over a century ago are still of relevance in the current knowledge and management of melanoma today. However, once the disease had spread there was no other effective treatment. Searches for adjuvant or neoadjuvant treatments have been pursued since the early part of the last century are still ongoing.

Interest in the detection, evaluation and treatment of melanoma and other cancers using antibodies has increased since the pioneering work of Pressman during the 1940s and 1950s (Pressman & Keighley, 1948; Pressman & Korngold, 1953), who used antibodies as carriers of radionuclides. He used ^{131}I labelled antibodies for the tumour targeting of osteogenic sarcoma. Since then many researchers have worked on improving the use of antibodies as mediators of imaging or treatment modalities for a range of medical conditions. However, one of the limitations endured was the limited production of antibodies with desired antigen specificity.

Then, in 1975 George Köhler and César Milstein of the Medical Research Council Laboratory of Molecular Biology in Cambridge described a way for making cell lines that secreted a single species of antibody (monoclonal antibody, Mab) with the desired specificity to antigen in large amounts – hybridoma technology. In the original method, a mouse was immunised repeatedly with desired antigen and the spleen, containing proliferating B cells, was removed. The B cells were then immortalised by fusion with a non-secreting myeloma cell, resulting in a hybridoma secreting large amounts of the desired antibody. Supernatants of cell lines that survived the selection procedure were then tested for binding to the original antigen. Alan Williams showed in 1977 that monoclonal antibodies could be raised against biologically interesting molecules and this triggered the development of a stream of useful monoclonal-based diagnostic procedures. It soon became apparent however that rodent monoclonal antibodies were unsuitable or ineffective for treating humans because they initiate human defence systems poorly and are themselves the target of an immune response that can greatly shorten their circulating half-life. This problem was addressed initially by using human lymphocytes to populate severe combined immunodeficient (SCID) mice. These mice could then be immunised to make human antibodies (Mosier *et al.*, 1988). However it was not until 1988 when Skerra and Pluckthün developed the phage display strategy for antibody production that this problem was successfully overcome. This will be discussed in more detail in section 1.7.3.

1.1.2   Epidemiology

The incidence of cutaneous melanoma has continued to rise over the last 50 years (MacKie *et al.*, 1997; Severi *et al.*, 2000; MacKie *et al.*, 2002) (Figure 1.1.2.1). It is now the 16th most common cancer in the UK in men and 18th most common in women (CRUK 2001). Using a mathematical model, it was predicted to become the 6th most common cancer in the USA in the year 2001

(Greenlee *et al.*, 2001) – due to lack of a national cancer registry in the USA the actual figures are not known. Although arguments exist as to whether the observed increase in incidence is true, or whether it is a reflection of improving diagnosis, it is generally acknowledged that a real increase does exist (Jemal *et al.*, 2001).
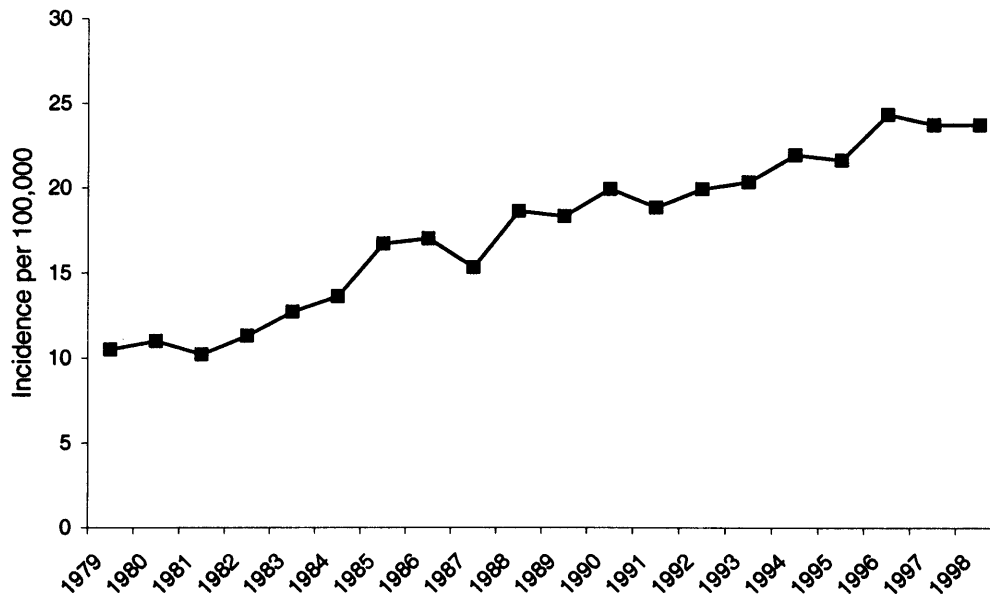
Figure 1.1.2.1 Incidence of primary cutaneous melanoma in Scotland 1979-1998 (Adapted from MacKie et al. 2002)

Rona MacKie's group (2002) found the increasing incidence was more prevalent in men than women and occurred in melanomas of all thicknesses in men. In women, the trend only seemed to be of melanomas thinner than 1.5mm increasing in incidence. Other studies' results reflected these findings, with a substantial increase in the number of thick lesions in men and thin lesions in both sexes (MacLennan *et al.*, 1992; Burton & Armstrong 1994). The number of new cases diagnosed in the UK is illustrated in Figure 1.1.2.2.
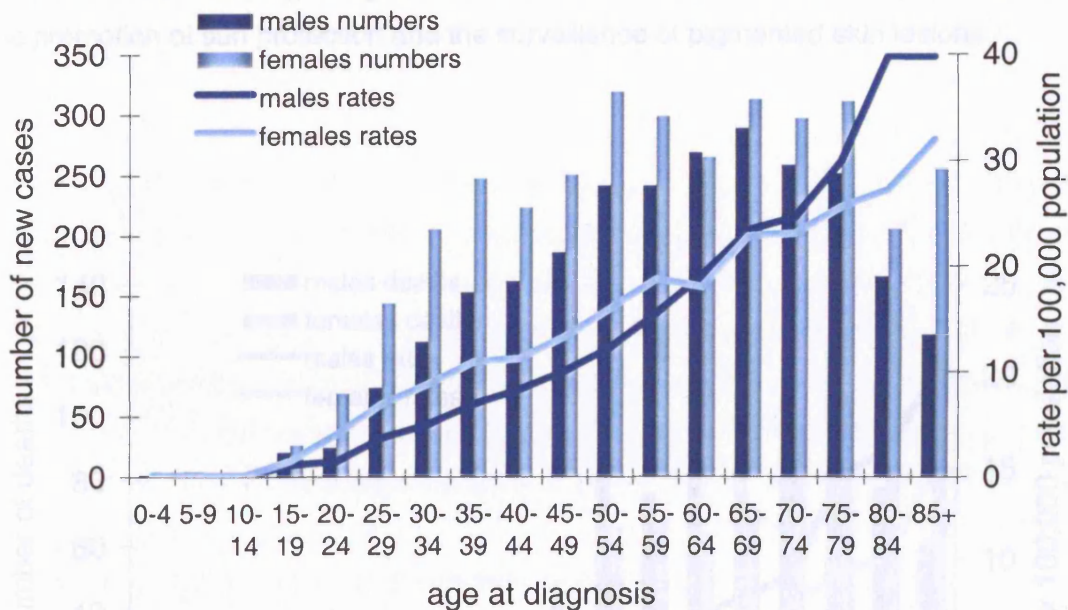
Figure 1.1.2.2 Number of new cases diagnosed and age specific rates per 100,000 population of melanoma, by sex, UK, 1999 (Source: Cancer Research UK website)

Overall mortality from melanoma is also rising; however this rise is mainly confined to men (La Vecchia *et al.,* 1999; MacKie *et al.,* 2000), which would be predicted from the rising number of thick lesions diagnosed in men. Actuarial survival for both sexes analysed by MacKie (1997) showed a divergence in survival between the sexes (men fairing worse) during the first five years after diagnosis and treatment, this difference being maintained over the next five years. They also clearly showed the steady and continuing death rate from melanoma during follow-up over 5-10 years, confirming that survival to 5 years is not a guarantee of cure. Recent UK mortality figures are illustrated below (figure 1.1.2.3).

Noting the variation in incidence between countries (La Vecchia *et al.,* 1999), Severi and co-workers (2000), using melanoma mortality data obtained from the World Health Organisation Cancer Mortality Data Bank, analysed the mortality trends between populations in different countries. The overall increase in mortality from melanoma was more pronounced in the older age groups (60-79). Downturns in mortality, essentially in women and starting with generations born just before the Second World War, were found in Australia, the Nordic countries and the USA.

Small decreases in rates were found in more recent generations in the UK and Canada. However in France, Italy and the Czech Republic mortality rates were still increasing. Their data suggests that populations are at different places on the melanoma mortality epidemic curve. The three trend patterns observed being in agreement with time differences between populations with respect to the promotion of sun protection and the surveillance of pigmented skin lesions.
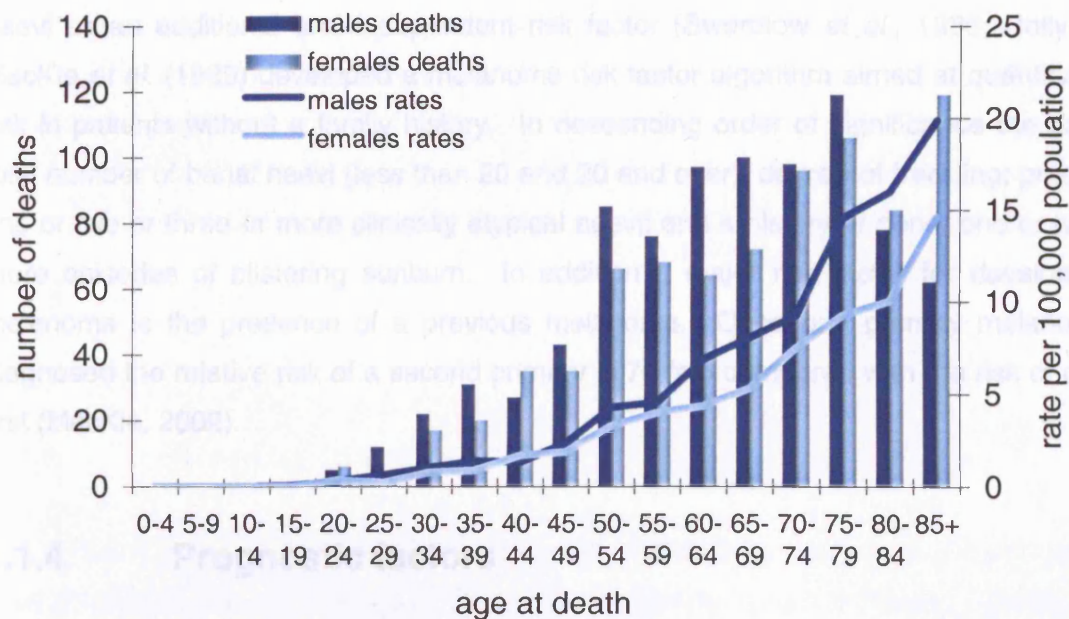
Figure 1.1.2.3 Number of deaths and age specific mortality rates per 100,000 population from melanoma, by sex, UK, 2001 (Source: Cancer Research UK website)

1.1.3 Aetiology

The process by which melanocytes are transformed into melanoma lies in the interplay between genetic factors and the ultraviolet (UV) spectrum of sunlight, although the nature of this relation remains unclear (Jhappan *et al.,* 2003). The division of risk factors into genetic and environmental is likely to be false, in that multifactorial genetic features determine response to environmental events, such as severe sunburn. It has been calculated that inappropriate sun exposure is responsible for two thirds of all melanomas (MacKie, 2002).

The major genetic feature increasing melanoma susceptibility so far identified is the presence of mutations of the *CDKN2A* or p16 gene found on chromosome 9 (Goldstein & Tucker, 2001). Around 5% of melanoma patients have a family history of one or more first degree relatives also being affected, and of these around 25% have had mutations identified in the *CDKN2A* gene. Other genetic defects have also been identified including mutations in the *CDK4* gene (Zuo *et al.*, 1996).

The strongest risk factor yet identified for sporadic melanoma in several large studies is the presence of large numbers of banal melanocytic naevi, with the presence of dysplastic or atypical naevi as an additional and independent risk factor (Swerdlow *et al.*, 1986; Holly *et al.*, 1987). MacKie *et al.* (1989) developed a melanoma risk factor algorithm aimed at quantifying melanoma risk in patients without a family history. In descending order of significance the risk factors are: total number of banal naevi (less than 20 and 20 and over); degree of freckling; presence of none, one or two or three or more clinically atypical naevi; and a history of none, one or two, or three or more episodes of blistering sunburn. In addition a major risk factor for developing a primary melanoma is the presence of a previous melanoma. Once one primary melanoma has been diagnosed the relative risk of a second primary is 70-fold compared with the risk of developing the first (MacKie, 2002).

1.1.4 Prognostic factors

Wallace Clark Jr divided the vertical growth of melanoma into 5 anatomic levels of invasion and found these levels to correlate with disease outcome (Table 1.1.4) (Clark, 1969). However, it has now been established that the "Breslow thickness" of the tumour in millimetres (Breslow, 1970) correlates more strongly with prognosis than the Clark's levels (Balch *et al.*, 2001a).

Level of Invasion	Layer of the skin
1	Above the basement membrane
2	Papillary dermis
3	Between papillary and reticular dermis
4	Reticular dermis
5	Subcutaneous tissue

Table 1.1.4 Wallace Clark Jr.'s levels of melanoma invasion (1969)

Various other factors have since been found to correlate with tumour progression. The most comprehensive publication to date has analysed the various proposed prognostic variables in 17,600 patients resulting in the largest prognostic factor analysis of melanoma ever conducted (Balch *et al.,* 2001a). The results of this study demonstrated that the two most powerful independent characteristics of the primary melanoma among all the variables analysed were tumour thickness and ulceration. Other statistically significant prognostic factors were patient age, site of the primary melanoma, level of invasion and sex. However, once the nodal status of the patient was taken into account, this became the most significant predictor and level of invasion and sex of the patient were no longer independent predictors of outcome. Clark's levels still do remain important, but only for tumours less than 1mm deep. Interestingly, there still are patients with thin non-ulcerated melanomas who fair badly and those with thick melanomas who fair well (Elder *et al.,* 1984; Burton & Armstrong, 1994). Clearly other factors come in to play in these situations. If high-risk patients could be better identified, treatment and follow-up regimes could be more individually tailored.

1.1.5   Staging of patients

On the basis of the study of 17,600 patients from 13 centres, Balch and co-workers published the Final Version of the American Joint Committee on Cancer (AJCC) Staging System for Cutaneous Melanoma in 2001 (Balch *et al.,* 2001b). This was an evidence-based system that reflected the dominant prognostic factors consistently identified in Cox multivariate regression analyses. The actual categories and groupings are shown in Tables 1.1.5.1 and 1.1.5.2. The most significant changes to the previous system are the inclusion of pathological staging of the patients by the use of sentinel lymph node biopsy and the inclusion of the presence or absence of ulceration of the primary tumour.

T Classification	Thickness	Ulceration Status
T1	≤ 1.0mm	a: without ulceration and level II/III
		b: with ulceration or level IV/V
T2	1.01-2.0mm	a: without ulceration
		b: with ulceration
T3	2.01 – 4.0mm	a: without ulceration
		b: with ulceration
T4	> 4.01mm	a: without ulceration
		b: with ulceration
N Classification	**No. Metastatic Nodes**	**Nodal Metastatic Mass**
N1	1 node	a: micrometastasis
		b: macrometastasis
N2	2-3 nodes	a: micrometastasis
		b: macrometastasis
		c: in transit met(s)/satellite(s) without metastatic nodes
N3	4 or more metastatic nodes or matted nodes or in transit met(s)/satellite(s) with metastatic node(s)	
M Classification	**Site**	**Serum lactate dehydrogenase**
M1a	Distant skin, subcutaneous or nodal metastases	Normal
M1b	Lung metastases	Normal
M1c	All other visceral metastases	Normal
	Any distant metastasis	Elevated

Table 1.1.5.1 Melanoma TNM classification (Adapted from Balch *et al.*, 2001(b))

	Clinical Staging			Pathological staging			5-year survival rates
	T	N	M	T	N	M	
0	Tis	N0	M0	Tis	N0	M0	-
IA	T1a	N0	M0	T1a	N0	M0	95%
IB	T1b	N0	M0	T1b	N0	M0	91%
	T2a	N0	M0	T2a	N0	M0	89%
IIA	T2b	N0	M0	T2b	N0	M0	77%
	T3a	N0	M0	T3a	N0	M0	79%
IIB	T3b	N0	M0	T3b	N0	M0	63%
	T4a	N0	M0	T4a	N0	M0	67%
IIC	T4b	N0	M0	T4b	N0	M0	45%
III	Any T	N1	M0				
		N2		There are no stage III subgroups for clinical			
		N3		staging			
IIIA				T1-4a	N1a	M0	67%
				T1-4a	N2a	M0	67%
IIIB				T1-4b	N1a	M0	52%
				T1-4b	N2a	M0	52%
				T1-4a	N1b	M0	54%
				T1-4a	N2b	M0	54%
				T1-4a/b	N2c	M0	-
IIIC				T1-4b	N1b	M0	24%
				T1-4b	N2b	M0	24%
				Any T	N3	M0	23%
IV	Any T	Any N	Any M1	Any T	Any N	Any M1	N/A

Table 1.1.5.2 Stage groupings for cutaneous melanoma (Adapted from Balch *et al.*, 2001 (b))

1.2 PRIMARY MELANOMA

1.2.1 The growth of melanoma

Cutaneous melanoma usually arises in the epidermis, where it may be confined for several months. Herlyn *et al.* described its proliferation in the epidermis as being in the radial growth phase (Herlyn *et al.*, 1987). If uninterrupted by therapy the tumour may progress into a vertical growth phase, whereby it invades the reticular dermis and even the subcutaneous fat (Clark *et al.*, 1969). With the exception of nodular melanoma, which does not show any significant radial growth pattern, all types of melanoma appear to progress through some degree of radial growth before the vertical growth begins. The vertical growth phase usually results in a nodule arising in the relatively flat radial growth phase lesion.

The radial growth phase represents the melanoma's capacity for autonomous growth and invasion of the papillary dermis, but not the capacity to metastasise. The vertical growth phase is said to result from a subpopulation of cells that has a growth advantage over the surrounding cells, and also possesses the potential to metastasise (Elder *et al.*, 1984, Clark *et al.*, 1989).

1.2.2 Subtypes of melanoma

1.2.2.1 Superficial Spreading Melanoma (SSM)

Superficial spreading melanoma is the most common subtype of melanoma, representing around 70% of all melanomas (Langley *et al.*, 1998). SSM frequently arise in a pre-existing naevus or a dysplastic naevus and are found most commonly on the legs of women and the backs of men (Mihm *et al.*, 1975). Early in their development, these melanomas are often flat or nearly flat, but as they become more invasive they may develop one or more nodules.

Figure 1.2.2.1 Nodule arising within superficial spreading melanoma

1.2.2.2   Nodular Melanoma (NM)

Nodular melanoma comprises 10% to 15% of all melanomas and is solely made up of cells showing a tumorigenic, vertical growth pattern (Langley *et al.,* 1998). It generally begins as a variably pigmented papule that grows rapidly to form a nodule. Because of the rapid growth, these lesions are often quite thick at the time of removal, hence have a poor prognosis. However, when controlled for thickness, survival is no worse than for other melanomas (Langley *et al.,* 1998).

1.2.2.3   Lentigo Maligna Melanoma (LMM)

Lentigo maligna melanoma was described by Hutchinson more than 100 years ago and is still occasionally referred to as Hutchinson's freckle. This type of melanoma represents about 10% of all melanomas. The term "lentigo maligna" is generally used to mean lentigo maligna melanoma in situ (confined within the epidermis). This form of melanoma usually occurs on the most chronically sun-damaged skin, usually the face, of older patients (mean age 65 years). The lesion begins as an unevenly pigmented macule that slowly grows peripherally, often reaching several centimetres in size. These lesions tend to remain non-invasive for a prolonged time, often many years. A papule may become apparent when the lesion becomes invasive.

1.2.2.4   Acral Lentiginous Melanoma (ALM)

Acral lentiginous melanoma is a distinct subtype of melanoma that arises on the hairless skin of the palms and soles, in and around the nails and on mucosal surfaces (oral, nasal, vaginal, and anorectal). Of these, melanoma on the soles is the most common (Langley *et al.,* 1998). This form of melanoma is uncommon and of similar incidence in all racial and ethnic groups, but is the predominant form in darker skinned races. ALM usually occurs in older patients (60 years plus) and the survival of patients has traditionally been poor, but this is likely to be due to late diagnosis. Subungual melanoma is a rare form of cutaneous melanoma and is often classified as a variant of the acral lentiginous type.
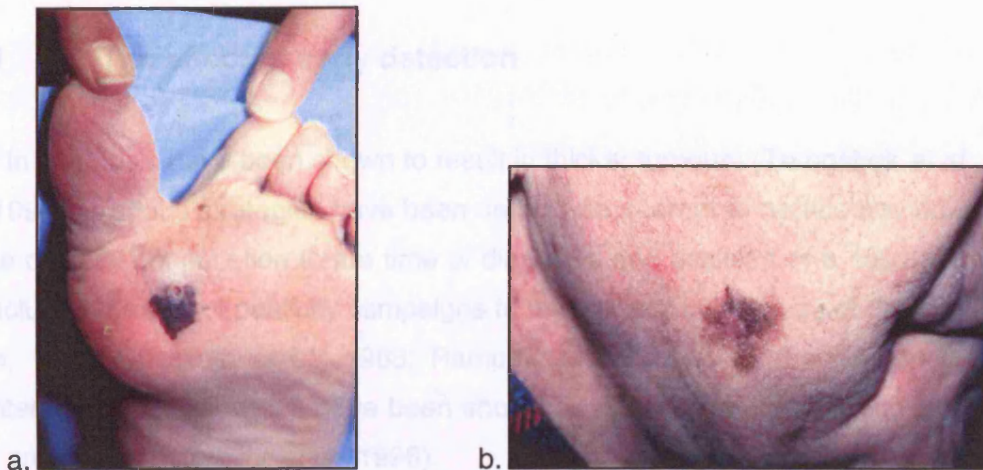
Figure 1.2.2.4   a. acral lentiginous melanoma on the sole of the foot; b. lentigo maligna melanoma on the cheek.

1.2.3 Problem diagnosis and diagnostic difficulties

Melanomas often present a diagnostic challenge. In general, malignant pigmented lesions exhibit disorganised and chaotic pigment patterns, whereas benign pigmented lesions tend to show order in colour, symmetry, regular borders and uniformity of surface characteristics (Mihm *et al.,* 1975). Important exceptions include small diameter, amelanotic and nodular melanomas. Rona MacKie (1986) devised a seven point checklist to aid diagnosis, which has been revised by Du Vivier *et al.,* (1991) and is shown in table 1.2.3. Lesions with any of the major features or three of the minor ones are suspicious of melanoma – this gives a diagnostic accuracy of around 80% (Du Vivier *et al.,* 1991).

Major Features	Minor Features
Change in size	Largest diameter $\geq$ 7mm
Change in shape	Inflammation
Irregular Colour	Oozing
	Change in sensation

Table 1.2.3 The revised 7-point checklist for melanoma diagnosis

1.2.3.1   The effect of early detection

Delays in diagnosis have been shown to result in thicker tumours (Temoshok *et al.*, 1984, Ibbotson *et al.*, 1995). Various strategies have been used in an attempt to reduce any delays incurred from the time of initial consultation to the time of diagnosis and excision of a suspicious lesion. These have included the use of publicity campaigns to raise public awareness of the condition (Doherty & MacKie, 1986; Cassileth *et al.*, 1988; Rampen *et al.*, 1989) to the existence of rapid access pigmented lesion clinics which have been shown to reduce the mean thickness of melanomas at time of presentation (Grover *et al.*, 1996).

1.3   HISTOPATHOLOGY

1.3.1 Histopathological features

The diagnosis of melanoma is made by the presence of proliferating atypical melanocytes along the dermal-epidermal junction that replace the basilar region and/or display a pagetoid spread within the epidermis (melanoma in-situ). The appearance of nests or aggregates of atypical melanocytes in the papillary dermis signals an invasive pattern, which in more advanced stages

form expansile nodules in the dermis (Slominski *et al.,* 2001). The most important determinant of prognosis in melanoma is the type of growth: in the radial growth phase melanoma mitoses are frequently seen in the epidermis but rarely in the dermis, melanoma aggregates do not become expansile and an inflammatory lymphocytic response is present in the papillary dermis. Vertical growth phase melanomas histologically show small expansile cell aggregates in the papillary dermis different in nature to those exhibited in the radial growth phase. They display increased cell numbers and occasional mitotic figures (Barnhill & Mihm Jr., 1998).

1.3.2 Histological parameters of prognostic significance in melanoma

Although many studies have been performed on prognostic modelling in melanoma no histological prognostic variable has yet been shown to have independent predictive value. However, a number of variables have been reported to modify the predictive meaning of thickness in some databases: Clark *et al.,* (1989) have suggested that it is the phase of tumour growth (i.e. radial or vertical growth) that is more important than thickness itself, although this has not yet been validated by other studies. It has been proposed that histogenetic subtype of tumour bears a relation to prognosis, however evidence suggests that prognosis in melanoma subtypes may largely be attributed to differences in tumour thickness. For example good prognosis in lentigo maligna melanoma may be attributed to the fact that they are usually thin at the time of excision (Koh *et al.,* 1984). Other histological factors implicated in prognosis include: evidence of tumour regression, tumour-infiltrating lymphocyte response and dermal mitotic activity (Carlson *et al.,* 2003).

1.3.3 Immunohistochemistry and melanoma

A variety of immunohistochemical markers may be used to aid the diagnosis of melanoma. The S100 antigen is the most commonly used immunohistochemical target, with a high sensitivity, found in almost all cases (Carlson *et al.,* 2003). However, S100 lacks sufficient specificity because it is also expressed in non-melanocytic cells. Another marker, the HMB-45 melanosomal antigen, is less sensitive, but highly specific for pigmented cells (Barnhill & Mihm Jr., 1998). The melanosomal protein MART-1/Melan-A is a further promising agent for the diagnosis of melanoma (Hofbauer *et al.,* 1998).

1.4   BIOLOGY OF MELANOMA METASTASIS

The molecular basis of human melanoma progression and the changes associated with the transition of melanoma cells from the radial growth phase to the vertical growth phase remain unclear. The production of metastases depends on the completion of a complex, multistep process involving the survival and growth of a unique subpopulation of cells with metastatic properties (Fidler *et al.,* 1990). At the core of this process lie the changing adhesive properties of the tumour cells, which dictate their interactions with the surrounding extracellular matrix and neighbouring cells.

During metastasis development cell-cell and cell-matrix interactions play a key functional role. Cell adhesion molecules are cell surface molecules which bind specifically to other cell surface molecules (ligands) on another cell. Structurally, many different kinds of molecules can mediate intercellular adhesion, but the majority of cell adhesion molecules known to date fall into three gene families: the integrins, the cadherins and the immunoglobulin superfamily (Chothia *et al.,* 1997). Adhesion molecules mediate various interactions and modulation of the expression of a number of adhesion molecules has been associated with metastatic spread in melanoma (Natali *et al.,* 1991; Bartolazzi *et al.,* 1994; Xie *et al.,* 1997; Johnson, 1999; Christofori & Semb, 1999; Thies *et al.,* 2002).

1.4.1 Interrelationships between cell adhesion molecules and their relationship to tumour progression

Cell adhesion molecules do not simply function as a molecular glue organising cells into static structural entities, but also support and direct the exchange of information between cells (Klymkowsky *et al.,* 1995). Adhesion molecules themselves actively participate in transducing signals into the cells. Observations suggest that changes in the adhesive properties of melanocytes and melanomas are involved in the upregulation of MCAM expression (Grimm *et al.,* 1995). This will be discussed in detail in the introduction to chapter 4.

1.4.2 Biological prognostic markers for primary melanoma

Some of the most promising molecular markers of melanoma prognosis include cell cycle biomarkers (Karjalainen *et al.*, 1998; Korabiowska *et al.*, 2000; Barzilai *et al.*, 1998), p16^{INK4a}/CDK2NA (Straume *et al.*, 2000; Straume *et al.*, 2002), MLSN-1 (Duncan *et al.*, 2001), transcription factor activator protein (AP)-2 (Karjalainen *et al.*, 1998), matrix metalloprotein (MMP)-2 (Vaisanen *et al.*, 1998), DNA content (ploidy) and fractional allelic loss analysis (Healy *et al.*, 1998; Umebayashi & Otsuka 1997). Other independent molecular biomarkers have prognostic significance in subgroups of melanoma patients such as *c-myc* gene amplification in acral lentiginous melanomas (Grover *et al.*, 1999). In addition, melanomas possess a repertoire of biosynthetic capacities represented by the production of hormones, growth factors and their receptors that may sustain and accelerate tumour development and progression (Slominski *et al.*, 2001).

1.4.2.1   Cell cycle markers

Proliferative activity correlates with metastatic potential and has significant prognostic value in several cancers (Quinn & Wright, 1990; Cross & Start, 1996; Tubiana & Courdi, 1989). For melanoma, five out of eight studies on large melanoma collectives (>450 patients with 5 years mean follow-up) have demonstrated a significant independent relationship between increasing mitotic count and decreasing survival (Sondergaard & Schou, 1985; Kopf *et al.*, 1987; Drzewiecki *et al.*, 1990; MacKie *et al.*, 1995; Barnhill *et al.*, 1996; Ostmeier *et al.*, 1999; Retsas *et al.*, 2002; Vossaert *et al.*, 1992). Ki-67/MIB-1 index, proliferating nuclear antigen (PCNA) and others are reliable and widely used methods of measuring proliferative index (growth fraction) (van Diest *et al.*, 1998) and have been demonstrated to be independent prognostic factors (Karjalainen *et al.*, 1998; Korabiowska *et al.*, 2000; Barzilai *et al.*, 1998). Most studies in melanoma have demonstrated that the Ki-67 index is a useful prognostic biomarker for melanoma progression with decreased overall survival and risk for metastatic disease (Straume *et al.*, 2000; Ramsay *et al.*, 1995; Korabiowska *et al.*, 2000; Vogt *et al.*, 1997; Boni *et al.*, 1996; Henrique *et al.*, 2000; Tran *et al.*, 1998). However this correlation appears to be related to melanoma thickness because high Ki-67 index in melanomas thicker than 1.5mm are at risk for metastases or death from melanoma (Straume *et al.*, 2000; Ramsay *et al.*, 1995; Vogt *et al.*, 1997; Boni *et al.*, 1996; Henrique *et al.*, 2000) but this is not the case for thin melanomas (Vogt *et al.*, 1997; Boni *et al.*, 1996).

1.4.2.2   p16^{INK4a}

P16^{INK4a}, also known as CDK2NA, inhibits the ability of cyclin-dependent kinases (CDK)-4 and –6 to activate critical substrates necessary for progression from G1 to the S-phase of the cell cycle (Castellano & Parmiani, 1999; Maelandsmo *et al.*, 1996; Bartkova *et al.*, 1996). As a tumour suppressor gene, p16^{INK4a} appears to play a fundamental role in melanoma progression (Karjalainen, 2001; Straume *et al.*, 2002), whereas aberrations (mutations, homozygous deletion) involving other products of CDK2NA locus such as p14, p15 and p18, are extremely rare in melanoma cell lines and sporadic melanomas (Castellano & Parmiani, 1999). Increasing loss of p16^{INK4a} expression is seen with melanoma progression (Straume *et al.*, 2000; Funk *et al.*, 1998; Sparrow *et al.*, 1998; Reed *et al.*, 1995; Talve *et al.*, 1997; Pavey *et al.*, 2002). This loss of p16^{INK4a} protein expression correlates with increased cell proliferation (Ki-67 index), advanced stage and metastases (Straume *et al.*, 2000).

1.4.2.3   Transcription factor AP-2

The transcription factor AP-2 is a 52kDa DNA-binding protein that is thought to inhibit tumour growth through the activation of p21. In a consecutive series of 369 stage I cutaneous melanoma patients, loss of AP-2 expression was significantly associated with low p21 expression, increased tumour thickness, high TNM category and recurrent disease (Karjalainen *et al.*, 1998). In this study, low AP-2 index was an important predictor of both recurrence-free survival and overall survival.

1.4.2.4   MLSN-1

MLSN-1 is a member of a novel family of at least three distinct putative calcium channel proteins that are distantly related to the transient receptor (Trp) calcium channel family (Hunter *et al.*, 1998). The regulation of intracellular calcium is widely used in biological signalling and appears to be a particularly important component of signalling pathways that affects cell cycle regulation and cell survival. By *in situ* hybridisation techniques on 150 patients with localised melanoma, loss of MLSN-1 mRNA independently predicted poor survival (Duncan *et al.*, 2001). However, it has not yet been demonstrated that loss of MLSN-1 expression is necessary for the acquisition of metastatic potential during the progression of melanoma (Duncan *et al.*, 2001).

1.4.2.5   MMP-2

Proteolytic enzymes, such as MMPs, are instrumental in the degradation of extracellular matrix components and are crucial in the first step of invasion of normal tissues by cancer cells. MMP-2, also known as Type 4 collagenase, is suspected of augmenting the metastatic capability of melanoma cells by degrading the basement zone and surrounding tissues (Ferrier *et al.*, 1998; Vaisanen *et al.*, 1996). Specifically, MMP-2 expression correlates with haematogenous metastases (Vaisanen *et al.*, 1996) but not tumour thickness (Vaisanen *et al.*, 1998) and in male patients particularly, high MMP-2 expression independently predicts for poor 5-year survival (Vaisanen *et al.*, 1998).

1.4.2.6   Chromosome analysis and DNA content

Gross chromosomal losses and gains characterise most human tumours including melanoma (Balaban *et al.*, 1986). Melanoma exhibits non-random chromosomal break points, specific aneusomies or specific allelic imbalances and metastasis is suppressed by specific chromosomes, implicating chromosomal instability in melanoma pathogenesis (Bastian *et al.*, 2000; Balaban *et al.*, 1986; Nelson *et al.*, 2000; Miele *et al.*, 1996; Udart *et al.*, 2001; Rao *et al.*, 2003). Furthermore, increasing fractional allelic loss correlates with poor survival and shortened disease-free survival (Healy *et al.*, 1998). Similarly, increasing DNA index (aneuploidy) predicts for both decreased disease-free survival and poor overall survival (Umebayashi & Otsuka, 1997; Scheistroen *et al.*, 1995; Kheir *et al.*, 1988).

1.4.2.7   MIA and S-100B

Melanoma inhibitory activity (MIA) and S-100B are proteins, which can be measured in the serum of melanoma patients to estimate disease progression or recurrence. MIA is an 11kd soluble protein, secreted by melanoma cells, that has been suggested to be predictive of disease relapse or response to chemotherapy in stage IV disease (Juergensen *et al.*, 2001). Recently Martenson *et al.*, have shown the independent prognostic value of serum S-100B as a marker, which is associated with clinical stage I and III disease (Martenson *et al.*, 2001). Retsas *et al.* have suggested that in metastatic melanoma S-100 might be a better prognostic factor than LDH, which is used in staging of the disease (Retsas *et al.*, 2002).

1.4.2.8   RT-PCR

Melanoma antigens recognised by T-cells-1 (MART-1) and tyrosinase, a key enzyme in melanogenesis, are specific for melanoma cells. Circulating melanoma cells positive for MART-1 or tyrosinase can be detected by reverse-transcriptase polymerase chain reaction (RT-PCR) to predict disease progression and metastasis (Curry *et al.,* 1998; Quereux *et al.,* 2001). Hoon *et al.* utilised a multiple mRNA marker (MM) RT-PCR to detect micrometastases in sentinel lymph nodes or the presence of melanoma cells in cerebrospinal fluid to predict disease relapse or development of central nervous system metastases (Bostick *et al.,* 1999; Hoon *et al.,* 2001). Furthermore Curry *et al.* have suggested that detection of MART-1 and tyrosinase positive circulating melanoma cells by RT-PCR could help to identify subgroups of patients who develop disseminated disease or local recurrence (Curry *et al.,* 1999).

1.4.2.8   Summary of prognostic factors

The staging and survival expectancy of primary cutaneous melanoma is principally based on tumour thickness and presence or lack of ulceration as well as on lymph node status. However, there is no single biological marker that can reliably predict the outcome of primary melanoma. As described, a wide variety of biological markers have been investigated and shown to be of importance in melanoma development and progression. However, as no single one has been shown to accurately predict outcome, further work is required in this field, including further investigation of markers already studies and discovery of novel prognostic markers.

1.5   HMW-MAA

High-Molecular Weight Melanoma-Associated Antigen (HMW-MAA), also known as mCSP (melanoma-associated chondroitin sulphate proteoglycan) and human NG2 homologue, is an established surface proteoglycan consisting of two non-covalently associated glycopolypeptides with molecular weights of 280 and 440 kDa (Wilson *et al.*, 1981; Bumol *et al.*, 1982). HMW-MAA is expressed on cell surface microspikes thought to be involved in migration and contact inhibition (Garrigues *et al.*, 1986; Schlingemann *et al.*, 1990). It is detected in 80-90% of surgically excised melanomas (Buraggi *et al.*, 1984; Giacomini *et al.*, 1984; Natali *et al.*, 1984) and expressed in 80-100% of primary melanoma cell cultures (Herlyn *et al.*, 1980; Herlyn *et al.*, 1985). Its expression levels in melanoma and normal tissues have been well characterised (Reisfeld *et al.*, 1982). Monoclonal antibodies directed against HMW-MAA have been produced by hybridoma techniques (Reisfeld *et al.*, 1987; Kupsch *et al.*, 1995; Kupsch *et al.*, 1999) and it has previously been used as a target for radioimmunoscintigraphy and immunotherapy (Buraggi *et al.*, 1985; Mittelman *et al.*, 1990; Siccardi *et al.*, 1990). HMW-MAA interacts with the extracellular matrix α4β1 integrin, collagen V and VI, plasminogen and its fragments such as angiostatin, MT3 matrix metalloproteinase, bFGF and intracellular signalling molecules (Garrigues *et al.*, 1986; Iida *et al.*, 1998; Eisenmann *et al.*, 1999; Iida *et al.*, 2001; Staub *et al.*, 2002). The protein appears to play a complex biological role in angiogenesis and cell spreading, tissue invasion and metastasis formation by melanoma. This multifunctional role may explain its very limited expression variability.

1.5.1 HMW-MAA Expression in Normal Tissues

Hellstrom *et al.* found HMW-MAA to be expressed on cultured melanocytes but not on normal epidermal melanocytes (Hellstrom *et al.*, 1983). They did, however, find it expressed at low levels on some small blood vessels or more specifically, tumour neovasculature and in wound healing, which would be beneficial for tumour targeting. Guinea pig studies have shown low levels of expression in hair follicles, sebaceous glands, some basal keratinocytes and several non-melanoma tumours (Liao *et al.*, 1987, Godal *et al.*, 1992; Behm *et al.*, 1996; Smith *et al.*, 1996; Schrappe *et al.*, 1991). A minor degree of expression on human basal keratinocytes has also been reported (Kupsch *et al.*, 1995, 1999). While these data show this antigen to be only *relatively* melanoma-specific, cross reactivity with normal tissues in these studies has been very low. Indeed, Pluschke et al. demonstrated very little HMW-MAA expression in normal tissues by RNA

blot (Pluschke *et al.*, 1996). A low level of binding to blood vessels is unlikely to interfere greatly with melanoma targeting. These data therefore suggest that a radioimmunopharmaceutical directed at HMW-MAA will bind to melanoma in a reliable fashion with very little cross-reactivity with normal tissues.

1.5.2 HMW-MAA Expression on Melanoma Cells

The level of antigen expression relates directly to its efficacy as a target for radioimmunoscintigraphy by determining the amount of antibody that accumulates in and around the tumour. For immunoscintigraphy to be successful, uniformity of antigen expression within a given tumour deposit would not be absolutely necessary, as any "hotspot" would be considered suspicious and warrant further investigation. In contrast, uniformity of expression would be important for therapeutic studies where it is important to target as many tumour cells as possible.

Fortunately, the degree of antigenic expression for HMW-MAA in metastatic melanoma deposits is relatively low in comparison to other tumour-associated antigens (Natali *et al.*, 1985; Kageshita *et al.*, 1991). There is also low heterogeneity of HMW-MAA expression in different deposits within the same individuals (Natali *et al.*, 1985). The expression is also similar in both primary and metastatic cutaneous melanomas (Kageshita *et al.*, 1991), with the exception being acral lentiginous melanomas, where the difference in expression is of prognostic significance (Kageshita *et al.*, 1992).

In vitro studies on the number of antigenic sites per cell have shown that HMW-MAA is present at levels of between $2x10^4$ and $1.8x10^6$ molecules per melanoma cell (Giacomini *et al.*, 1985; Shockley *et al.*, 1990). The density of HMW-MAA on a wide variety of both cultured melanoma cells and melanoma specimens is comparable to that of other potential target antigens such as p97 ($5x10^4$ to $5x10^6$ molecules per melanoma cell) (Houghton *et al.*, 1992) and gangliosides GM2, GD2, GT3 and 9-O-Ac-GD3 ($8x10^6$ to $3x10^8$ molecules per melanoma cell) (Hamilton *et al.*, 1993). These data suggest that a radioimmunopharmaceutical directed at HMW-MAA should be able to bind melanoma cells in sufficient amounts to be easily detected by RIS. This has been demonstrated in extensive immunoscintigraphic studies targeting HMW-MAA in melanoma patients (Buraggi *et al.*, 1985; Lotze *et al.*, 1986; Cerny *et al.*, 1987; Siccardi *et al.*, 1990).

Correlations have not been found between HMW-MAA staining and survival in for most melanoma subtypes (Natali *et al.,* 1985; Odili, Thesis 2002), however one group found a correlation between its expression and prognosis in primary acral lentiginous melanomas (Kageshita *et al.,* 1993). The expression of HMW-MAA has been shown to vary according to the stage of disease. Siccardi *et al.* described a possible down-regulation of antigen expression as a cause for sub-optimal immunoscintigraphic images obtained from patients with stage IV disease (Siccardi *et al.,* 1990).

1.6   DIAGNOSIS OF METASTASES

The ideal method of detecting recurrent disease would be highly sensitive and specific, whilst allowing whole body examination. A thorough patient history and physical examination have been shown to be more effective in detecting metastatic disease than any radiological investigation (Weiss *et al.,* 1995). In a German cohort of 661 patients 74% of synchronous metastases were first detected by clinical examination, which proved to be more sensitive than ultrasound and chest radiography (Hofmann *et al.,* 2002). Currently the routine investigations for the detection and evaluation of secondaries recommended include: chest radiography, liver ultrasound, computed tomography of the chest, abdomen and pelvis, lactate dehydrogenase levels and a full blood count (Roberts *et al.,* 2002).

1.6.1 Lactate dehydrogenase (LDH)

Serum LDH has been included in the latest AJCC staging classification of melanoma (Balch *et al.,* 2001b). It was found to be amongst the most predictive independent factors of diminished survival in all published studies when it was analysed in a multivariate analysis, even after accounting for the site and number of metastases. Therefore, although not diagnostic of metastasis on its own, if elevated, patients with metastases are upstaged to M1c, regardless of the site of metastasis.

1.6.2 Chest radiography (CXR)

Chest x-ray is recommended for stage IIB patients and above as a screening tool (Roberts *et al.*, 2002). However, in the Geman study by Hofmann et al. only five out of 31 synchronous metastases were detected by CXR (Hofmann *et al.*, 2002). In another study (Terhune *et al.*, 1998) 130 patients had a suspicious CXR out of a total of 876 patients with clinically localised melanoma. Of these, only one had a pulmonary metastasis (0.1%). These findings suggest that CXR is of little value in asymptomatic patients with clinically localised melanoma.

1.6.3 Ultrasound

Ultrasound B-scan (brightness-scan) has proved to be a useful tool in the detection of regional recurrent and in-transit disease in melanoma patients (Voit *et al.*, 2002). It is a fast, reliable and inexpensive staging investigation that may be performed regularly. One advantage is the ability to combine it with fine needle aspiration cytology of a suspect lesion, thus obtaining a cytological sample (Basler *et al.*, 1997). Ultrasound may also be used for the marking of metastases in stage III and IV patients with solitary lesions to facilitate subsequent surgical removal (Voit *et al.*, 2001).

1.6.4 Computed Tomography

The UK guidelines for the management of cutaneous melanoma, published in 2002 (Roberts *et al.*, 2002) suggest CT as a staging investigation for any patient with intermediate or high risk of recurrence, i.e. stage IIB or over. At MD Anderson Hospital, in a group of patients most of who had lesions deeper than Clark's level III, 17 out of 122 (14%) CT scans detected subcutaneous metastases (Shirkhoda *et al.*, 1987). CT is usually used for looking for regional and distant metastases, offering greater resolution than ultrasound. However, high false positive rates have been reported in asymptomatic patients, suggesting the requirement for cytological confirmation of any lesions identified (Buzaid *et al.*, 1995).

1.6.5 Magnetic Resonance Imaging (MRI)

Magnetic resonance imaging avoids the exposure to ionising radiation. It produces superior soft tissue resolution than CT and has been shown to distinguish melanoma from other tissues (Takahashi *et al.*, 1992). Its downfalls are its cost, the discomfort to the patient and its lack of availability.

1.6.6 Positron Emission Tomography (PET)

PET is a non-invasive, high resolution imaging modality that is aimed at detecting micrometastases based on abnormal cellular metabolic activity, rather than relying on structural changes. It works on the basis that malignant cells have a higher rate of utilisation of glucose than normal cells (Warburg *et al.*, 1930). PET scanners have been developed that are capable of acquiring whole-body images in a short timeframe, combined with full three-dimensional image reconstruction. Positron-emitting radiopharmaceuticals (commonly ^{18}F-FDG) can be used to target malignancy at an early stage. A review of the literature by Prichard *et al.* showed the sensitivity of PET in melanoma to be 74-100% and specificity to be 67-100% (Prichard *et al.*, 2002). These figures were better than CT or MRI for all body regions apart from the thorax. However, ^{18}F-FDG is a non-specific metabolite agent that reflects increased glucose metabolism and increased anaerobic glycolysis regardless of the underlying cause. It is therefore liable to high uptake in any cell with increased glucose utilisation or tissue oxygenation, or when there is increased regional blood flow or an inflammatory reaction (Wahl *et al.*, 1990). In addition, PET scanning is expensive, its availability is limited and imaging is restricted by the size of tumour deposit, which must be at least 5mm in diameter (Holder *et al.*, 1998). It also provides a less accurate anatomical localisation than other imaging modalities such as CT and typically requires follow-up with conventional imaging.

1.6.7 Sentinel node biopsy

The popularity of sentinel node biopsy stems from the work of Alistair Cochran and Donald Morton (Cochran *et al.*, 1989; Morton *et al.*, 1992). It is a process whereby the first draining, or sentinel, lymph node from the site of a primary melanoma is removed and studied histopathologically for the presence of metastasis. Should any metastases be present, the patient subsequently undergoes

a full lymph node dissection of the appropriate area. No data have yet shown this procedure to confer any survival benefit. This along with the associated morbidity of the procedure has resulted in the technique not yet being universally accepted. However, it has proven to be a highly accurate method of determining prognosis (Balch *et al.*, 2001b). As regional metastases are uncommon in early melanoma, the procedure is not recommended until patients have at least stage II disease (Roberts *et al.*, 2002).

Investigation	Advantages	Disadvantages	Cost (£)	
Chest X-ray	Cheap, widely available	Very low sensitivity	£34	single film
			£51	multiple views
Ultrasound	Widely available, fast, reliable		£85	single region
			£130	whole abdomen
Computerised Tomography (CT)	Widely available, sensitive	Low specificity	£250	single region
			£350	whole body survey
			£80	contrast + rescan
Magnetic Resonance Imaging (MRI)	Avoids ionising radiation, sensitive and relatively specific	Expensive, not widely available, uncomfortable for patients	£400	single region
			£650	two or more regions
			£100	contrast + rescan
Positron Emission Tomography (PET)	Highly sensitive	Not specific, expensive, limited availability, tumours must be > 5mm	£500	head
			£700	whole body survey
Bone scan	Relatively sensitive	Not specific, expensive, limited availability	£200	whole body survey
Lymphoscintigraphy (LSG)	Sensitive	Limited availability, non-specific	£120	single region
Immunoscintigraphy (ISG)	Sensitive, specific	Limited availability	£200	single region

Table 1.5 Relative costs of imaging techniques (Costs include interpretation of the images by a radiologist. Costs are correct for February 2002. Prices courtesy of Department of Radiology and Department of Nuclear Medicine, Mount Vernon Hospital, Northwood, United Kingdom).

1.7   ANTIBODIES

1.7.1 Antibody structure

IgG antibody is a glycoprotein with a molecular weight of 150,000Daltons. The molecule consists of two identical heavy chain-light chain heterodimers linked by disulphide bridges to form a Y-shaped structure (Figure 1.6.1). Each heavy chain comprises one variable (V) and three constant immunoglobulin (Ig) domains, whereas light chains consist of a single variable and a single constant Ig domain. Each domain consists of about 110 amino acids.
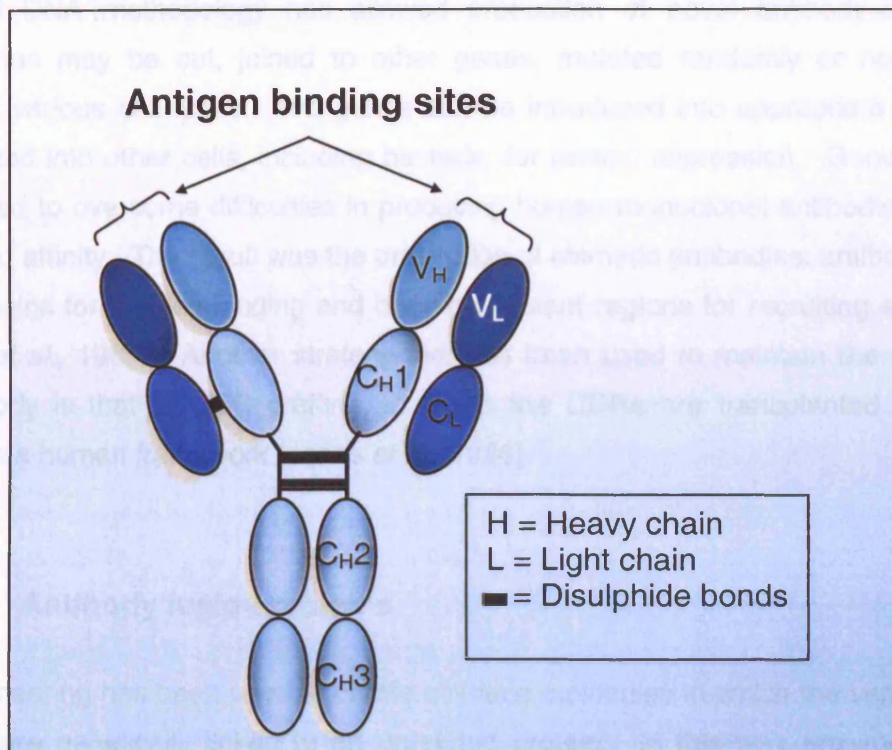
Figure 1.6.1 Diagram of whole monoclonal antibody IgG$_1$. V_L – variable light chain, V_H – variable heavy chain, C_H – constant heavy chain.

From a functional perspective the important features are the antigen-binding site (Fab) and the Fc region (fragment crystallisable), which is responsible for initiating host-defence mechanisms. The Fc region is the stem of the Y-structure and the two antigen binding sites are at the tip of the

outstretched arms. The hinge region of the antibody is flexible so the antigen-binding sites are widely and variably separated, permitting the linking of two identical antigens by the same antibody (the avidity effect). The antigen-binding site is formed by the apposition of two globular variable domains, one derived from the heavy chain (V_H) and the other from the light chain (V_L). The folds of each of the V domains form three short loops of amino-acids – the hypervariable loops or complementarity determining regions (CDRs).

1.7.2 Chemical modification of monoclonal antibodies

1.7.2.1 Genetic modification of monoclonal antibodies

Recombinant DNA methodology has allowed production of novel antibody-derived proteins. Antibody genes may be cut, joined to other genes, mutated randomly or non-randomly and expressed in various cell types. The genes can be introduced into appropriate plasmid vectors and transfected into other cells, including bacteria, for protein expression. Genetic manipulation has been used to overcome difficulties in producing human monoclonal antibodies of the desired specificity and affinity. The result was the production of chimeric antibodies: antibodies with rodent variable domains for antigen-binding and human constant regions for recruiting effector functions (Neuberger *et al.*, 1985). Another strategy that has been used to maintain the specificity of the rodent antibody is that of CDR grafting, in which the CDRs are transplanted from the rodent antibody onto a human framework (Jones *et al.*, 1986).

1.7.2.2 Antibody fusion proteins

Genetic engineering has been used to create chimeric molecules in which the variable domains of an antibody are genetically linked to an unrelated protein. In this way enzymes or toxins, for example, may be given novel binding specificities and can be produced in bacteria (Gross *et al.*, 1989). The approach is made easier when the antibody moiety is expressed as a single chain Fv (scFv), an antibody fragment with variable, but without constant domains (see below). Previous work (Odili, Thesis 2002) in our laboratory has involved the fusion of a Staphylococcal protein A molecule onto a single chain Fv antibody fragment. This has been used to invoke both cell-mediated and antibody mediated cytotoxicity against human melanoma xenografts in nude mice (Joshi *et al.*, 2003).

1.7.3 Phage display and antibody libraries

Genetic engineering using antibody phage display techniques (Skerra & Plukthun, 1988) has allowed rapid production of large numbers of antigen-specific phage (McCafferty *et al.*, 1990). Filamentous bacteriophage attach to the surface of bacteria and inject their single-stranded DNA through the bacterial wall. The infected bacterium continues to divide distributing copies of the viral DNA onto its progeny, which assemble and extrude perfect replicas of the infecting phage. After overnight incubation, one millilitre of the bacterial culture supernatant contains over 10^{11} progeny phage particles. When these phage DNA are used to infect bacteria, they produce progeny phage particles that not only display the appropriated antibody but also contain a single copy of the antibody gene and are still able to infect bacteria almost as efficiently as unmodified phage. Antibodies displayed on the surface of phage are fully functional and will still bind their antigen specifically. Phage antibodies with the desired specificity can be isolated from a mixed population because of their ability to bind antigen (McCafferty *et al.*, 1990).

Phage display libraries enable the whole process of the humoral immune system (selection of suitable antibody, amplification and affinity maturation) to be reproduced *in vitro*, resulting in the production of high affinity human monoclonal antibodies and their fragments (Winter & Milstein, 1991; Chester *et al.*, 1994). The bacteriophage with desired antigen reactivity are readily isolated by repeated rounds of selection using immobilised antigen (Clackson *et al.*, 1991). Kretzschmar and von Rüden have estimated that phage display has provided approximately 30% of all human antibodies now in development (Kretzschmar & von Rüden, 2002).

Verhaar *et al.* were the first to suggest that scFvs isolated by phage display are superior to scFvs constructed from Mabs for the *in vivo* targeting of tumours (Verhaar *et al.*, 1995). This idea arose from the results of using radiolabelled phage displayed-isolated scFv in a mouse study, which located tumour lesions more successfully than Mab-derived scFvs. Richard Begent's group (Begent *et al.*, 1996) obtained better images using phage displayed-isolated scFv in patients than Larson *et al.* with a Mab-derived scFv, suggesting that Verhaar et al.'s mouse data might be predictive for the image quality in patients (Larson *et al.*, 1997). More recently, we have shown that light chain shuffling (Clackson *et al.*, 1991; Kang *et al.*, 1991) of a mouse Mab derived anti-HMA-MAA scFv can result in significant improvements of scFv yield and melanoma binding with retention of high melanoma specificity *in vitro* (Kupsch *et al.*, 1995).

1.8 RADIOIMMUNOSCINTIGRAPHY

1.8.1 Introduction

Radio-immunoscintigraphy is the use of radiolabelled tumour-specific antibodies or their fragments to detect tumour. The antibodies act as carriers of radionuclides that enable external imaging of disease throughout the body by gamma camera imaging (Larson *et al.*, 1984). Melanoma seems ideally suited to antibody-mediated targeting as a number of melanoma antigens have been well characterised (Carrel *et al.*, 1980; Houghton *et al.*, 1982; Reisfeld & Ferrone, 1982; Houghton *et al.*, 1986; Herlyn & Koprowski, 1988). Monoclonal antibodies (Mabs) against melanoma antigens have been labelled with radioisotopes and shown to localise to melanoma in patients (Buraggi *et al.*, 1984; Carrasquillo *et al.*, 1984; Buraggi, 1986). The majority of these studies used Mabs directed against high molecular weight melanoma-associated proteoglycan (HMW-MAA). HMA-MAA is a well-characterised melanoma surface antigen with limited expression by normal tissues (Pluschke *et al.*, 1996; Kupsch *et al.*, 1999), and low heterogeneous expression between patients (Natali *et al.*, 1985). It has also been used as a target for melanoma therapy (Mittelman *et al.*, 1995a). It is expressed in up to 90% of cutaneous melanomas, and with similar frequency in both primary and metastatic lesions (Kageshita *et al.*, 1991).

1.8.2 Melanoma as a target for RIS

There are several rationales for using RIS to image melanoma and as a result melanoma has been the subject of numerous RIS studies in patients. Metastatic lesions often go undetected by physical examination and conventional diagnostic means: in one study 30% of patients developed metastases despite negative findings on physical examination (Schmelter *et al.*, 1986). The disseminated distribution of metastatic melanoma makes RIS a useful adjunct offering a more complete staging of patients before surgery than conventional imaging and allows the location of disease outside the surgical field. It can be used to identify patients who could benefit from further surgery (i.e. regional lymph node dissection for nodal metastases, or hyperthermic isolated lymph node perfusion for patients with in-transit metastases) or aggressive biochemotherapy. Further extension of radiolabelled antibodies towards treatment of disease would offer an improved method of delivering high doses of radiation to lesions throughout the body. Finally in one study

(Bender *et al.,* 1997) it was found that patients who had undergone repeated imaging by means of RIS almost doubled their chances of survival compared with patients imaged only once, suggesting that RIS itself may have therapeutic potential.

1.8.3 Comparison with Other Imaging Modalities

Current investigations (CT, MRI, PET) are not specific for melanoma and biopsy remains the only definitive means of identifying the pathology of a lesion. The sensitivity of RIS using anti-HMW-MAA antibody preparations for the detection of known metastasis is around 75%, comparing favourably with standard diagnostic methods (Kang & Yong, 1998). RIS is able to survey the entire body in a single procedure, detecting around 25% of clinically occult metastasis in one study (Siccardi *et al.,* 1990).

Advantages of antibody-mediated imaging over other imaging modalities have previously been demonstrated (Begent *et al.,* 1996). Antibody imaging has the potential to exceed the performance of CT and MRI scans, because it depends on the contrast between normal tissue, tumour and the amount of localised radionuclide and is not limited by size of deposit, whereas CT and MRI depend on the resolution of the imaging device. CT scans also define abnormality based on size and site of the pathological lesion. PET, like immunoscintigraphy provides little anatomic information but can be used to scan the entire body. However, false positive rates with PET are high, and this form of imaging cannot be used to detect microscopic deposits present in the early stages of the disease (Tyler *et al.,* 2000). High sensitivity in imaging is important when selecting patients for appropriate treatment, as chemotherapy appears to confer a survival benefit if it is administered when tumour deposits are small (Moertel *et al.,* 1994; Glimelius & Pahlman, 1992).

1.8.4 Clinical Experience

The first clinical investigations with radiolabelled anti-melanoma antibodies were conducted by Larson et al., who were able to visualise 80% of lesions larger than 1.5cm in six patients with metastases (Larson *et al.,* 1983). Siccardi et al. undertook an Italian multicentre study analysing the efficacy of [99m]Tc- and ^{111}In-labelled F(ab')$_2$ fragments reactive against high molecular weight melanoma associated antigen to radioimage 254 patients with known melanoma metastases (Siccardi *et al.,* 1986). Three hundred and seventy seven of the 412 known lesions were identified

using RIS. However an additional 127 previously occult lesions were also identified using RIS. As well as demonstrating the potential for the use of RIS in melanoma, Siccardi's group also confirmed the reliability of the technique between 10 nuclear medicine departments.

Later Siccardi carried out a large European multicentre trial (Siccardi *et al.*, 1990), in which 493 patients were investigated with the murine monoclonal antibody 225.28S recognising HMW-MAA. A total of 728 lesions, 70% including 123 previously occult metastases, were visualised by radioimmunoscintigraphy. About 70% of the lesions not visualised were less than 2cm in size. Other radioimmunoscintigraphic studies that have been performed are summarised in table 1.8.4 below.

Authors	Number of patients	Sensitivity (%)	Specificity (%)	Accuracy (%)
Siccardi *et al.* (1990)	493	79	96	84
Siccardi *et al.* (1986)	254	84	92	86
Feggi *et al.* (1993)	85[a]	83	99	
Feggi *et al.* (1993)	50[b]	62	93	
Eary *et al.* (1989)	27	81	100	
Cerny *et al.* (1987)	25	59		
Pyrhönen *et al.* (1990)	23	68	100	
Murray *et al.* (1987)	21	60		
Lamki *et al.* (1990)	20	79		
Blend *et al.* (1992)	12	91		
Larson *et al.* (1983)	6	80	100	

Table 1.8.4 Summary of results from studies on radioimmunodetection of cutaneous melanoma. The performance characteristics of lesion detection.
[a] Follow-up patients, [b] pre-operative patients

Although RIS for melanoma appears attractive, its routine use has not yet become established because it has not yet been refined enough to be superior to existing imaging techniques such as PET. A wide variation in overall sensitivity for melanoma metastases has been reported as shown in the table above. Investigations have mainly focussed on two antigens, HMW-MAA (48 studies) and p97 (10 studies). Sensitivity appears to be largely independent of the antigen studied.

1.8.5 Limitations of RIS with monoclonal antibodies

One of the main limitations to the routine use of RIS for metastatic melanoma is non-specific accumulation of the whole monoclonal antibody (IgG) in normal organs. Binding of IgG to Fc receptors present on normal tissues is a factor resulting in non-specific accumulation (Herlyn *et al.,* 1983; Buchegger *et al.,* 1986; Herlyn *et al.,* 1986). The Fc receptors are naturally present on macrophages (especially in the lungs, kidneys, and liver) as well as other cells involved in antibody metabolism and clearance (Carone *et al.,* 1979; Mizoguchi, 1979; Sancho, 1984). This results in a poorer contrast and reduces the efficacy of the technique. Therefore, the detection of metastases in tissues with high non-specific accumulation is hampered (Siccardi *et al.,* 1990). Non-specific accumulation would also result in toxicity to tissues such as the kidneys, should a toxic agent be attached to the antibody for therapy.

In addition, the relatively large size of whole monoclonal antibodies also leads to long circulatory half-lives and prolonged antibody retention in the blood (Adams, 1998c). The large size of the Mabs molecules can hinder their circulation into extra-vascular tumour sites and results in poor tumour penetration (Juweid *et al.,* 1992). Clearance from normal tissues is slower, especially in blood-rich organs such as the liver, spleen, or kidneys, reducing the contrast obtained between tumour and normal tissue (Yokota *et al.,* 1992). Therefore, there is a considerable delay in reaching high tumour to normal tissue ratios of antibody concentration. Reasonable contrast would take 6-7 days to develop (Siccardi *et al.,* 1990) and is thus incompatible with the use of ^{99m}Tc-labelling, with a half-life of 6 hours.

Monoclonal antibodies of murine origin elicit immune responses in human subjects (Rosen *et al.,* 1987; Schulz *et al.,* 1988; Van Kroonenburgh *et al.,* 1988). Multiple administrations of murine Mab can result in the development of human-anti-mouse antibodies (HAMA) in > 90% of patients. This results in the formation and deposition of immune complexes in normal tissues (lung, liver, spleen, kidney) (Torres, 1993). The HAMA response frequently results in the accelerated clearance of Mabs from the blood (Dillman, 1994). This hampers efficient tumour localisation and limits the number of doses that can be administered. Antibody fragments are less immunogenic (Reynold *et al.,* 1989) and the production of humanised Mabs and antibody fragments should eliminate this problematic HAMA response characteristic of murine antibodies (Clackson *et al.,* 1991; Winter *et al.,* 1994). Antibody fragments have therefore formed the focal point for improving the tumour to normal tissue contrast in melanoma antibody localisation studies (Kang *et al.,* 1999, Kang *et al.,* 2000; Pacifico *et al.,* 2003).

1.8.6 Antibody fragments

Antibody fragments similar to those generated by proteolytic cleavage can be generated from shortened versions of the heavy and light chains. The modified genes can then be expressed in bacteria (Skerra & Pluckthün, 1988) to produce functional Fv or Fab, which is then easily purified. ScFvs are molecules in which the heavy and light chain V domains are contiguously linked by a short peptide between the C-terminus of one V-domain of the Fv fragment and the N-terminus of the other that does not seriously affect antigen binding (Figure 1.8.6). The antibody can then be expressed as a single protein from a single gene rather than as two chains that must subsequently associate. ScFvs represent the minimal portion of an antibody that can still retain full antigen binding. The scFv is a continuous polypeptide molecule of approximately 28 kDa. The single stranded nature of the peptide facilitates production as recombinant protein and increases stability of the molecule in comparison to isolated Fvs lacking a linker peptide (Glockshuber *et al.,* 1990).

ScFvs have several further advantages over their parent monoclonal antibodies (Colcher *et al.,* 1990, Begent *et al.,* 1996). They have improved tumour penetration, retain specific binding to antigen but have very short half-lives *in vivo* due to their small size (Plückthun *et al.,* 1997; Adams *et al.,* 1998a; Colcher *et al.,* 1999). The short residence time in the circulation and normal tissues results in good tumour to normal tissue ratios, but simultaneously limits the maximum amount of scFv that can accumulate in the tumour.
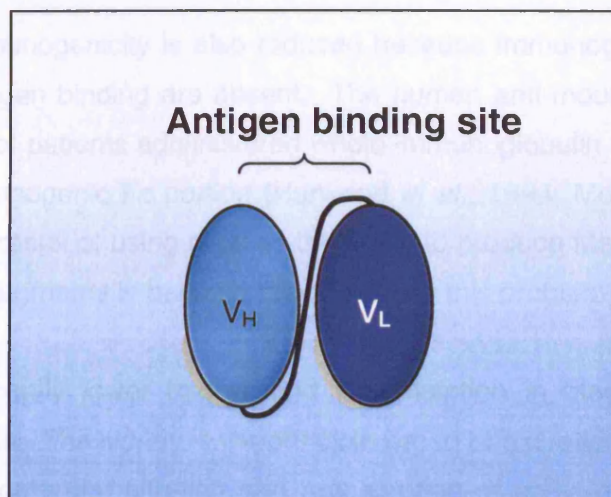
Figure 1.8.6 Diagram of single chain (Fv) antibody fragment, demonstrating linker peptide between the C-terminus of one V-domain and the N-terminus of the other.

1.8.7 Pharmacokinetics of scFvs

Colcher et al. reported the first pharmacokinetic study of radiolabelled anti-tumour scFv in a tumour bearing immunodeficient mouse model (Colcher *et al.*, 1990). They showed that the rapid elimination of scFv from the circulation followed a biexponential clearance curve. A number of other investigators have since confirmed this observation (Adams *et al.*, 1993; King *et al.*, 1994). The rapid elimination of scFv from the circulation facilitates high contrast imaging of radiolabelled scFv that remains bound to the target of choice.

1.8.8 Antibody Fragments in Immunoscintigraphy

Reduction in background accumulation and improved tumour targeting has been achieved using scFvs (Colcher *et al.*, 1990; Colcher *et al.*, 1998). The lower molecular weight of scFvs allows rapid plasma clearance and improved tumour penetration, resulting in higher tumour-to-normal tissue ratios at early time points *in vivo* (Begent *et al.*, 1996). The reasons for the faster dynamics are not clearly understood but most probably relate both to the smaller physical size of the molecule and to the fact that they lack the Fc portion of the antibody, which promotes uptake and metabolism by phagocytic cells (Holliger *et al.*, 1997).

The problem of immunogenicity is also reduced because immunogenic protein domains that are not required for antigen binding are absent. The human anti-mouse antibody (HAMA) response seen in 50% - 90% of patients administered whole immunoglobulin is reduced as these molecules lack the highly immunogenic Fc portion (Harwood *et al.*, 1994; Moffat *et al.*, 1996). This HAMA response is a direct result of using mice as the hosts to produce Mabs. It has been suggested that production of scFv fragments in bacteria may eliminate this problem altogether.

ScFvs show significantly lower background accumulation in blood-rich organs but high renal accumulation remains. The kidney is the principle organ of excretion, and the kidney accumulation is consistent with glomerular filtration and reabsorption of scFv in the renal tubules, which is a common problem with proteins of similar molecular size (Takakura *et al.*, 1990). Renal uptake does not appear to interfere with tumour imaging in patients but would limit the capability of detecting small tumours near the kidney (Begent *et al.*, 1996).

In terms of overall toxicity, scFvs are well tolerated in patients after injection. In a clinical trial using ^{123}I-labelled scFv antibody to carcinoembryonic antigen (CEA) no serious side effects were seen (Begent *et al.,* 1996). One out of nine patients suffered hot flushes starting six hours after the injection and lasting one hour. No adverse effects were observed when using the Common Toxicity Criteria (Operation Manual, CRC 1986). In particular there was no evidence of toxicity to the kidneys, liver or bone marrow, which were the tissues exposed to the highest doses of radioactivity. Other patient trials have supported this evidence (Mayer *et al.,* 2000). This contrasts with Mab therapies such as Mylotarg, associated with relatively high incidences of myelosuppression, hyperbilirubinaemia and elevated hepatic transaminases (Sievers *et al.,* 2001a). A post-infusion syndrome of fevers and chills was observed in two thirds of patients treated with Mylotarg (Sievers *et al.,* 2001b).

1.8.9 Strategies to improve targeting

1.8.9.1 Increasing the dose

Adams *et al.* (1995) studied the intravenous administration of radioiodinated scFv at a range of doses. The scFv bound in a dose-dependent manner, but at 4 and 24 hours both doses gave proportionately similar levels of binding, relative to the amount injected. These results implied that injecting large doses at high concentrations could counteract the rapidity of scFv clearance.

1.8.9.2 Use of Multimers

ScFvs show rapid blood clearance, excellent tumour penetration, possible reduced immunogenicity and higher tumour-to-normal tissue ratios (except in the kidneys). However due to their small size and monovalency, they clear the body too rapidly to allow for sufficient tumour uptake and retention for optimal imaging and therapeutic applications (Larson *et al.,* 1997; Pavlinkova *et al.,* 1999). Multimerisation has been explored as an approach for increasing size and valency, two factors that enhance tumour targeting. Higher molecular weight fragments exhibit extended persistence in the serum, resulting in longer availability for binding to tumour cells (Goel *et al.,* 2001). Increased antibody valency results in an 'avidity' effect, leading to increased retention of antibody by the tumour, thereby improving tumour targeting (Pluckthun *et al.,* 1997).

Recombinant fragments with two or more binding sites have been made in several ways, for example: by chemical cross-linking hinge cysteine residues (Shalaby *et al.,* 1992), by including a

C-terminal peptide that promotes dimerisation (Pack & Pluckthün, 1992) or by varying the linker length (Holliger *et al.*, 1993). Studies have shown increasing the valency of the scFv to improve targeting (Wu *et al.*, 1996) however this is at the expense of increasing the size of the resultant molecule. The scFvs are then expressed as dimers in eukaryotic cells (FitzGerald *et al.*, 1997; Plückthun *et al.*, 1997) or produced as monomer in *E. coli* prior to dimerisation *in vitro* (Glockshuber *et al.*, 1990; Cumber *et al.*, 1992; Kipriyanov *et al.*, 1994). The dimeric molecule is relatively flexible, mimics the avidity of a whole monoclonal antibody yet maintains a relatively small size (~56kDa) (Adams *et al.*, 1993; McCartney *et al.*, 1995).

1.8.9.3 Use of a cocktail of antibodies to different antigenic epitopes

Antibody cocktails have been tested in human melanoma-bearing nude mice and produced a slight increase in the specific tracer localisation in melanoma lesions compared with injection of a single antibody (Matzku *et al.*, 1989). Wahl *et al.* evaluated a lymphoscintigraphic approach by administering a cocktail of two ^{131}I-labelled monoclonal antibodies to melanoma antigens at the site of the primary lesion, or its post-operative scar in 11 patients with stage I or II disease, demonstrating the potential usefulness of the method (Wahl *et al.*, 1990).

1.8.9.4 Tumour targeting Efficiency

A number of strategies have been shown to affect the efficiency of *in vivo* tumour targeting. These include affinity, valency (avidity), k_{off}, and antigen expression levels (Adams *et al.*, 1999). Other parameters that could be equally important include the mouse model (tumour size, site of implantation), route of antibody administration, radiolabelling method, *in vivo* stability of the radiolabel and the antibody itself, or the nature of the target antigen (Adams, 1998c). It is possible that more than one factor could determine the efficiency of *in vivo* tumour targeting in a complex manner. If this were the case then a single, generally applicable strategy for efficient tumour targeting may not exist. The importance of scFv thermal stability for the efficiency of *in vivo* tumour targeting has been demonstrated in a recent study (Willuda *et al.*, 1999). The authors showed a clear advantage in tumour targeting of two thermally stabilised anti-EGP-2 scFvs in comparison with its parent scFv in spite of virtually identical affinities. Several previous studies have considered the effect of affinity on targeting but there is still considerable debate as to the impact of affinity maturation (Adams *et al.*, 1998a; Adams *et al.*, 1999; Schier *et al.*, 1996; Nielsen *et al.*, 2000).

1.9   MELANOMA THERAPY

1.9.1 Traditional therapies

1.9.1.1   Surgery

Surgery has remained the mainstay of treatment for primary melanoma since Hunter's day (Bodenham, 1968; Ross & Balch, 1998). The procedure involves en bloc removal of the entire tumour with a margin of normal skin and subcutaneous tissue. Further wider excision is then carried out depending on the Breslow depth of the tumour (Roberts *et al.*, 2002). Varying the width of excision margins has been shown to effect local disease recurrence however has no impact on survival (Veronesi *et al.*, 1988; Balch *et al.*, 1993; Ng *et al.*, 2001; Lens *et al.*, 2002). Early excision of the primary lesion is the only treatment that is capable of achieving cure (Balch *et al.*, 2001a).

Surgical resection with a generous margin may be carried out for local recurrence. It is the simplest and most effective form of therapy for local recurrence (Karakousis *et al.*, 1998). Lymph node dissection is performed after a positive sentinel lymph node biopsy or when clinically or radiologically detected nodes are found (Balch *et al.*, 1998).

1.9.1.2   Chemotherapy

Melanoma metastases do not respond well to chemotherapies. Trials are continuously underway to seek novel agents or combination drugs that improve both survival and disease-free interval. Dacarbazine (DTIC) is the most active single agent used in patients with metastatic melanoma, inducing a partial response in <20% of patients and a complete response in <5% (Legha *et al.*, 1996). Responses however, are usually short-lived, ranging from 3 to 6 months, although longer-term remission can occur in a limited number of patients who attain a complete response. Multi-drug regimens containing DTIC have produced higher partial response rates (20-45%) and similar complete response rates than other agents, but none have proven superior to DTIC alone (Legha *et al.*, 1989; McClay *et al.*, 1992; Legha *et al.*, 1996).

1.9.1.3   Radiotherapy

Melanoma is a relatively radio-resistant tumour but palliative radiation may alleviate symptoms in some patients. Radiotherapy may also be the treatment of choice for patients who are not surgical candidates, either because of the size or location of the metastases or other physical limitations. Retrospective studies have shown that patients with multiple brain metastases, bone metastases and spinal cord compression may achieve symptom relief and some shrinkage of the tumour with radiation therapy (Rate *et al.*, 1988; Herbert *et al.*, 1991). The most effective dose-fractionation schedule for palliation of melanoma metastasis to bone or spinal cord is unknown, but high-dose-per-fractionation schedules are sometimes used to overcome tumour resistance.

1.9.1.4   Biological Therapies

Work by Kirkwood *et al.* has demonstrated that high dose alfa-2b interferon may improve overall survival in melanoma (Kirkwood *et al.*, 1996, 2001). Other studies have contradicted Kirkwood's results (Cascinelli *et al.*, 2001), even when combining interferon with chemotherapy (Proebstle *et al.*, 1998). Unfortunately interferon treatment is associated with significant side effects and a considerable proportion of patients who are started on the treatment withdraw for this reason (Kirkwood *et al.*, 2001; Cascinelli *et al.*, 2001).

1.9.2 Experimental Therapies

1.9.2.1   Vaccines

The poor response to chemotherapy and the toxicities associated with biologic therapy have directed treatment towards forms of immunotherapy with fewer side effects. Melanoma is the most immunogenic solid tumour making it an ideal target for specific immunotherapy (Herlyn *et al.*, 1988). Laboratory studies show that blood from melanoma patients contains antibodies against tumour antigens (Morton *et al.*, 1968; Morton *et al.*, 1970) as well as cytotoxic T-lymphocytes that can destroy melanoma cells *in vitro* (Mukherji *et al.*, 1990). Between 3-15% of all cutaneous melanoma are first diagnosed as lymphatic or visceral metastases without evidence of a primary tumour, suggesting that the immune system may have induced complete regression of the primary lesion (Morton *et al.*, 1991).

Experience with vaccines in melanoma is limited to early clinical trials. Several phase II trials have shown enhanced disease-free and overall survival of patients who develop a humoral and/or cellular response to a melanoma vaccine but this has not been shown for phase III trials (Ollila *et al.*, 1998b). Two randomised immunotherapy trials with tumour cell-based vaccines failed to show a significant impact on disease-free survival (DFS) or overall survival (OS) (Eggermont *et al.*, 2000). A trial using GM-CSF in stage II and III melanoma patients demonstrated a prolonged disease-free survival compared with historical matched controls (Spitler *et al.*, 2000). Only one patient experienced side effects and that was at the injection site, suggesting this as a safe therapy suitable for further evaluation in a prospective randomised controlled clinical trial.

Early results combining cytoreductive surgery with CancerVax vaccine (against melanoma) appear to show a survival benefit when compared to patients undergoing surgery alone or surgery plus alternative adjuvant therapy (Morton *et al.*, 1993b; Jones *et al.*, 1996; Morton *et al.*, 1996; Hsueh *et al.*, 1998). Resection of visible tumour masses is required to remove the cells producing immunosuppressive factors, thereby allowing the vaccine to be effective. This suggests that for treatment of solid neoplasms a combined modality approach to metastatic disease may be the best option. However, detection of micrometastases for vaccine therapy holds the most promise.

1.9.2.2 Monoclonal Antibodies and Antibody Fragments in Melanoma Therapy

Antibodies can selectively target tumour cells in patients, and a number of Mabs are currently tested in advanced clinical trials (Scott *et al.*, 2001; Phan *et al.*, 2003; Bettinotti *et al.*, 2003), whilst some have been approved for use in cancer therapy (Anderson *et al.*, 1997). Dillman *et al.* administered anti-melanoma antibodies producing regression: four of the twenty-one patients treated with the anti-GD3 Mab, R24, had objective responses at a variety of dose levels in a phase I trial (Dillman *et al.*, 1994). Toxicity included pruritis and urticaria. Mittleman *et al.* used antiidiotype antibodies that mirror the HMW-MAA antigen and demonstrated the induction of endogenous human HMW-MAA antibodies and some tumour responses and improved patient survival (Mittleman *et al.*, 1990). In a later study, the development of anti-HMW-MAA immunity preceded the reduction in the size of metastatic lesions suggesting that the anti-HMW-MAA immunity elicited has a beneficial effect on the clinical course of the disease in patients with melanoma (Mittelman *et al.*, 1994)

Single chain Fv antibody fragments have also been used for immunotherapy, often used as bispecific scFvs (dual function scFvs, such that one arm of the scFv recognises the tumour whilst the other arm recognises either an effector cell or a toxic molecule). A number of studies have demonstrated the ability of bispecific fragments to accurate localise to tumours and result in tumour destruction (Holliger *et al.,* 1997).

1.10   AIMS

As outlined previously in this introductory chapter, problems are faced in melanoma throughout the course of the disease: from initial prevention and early diagnosis to the treatment of metastatic disease. Advancements in understanding at any stage of the disease process will be of benefit to melanoma patients. Bearing this in mind, this thesis aims to address several of the dilemmas faced in improving patient outcome in melanoma. It deals with secondary prevention (early detection), estimation of prognosis and early detection of metastases. The thesis is divided into four results chapters. The aims for each are as follows:

- To establish whether an early detection strategy is effective in improving outcome for melanoma patients. Does an early detection clinic lead to better patient survival and disease control?
- To discover novel prognostic markers for the outcome of primary melanoma with the use of a tissue microarray.
- To improve targeting of melanoma xenograft-bearing nude mice using a cocktail of single chain (Fv) antibody fragments.
- To determine the improvement in tumour targeting by using single chain (Fv) antibody fragment multimers.

CHAPTER 2

MATERIALS AND METHODS

2.1 MOLECULAR BIOLOGY TECHNIQUES

Unless otherwise specified, all "standard methods", "established techniques" or "standard formulations" referred to in sections 2.1 and 2.2 were obtained from "Molecular Cloning: A Laboratory Manual" (Sambrook, 1989). Unless otherwise specified, all "standard methods", "established techniques" or "standard formulations" referred to in subsequent sections were obtained from "Antibodies: A Laboratory Manual" (Harlow, 1988).

2.1.1   Bacteria Culture Media

Luria Broth (LB) base (GibcoBRL) was used for all bacterial culture. 25g of broth base was added per litre of distilled H_2O and autoclaved at 15psi, 120°C for >20 minutes before use.

2.1.2   Technique of Bacterial Culture

Transformed bacteria were stored as glycerol stocks at -80°C (500µl bacterial culture with 500µl sterile glycerol (BDH)). All scFvs were isolated from secreted proteins in bacterial supernatants. For bacterial cultures, the glycerol stocks were stab-sampled with a sterile pipette tip. The pipette tip was ejected into a 50ml polypropylene tube containing 10 - 20mls of LB media + 1ml 100µg/ml ampicillin (Boeringer Mannheim) + 10mls 40% D-glucose (LB_{GA}) for large-scale cultures (1.5ml LB_{GA} for small-scale cultures). The inoculated culture was placed overnight in a rotary shaker at 30°C and 250rpm. Temperatures above this resulted in poor bacterial growth and scFv expression.

The following morning the overnight cultures (ONCs) were diluted 1/100 in LB_{GA} and shaken at 250rpm for 6 hours at 30°C to an OD_{600} of approximately 1.5. Large-scale inductions involved a 1l culture produced from 10 - 20ml ONC. For induction of scFv expression, all bacteria were cultured in LB media + 1ml ampicillin (100µg/ml) with 50mls 40% D-glucose + isopropyl-thio-galactoside (IPTG) (LB_{IA}). Optimum conditions for induction of individual scFvs in large and small-scale bacterial culture have been determined empirically (Kang, MD thesis 1997; Gundogdu, unpublished).

At 6-8 hours the cells were spun down (5000 rpm, 18 minutes) and the supernatant discarded. The bacteria were resuspended in 1-3l of LB with 1ml 100µg/ml ampicillin and 100µM IPTG per litre. 2.5l baffled flask(s) each containing 1l broth were used. Cultures were then shaken overnight (16-18 hours) at 30°C and 250 rpm. Further purification was required before subsequent use.

2.2 PROTEIN PURIFICATION TECHNIQUES

2.2.1 Storage of Bacterial Clones

As stated above, all bacterial clones were stored at -80°C as glycerol stocks. 500µl of an overnight bacterial culture was mixed with 500µl of sterile glycerol, vortexed briefly to mix, then stored in a Cryovial^{TM} (Nalgene) at -80°C.

2.2.2 Preparation of Bacterial Supernatants

All the scFvs were purified from bacterial supernatants. After a standard induction (see above), the cultures were centrifuged at 10,000 rpm (Beckman centrifuge, JLA 10-500 rotor) for 27 minutes at 4°C and the supernatant filtered (0.2µm filter − Nalgene). Sodium azide (NaN$_3$ 0.05%), a bacteriostatic agent, was added to the bacterial supernatant. 200µM phenyl-methyl-sulphonyl-fluoride (PMSF) was also added to reduce proteolysis. All supernatants were then concentrated before use (see below).

2.2.3 Protein Concentration

In order to improve the recovery of scFv from the supernatant, ultrafiltration was used to reduce the volume of the supernatants. This was achieved by using a Mini-Ultrasette^{TM} tangential flow device (Filtron) with a 10kDa molecular weight cut-off to prevent loss of scFv through the membrane. The Mini-Ultrasette^{TM} filter was set up and used according to the manufacturer's

instructions. Using this system the bacterial supernatant was concentrated to a volume of around 100mls.

2.2.4　　Dialysis

The supernatant was transferred to a length of dialysis membrane tubing, which was clamped at each end. The dialysis membrane was immersed in 2l PBS overnight at 4°C, to remove small molecular particles from the hypertonic supernatant.

2.2.5　　Biologic LP Platform for Column Chromatography

All liquid chromatography was carried out using a Biologic LP Chromatography Platform™ (Bio-Rad). This equipment included a programmable peristaltic pump and fraction collector, conductivity, UV absorbance meters and an electronic mixer valve for accurate proportioning of reagents. All purifications were carried out at room temperature.

2.2.6 Purification of scFvs by Immobilised Metal-ion Affinity Chromatography (IMAC)

A poly-histidine oligopeptide (6 histidine residues) has a high affinity for certain metal ions including Zn^{2+}, Cu^{2+} and Ni^{2+}. Proteins incorporating a poly-histidine sequence can therefore be affinity purified by selective binding to metal ions immobilised on a solid medium (IMAC). The development of His-trap gels (Amersham Pharmacia Biotech) able to chelate metal ions has enabled the purification of numerous proteins including scFv, in a column system based on this principle (Casey 1995).

Three pre-packed His-trap columns arranged in series were used according to manufacturer's instructions. They were washed with distilled water to remove storage buffer. 1ml of 0.1M $CuSO_4$ (BDH) in distilled H_2O was loaded onto the top column at 1ml/min. The top column was then washed with distilled H_2O to remove any unbound Cu^{2+}. This was then reconnected to the rest of the His-trap columns. 40mls of start buffer was then run through the column.
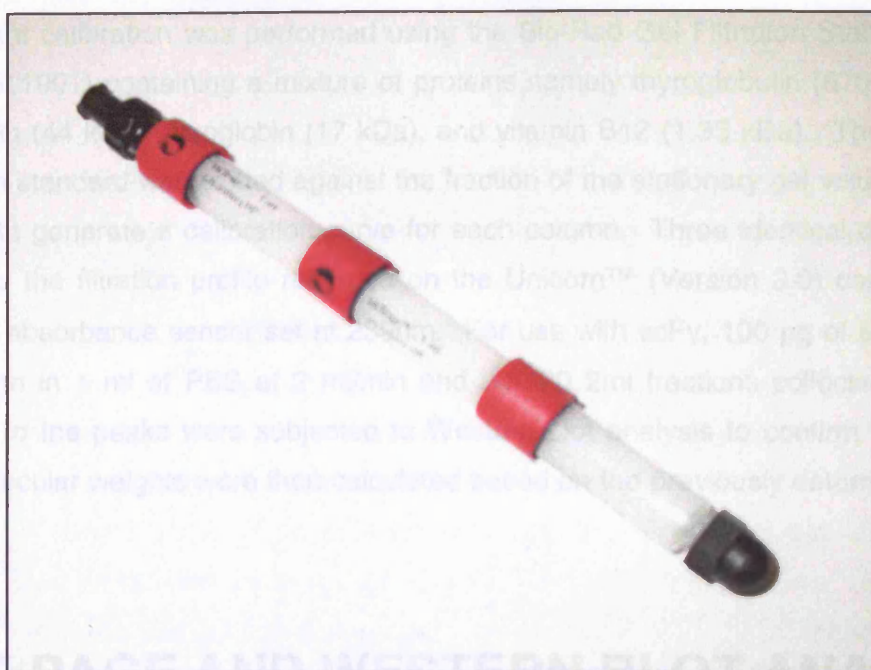
Figure 2.2.6 Three pre-packed His trap columns arranged in series

0.5M sodium chloride and 0.01M imidazole were added to the dialysed and concentrated supernatant in PBS, to reduce non-specific binding of protein to the gel. The solution was then filter sterilised before loading onto the column at 2ml/min. After loading, the column was washed with a further 10ml start buffer to remove any unbound protein. Competitive elution was then carried out with a stepped gradient of imidazole (Sigma Aldrich) in PBS at 2ml/min. 20ml of 30mM, 50mM and 200mM imidazole in PBS (+0.5M NaCl, pH 7.5) was used. Fractions containing scFv, as determined by Western blot were pooled ($\approx$ 20mls), concentrated using a centrifugal concentrator (Vivascience, Germany) to $\approx$ 1ml, and then analysed on SDS-PAGE (see below). To regenerate the column, Cu^{2+} was stripped from the column with 20ml 0.1M EDTA in PBS at 2ml/min. The column was then washed with 40mls distilled H_2O at2ml/min. Following that, the column was washed through with 20% ethanol and stored at 4°C, ready for re-use.

2.2.7   Analysis of scFv by Gel Filtration

The monomeric or multimeric nature of the scFv constructs was investigated using low-pressure gel filtration on the Pharmacia LKB system, the ÄKTA *FPLC*™ system (Amersham Pharmacia Biotech, Uppsala, Sweden). A pre-packed HiPrep™ Superdex 75 XK 16/60 (Amersham Pharmacia

Biotech) was equilibrated using 2 column volumes of PBS as per the manufacturer's instructions. Molecular weight calibration was performed using the Bio-Rad Gel Filtration Standard (Hercules, USA; Cat # 1511901) containing a mixture of proteins namely thyroglobulin (670 kDa), IgG (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B12 (1.35 kDa). The log (molecular weight) of each standard was plotted against the fraction of the stationary gel volume available for diffusion (K_{av}) to generate a calibration curve for each column. Three identical calibrations were carried out and the filtration profile recorded on the Unicorn™ (Version 3.0) computer package linked to a UV absorbance sensor set at 280nm. For use with scFv, 100 μg of scFv was loaded onto the column in 1 ml of PBS at 2 ml/min and 60-120 2ml fractions collected. The sample corresponding to the peaks were subjected to Western blot analysis to confirm the presence of scFv. The molecular weights were then calculated based on the previously determined calibration curve.

2.3 SDS-PAGE AND WESTERN BLOT ANALYSIS

2.3.1   Gels

All protein separation was performed using a 12% or 15% Sodium Dodecyl Sulphate-Polyacrylamide Gel (SDS-PAGE) with a 4% stacking layer, prepared from a 30% acrylamide solution (Boehringer Mannheim) according to standard methods, unless otherwise stated (see table 2.3).

Component	Stacking gel	Separating gel	
	4%	12%	15%
Acrylamide/Bis (30%)	1.3ml	4.0ml	5.0ml
Distilled H_2O	6.1ml	3.4ml	2.4ml
3.0M Tris-HCl, pH 8.8	-	2.5ml	2.5ml
0.5M Tris-HCl, pH 6.8	2.5ml	-	-
10% SDS	100μl	100μl	100μl
Ammonium persulphate (100mg/ml)	50μl	50μl	50μl
TEMED	10 μl	10 μl	10 μl
TOTAL VOLUME	10ml	10ml	10ml

Table 2.3 Formulation of SDS-PAGE Gels

2.3.2   Buffers

All buffers used for Western blot and SDS-PAGE analysis followed standard formulations. All electrophoresis was carried out in 1x concentration electrophoresis *running buffer*, made up from a 5x concentration stock solution (235mM Tris base (3g/l), 192mM glycine (14.4g/l), 0.1% SDS (1.0g/l) and 1l distilled water). Transfer of protein to nitrocellulose for Western blot was achieved in 1x concentration *transfer buffer* (25mM Tris base (3.03g/l), 192mM glycine (14.4g/l), 20% methanol (200ml/l) and 800ml distilled water). *Blocking buffer* for nitrocellulose filters was made from skimmed milk (Marvel^{TM} supplied by Tesco supermarkets) made as 15g of skimmed milk (3%) + 2.5ml of 10% NaN_3 + 50ml of 10x PBS stock + 447.5 ml distilled water. Antibodies used to stain the nitrocellulose were made up in *incubation buffer* composed of 6.6ml of 3% BSA (bovine serum albumin) + 10ml of 10% Tween-20 + 100ml of 10x PBS + 883ml of distilled water. The 5x *sample buffer* was composed of 1.0ml of Tris HCl pH 6.8, 1% glycerol, 2% SDS, 0.05% bromophenol blue with or without a reducing agent (dithiothreitol (DTT) or β-mercaptoethanol).

2.3.3   SDS-PAGE Analysis

The method used for SDS-PAGE analysis of protein was as previously published (Molecular Cloning, A Laboratory Manual, 1989). Protein samples were prepared in 1.5ml microcentrifuge tubes. Volumes of 10 - 20μl of sample were mixed with 7μl of 5x *sample buffer* ± reducing agent and heated to 95°C for 3 minutes. 25μl of each of the samples (total volume usually 27μl) was loaded onto the gel. The Mini-Protean III™ gel electrophoresis system (BioRad) was used for all experiments. Protein separation was achieved by electrophoresis at 250 volts for approximately 30 minutes. A protein stain was prepared according to published methods. Approximately 1g of Coomassie-brilliant blue dye (Pierce) was dissolved in 200ml 10% glacial acetic acid, 45% methanol and 45% dH_2O (20ml: 90ml: 90ml respectively). The gel was allowed to stain for approximately 40 minutes and then de-stained in the same mixture of acetic acid, methanol and water without Coomassie dye, for approximately 24 hours. Kaleidoscope protein standards™ (BioRad) or Precision protein standards™ (BioRad) were used in all experiments to show relative molecular weights unless otherwise indicated (Figure 2.3.3). SDS-PAGE was used to quantify the yield of scFv at all stages of purification. A quantitative assessment of the protein loaded was obtained using standard ovalbumin (Sigma Aldrich) samples. Three standard solutions were made: 1μl, 3μl and 5μl in 20μl distilled H_2O.

Kaleidoscope Marker   **Protein Standards**

kDa				kDa
205		Myosin		250
123		ß-galactosidase		150
				100
79		Bovine serum albumin		75
				50
40.8		Carbonic Anhydrase		37
31.6		Soybean trypsin inhibitor		25
16.9		Lysozyme		15
6.6		Aprotinin		10

A   B

5 µl Kaleidoscope prestained standards run on 4-20% ready gel. Molecular weight (kDa) and protein standards are shown alongside corresponding bands.

Protein Standards showing A) stained and B) unstained markers.

Figure 2.3.3 Protein Standards for Molecular Weight Determination

2.3.4   Western-blot Analysis

Western blot analysis of proteins was carried out according to standard methods. Protein samples and gels were prepared as for SDS-PAGE analysis and electrophoresis was performed using the Mini-Protean III™ system (BioRad) at 250 volts for 30 minutes. However only 1 - 2 µl of sample protein was loaded. Transfer of the separated proteins onto nitrocellulose membrane (Hybond™ Amersham International) was achieved using a semi-dry electroblotting unit (Z34,050-2 Sigma Aldrich). Six pieces of Whatman™ 3MM filter paper measuring 7 x 9cm were soaked in *transfer buffer*. Three pieces of pre-soaked filter paper were placed on the bottom (anode) plate of the blotting unit, followed by the nitrocellulose membrane. The gel was placed onto the nitrocellulose membrane and then a further three pieces of filter paper were placed on top of this. A roller was used to gently ease out any air bubbles from between the papers. The top (cathode) electrode plate was placed on top of the stack, closing the blotting unit. Transfer was achieved at 100V and 200mA for 1 hour.

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Semi-dry blotter used for Western transfer
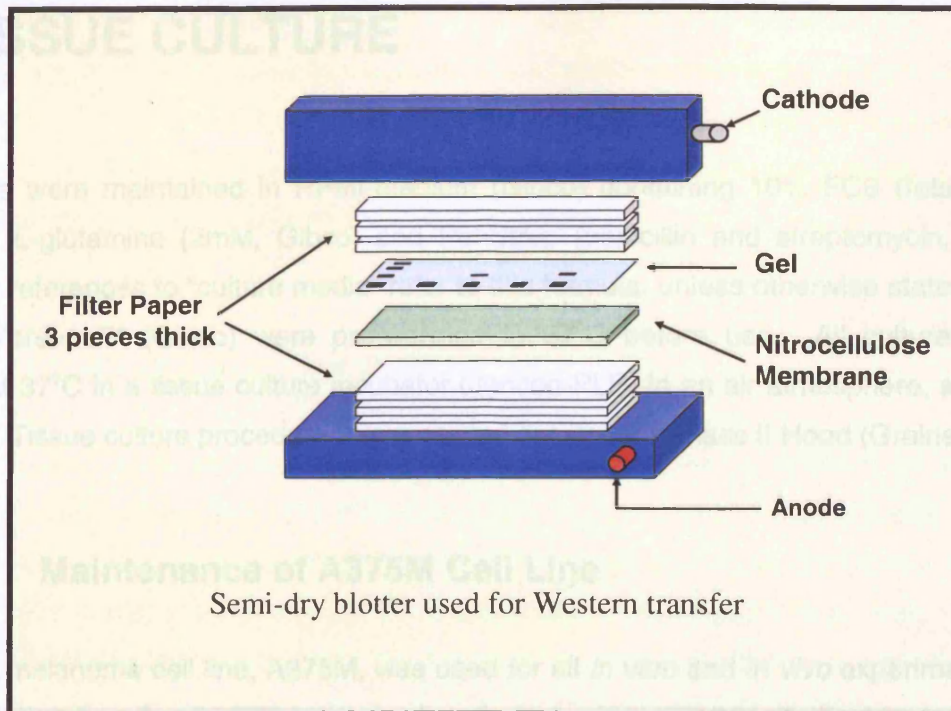
Figure 2.3.4 Semi-Dry Western Blotting Unit (reproduced with permission – Odili, Thesis, 2002)

The nitrocellulose membrane was place in *blocking buffer* with NaN_3 and left on a shaker overnight at room temperature. After blocking, the nitrocellulose membrane was washed briefly with PBS containing 0.5% Tween (PBS-Tween). Detection of all scFvs possessing *c-myc* sequences was achieved with the anti-*c-myc* monoclonal antibody 9E10. Rabbit anti-human kappa light chain™ (Sigma Aldrich) antibody was used to detect kappa light chains and Tetra-His Mouse IgG™ (Quagen) was used to detect the histidine (His-6) tail. The antibodies were applied to the nitrocellulose membrane as a hybridoma supernatant (9E10) or in a 1:1000 dilution in *incubation buffer* for 4-16 hours at room temperature.

The nitrocellulose membrane was washed in PBS-Tween and then rabbit anti-mouse Fc-specific conjugate (Sigma Aldrich) at 1:1000 dilution in *incubation buffer* was applied at room temperature for at least 1 hour. Rabbit anti-mouse Fc-specific conjugate was used for most experiments, unless otherwise stated. The nitrocellulose membrane was washed again in PBS-Tween. The alkaline phosphatase conjugate substrate (BioRad) was prepared from two components, which were applied to the nitrocellulose membrane, and the colour change allowed to proceed. Washing the nitrocellulose membrane in tap water terminated the reaction.

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2.4 TISSUE CULTURE

All cell lines were maintained in RPMI-medium (Gibco) containing 10% FCS (fetal calf serum, Gibco) and L-glutamine (2mM, Gibco) and PenStrep (penicillin and streptomycin, Gibco). All subsequent references to "culture media" refer to this formula, unless otherwise stated. All media, PBS and Versene™ (Gibco) were pre-warmed to 37°C before use. All culture flasks were incubated at 37°C in a tissue culture incubator (Jencon-PLS) in an air atmosphere, supplemented to 5% CO_2. Tissue culture procedures were carried out under a Class II Hood (Greiner).

2.4.1   Maintenance of A375M Cell Line

The human melanoma cell line, A375M, was used for all *in vitro* and *in vivo* experiments involving cultured melanoma cells. A375M is an amelanotic melanoma cell line, derived from a 54-year old patient with metastatic melanoma. The cell line was obtained from the European Catalogue of Human and Animal Cell Culture (ECACC) and grown using standard tissue culture techniques, Briefly, frozen stocks (10^6 cells) kept in cryosuspension in liquid nitrogen, in 1ml dimethyl sulphoxide (Sigma Aldrich)/FCS (1:9) were thawed rapidly in a 37°C water bath, resuspended in 10ml of *culture media* and pelleted at 1000rpm for 5min in a bench-top centrifuge. The pellet was resuspended in 1ml *culture media* and added to a T-25 culture flask (Greiner). At confluence (3-4 days at 37°C), the *culture media* was aspirated off and the cells washed with 10ml PBS. The cells were released by incubation in 1:5000 Versene (Gibco) at 37°C for around 10min. The A375M cells adhere firmly to the flask, and release of the cells usually required a vigorous tap to the side of the flask. An equal amount of *culture media* was added to stop Versene digestion and the cells pelleted at 1000rpm for 5 minutes. When passaging cells, the pellet was resuspended in a small volume of *culture media* prior to seeding T-75 flasks (Greiner) containing 20ml *culture media*. Typically, cells were diluted 1:5 before seeding the flasks. When larger cell numbers were required, the cells were passaged into T-175 flasks (Greiner) containing 40ml *culture media*. Cells were allowed to reach confluence (at 3-4 days) at 37°C.

2.4.2 Cell ELISA plate preparation

Since purified HMW-MAA is not readily available, *in vitro* analysis of scFv-antigen interaction was carried out using cell ELISA plates seeded with A375M melanoma cells. Cells from a single T-75

flask at confluence (approximately 8×10^6 cells) were harvested and resuspended in 50ml *culture media*. This suspension was used to seed ten 96-well flat-bottomed tissue culture plates (Falcon) aliquoting 50μl per well (around 8×10^3 cells). The plates were incubated for 3-4 days ($37^{\circ}C$, 5% CO_2) until 80% confluent. The *culture media* was then aspirated off and the plates dried overnight in a $37^{\circ}C$ incubator, before storage at $4^{\circ}C$.

2.4.3   Technique of Cell ELISA

Cell ELISA plates were typically set up using two-fold dilutions of antibody or scFv in RPMI medium (GibcoBRL) containing 10% FCS and 0.05% sodium azide. Total volumes of 50μl / well were employed. Outer lanes on the plate were not used due to inconsistency in the results obtained in these wells. For scFvs, 90μl 9E10 supernatant was added to each well. 10-50μl of the scFv or antibody was then loaded into the first well of each row, titred out and incubated for 2 hours at room temperature. The wells were washed 3 times with 200μl PBS per well. Between washes the wells were blotted to remove remaining scFv and media. 50μl of a 1:5000 dilution of rabbit anti-mouse horseradish peroxidase (HRP) conjugate (Dako) was added, and after further 1-hour incubation at room temperature, the HRP conjugate was washed off in the same way. Following this wash, all remaining media was aspirated from the wells and 100μl of OPD™ peroxidase substrate (Sigma Aldrich) prepared according to manufacturer's instructions, was added to the wells. The plate was stored in the dark for 5-15 minutes, until a colour reaction occurred which was terminated using 50μl 2M sulphuric acid. All plates were read using a Microplate Reader™ (BioRad).

2.4.4   Maintenance of 9E10 Hybridoma Cell Lines

The 9E10 monoclonal antibody directed against the c-*myc* epitope was prepared as a hybridoma supernatant. The cell line was obtained from the American Tissue Culture Collection (ATCC) and maintained according to established culture techniques. Cells were cultured from frozen stocks by the same technique employed for melanoma cells (see above). Cells were passaged from T-25 to T-75 and then T-175 flasks containing 100ml of culture media. The hybridoma cells were only mildly adherent and could be detached from the flask using gentle tapping without using Versene™. Once in T-175 flasks, the medium was harvested when depleted and replaced with 100ml culture medium and the process repeated. By this technique, many litres of hybridoma

supernatant was be prepared. The harvested supernatant was filter-sterilised (0.2μm filter, Dow-Corning) and 0.05% sodium azide added as a bacteriostatic agent before storage at 4°C.

All 96 wells are loaded with 50 μl 9E10 hybridoma supernatant. ■

Columns
1 2 3 4 5 6 7 8 9 10 11 12
Rows
A B C D E F G H

Wells in first column are loaded with ScFv made up to 50 μl with media □

Columns
1 2 3 4 5 6 7 8 9 10 11 12
Rows
A B C D E F G H

Volume in each well of first column is now 100 μl

Mix gently with multichannel pipette

Columns
1 2 3 4 5 6 7 8 9 10 11 12
Rows
A B C D E F G H

TWO-FOLD DILUTIONS

Take out 50 μl aliquot from each well of first column and add this to the next well in the second column. Repeat the process until column 12 is reached.

INCUBATE AT ROOM TEMPERATURE FOR 1 HOUR

Wash with media (blot at end of each wash).

Load each well with 50 μl of anti-mouse IgG-HRP conjugate (Dako) at 1:1000

RT 1 hour

Wash with media (blot at end of each wash).

Load 100 μl/well OPD peroxidase substrate (Sigma Aldrich)

RT 5-10 mins.

Stop reaction with 2.0 M H₂SO₄
READ PLATE at 490 nm

Figure 2.4.3 Technique of Cell ELISA (reproduced with permission Hamilton, Thesis, 2001)

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2.5 RADIOLABELLING TECHNIQUES

Antibody fragments were iodinated with iodine-125 (Amersham International) using IODO-GEN® (1,3,4,6-tetrachloro-3α, 6α-diphenyl glycouril) pre-coated iodination tubes obtained from Pierce Chemical Company, USA. Radioactivity was monitored using a Mini-Monitor™ (Mini-Instruments) calibrated for gamma radiation. Counts were measured as counts per second (cps) at 30 cm from the source of radioactivity to obtain an estimate of the iodine-125 activity in MBq.

2.5.1 Buffers

Phosphate buffer (pH 7.5) was used for all iodination reactions. To make 100 ml of 1M phosphate buffer, 81 ml of 1 M Na_2PO_4 was mixed with 19 ml of 1 M $NaHPO_4$ and adjusted with NaOH to pH 7.5. IODO-GEN® stop buffer was composed of 2.0 mg/ml D-tyrosine in 0.05 M phosphate buffer pH 7.5 + 10% glycerol (BDH) + 0.1% xylene cyanol (BDH).

2.5.2 Iodination of Antibodies

All reactions were carried out in a dedicated fume hood lined with 3 mm of lead-sheet and a gamma shield (Scot Lab). A lead apron, sleeve protectors (Jencon-PLS), facemask, goggles and double layers of latex gloves were worn for all iodination reactions. Standard safety precautions for safe disposal of all radioactive waste were observed in accordance with regulations for Mount Vernon Hospital, Northwood, Middlesex.

Radiolabelling was carried out using the direct iodination method (Markwell 1978). All reactions were carried out at room temperature. Approximately 50 - 100 μg in 100 μl PBS antibody was placed in a 1.5ml polypropylene Eppendorf tube. ^{125}I (Amersham) as NaI was added immediately to the IODO-GEN® (Pierce) pre-coated tube. In general, 500 μCi of $Na^{125}I$ was used per 100 μg of scFv for radiolabelling. The reaction was allowed to proceed for around 5-10 minutes. 50 μl IODO-GEN® stop buffer was then added to the mixture to stop the reaction. The scFv and $Na^{125}I$ were then transferred to a PD10 column (G-25 Sephadex™ Pharmacia Biotech) to separate iodinated protein from unincorporated iodine (see below).

2.5.3 Separation of Unincorporated Iodine-125

A pre-packed PD10 column (Pharmacia Biotech) containing 10 ml of Sephadex™ G-25 was equilibrated with 100 ml of PBS +1% BSA + NaN₃. Equilibration with 1% BSA was carried out to reduce the amount of non-specific binding of radiolabelled protein. The column was washed with 100 ml PBS and stored at 4°C until use. To separate unincorporated iodine from the iodinated protein, the mixtures from the iodination reactions were loaded on to the PD10 columns. A tension net at the top of each PD10 column prevented the gel from drying out and ensured that the same volume of fluid loaded onto the top of the column dripped out at the bottom. 300 µl aliquots of PBS were used to run the mixture through the PD10 column. A total of 20 fractions were collected from each column and the radioactivity in each fraction measured. Ultrafiltration after radiolabelling (Chapter 6) was performed using VectaSpin Micro™ centrifuge tube filters (Whatman, Maidstone, Kent) to concentrate the radiolabelled scFv to the original volume used previous to radiolabelling.

2.5.4 Estimation of Incorporation of Iodine-125

The fractions collected from the PD10 column with the highest counts (usually fractions 10 – 13) were assumed to contain the radiolabelled protein and were pooled. Counts in these fractions were expressed as a percentage of the total counts in all fractions + the PD10 column. The amount of radioactivity incorporated into the protein sample was assumed to be the same percentage of the total iodine used in the reaction (see individual results chapters for values obtained).

2.6 DETERMINATION OF ANTIBODY AFFINITY

2.6.1 Preparation of Antibody and Human Melanoma Cells

ScFvs were prepared as described in section 2.2. The Mab LHM2 was a gift of Prof. I. Leigh (Queen Mary and Westfield Medical College, London). Dried cell ELISA plates made from the human melanoma cell line A375M were used for all experiments (Section 2.4).

2.6.2   Experimental Technique

Binding affinities were calculated according to the methods of Schodin and Kranz (Schodin, 1993) using a competitive inhibition assay. The method used was based on the binding affinity of LHM2 Mab and the inhibition of LHM2 binding with various concentrations of scFv preparations. The wells of cell ELISA plates were initially loaded with 500 ng of LHM2 IgG (parent mouse monoclonal antibody) per well and serial dilutions of various scFvs were carried out across the plate from 500 to 1 ng per well. LHM2 IgG binding was detected using rabbit anti-mouse IgG-HRP conjugate (Dako), which does not cross-react with scFv. The ELISA was completed as previously described (Section 2.4) and the plate read at 490 nm.

2.6.3   Affinity Determination

The affinity constants were calculated on the basis of the equation described by Cheng (Cheng *et al.*, 1973).

Cheng equation: $K_{d(I)} = [I]_{50\%} / (1 + ([LHM2] / K_{d[LHM2]}))$

$K_{d[scFv]}$: binding affinity of scFv (M)

I : unlabelled inhibitor (such as scFv)

$[I]_{50\%}$: concentration of inhibitor that yields 50% binding inhibition (nM)

[LHM2] : concentration of ^{125}I-labeled Mab (nM)

$K_{d[LHM2]}$: binding affinity of LHM2 Mab ($1.6 \times 10^{-8}M$)

Affinity Curves were constructed by plotting the OD against scFv concentration using Origin™ software (version 4.0, Microcal™).

2.7 ANIMAL MODEL

Localisation of radiolabelled scFv was carried out in athymic mice bearing xenografts of human melanoma. The human melanoma cell line A375M was used for all experiments (Section 2.4). The methods used for implantation were based on the method of McCready (McCready *et al.*, 1989).

2.7.1 Preparation of Tumour Cells

A375M tumour cells were used for all animal experiments. Cells were cultured as described in Section 2.4. Cells were harvested from confluent flasks under sterile conditions at room temperature within 1 hour of inoculation.

2.7.2 Tumour Production in the Mice

Immuno-deficient female Balb/c nu mice were bred from existing animal stocks maintained at the Gray Cancer Institute. The mice were housed in MB1 plastic cages (North Kent Plastics) holding a maximum of 20 mice and fed on a diet of standard expanded pellets (B & K Universal). They were implanted with tumour cells when they reached between 7 to 10 weeks of age. Standard precautions were observed when handling immuno-compromised animals. Hands and forearms were scrubbed with antiseptic agents before touching the animals. All clothing except underwear was removed before entering the animal house and surgical hats, masks, dedicated (clean) overalls, latex gloves and operating boots were worn at all times. A clean operative field was established by laying tissue paper onto the bench-top and spraying this with 70% ethanol. The cell suspension was drawn up into a 1 ml syringe (NHS supplies) and a 23 G needle (Microlance). The mice were anaesthetised in anaesthetic jars containing tissue soaked with Enflurane™. A subcutaneous injection of ≈100 µl of the cell suspension (≈10 million cells) was made into the right flank of each mouse. Palpable tumours were visible as early as 1 week after injection. Tumour take was successful in 80 - 90% of mice. Tumours were allowed to grow until they reached 7 mm +/- 1 mm mean geometric diameter before the mice were used for experiments (Figure 2.7). Mice suffering from abscesses and infection were excluded.
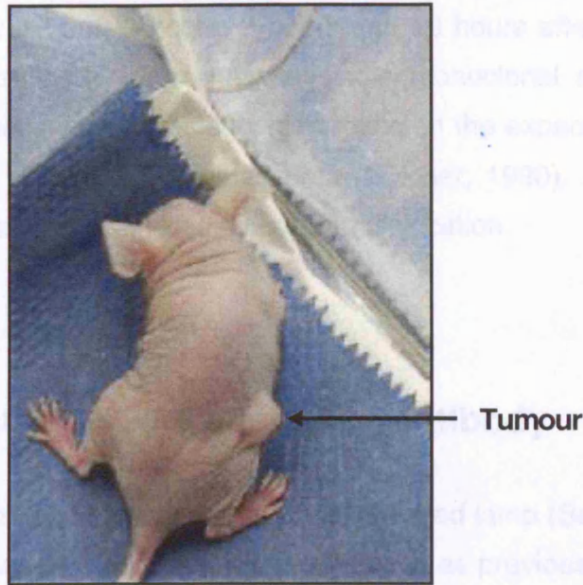
Figure 2.7.2 Balb/c nu Mouse Bearing Human Melanoma Xenograft 2 weeks after subcutaneous injection with A375M melanoma cells into right flank. The tumour measures 7 mm (+/- 1 mm).

2.8 ANTIBODY BIODISTRIBUTION AND PHARMACOKINETICS

2.8.1 Design of Experiments

Freshly radiolabelled antibody fragment was injected by tail vein injection. Each mouse was injected with ≈ 0.5 µg of antibody fragment at an activity of ≈ 3 MBq/µg. Efforts were made to ensure that the absolute amount of radiolabelled antibody used in each experiment was similar. However, there was still some variation due to differences in the specific activity achieved with each iodination reaction. On average, each mouse received between 0.011-0.037 MBq (0.3-1.0 µCi) of radioactivity representing 0.5 µg of antibody fragment. No specific steps were carried out to remove endogenous pyrogen, trace amounts of Cu^{2+} or NaN_3 in the radiolabelled preparations before injection. However, no adverse effects of any kind (directly attributable to the injected solutions) were observed in the mice for the duration of any of the experiments

For localisation experiments, time points were selected which reflected the expected distribution of antibody and antibody fragments based on previously published data (Colcher, 1990). Four time points were selected: 1 hour, 3 hours, 6 hours and 18 hours after injection. This was to enable comparison of this antibody fragment with whole monoclonal antibody. For pharmacokinetic experiments, time points were selected which reflected the expected blood clearance of antibody fragments based on previously published data (Colcher, 1990). At these time points, groups of between 4 and 5 mice were sacrificed by cervical dislocation.

2.8.2   Injection of Radio-labelled Antibody

Mice were warmed for 2 to 3 min under a 250 W infrared lamp (Salamander) at 30 cm to increase tail vein dilation. Enflurane™ anaesthesia was induced as previously described (Section 2.7.2) and tail vein injections carried out using a 26 G needle (Microlance). The mice were returned to their cages after recovering from anaesthesia. In order to ensure reproducibility of the administered dose, all radiolabelled antibodies/antibody fragments were made up to a volume of 100 μl/mouse. After injection, haemostasis of the venepuncture site was achieved by digital pressure applied for 1 - 2 minutes. The mice were returned to their cages and left on tissue paper in the prone position to recover from the anaesthesia.

2.8.3   Sample Collection

At the pre-determined time points, mice were sacrificed by cervical dislocation and tissue specimens obtained immediately. The whole tumour, both kidneys, both lungs, the liver, the spleen, both quadriceps muscles and both femurs were collected. A blood sample was obtained by cardiac puncture. Samples were placed into scintillation tubes (BDH) and then weighed for analysis.

2.8.4   Sample Analysis

Scintillation tubes were loaded onto a gamma counter (CompuGamma CS, LKB Wallace) and activity measured in counts per minute (cpm) over 3 minutes with a window set at 111 to 140keV.

Three calibration samples of the radio-labelled antibody fragment equal to that injected per animal were also loaded to allow calculation of the percentage of the injected dose in each sample (%ID).

After counting, the sample weight was determined and the %ID/g of tissue was calculated:

$$\text{\%ID/g} = \frac{\underline{\text{cpm in sample X 100}}}{\textbf{cpm in calibration sample x sample weight (g)}}$$

Tumour to normal tissue ratios were calculated for each mouse as follows:

$$\text{T:NT} = \frac{\underline{\text{\%ID/g of tumour}}}{\textbf{\%ID/g of normal tissue}}$$

Errors were calculated as standard errors of the mean (SEM) of the measurements obtained from groups of 3 or 5 mice, using the computer program JMP™ (version 3.1.5, SAS Institute).

$$\text{SEM} = \text{SD} / \sqrt{N}$$

N = number of measurements

SD = standard deviation

Curve fitting was carried out using Origin™ software (version 4.0, Microcal™), and enabled calculation of the circulatory half-lives ($t1/2\alpha$ and $t1/2\beta$).

2.9 PIGMENTED LESION CLINIC AUDIT

2.9.1   The pigmented lesion clinic

A weekly PLC was started in January 1993 at Mount Vernon Hospital. Consultants in plastic surgery as well as two to three plastic surgery skin cancer research fellows staffed the weekly clinic. It was run such that general practitioners with a reasonable suspicion of the presence of a melanoma or non-melanoma skin cancer may refer patients. It operated on a "walk-in" basis, with

no appointments made so that patients could turn up to the clinic and as long as they provided their GP's letter of referral, they would be seen. After consultation, patients with truly suspicious lesions were offered immediate excision under local anaesthesia in an operating theatre that ran in parallel with the clinic. Lesions that were not suspicious of being skin cancers, but merited excision, were given appropriate priority on the hospital waiting list. If the patient presented with a benign lesion they could be reassured and discharged. All patients seen had a standard proforma completed, which allowed immediate feedback to the patient's GP regarding the outcome of the consultation. If surgical excision was performed the histology result was forwarded to the G.P. within seven working days.

2.9.2 Audit Design

An audit of all melanoma patients attending the regional melanoma centre, serving both a rural and urban population of 1.6 million, based at Mount Vernon Hospital, between 1991-1996 was performed. Before the establishment of the PLC, referral of such lesions was made by letter from the G.P. to the outpatient department of plastic surgery. Even after introduction of the PLC, many referrals still continued via this route. Patients were split into three groups: "pre-PLC", those diagnosed before the establishment of the PLC (January 1991 – December 1992); "non-PLC" patients, those diagnosed January 1993 – December 1996 via routine, existing outpatient clinics; and "PLC" patients diagnosed via the PLC January 1993 – December 1996. All melanomas diagnosed by the pathology services at Mount Vernon Hospital over the study period were sought on the hospital pathology department computer and from hand-written records and relevant case notes retrieved from the hospital's medical records department. A proportion of patient records were incomplete. Further details were initially obtained via the Mount Vernon Hospital Cancer Registry. Following that, letters were written to GPs also involved in the patients' care. The letters included a questionnaire about the missing details from the patients' notes and a stamped, self-addressed envelope, to ensure a maximum response rate. After 5 weeks, those GPs who had not replied were contacted via the telephone.

A database was created using Microsoft Access™. Patients' demographic details were recorded as well as information on their primary melanomas and recurrences. The end-point for the study was taken to be July 2002. Information about the melanomas included: Breslow depth, Clark's level, site of primary melanoma, subtype of melanoma, information on metastases (both local and regional) and disease-free intervals.

2.9.3 Statistical analysis

Kaplan Meier univariate survival curves were plotted from the database and analysed using the statistical package, JMP©. Log Rank tests were performed on the data, which was then analysed using Chi-squared tests to obtain p values. The level of statistical significance accepted was $p < 0.05$. Graphs were constructed using Origin© and Microsoft Excel©.

2.10   TISSUE ARRAY

Tissue array is a high throughput screening technique that may be used to ascertain patterns of protein expression in a large number of tissue specimens simultaneously. Using this strategy, small tumour biopsies are retrieved from selected regions of archival tissue blocks, and multiple such cylindrical samples are then precisely arranged in a new paraffin block, which may be cut and stained repeatedly.

2.10.1   Components of the tissue arrayer

The Tissue Arrayer™ (Beecher Instruments, Maryland, USA) (Fig 2.10.1) consists of a base plate, receptacle for the array block, a donor block bridge, two thin-walled stainless steel hollow borer needles and turret for switching between the two borer needles, as well as an X-Y precision guide. The X-Y guide moves by turning the knobs on the ends of the steel arms, and the micrometers display either the absolute or relative position of the guide.

2.10.2   Patients

The records of melanoma patients with a minimum 7-year follow up (range 7 – 10 years) were sought from the pathology records of Mount Vernon Hospital. A database of 222 primary melanoma patients was created and relevant paraffin-embedded specimens retrieved from the pathology archives at Mount Vernon Hospital. The database contained information on patient

demographics, melanoma disease course and patient outcome. Details of established clinicopathological predictors of prognosis including Breslow depth, Clark's level, site of melanoma, patient age, patient sex and presence of ulceration were incorporated into the database.
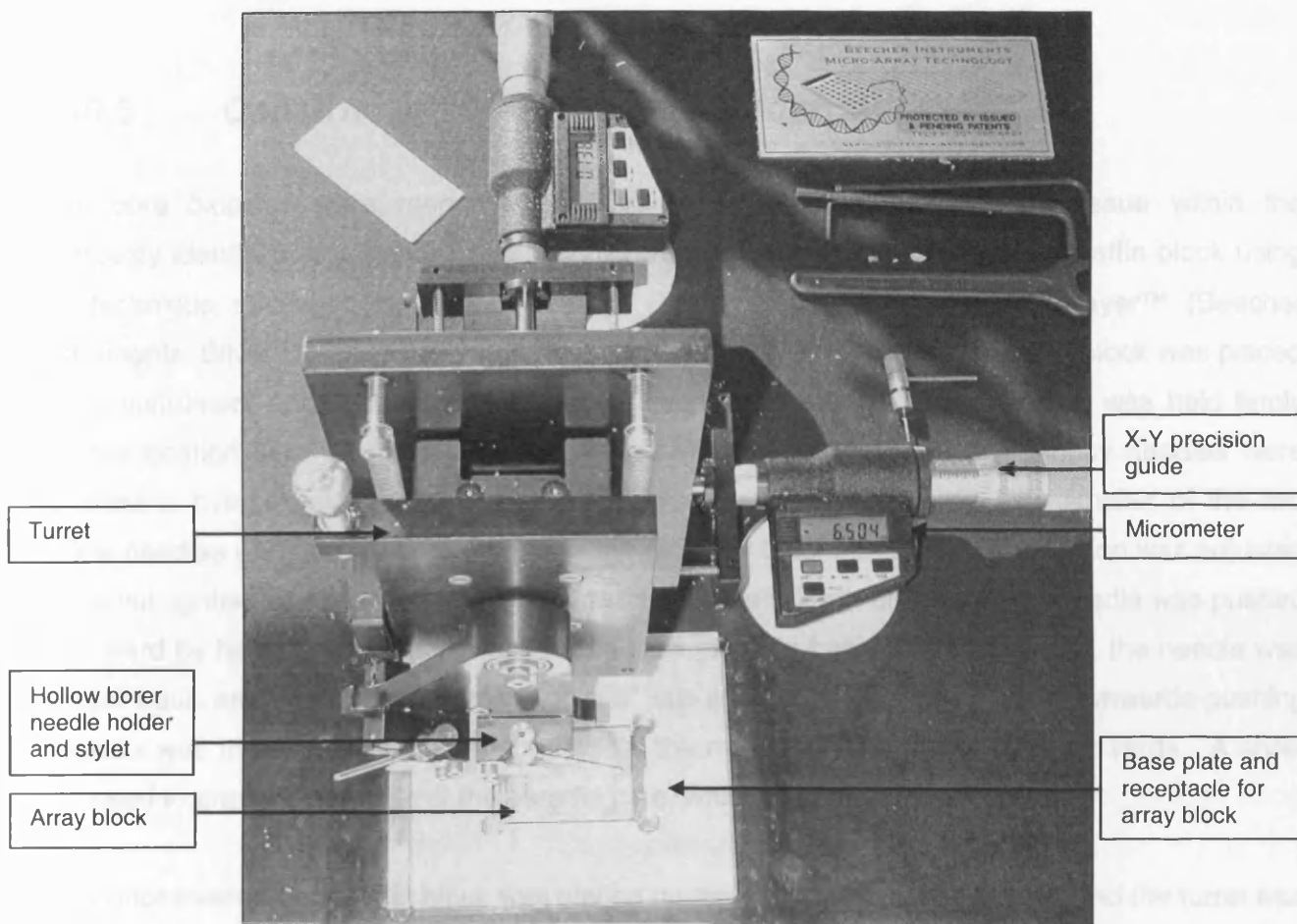
Figure 2.10.1 Tissue microarrayer™

2.10.3   Preparation of donor blocks and slides

All blocks were sectioned and stained with Haematoxylin and Eosin (H&E). A consultant histopathologist reviewed the slides for quality and presence of melanoma. Specimens with insufficient melanoma or of poor quality were not included in the array. Areas of melanoma, distinct from surrounding normal skin, were identified and marked for subsequent sampling.

2.10.4   Preparation of the (recipient) array block

A blank paraffin block was prepared as a recipient for the tissue samples. Regular paraffin was melted and poured over a mould; a block holder was placed on top of the melted paraffin, the complex cooled and the wax removed. Irregularities were cut from the plastic block holders and it was ensured that there were no holes in the paraffin caused by air bubbles during preparation of the block.

2.10.5   Construction of the tissue microarray

Four core biopsies were randomly obtained from the paraffin-embedded tissue within the previously identified and marked area. Cores were transferred to a recipient paraffin block using the technique described by Kononen *et al.* (1998) using a Tissue Microarrayer™ (Beecher Instruments, Silver Spring, Maryland, USA). Briefly, the empty recipient paraffin block was placed in the instrument and secured by tightening attachment screws. This complex was held firmly against location bars by magnets built into the microarrayer. The tissue biopsy needles were adjusted to overlie the start position and the micrometers set at zero. The smaller of the two hollow needles was used to create holes in the recipient block. Initially, a depth stop was adjusted and a nut tightened to stop the needle at a predetermined depth of 4mm. The needle was pushed downward by hand to the required depth and then, using a handle on the needle, the needle was twisted back and forth several times to "drill" out a core of paraffin. The downwards-pushing pressure was then released and springs within the machine pulled the needle upwards. A stylet was used to empty the needle of the paraffin core, which was then discarded.

The donor melanoma paraffin block was placed on top of the array block holder, and the turret was moved to switch the larger needle into a vertical sampling position. The second, wider bore, needle was then moved into position. The H&E slide and the corresponding tissue block were aligned to highlight the previously marked area from which to obtain the biopsy and the slide-block complex was manually held in position on top of the sampling bridge. The slide-block complex was moved under the sampling needle, and the larger bore needle used to retrieve the sample. The H&E slide was removed and the needle pushed down to retrieve the biopsy sample. Once the sample was retrieved, the donor block was removed and the needle pushed down until its tip reached the hole in the recipient array block. Whilst holding this position, the stylet was used to insert the tissue core into the hole created with the smaller needle. The needles were then

advanced using the micrometers, and the process repeated to construct the whole array. Spacing between adjacent samples on the array was 0.8mm, and a sampling needle with a calibre of 0.6mm was used. The four cores from each specimen were arranged adjacently. An Excel file (Microsoft, USA) was simultaneously constructed with the biopsy coordinates during the construction of the array.

Once complete, the array block surface was smoothed and levelled. This was achieved by placing it in a warm chamber (37°C) for 10-15 minutes. This served to warm up the paraffin, promoting adherence of the tissue biopsies to the walls of the holes in the array block, and made the paraffin flexible to handle. After the block was warmed, a glass microscope slide was placed on top of the array block, and even pressure applied, to push all tissue cores in the array to the same level. The block was then ready for sectioning. Five μm thick sections of the paraffin-embedded specimens were mounted on aminopropyl triethoxysilane-coated poly-l-lysine slides, dried for 2 hours at 60°C, and used for staining.

2.11   IMMUNOHISTOCHEMISTRY

2.11.1   Immunohistochemistry of tissue array slides

The master block was sectioned and slides of 5μm thickness produced. Slides were stained using the Dako Envision method (DAKO Ltd., Cambridgeshire, UK). Formalin fixed paraffin embedded sections were dewaxed in xylene for five minutes prior to rehydration through graded alcohols (100, 90 70% industrial methylated spirits) to water. Antigen retrieval was performed on the tissue by placing the slide in a bath of 10mM citric acid or EDTA according to Table 2.11.1 below, in a microwave oven (2450MHz Panasonic microwave, 800W). The volume of fluid was topped up to its original level and the slides were then left to stand for 20 minutes at room temperature before being washed well in running tap water. Slides were then transferred to a Dako Autostainer (DAKO, UK) containing Peroxidase block (DAKO, S2023) with the detection reagents (Chemmate or Envision HRP, DAKO K4006 and K5007) and the relevant antibody added (see Table 2.11.1). The Autostainer programme included 5 minutes in peroxidase block, 1 hour incubation with primary antibody at various dilutions, 30 minute's incubation in Envision polymer and 5 minutes in

Vector Labs SG horse radish peroxidase (HRP) or alkaline phosphate (AP) substrate (Vector Labs, Peterborough, UK SK-4700). When the programme was complete stained slides were removed from the machine and counterstained with Nuclear fast red (Vector Labs UK H3403) for 60 seconds. Slides were then washed in water, dehydrated in graded alcohols (70, 90, 100%), cleared in xylene and mounted in DPX (Surgipath Europe Ltd 08600E). Each staining run incorporated a positive control slide as recommended as a positive control by the primary antibody manufactures (Table 2.11.1). A negative control was also included with the anti-human antibody substituted for an isotype-matched control antibody at the same concentration.

2.11.2   Assessment of staining

The slides were scored by two independent observers without prior knowledge of the clinical outcome for staining positivity and stain intensity. Cores were scored as positive if any degree of antibody staining was evident within the melanoma cells. The tissue array was then scanned in using an Axioscope Transillumination microscope (Zeiss) connected to a 2A3ccD colour camera (JBC, UK) and a semi-quantitative scoring system was constructed for staining intensity based on the variation between the specimens. Specimens were classified as negative, weak, moderate or strong positive staining, as compared with a control section (Table 2.11.1). The stain intensity score was taken as the strongest staining sample from the four biopsies that represented each tumour.

2.11.3   Statistical analysis

Assessment of association between markers and clinicopathological features was carried out using Chi-squared analysis. Univariate analysis was undertaken using the Kaplan Meier method with the JMP® statistical package (Version 4.0, SAS Institute, USA). Log Rank tests were performed on the data, which were then analysed using Chi-squared tests to obtain p values. Cox multivariate analysis was performed using the Statistical Package for Social Sciences (SPSS) (SPSS Inc., Chicago, Illinois, USA). The level of statistical significance accepted was $p < 0.05$.

Antibody	Positive control	Pre-treatment	Source of antibody	Dilution in TBS	Method of detection
AP-2	Breast Carcinoma	3x 5min microwave in 10mM citric acid pH6	Neomarkers	1:50	Envision HRP
β-catenin	OP/PR +ve breast carcinoma	3x 5min microwave in 10mM citric acid pH6	Novacastra, UK	1:100	Envision HRP
Bcl-2	Tonsil	2x 5min microwave in 10mM citric acid pH6	Dako, UK	1:40	Chemmate ALP
CD44v3	Tonsil	3x 5min microwave in 10mM citric acid pH6	Novacastra, UK	1:25	Envision HRP
CEACAM 1	Liver	3x 5min microwave in 10mM citric acid pH6	Novacastra, UK	1:50	Envision HRP
E-Cadherin	Tonsil	3x 4min microwave in 10mM citric acid pH6	Novacastra, UK	1/35	Envision HRP
IGF-1R	Breast carcinoma	5x 4min 1mM EDTA pH8	Neomarkers UK	1/50	Envision HRP
Ki-67	Tonsil	3x 4min microwave in 10mM citric acid pH6	Dako, UK	1/200	Chemmate ALP
MCAM	Appendix	4x 4min microwave in 10mM citric acid	Dako, UK	1/50	Envision HRP
MG50	Breast Carcinoma	3x 4min microwave in 10mM citric acid	University of Southern California, USA	1/1000	Chemmate HRP
NCAM	Neuro-blastoma	3x 4min microwave in 10mM citric acid	Novacastra	1/100	Envision HRP
Nm23	Breast Carcinoma	None	Novacastra	1/250	Envision HRP
P-Cadherin	Skin	3x 4min microwave in 10mM citric acid pH6	Novacastra	1/50	Envision HRP

Table 2.11.1 Antibodies used for antigen retrieval and tissue array staining. Positive control slides were obtained from the Mount Vernon Hospital pathology department and treated and stained in parallel

CHAPTER 3

THE EFFECT OF AN EARLY DETECTION PIGMENTED LESION CLINIC ON MELANOMA PATIENT OUTCOME

3.1 INTRODUCTION

In an attempt to eliminate intra-hospital delays, as well as delays in the bureaucracy of patient referral, pigmented lesion clinics (PLCs) were established across the UK in the 1980s and early 1990s. The aim was to diagnose and excise melanomas while they are thinner, and hence of better prognosis.

3.1.1 Trends in incidence of melanoma

Cutaneous melanoma is among the most rapidly increasing cancers among Caucasians (Ries *et al.*, 2000; Jemal *et al.*, 2001), although the rate of increase in the incidence appears to be declining, except for men over 60 (Severi *et al.*, 2000, Jemal *et al.*, 2001). As the incidence of melanoma has continued to rise, the number of thick lesions has remained relatively stable or increased, and the thin lesions have risen to an even greater extent (Doherty & MacKie, 1986; Burton *et al.*, 1993; Burton & Armstrong, 1994; MacKie *et al.*, 1997; Jemal *et al.*, 2001). There is no consensus as to whether the observed increase in incidence and pattern of incidence is true or artefact and due to other factors. Arguments cited include the following:

3.1.1.1 An increase in the proportion of excised pigmented skin lesions being submitted for histopathological examination

This hypothesis has been addressed in a study by Burton & Armstrong who found that the ratio of total patients referred to two melanoma units to all skin lesions excised remained constant over two years, despite a dramatic increase in the number of patients referred (Burton & Armstrong, 1994). In addition, the ratio of the number of skin lesions diagnosed histopathologically to the total number of patients having skin lesions treated remained constant, even though the number of melanomas diagnosed by the pathologists doubled. Evidence from this study suggests there has been no increase in the proportion of excised pigmented skin lesions being submitted for histopathological examination.

2001). More importantly, mortality has increased each decade (Jemal *et al.*, 2001) and there was no major change in the International Causes of Death code for melanoma to account for its increase. On balance, therefore, it appears that lead-time bias is unlikely to explain more than a modest part of the observed increase in melanoma incidence.

A further argument that supports the above trends reflecting true increases in incidence is the fact that sun exposure, as measured by sun burning and the regular use of solariums, has increased in the past decade (Robinson *et al.*, 1997; Westerdahl *et al.*, 2000); ultraviolet exposure being known to be the most important risk factor to melanoma development (Gilchrest *et al.*, 1999; Jhappan *et al.*, 2003).

Thus it would appear that the observed increasing incidence in melanoma is not a result of artefacts. Melanoma is now the 16th most common cancer in the UK in men and 18th most common in women (CRUK 2001). Using a mathematical model, it was predicted to become the 6th most common cancer in the USA in the year 2001 (Greenlee *et al.*, 2001) – due to lack of a national cancer registry in the USA the actual figures are not known. The lifetime risk of developing a melanoma has been predicted at one in 68 (Rogers *et al.*, 2002) with some authorities predicting a risk of 1 in 20 by the year 2020 (CRUK, 2001).

3.1.2   Melanoma mortality

Overall mortality from melanoma is also rising, however this rise is mainly confined to men (La Vecchia *et al.*, 1999; MacKie *et al.*, 2000,), which would be predicted from the rising number of thick lesions diagnosed in men. Actuarial survival for both sexes analysed by MacKie showed a divergence in survival between the sexes (men faring worse) during the first five years after diagnosis and treatment (5-year survival for women ~82%, men ~78%), this difference being maintained over the next five years (MacKie *et al.*, 1997). They also clearly showed the steady and continuing death rate from melanoma during follow-up over 5-10 years, confirming that survival to 5 years is not a guarantee of cure. A follow-up study by MacKie *et al.* showed the mortality of men from melanoma in Scotland to have been the same in 1998 as it was in 1988 (MacKie *et al.*, 2002). However there was a rise in mortality over 1993 – 1997, the period of interest for this thesis. Mortality in women remained fairly constant, although fell from 1.9 to 1.85 per 100,000 (not significant) and in women younger than 65 years from 1.3 to 1.5/100,000 per year.

3.1.3 Improving outcome

On the background of a rising incidence and mortality, various strategies have been investigated in an attempt to improve outcome in melanoma patients. These have included: varying the width of excision, sentinel lymph node biopsy and adjuvant therapies.

3.1.3.1 Varying the Width of Excision

It has been recommended that a lesion suspected of being a melanoma be excised as a full-thickness skin biopsy to include the whole tumour with a 2-5mm clinical margin of normal skin laterally and with a cuff of subdermal fat (Newton Bishop *et al.*, 2002). This allows confirmation of the diagnosis, such that subsequent definitive treatment can be based on Breslow thickness. The World Health Organisation (WHO) conducted a randomised control trial comparing excision margins of 1cm and 3cm for melanomas up to 2mm thick (Veronesi *et al.*, 1988). No local recurrences were seen in patients with melanomas less than 1mm in depth with either excision margin and therefore a 1cm margin was deemed safe and appropriate for these lesions. Four patients with tumours between 1 and 2mm thick developed recurrences with excision margins of 1cm, leading the authors to be cautious in recommending 1cm margins for this group and therefore suggested 2cm margins may be more appropriate until further follow-up is completed. The Intergroup Melanoma Trial compared 2cm versus 4cm margins of excision for lesions of 1-4mm in depth (Balch *et al.*, 1993). No difference was seen between the two groups in either local recurrence or survival in patients with lesions up to 4mm in depth. No randomised studies have been performed on patients with tumours deeper than 4mm. Despite improvements in local recurrence rates, no published series to date has demonstrated that varying the width of excision has an impact on survival.

3.1.3.2 Sentinel Lymph Node Biopsy

Sentinel lymph node (SLN) biopsy was developed as a means of identifying and excising the lymph node draining the skin in which the melanoma arises (Morton *et al.*, 1992). The procedure is usually carried out at the same time as definitive (wider) excision of the primary tumour. Patients with positive SLN proceed to excision of lymph nodes in the relevant nodal basin. SLN biopsy has been shown to be of importance in the accurate staging and prediction of prognosis of melanoma patients, so much so, that it is now recommended as a requirement to define

homogeneous patient populations for current clinical trials (Balch *et al.*, 2001a & b). Although elective lymph node dissection has never shown an improvement on survival, it may be argued that it would have been difficult to show benefit because only about 20% of patients with intermediate thickness melanomas have sub clinical nodal metastasis. Therefore, 80% could not possibly benefit (McMasters et al., 2001). Furthermore, in lymph node dissections not directed by lymphoscintigraphy, the incorrect nodal basin could be chosen for surgery in up to 32% of cases (Norman *et al.*, 1991), making the benefit of node removal even harder to demonstrate. When only patients who had actual nodal metastases were considered, the 5-year survival rate was 48.2% in patients who had immediate lymph node removal that showed occult metastases, compared with 26.6% in those in whom node dissection was delayed until the appearance of overt regional metastases (Cascinelli *et al.*, 1998). Therefore, the used of SLN biopsy may identify patients who could benefit from lymph node dissection. The final evidence needed that SLN biopsy may have a bearing on outcome awaits the completion of the large prospective randomised control - the Multicentre Selective Lymphadenectomy Trial (MSLT).

3.1.3.3 Adjuvant Therapy

As discussed in the introduction to this thesis, there are no conventional adjuvant therapies of proven benefit for melanoma as yet. Despite the evaluation of many chemotherapy drugs in metastatic melanoma no single agent demonstrates a response rate of greater than 25% (Lotze *et al.*, 2001). Dacarbazine (DTIC) remains the most commonly used single agent and the standard for randomised studies evaluating new combinations (Flaherty & Gadgeel, 2002). Among 1,900 patients evaluated with single agent DTIC treatment, response rates were in the range of 10% to 20% with median durations of response of 4-6 months, median survival of 6-9 months and a 5-year survival of 2% (Middleton *et al.*, 2000). Combination chemotherapy regimes have been unable to reproduce promising response rates from single institution phase II studies in larger multi-institutional studies and none has demonstrated a survival advantage over single agent DTIC (Flaherty & Gadgeel 2002).

3.1.3.4 Biological therapies

Modulation of the immune response by interferon alpha is based on its ability to stimulate the anti-tumour activity of natural killer cells and expression of histocompatability antigens, leading to increased susceptibility of tumour cells to be killed by immune effector cells (Kirkwood *et al.*,

1991). In the USA, following the ECOG 1684 trial the "Kirkwood" high-dose alpha interferon regime is regarded as standard therapy for patients at high risk of recurrence (Kirkwood *et al.,* 1996), although the ECOG 1690 trial was non-confirmatory as to the survival advantage achieved (Kirkwood *et al.,* 2000). Another study comparing one year's treatment with high-dose interferon with a vaccine to gangliosides (anti-GM2) was closed after interim analysis because of apparent better survival of the interferon-treated group (Kirkwood *et al.,* 2001). In metastatic melanoma, interferon alpha, as a single agent produces a response rate of approximately 15% and median response durations in the range of 6 to 9 months (Kirkwood 1991). Although interferon is licensed for use in the UK, it is not yet recommended as standard treatment, on the basis of a second confirmatory trial considered necessary and not least because of the side effects experienced (Roberts *et al.,* 2002).

Apart from high dose alpha interferon (Kirkwood *et al.,* 1996, 2001) none of the above therefore has yet been shown to improve survival in melanoma patients in any prospective randomised controlled trial. However, survival has been improved in other cancers, such as breast and colorectal cancer (Moss *et al.,* 1994; Niv *et al.,* 2002) by early detection strategies. With the knowledge of the growth and natural history of melanoma progression, early detection and treatment should offer great potential for reducing mortality from melanoma.

3.1.4   Growth of melanoma

Melanoma usually arises in the epidermis, where it may be confined for several months. Herlyn *et al.* described its proliferation in the epidermis as radial; hence during this period it is referred to as being in the radial or horizontal growth phase (Herlyn *et al.,* 1987). If uninterrupted by therapy the tumour may progress into a vertical growth phase, whereby it invades the reticular dermis and even the subcutaneous fat (Clark *et al.,* 1969). With the exception of nodular melanoma, which does not show any significant radial growth pattern, all types of melanoma appear to progress through some degree of radial growth before the vertical growth begins. The vertical growth phase usually results in a nodule arising in the relatively flat radial growth phase lesion.

It has been suggested by some authors that the radial growth phase represents the melanoma's capacity for autonomous growth and invasion of the papillary dermis, but not the capacity to metastasise. The vertical growth phase is said to result from a subpopulation of cells that has a growth advantage over the surrounding cells and also possesses the potential to metastasise

(Elder *et al.*, 1984, Clark *et al.*, 1989). This natural history of melanoma would suggest that a thicker tumour has been present for longer than a thin tumour and that consequently any delay in diagnosis would result in a more advanced tumour and hence a poorer prognosis. Thus the earlier the excision, the more likely the lesion is to be in its radial growth phase and excision at this stage should therefore not only improve survival but also has the potential to achieve cure.

3.1.5   Melanoma thickness

It has long been established that patients with thin melanomas have a much improved survival rate than those with thick lesions (Breslow 1970) and this remains the single most important prognostic indicator of primary melanoma (Barnhill *et al.*, 1996; Balch *et al.*, 2001; Ng *et al.*, 2001; Osborne & Hutchinson 2001). In a recent extensive study, Balch and colleagues merged the data from 13 prospective databases containing information on 30,450 melanoma patients with the aim of updating the American Joint Committee on Cancer (AJCC) Melanoma Staging System (Balch *et al.*, 2001a). The data items used from each group's database were those already known to be independent prognostic factors, were generally available from the databases and were relevant to validating the AJCC Melanoma Staging System (Balch *et al.*, 2001a & b). 17,600 patients (58%) had information available for all the factors required, and 13,581 were patients with primary localised melanoma. In a multivariate analysis of the 13,581 patients with localised melanoma, the most powerful independent characteristic of the primary melanoma among all the prognostic variables was tumour thickness (p<0.00001) – the second most important was ulceration. Thin (<1.0mm), non-ulcerated melanoma has a much-improved outcome – the associated 5-year survival probability is better than 95% (Balch *et al.*, 2001a), whereas a patient with a non-ulcerated melanoma over 4mm thick has a 5-year survival chance of 67.4% (see Table 1.1.5.2 in chapter 1).

3.1.6   Diagnostic difficulties

Misdiagnosis of melanoma is inevitable both by GPs and specialists. In an Australian study, the diagnostic accuracy of GPs was found to be around 45% (Paine *et al.*, 1994) and Cassileth *et al.*'s study comparing dermatologists with non-dermatologists found the diagnostic accuracy of the non-dermatologists to be only 38% (Cassileth *et al.*, 1986). Morton and MacKie compared the diagnostic accuracy of consultant, senior registrar and registrar dermatologists (Morton and MacKie 1998). Their diagnostic accuracy was 80%, 62% and 56%, respectively. The number of

false positives was not significantly higher amongst the trainees, indicating that their decreased accuracy could not be explained by them erring on the side of caution. These data demonstrate the improved diagnostic accuracy of specialists over general practitioners. The establishment of a pigmented lesion clinic staffed by specialists therefore provides an easy-access specialist diagnostic service, as well as a rapid-referral treatment service.

3.1.7   Diagnostic delays

Despite the unique occurrence of melanoma in often easily visible sites, delay on the part of the patient in presenting a suspicious lesion to a doctor has been shown to make a major contribution to delay in diagnosis. Delays by patients with cancer symptoms are most often due to lack of knowledge about the seriousness of the condition (Temoshok *et al.*, 1984; Doherty & MacKie 1986; Cassileth *et al.*, 1988; Rampen *et al.*, 1989), anatomic site (the upper and lower back) and patient denial (Temoshok *et al.*, 1984). The fact that patient lack of knowledge about the condition has been found to result in delays in seeking medical attention has led some to propose that a pigmented lesion clinic will not be effective in improving survival, unless it is accompanied be a publicity campaign (The Southampton Melanoma Group 1986; Severi *et al.*, 2000). Temoshok and colleagues demonstrated a statistically significant positive correlation between time delay and tumour thickness (Temoshok *et al.*, 1984), however their results were contradicted by a more recent study (Richard *et al.*, 1999), which found a negative correlation between tumour thickness and the delay to seek medical attention. On examination of the data, the authors discovered that the negative correlation achieved was the result of a few patients with the most aggressive tumours presenting early and when the analysis was repeated without these, the delays incurred by the patients did have an impact on the rest of the melanoma patient study population.

Diagnostic difficulties and delays in patients seeking medical attention are often compounded by delays due to the bureaucracy of referral to hospital and further delays within the hospital healthcare system. In normal practice, GP letters of referral are reviewed by the consultant to whom they were referred, and then the patients are prioritised according to the content of the letter. Thus, if the GP suggests the possibility of a diagnosis of melanoma, the patient will be given an urgent appointment. If, however, it is not clear that a melanoma is suspected, or, of course, that the GP has diagnosed another lesion altogether, a lower priority will be given (Ibbotson *et al.*, 1995). Ibbotson has shown that this to leads to severe delays resulting in patients

with previously unsuspected melanomas presenting with thicker tumours. Hence further potential delays may occur even after initial GP referral.

3.1.8   Previous work

A previous audit into the PLC at Mount Vernon (Grover *et al.*, 1996) had demonstrated a reduction in the mean thickness of melanoma in patients presenting to the PLC when compared with both melanomas diagnosed before the establishment of the PLC, and with melanomas diagnosed via existing routes of referral in parallel with the PLC. However, the impact of the PLC on patient outcome, in terms of survival and disease-free interval, was not measured.

3.2   AIMS

This study aimed to investigate the effect of a pigmented lesion clinic on survival in melanoma patients. The outcome would be compared with 2 groups: those diagnosed with melanoma before the establishment of the PLC ("pre-PLC") and those diagnosed via normal routes of referral ("non-PLC"). As well as survival, other measures were evaluated, including differences in tumour depth, length to both local and regional recurrence, and disease control.

3.3   METHODS

A weekly PLC was started in January 1993 at Mount Vernon Hospital in an attempt to overcome the delays in diagnosis and treatment of melanoma. Consultants in plastic surgery, as well as two to three plastic surgery trainees staffed the weekly clinic. General practitioners with a reasonable suspicion of melanoma or non-melanoma skin cancer were able to refer patients to the PLC. It was run on a "walk-in" basis, with no appointments made. As long as patients had a GP letter of referral, they are eligible to attend the clinic. After consultation, patients with truly suspicious lesions were offered immediate excision under local anaesthesia in an operating theatre running in parallel with the clinic. Lesions that were not suspicious of being melanoma, but merited excision, were given appropriate priority on the hospital waiting list. If the patients presented with benign lesions they were reassured and discharged.

An audit of all melanoma patients attending the regional melanoma centre, serving both a rural and urban population of 1.6 million, based at Mount Vernon Hospital, between 1991-1996 was performed. Before the establishment of the PLC, referrals of such lesions were made by letter from the GP to the outpatient department of plastic surgery. Even after introduction of the PLC many referrals still continued via this route. Patients were split into three groups: "pre-PLC", those diagnosed before the establishment of the PLC (January 1991 – December 1992); "non-PLC" patients, those diagnosed January 1993 – December 1996 via routine, existing outpatient clinics; and "PLC" patients diagnosed via the PLC January 1993 – December 1996. All melanomas diagnosed by the pathology services at Mount Vernon Hospital over the study period were sought on the hospital pathology department computer and from hand-written records and relevant casenotes were retrieved from the hospital's medical records department. A proportion of patient records were incomplete. Further details were initially obtained via the Mount Vernon Hospital Cancer Registry. Following that, letters were written to GPs also involved in the patients' care. The letters included a questionnaire about the missing details from the patients' notes and a stamped, self-addressed envelope, to ensure a maximum response rate. After 5 weeks, those GPs who had not replied were contacted via the telephone.

A database was created using Microsoft Access™. Patients' demographic details were recorded including patient sex, patient age at diagnosis and route of referral. Details of the primary tumour that were recorded were: subtype of tumour, site of primary tumour, Breslow depth, Clark's level, tumour ("T") classification (see section 1.1.5) and presence of ulceration. Metastatic tumour details that were recorded were: site of recurrence (local or regional), disease-free interval and extent of nodal involvement. In addition, date and cause of death were recorded and the time to

outcome could therefore be calculated. The end-point of the study for those patients still alive was taken to be July 2002.

A significant number of patient records were incomplete. Further details were initially obtained via the Mount Vernon Cancer Registry. Following that letters were written to GPs also involved in the patients' care. The letters included a questionnaire about the missing details from the patients' notes. A stamped, self-addressed envelope was also included, to ensure a maximum response rate. After 5 weeks, those GPs who had not replied contacted via the telephone. Kaplan Meier univariate survival curves were plotted and analysed using the statistical package, JMP©. Log Rank tests were performed on the data, which were then analysed using Chi-squared tests to obtain p values. The level of statistical significance accepted was p<0.05. Graphs were constructed using Origin© and Microsoft Excel©.

3.4   RESULTS

3.4.1   Patients

A total of 290 melanomas were diagnosed over the study period. One hundred and seventy one patient casenotes were incomplete and letters were sent to the relevant GPs. After both letters and telephone calls, a total of 13 patients' details were incomplete to varying degrees (none to a degree that prohibited further analysis). 96% of patients in the study were still with the same GP practice up to 10 years after initial treatment at the hospital, suggesting a stable population on which the study was performed, within an identical catchment area.

The clinicopathological variables of the patients included are shown in Table 3.4.1.1. Of the 290 melanomas diagnosed in the whole study population (irrespective of route of referral), 62% were diagnosed in women (n=180), 38% in men (n=110) (Figure 3.4.1.1). A total of 4399 patients attended the PLC over the study period (1993-1996), with an average attendance of 28.2 patients per clinic. The melanomas diagnosed accounted for 2.1% of all referrals. 748 non-melanoma skin cancers were treated via the PLC. The distribution of melanoma patients between the three groups is illustrated in Table 3.4.1. Seventy-eight melanomas were diagnosed before the establishment of the PLC ("pre-PLC"), 119 were diagnosed via normal routes of referral once the PLC was established (1993-1996; "non-PLC"), and the remaining 93 melanomas were diagnosed via the PLC (Table 3.1.4.2).

Figure 3.4.1.1 Proportion of male and female melanoma patients over study period

Variable	Results
Number of patients in cohort	290
Mean age at diagnosis	58.14 (range 18-97)
♀: ♂	1.6:1
Site of melanoma (%) [♀: ♂]:	
Head & neck	49 (16.9 %) [1:1.08]
Trunk	70 (24.14 %) [1:1.45]
Upper limb	51 (17.59 %) [1.2:1]
Lower limb	120 (41.38 %) [5:1]
Tumour subtypes represented (%):	
Superficial spreading	157 (54.14 %)
Lentigo maligna melanoma	22 (7.59 %)
Nodular melanoma	95 (32.76 %)
Acral lentiginous	7 (2.41 %)
Other	9 (3.1 %)
Mean Clark's level	3.49 (range 2-5)
Mean tumour thickness	2.56 mm (range 0.1 - 35mm)
Median tumour thickness	1.6 mm
Number of ulcerated melanoma (%)	60 (20.7 %)
Number developing regional recurrence (%)	83 (28.62 %)
Number of patients surviving in cohort (%)	161 (55.5 %)

Table 3.4.1.1 Details of patients and melanomas included in the study.

Route of referral	Number of melanomas diagnosed
Pre-PLC	78
PLC	93
Non-PLC	119
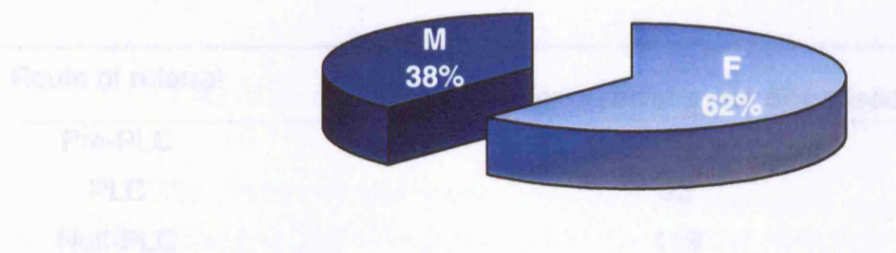
Table 3.4.1.2 Distribution of melanomas within study
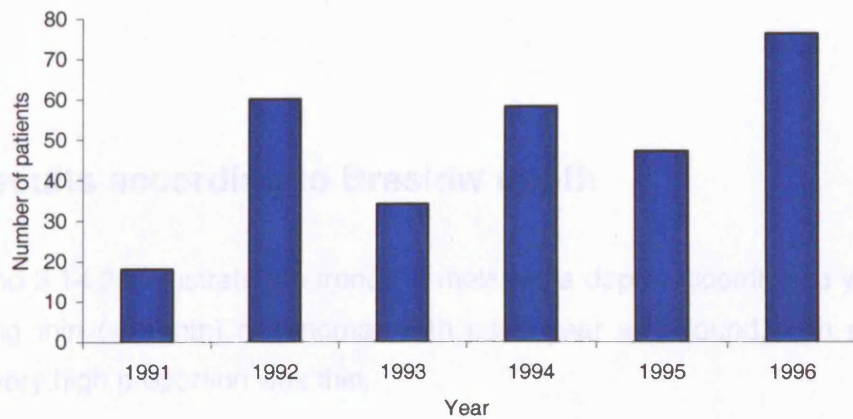
Figure 3.4.1.2 Number of melanomas diagnosed each year of the study period

The number of melanomas diagnosed each year varied, but the general trend was of an increasing incidence (Figure 3.4.1.2). The proportion of melanomas diagnosed via the PLC, compared with parallel routes of referral increased after its establishment, with the highest proportion being diagnosed in 1995 (Figure 3.4.1.3).
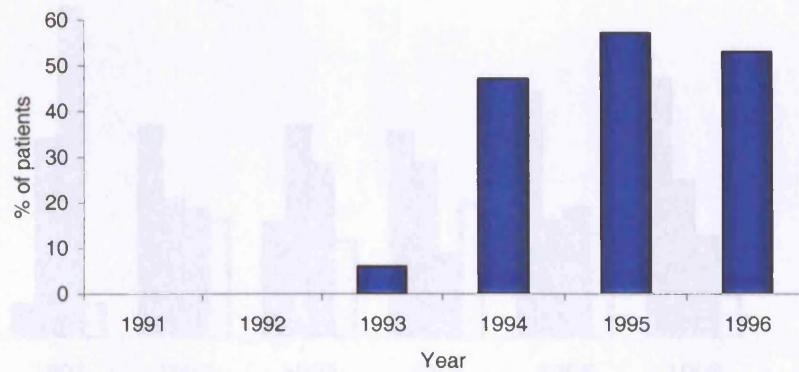
Figure 3.4.1.3 Proportion of melanomas detected via the PLC over the study period. The PLC was established in 1993.

At best, when the GP suspected a diagnosis of melanoma, the time from GP visit to specialist consultation was two weeks. After consultation a further delay of around a week was incurred in arranging the surgery, resulting in a minimum delay of three weeks between GP diagnosis and excision. If there was no suspicion of malignancy by the GP patients waited up to 22 weeks before review by a specialist. This then translated into a delay of 23 weeks until excision. The PLC

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however, conferred a maximum time delay of one week and a minimum of same-day consultation and excision.

3.4.2　　Results according to Breslow depth

Figures 3.4.2.1 and 3.14.2.2 illustrate the trends in melanoma depth according to year of study. A trend of increasing thin (<1.0mm) melanomas with each year was found, with an exception in 1992, in which a very high proportion was thin.

When comparing the percentage of thin melanomas in the pre-PLC group with all melanomas diagnosed between 1993-1996, there were a higher proportion of thin melanomas seen in the latter group. However, this was not statistically significant (x^2 3.76, p=0.2880) (Figure 3.4.2.3).
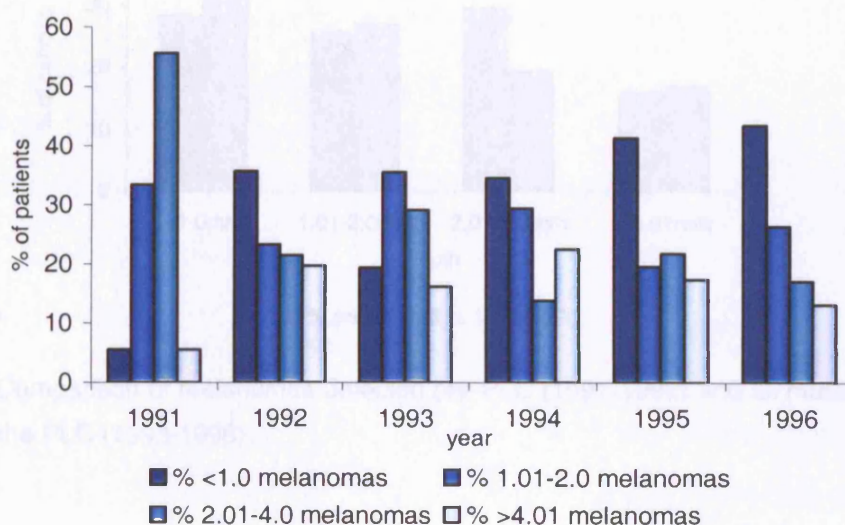
Figure 3.4.2.1 Proportions of melanomas of varying thickness according to year.
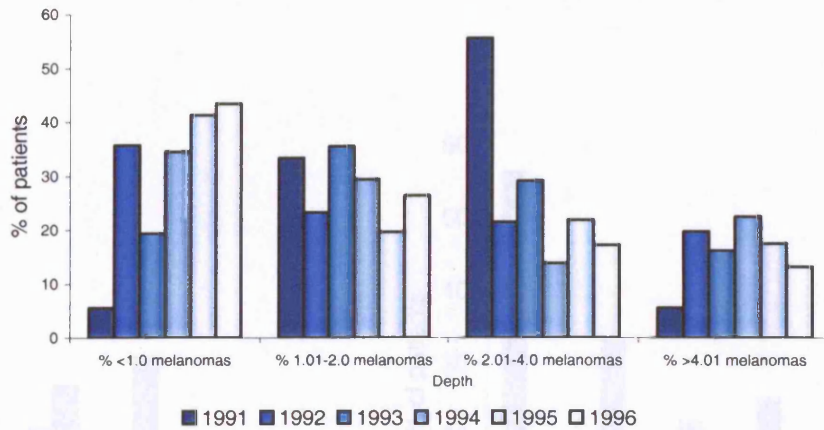
Figure 3.4.2.2 Proportions of melanomas of varying thickness according to year.
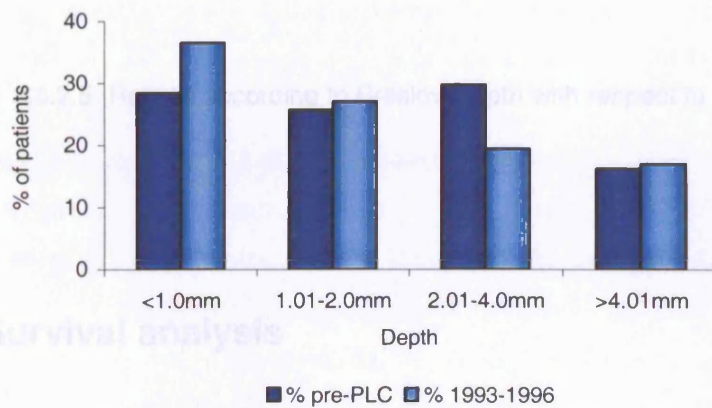
Figure 3.4.2.3 Comparison of melanomas detected pre-PLC (1991-1992) and all melanomas detected after the inception of the PLC (1993-1996)

There was a significant difference in the thickness of melanomas seen in the PLC when compared with both the pre-PLC group and the non-PLC groups (pre-PLC vs. PLC, χ^2 17.23, p=0.00063; non-PLC vs. PLC, χ^2 31.9, p=0.00000085) (Figs. 3.4.2.4 & 3.4.2.5).
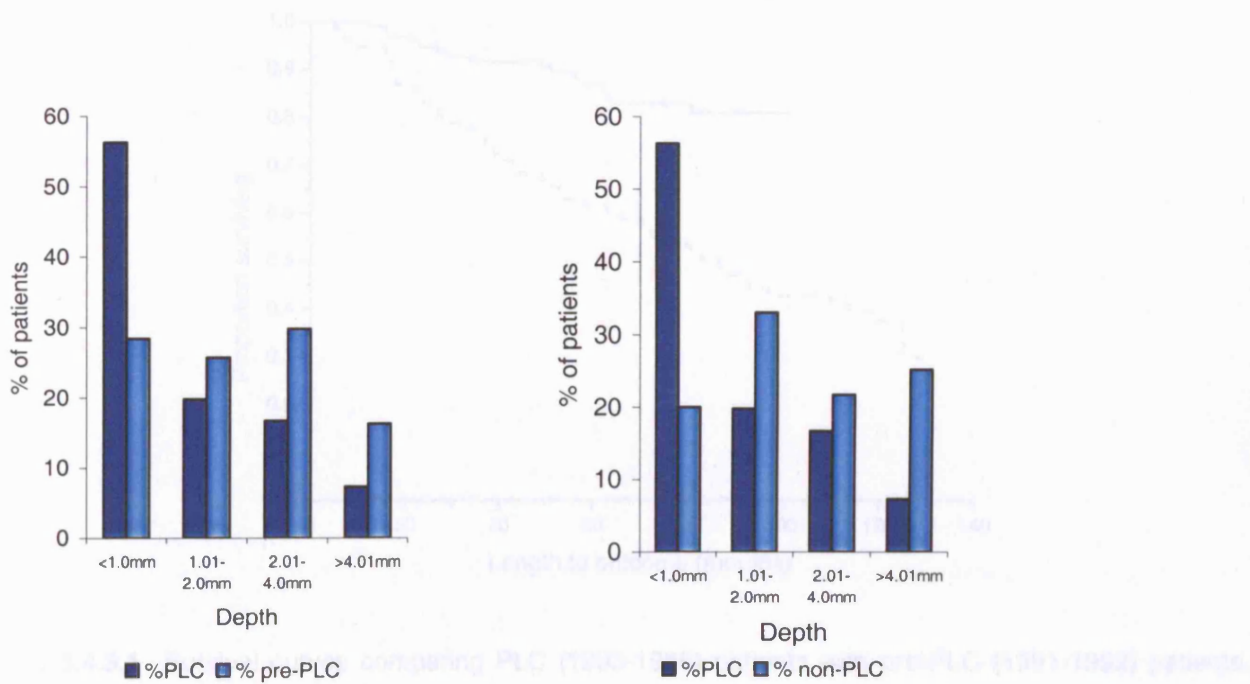
Figures 3.4.2.4 & 3.4.2.5 Results according to Breslow depth with respect to route of referral.

3.4.3   Survival analysis

Survival curves were plotted and Log Rank tests were performed on the data, which was then analysed using Chi-squared tests. A highly significant improvement in survival was shown when comparing pre-PLC with PLC patients (x^2 18.1924, p<0.0001) (Fig. 3.4.3.1). At 5-years post-diagnosis, 62% of pre-PLC patients were alive, compared with 86% of PLC patients.
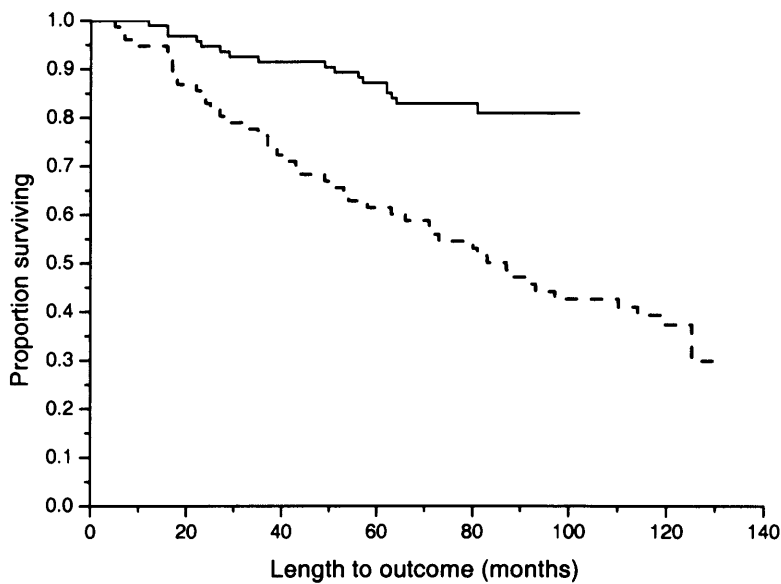
Figure 3.4.3.1 Survival curves comparing PLC (1993-1996) patients with pre-PLC (1991-1992) patients. Log rank χ^2 18.1924, p<0.001. ———— PLC patients, - - - - pre-PLC patients.

It was possible that this was the result of a "creaming-off" effect, whereby the PLC had purely been seeing better prognosis melanomas for reasons discussed in section 3.5.3 below. Taking this potential confounding factor into account, the analysis was repeated, this time comparing the "pre-PLC" group with all those patients diagnosed with melanoma after the inception of the PLC, irrespective of route of referral. Figure 3.4.3.2 illustrates that even when comparing the "pre-PLC" group with all those patients diagnosed with melanoma after the inception of the PLC, irrespective of route of referral, there is still a statistically significant difference in survival (χ^2 4.3577, p=0.0368). This suggests that the previous result cannot simply be explained by a "creaming-off" effect. The 5-year survival for the patients seen after the inception of the PLC, irrespective of route of referral, is 72% compared with the pre-PLC 5-year survival of 62%.
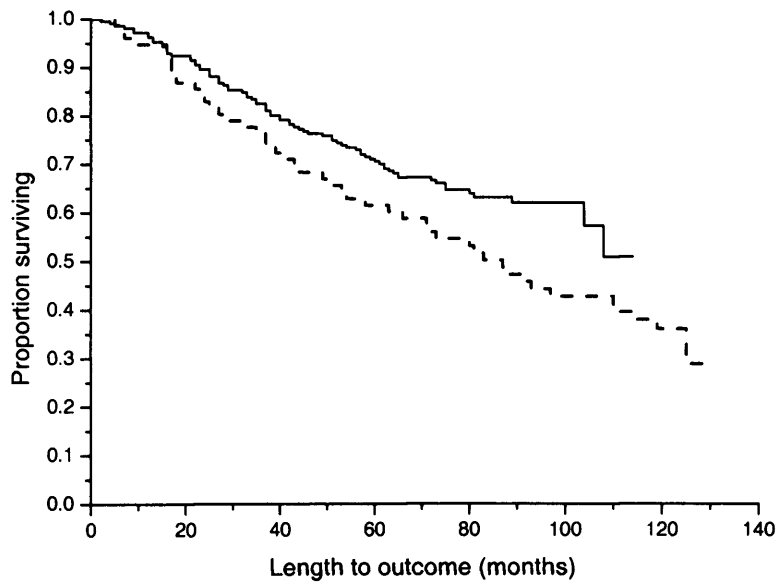
Figure 3.4.3.2 Survival curves comparing all melanoma patients 1993-1996, irrespective of route of referral compared with pre-PLC patients. Log Rank χ^2 4.3577, p = 0.0368   - - - - - pre-PLC patients

All 1993-1996 patients ———
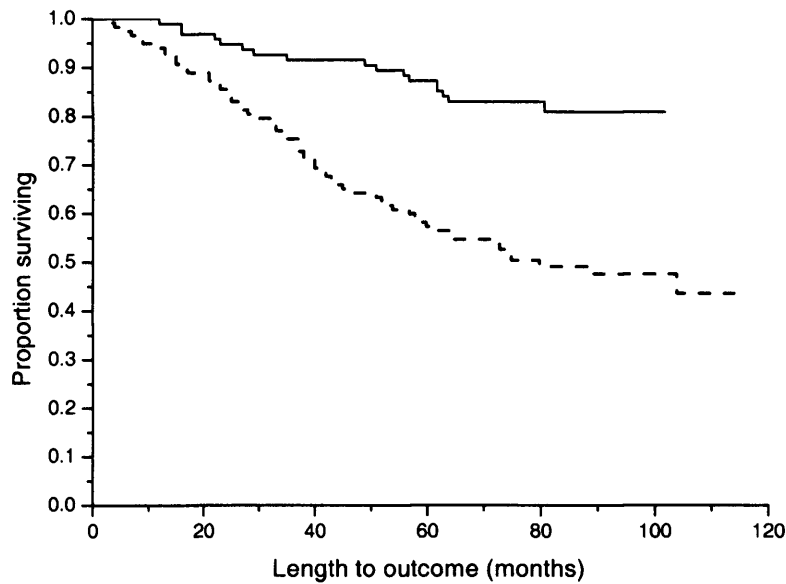
Figure 3.4.3.3 Survival curves comparing PLC and non-PLC melanoma patients (both 1993-1996). Log Rank χ^2 21.4185, p<0.0001.   ——— PLC patients - - - - non-PLC patients

When the survival of non-PLC patients is compared with the survival of PLC patients, the PLC patients still show an improved survival (χ^2 21.4185, p<0.0001) (Fig. 3.4.3.3). The 5-year survival of non-PLC patients is 59%, compared with 86% for the PLC patients. Figure 3.4.3.4 demonstrates that there was no difference in outcome between the pre-PLC and non-PLC patients.
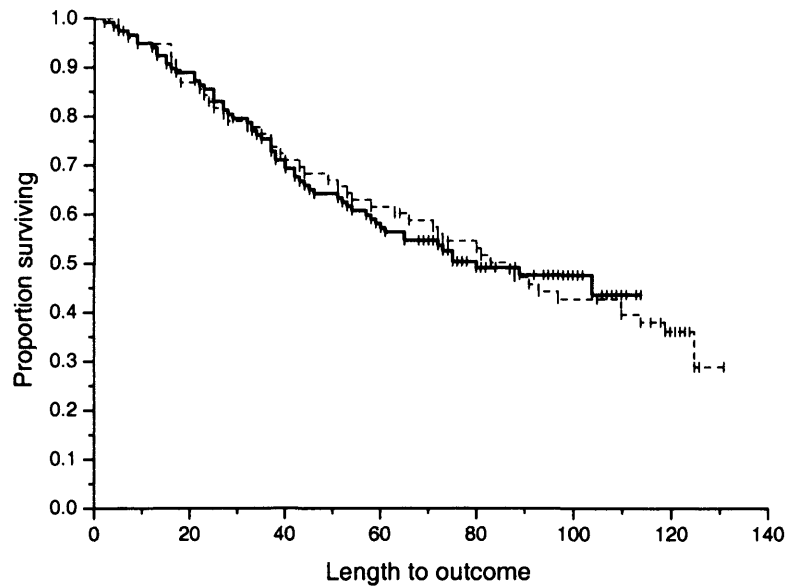
Figure 3.4.3.4 Survival curves comparing pre-PLC and non-PLC melanoma patients. Log Rank χ^2 0.0074, p = 0.9315 ———— non-PLC patients - - - - - pre-PLC patients

3.4.4   Recurrence rates

Both local and regional recurrence rates over the study period are summarised in Table 3.4.4. Patients diagnosed in the PLC developed far fewer regional and local recurrences than those seen either before the establishment of the PLC or in routine outpatients in parallel to it.

Regional recurrence	Number of patients		
	Number	in group	% Recurrence
pre-PLC	29	78	37.18
non-PLC	47	119	39.50
PLC	13	93	13.98
Total	89	290	30.69
1993-1996	60	212	28.30

Local recurrence	Number of patients		
	Number	in group	% Recurrence
pre-PLC	10	78	12.82
non-PLC	23	119	19.33
PLC	6	93	6.45
Total	39	290	13.45
1993-1996	29	212	13.68

Table 3.4.4 Rates of regional and local recurrence.

3.4.5   Regional recurrence rates

Each year there were consistently fewer lymph node recurrences in those patients whose melanomas had been diagnosed via the PLC (Fig. 3.5.1). However this difference did not reach significance (χ^2 5.77, p=0.12), possibly due to the low numbers of patients with recurrences: in 1994 and 1995 there were 9 recurrences in the non-PLC group both years compared with 2 and 3 in the PLC group, respectively. A comparison of the proportion of recurrences between subgroups is shown in Table 3.5.1.
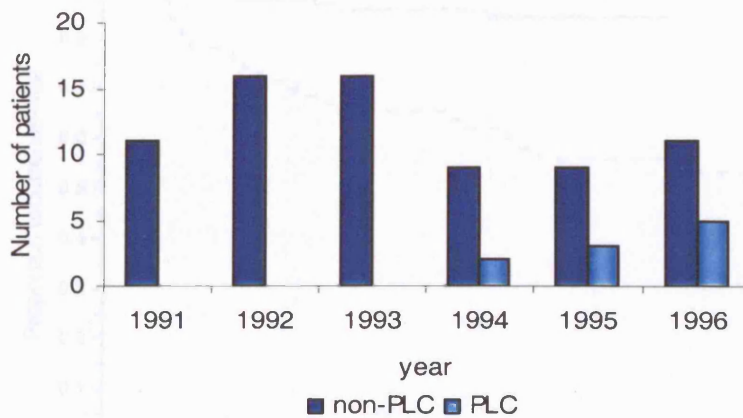
Fig. 3.4.5.1 Number of lymph node recurrences according to route of referral.

An increase in length to lymph node recurrence was observed when comparing those patients diagnosed "pre-PLC" with those diagnosed via the PLC (χ^2 13.8487, p=0.0002) (Fig. 3.4.5.2). In a similar manner to that employed when analysing patients survival, to ensure that this highly statistically significant improvement in regional recurrence rates was not a result of having creamed off the better prognosis melanomas into the PLC group, the analysis was repeated comparing the pre-PLC patients with all melanoma patients diagnosed between 1993-1996, irrespective of route of referral. Again, a statistically significant difference between the groups was observed (χ^2 5.2773, p=0.0216) (Fig. 3.4.5.3). When the length to recurrence of non-PLC patients was compared with the PLC patients, the statistically significant improvement in outcome of the PLC patients was maintained (χ^2 17.0164, p<0.0001) (Fig. 3.4.5.4).

Regional recurrence	Chi-squared	P-value
PLC - pre-PLC	12.32	0.00045
PLC - non-PLC	16.75	0.000043

Table 3.4.5.1 Comparison of rates of recurrence between subgroups

108
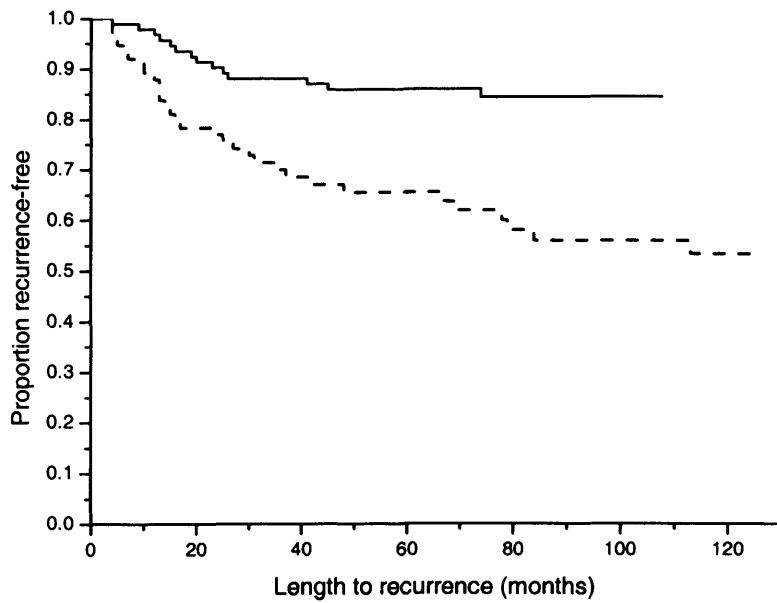
Figure 3.4.5.2 Regional recurrence curves comparing PLC and pre-PLC patients. Log Rank χ^2 13.8487, p = 0.0002 ——— proportion of PLC patients regional recurrence-free - - - - proportion of pre-PLC patients regional recurrence-free.
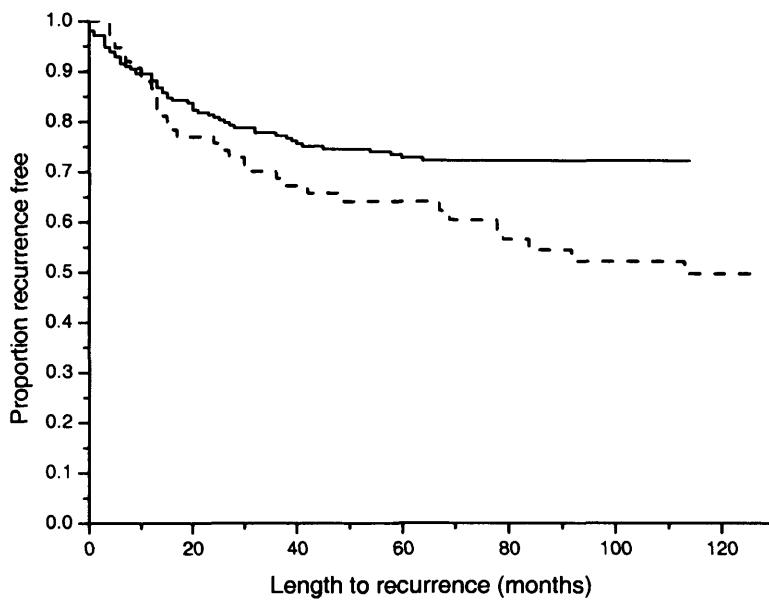
Fig. 3.4.5.3 Comparison of length to nodal recurrence between pre-PLC (1991-1992) and all melanoma patients 1993-1996. Log Rank χ^2 5.2773, p = 0.0216. ——— proportion of all 1993-1996 patients regional recurrence-free - - - - proportion of pre-PLC patients regional recurrence-free.
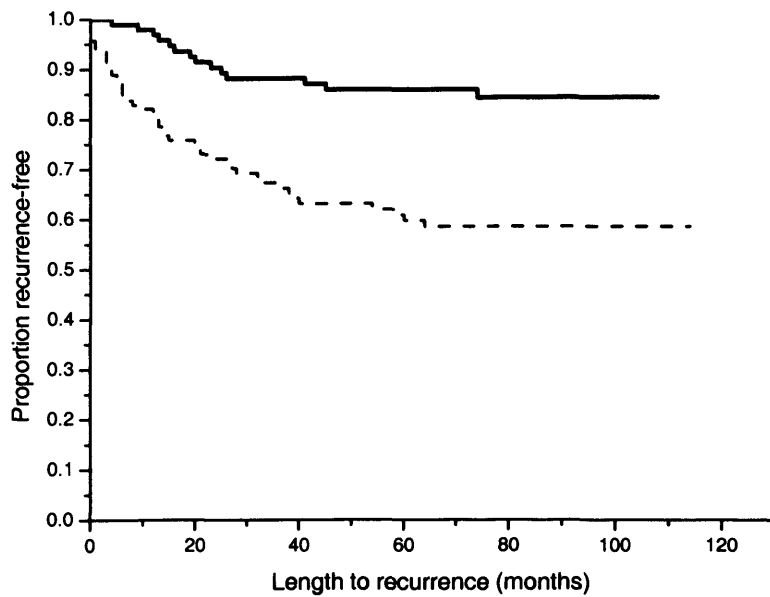
Figure 3.4.5.4 Comparison of length to nodal recurrence between non-PLC and PLC melanoma patients (both 1993-1996). Log Rank χ^2 17.0164, p <0.0001 ———— proportion of PLC patients regional recurrence-free, - - - - - proportion of non-PLC patients regional recurrence-free.

3.4.6   Local recurrence rates

Analysis of local recurrence rates demonstrated a significant difference between pre-PLC patients with PLC patients (χ^2 6.4883, p=0.0109). On this occasion, however, the difference between pre-PLC patients and all those diagnosed between 1993-1996 was not statistically significantly different (χ^2 0.8076, p=0.3688). However, a significant difference was shown when comparing non-PLC patients with PLC patients (χ^2 18.4898, p<0.0001) (Results summarised in Table 3.4.6.1).

Analysis performed	Log Rank χ^2	P value
pre-PLC vs. PLC	6.4883	0.0109
pre-PLC vs. all 1993-1996	0.8076	0.3688
non-PLC vs. PLC	18.4898	0.0001

Table 3.4.6 Comparison of length to local recurrence between subgroups

3.4.7   Disease control

The presence of any recurrence was then analysed under the heading "disease control"; i.e. whether the patients developed either a local or regional recurrence. The groups were compared in a similar way to above. The results are summarised in table 3.4.7.

Groups compared	χ^2 Value	P value
pre-PLC vs. PLC	20.1555	<0.0001
pre-PLC vs. all 1993-1996	1.9520	0.1624
non-PLC vs. PLC	88.1080	<0.0001

Table 3.4.7 Comparison of development of disease recurrence between groups.

3.5   DISCUSSION

Pigmented lesion clinics are dedicated to the facilitation of rapid referral to a specialist for clinically suspicious lesions. They have become popular in the last 20 years because of concerns about the increasing incidence of melanoma and the need to provide primary care physicians with a form of rapid referral system. To date no published study has looked at the effect of a pigmented lesion clinic on survival, recurrence rates and disease-free interval in melanoma. The PLC at Mount Vernon differs from some other units in that it is run on a "no-appointment" basis; other units require appointments to be made prior to attendance.

3.5.1   Demographics and melanomas

In this study 290 patients with melanoma were reviewed in total. The female to male ratio was 1.6:1, which compares with other data on sex distribution of melanoma (1.5:1 in MacKie & Hole 1992; 1.8:1 in MacKie *et al.,* 1992; 1.5:1 in de Rooij *et al.,* 1995). The site distribution of melanomas was predominantly the legs in women and the trunk in men. This pattern followed that established in the literature and corresponds to the sites most often exposed to the sun in each sex (Mihm MC et al., 1975; Koh 1991; MacKie 1998; Langley *et al.,* 1998; Smolinski *et al.,* 2001). The variation in subtype of melanoma was similar to that described elsewhere, with a predominance of superficial spreading melanomas (Mihm *et al.,* 1975; McGovern *et al.,* 1973). However, in this series, there were a relatively high proportion of nodular melanomas, at 32.76% compared with other series, which describe frequencies of 15% - 30% (Mihm *et al.,* 1975; Clark *et al.,* 1975; McGovern *et al.,* 1973).

3.5.2   Melanomas diagnosed via the PLC

2.1% of diagnoses made via the PLC over the study period were melanomas. This compares with 1.6% in Florence (Carli *et al.,* 2002), 2.17% in Leicester (Graham-Brown *et al.,* 1990), 2-5% in Edinburgh (Herd *et al.,* 1995), 2.6% at St. George's Hospital, Tooting (Mallett *et al.* 1993, Bataille *et al.* 1999), 3.1% seen in Dublin's PLC (O'Connor & Barnes, 1994) and 5.65% in Duff *et al's* study of the PLC at The Frenchay hospital in Bristol (Duff *et al.,* 2001). An explanation for the relatively low proportion of melanomas being diagnosed at early detection pigmented lesion clinics may be

the result of GPs being less discriminating in their diagnoses (i.e. referring a large number of benign naevi), or not utilising the PLC appropriately. Both of these factors will be discussed in depth below.

In terms of other PLC studies, O'Connor and Barnes reported melanoma patients seen at a PLC in Dublin. Eighty per cent of PLC patients' melanomas were 1.5mm or less in depth (O'Connor & Barnes, 1994). In their analysis, however, they compared their PLC data with data from other studies, rather than their own non-PLC data. Thus it is difficult to interpret the significance of this finding. A 9-year retrospective audit of the PLC at St. George's Hospital, Tooting (Bataille *et al.,* 1999) did not find any change in thickness of melanomas over time. No comparison was made with melanoma patients seen before the advent of the PLC or with "non-PLC" patients. Duff *et al.*'s review of the pigmented lesion clinic at The Frenchay Hospital in Bristol (Duff *et al.,* 2001) looked at the trend of melanoma thickness over time, but again did not use a control group with which to compare. There was no significant reduction in mean Breslow thickness with time, although a trend in that direction was observed.

3.5.3   Under-utilisation of PLCs

Of the 212 melanomas diagnosed 1993-1996, only 43.87% were diagnosed via the PLC. The maximum proportion of melanomas diagnosed via the PLC over the study period was 57.4% of the total, in 1995. The pigmented lesion clinic was designed to be a rapid referral system enabling melanomas to be diagnosed with as minimal a time delay as possible, however, this illustrates a marked under-utilisation. Other published series on PLCs have noted similar findings (Melia *et al.,* 1995, Osborne *et al.,* 1998, Osborne & Hutchinson 2001).

Reasons for this under utilisation may be because GPs see the PLC as a diagnostic service, rather than a treatment or rapid referral system as suggested by Herd *et al.* (Herd *et al.,* 1995). There is also an argument that GPs preferentially refer less suspicious lesions to the PLC rather than routine outpatients. If a GP is certain of the diagnosis of a melanoma, they may be more confident of writing a letter of referral to dermatology or plastic surgery consultants' outpatient clinics, such as in the case of a nodular melanoma. Whereas, if they are unsure of the diagnosis, as may be the case for thin, early melanomas, they may rather preferentially refer to the PLC, so as not to have to commit themselves to a definitive diagnosis. In this way, if they felt the lesion unlikely to be a melanoma, but were not completely sure, the patient will nevertheless be seen quickly.

Therefore, if the lesion did in fact turn out to be a melanoma, they would not be responsible for any delays incurred. In a letter to the Lancet Ibbotson *et al.* from Newcastle, whose department does not run a PLC, reviewed GP referral letters (Ibbotson *et al.,* 1995). Those patients whose GPs suspected melanoma were seen within a median of 13 days and the melanomas had an average thickness of 0.9mm, but in those in which the GPs did not suspect melanoma, the median time to be seen was 69 days and the mean thickness was 3mm. This supports the notion that GPs may preferentially refer cases in which they are uncertain of the diagnosis, as in early melanomas, to PLCs, rather than outpatient departments, to minimise potential delays incurred as the result of misdiagnoses. Finally, under-use of the PLC could also be due to new GPs to the area being unaware of its existence, and those already established forgetting to refer to the PLC, if not done regularly.

The consequence of not using the PLC, and referring to normal outpatient clinics, is of course a delay in time to excision of the melanoma. In order to encourage the use of the PLC as the sole referral point for suspicious skin lesions, annual reminders could be sent out – although this may encourage an increase in referral of benign lesions (Whitehead *et al.,* 1989). To minimise the referral of benign lesions, regular updates as to the presenting features of melanoma could be sent to GPs. In Scotland the quality of the referral letters rose noticeably after the GPs were updated about the presenting features of melanoma prior to a publicity campaign. Most of their referral letters now clearly indicate the degree of suspicion that the lesion is a melanoma and offer appropriate differential diagnosis (MacKie & Hole 1992).

3.5.4   Trends in melanoma incidence

A general increase in the total number of melanomas diagnosed each year was found, and the observed trend of increasing proportions of thin melanomas being diagnosed with each year mirror results of other studies (Doherty & MacKie 1988; Burton *et al.,* 1993; Burton & Armstrong 1994; Herd *et al.,* 1995; MacKie *et al.,* 1997; Jemal *et al.,* 2001; MacKie *et al.,* 2002). This effect has been put down to an increasing public awareness of the dangers of increased sun exposure, more vigilance in the community and therefore earlier presentation to physicians (Herd *et al.,* 1995; MacKie *et al.,* 1997).

3.5.5   Melanoma thickness and survival

The single most important variable in melanoma prognosis remains maximum tumour thickness (Breslow 1970, 1975; Barnhill *et al.,* 1996; Balch *et al.,* 2001; Ng *et al.,* 2001; Osborne & Hutchinson 2001). Efforts focused on the early detection of melanoma are based on the assumption that early melanomas will be thinner, as a result of their natural history, and so have a better prognosis. Thus the aim of a PLC is to provide a method for early detection and surgical intervention in melanoma thereby contributing to an improved survival and a reduction in mortality from this condition. Other PLC-based studies have therefore investigated tumour depth as a measure of survival (Mallett *et al.,* 1993; Herd *et al.,* 1995; Kirkpatrick *et al.,* 1995; Grover *et al.,* 1996). In this study, a statistically significant improvement in both tumour depth and survival was found for the melanoma patients diagnosed via the PLC when compared with those diagnosed before its inception. The thinner tumours in the PLC group bear out the results of a previous study from the PLC at Mount Vernon Hospital that suggested that this might affect improved survival for those patients being diagnosed in the PLC (Grover *et al.,* 1996). Other previous studies that also found a decrease in thickness in PLC-diagnosed tumours include those by Herd, O'Connor & Barnes and Osborne & Hutchinson (Herd *et al.,* 1995; O'Connor & Barnes 1994; Osborne & Hutchinson 2001). However, these studies compared their PLC groups with patients from other studies (O'Connor & Barnes, 1994) or only those from other clinics that ran in parallel with the PLC, rather than "pre-PLC" patients as well (Herd *et al.,* 1995; Osborne & Hutchinson 2001). Some studies investigating the effect of a PLC on tumour depth have found no statistically significant improvement in tumour thickness (Mallett *et al.,* 1993, Kirkpatrick *et al.,* 1995), although in one of these studies, trends toward thinner melanomas were seen in the PLC group (Kirkpatrick *et al.,* 1995).

No other study has so far shown an improvement in survival in melanoma patients whose tumours were diagnosed via a PLC. As discussed above, the influence of this improvement being the result of a "creaming off" the better prognosis melanomas into the PLC group was investigated by comparing the pre-PLC group with all melanoma patients diagnosed between 1993-1996, irrespective of route of referral. This comparison still showed an improvement in the PLC group, thereby suggesting that a "creaming off" effect alone could not explain the survival benefit. As well as demonstrating the better prognosis of PLC patients when compared with pre-PLC patients, a significant improvement in survival is also shown when comparing them with non-PLC patients, which has been shown in the two other PLC studies (Herd *et al.,* 1995; Osborne & Hutchinson 2001). The present study, however, is the first to show an improvement in survival compared with both a parallel control group and an historical one.

3.5.6　Non-metastasising forms of thin tumours

Some authors have suggested a form of melanoma may exist, which, if not detected, would either regress spontaneously or not progress and would not metastasise (Clark *et al.*, 1989; Burton *et al.*, 1993). Burton and Armstrong compare it with the situation in breast cancer in which the recent increase in incidence has been attributed to the introduction of screening mammography (Burton & Armstrong 1994). There is concern that many of these cancers would not have become clinically apparent during the lifetime of the patient. This is in accordance with the findings of autopsies on women not previously diagnosed with breast cancer; and autopsy and biopsy studies on women with breast cancer, which have detected a much higher prevalence of the disease than the known incidence of first and bilateral breast cancer would have predicted (Neilsen *et al.*, 1987). Burton proposes that there is a pathway of development of cutaneous melanoma, which leads only to local invasion and precludes the development of metastatic potential (Burton *et al.*, 1993). If this were true, then pigmented lesion clinics and screening of melanoma would lack specificity for fatal disease. This has led some authorities to propose that early excision will have no real impact on survival, as they argue that thin lesions may well remain static indefinitely, not undergo a vertical growth phase and subsequently have no substantial effect on mortality (Swerlick & Chen 1996). In this study, however, it appears that the reduction in tumour depth in the PLC-diagnosed melanomas does have an impact on survival, contradicting such authors' arguments.

In addition, the hypothesis that some thin lesions would never have the capacity to metastasise is impossible to prove, as the entire lesion must be excised and examined pathologically to establish the diagnosis and growth phase. Therefore one cannot predict the future behaviour of these lesions if left *in vivo*. MacKie suggests that long-term follow-up of patients who have had thin primary tumours excised is needed to establish whether they are truly cured, or whether disseminated tumour cells are present even at an early stage that will eventually develop into metastatic deposits and cause the patient's death (MacKie 1997). The fact that patients who have such lesions excised whilst still in the radial growth phase have later developed metastases indicates that conventional microscopic examination is not enough to positively exclude metastatic potential, and therefore this alone justifies the removal of early invasive (Clark's level 2) primary melanomas. Besides, even if a proportion of melanomas were not to progress, the benefit of increased sensitivity at the expense of specificity, is undoubtedly worth gaining for those patients with the progressive form of the disease.

3.5.7 Lead-time bias

The evaluation of screening programmes such as that for breast cancer has led to awareness of the confounding factor of lead-time bias. Patients who are diagnosed at an early stage in their illness may appear to survive longer than those diagnosed at a later stage only because the diagnosis is established for a longer period of time. It may be argued that the improvement in survival of the PLC group has arisen due to lead-time bias. The melanomas diagnosed via the PLC have been shown to be thinner, and whether or not this is due to lead-time bias, excision at this stage offers patients the only chance of cure. Thus any effect of lead-time bias is diluted, as the same patients diagnosed at a later stage may have missed the potential of being cured.

In addition, if lead-time bias were to be a factor in explaining the thinner melanomas being diagnosed in the PLC, it would be expected that there would be a decrease in the number of melanomas being diagnosed after a sufficient time lag for the previously expected time of diagnosis. There would also be a decrease in the number and proportion of thick lesions being diagnosed after a time period. It has been shown in this study that the number of melanomas diagnosed each year actually continues on an increasing trend, and there is no overall decrease in the thickness of melanoma diagnosed with time (Figure 3.4.1.2). This reasoning does assume a relatively stable incidence of melanoma in the population. Despite other studies (Doherty & MacKie 1988; Burton *et al.,* 1993; Burton & Armstrong 1994; MacKie *et al.,* 1997; Jemal *et al.,* 2001) showing the incidence of melanoma continuing to rise; they also show that this rise is now levelling off, so this argument is still valid. Thus the impact of lead-time bias is again negated, or at least much reduced. Finally, discussions on lead time bias also do not allow for the fact that a clone of cells with full metastatic potential might develop in an untreated initially in-situ primary melanoma. Some of these cells could move from the primary site before the primary tumour is eventually excised. This justifies the detection and excision of melanomas as early as possible, however much the influence of lead-time bias.

3.5.8 Length-time bias

Slow-growing tumours are detectable for longer at an early stage than fast-growing ones and will therefore be preferentially identified by any early diagnosis strategy. Therefore it would seem that by looking at the decrease in melanoma depth alone, there is a chance that the benefits of the PLC are due to an increased number of slow-growing melanomas being diagnosed. However, if

this were the case, any impact on survival would most likely be minimal, as the slow-growing tumours would also be of low metastatic potential. It has been shown here that survival has improved in those diagnosed via the PLC (Figures 3.4.3.1-3). Therefore, length-time bias is unlikely to have occurred.

3.5.8   Recurrent disease

There were fewer lymph node recurrences in the PLC group when compared with both the pre-PLC and the non-PLC groups (χ^2 12.32, p = 0.0005; χ^2 16.75, p = 0.0005 respectively). A statistically significant improvement was demonstrated in the length of time elapsing before the occurrence of regional metastases in those patients diagnosed with melanoma via the pigmented lesion clinic when compared with those diagnosed prior to its inception and with patients diagnosed via existing routes of referral. This was on a background of population-based studies that have shown overall mortality from melanoma in the UK over the study period to stay constant and actually increase for men (La Vecchia *et al.,* 1999; MacKie *et al.,* 2002). This would suggest that the disease-free interval for the study population would be expected to have at best remained stable, however an improvement in disease-free interval in the PLC patients over the study period was observed. Metastases form once the melanoma has transformed from the radial into the vertical growth phase (Elder *et al.,* 1984). Therefore, if excision is performed at the earliest opportunity there is a chance of cure as the lesion may be excised before it has reached the vertical growth phase and therefore before it has developed the capacity to metastasise.

Local recurrence is believed to be an indicator of biological aggressiveness and is also used clinically to assess the adequacy of surgical treatment (Karakousis *et al.,* 1998; Ng et *al.,* 2001). Despite the improved survival and increased length to recurrence in the 1993-1996 melanoma patients compared with those diagnosed prior to the establishment of the PLC, this improvement did not reach significance (Table 3.4.6). The PLC group did however have a significant improvement in length to local recurrence when compared with the "pre-PLC" group and the "non-PLC" group. This was probably due to the relatively small number of patients that developed local recurrences, thus statistical tests not reaching significance.

3.5.10   Delays

The next point of discussion is the timing of the excision. PLCs work on the rationale that the earlier the tumour is excised, the better the outcome. PLCs aim to minimise any delays, but, of course, can have no impact on patient delay in seeking medical attention, which has been shown to be of importance (Temoshok *et al.,* 1984; Doherty & MacKie 1986; Rampen *et al.,* 1989).

Significant delays often occur after consultation of the primary care physician. The delays are often due to a combination of the bureaucracy of the healthcare system and either the failure of the primary care physician to recognise a lesion as suspicious (Cassileth *et al.,* 1988; Rampen *et al.,* 1989; Krige *et al.,* 1991) or failure to convey this point in the letter of referral (Ibbotson *et al.,* 1995). The difference in time between referral from the GP to the consultation between routine clinics and the PLC in this study also depended upon the suspected diagnosis by the GP. At best, when the GP suspected a diagnosis of melanoma, the time from GP visit to specialist consultation was two weeks. After consultation a further delay of around a week was incurred in arranging the surgery, resulting in a minimum delay of three weeks between GP diagnosis and excision. On the other hand, if there was no suspicion of malignancy by the GP patients may have had to wait up to 22 weeks before review by a specialist. This then translated into a delay of 23 weeks until excision. The PLC however, conferred a maximum time delay of one week and a minimum of same-day consultation and excision. The PLC thus offers a safety net for those instances in which previously unsuspected lesions would be picked up without the time delay that would have otherwise been incurred. Osborne et al. examined the mean referral interval between GP and clinic appointment, comparing PLC and non-PLC patients in Leicester, UK (Osborne *et al.,* 1998). The overall mean referral interval for the 11-year study period was 7 days, compared with 19.5 days to plastic surgery and 38.4 days to dermatology routine outpatient clinics, showing a clear reduction in delay between referral and appointment. Of course delays due to misdiagnosis by hospital specialists will not be altered by a pigmented lesion clinic. However, hospital specialists have an improved diagnostic accuracy when compared with primary care physicians (Paine *et al.,* 1994) and trainees (Morton & MacKie 1998).

3.5.11   Publicity campaigns

Tactics used to overcome the patient delay factor are usually in the form of publicity campaigns involving local media, leaflets and posters. Both Healsmith *et al.* and Graham-Brown *et al.*

experienced an increase in the number of melanomas seen after a publicity campaign, and a rise in the proportion of thin lesions, although this did not reach statistical significance in either study (Graham-Brown *et al.,* 1990; Healsmith *et al.,* 1993). After a Scottish publicity campaign in 1985, MacKie & Hole found a significant increase in the numbers of melanomas diagnosed, mainly confined to thin, good prognosis lesions (MacKie & Hole 1992). There was also a reduction in the number of thick melanomas in women, but not men, implying that the campaign succeeded in delivering the message of early detection to women but not men. In a later study by MacKie *et al,* a reduction in the annual mortality rate from melanoma was reported among women living in the whole of Scotland (MacKie *et al.,* 1997). This reduction, however, occurred almost immediately after the start of the intervention. Given the good survival for melanoma in the first five years, it is debatable whether this was a result of the 1985 campaign. In contrast, Herd *et al.,* did not find any significant change in the rate of diagnosis of melanoma in Edinburgh after 2 publicity campaigns (Herd *et al.,* 1995). A later study on melanoma mortality trends between different populations also did not find a correlation between improvements in mortality with local and national melanoma prevention campaigns (Severi *et al.,* 2000).

In conclusion, studies into the effectiveness of publicity campaigns are inconsistent, and when showing an improvement in melanoma diagnosis it is usually short-lived. The downside of publicity campaigns is the massive increase in attendance of pigmented lesion clinics, often with a disproportionate number of benign lesions (Whitehead *et al.,* 1989). However, de Rooij and colleagues found that by concentrating public awareness and GP education purely on melanoma, rather than including non-melanoma skin cancer, increases in the detection rates of melanoma and lesions suggestive of melanoma (such as dysplastic naevi) were achieved (de Rooij *et al.,* 1995).

3.5.12   PLCs and publicity campaigns

Some authors argue that PLCs only result in reducing the mean depth of melanoma at presentation (and hence mortality) if such a publicity campaign is instigated. Mallett *et al.* compared the thickness of melanomas excised in two neighbouring health authorities, only one of which was served by a PLC (Mallett *et al.,* 1993). Following a Cancer Research Campaign to raise public awareness of melanoma, the PLC had an increase in the number of melanomas seen, compared with other clinics, in which no increase was seen. A consistently greater number of thin melanomas in patients attending the PLC compared with normal outpatient clinics were also seen,

but this was only after the publicity campaign. No statistical analysis was performed on the data in this study; hence its significance is unknown. The authors concluded that a PLC might only be of benefit if there is a concurrent publicity campaign, although it does nevertheless fulfil a demand from both GPs and patients. When Melia's group studied data collected from 6 UK district health authorities' PLCs that took part in the Cancer Research Campaign's (CRC) early detection intervention (1987-1989) and compared it with two districts that did not take part, no significant difference in annual mortality rates were found, leading them to conclude that awareness campaigns were not effective in PLCs and other strategies ought to be pursued (Melia *et al.*, 1995 & 2001).

In this pigmented lesion study there has been a significant improvement in survival despite the lack of a local publicity campaign since the inception of the PLC. Thus it would appear that although a publicity campaign may be of benefit in increasing the number of melanomas diagnosed and the proportion of thin melanomas, it is not essential. With the continued increase in public awareness campaigns each spring and summer, the effects of individual campaigns are harder to evaluate. The earlier study on the pigmented lesion clinic at Mount Vernon Hospital found an increased number of attendees over the spring and summer months (Grover et al., 1996), which has been reflected in the personal experience of the author.

3.5.13   Alternative strategies

In contrast to pigmented lesion clinics that operate in the U.K. and some other European countries, in the United States members of the public are encouraged to seek advice directly, bypassing GPs. "Skin Cancer Fairs" or "Free Melanoma Diagnostic Clinics" (Koh *et al.*, 1996) are set up for varying periods of time in different States. These clinics are free, and run on the principle that diagnosis and advice is offered, but no treatment is carried out. The screening days have proven to be popular, and it has been found that those who take advantage of them have an increased number of melanoma risk factors when compared with the general population (fair skin, fair hair and large numbers of naevi). Melanomas detected at these fairs and subsequently excised have been shown to be thinner than those of the population at large. The structure of these screening days does not, however, allow for changes in mean thickness or in melanoma-associated mortality to be assessed.

3.5.14   Further points of discussion

In addition to the detection of melanomas, 748 non-melanoma skin cancers were diagnosed and treated after presentation to the PLC. This made the overall excision rate of skin cancer to be 24% for all patients referred for surgery, although a significant number of benign lesions were excised as a result. The proportion of benign lesions excised compares with that found in other PLC studies, which have shown ranges of 58% - 94% (Bataille *et al.,* 1999; Mallett *et al.,* 1993; Kirkpatrick *et al.,* 1995; Duff *et al.,* 2001). With regard to melanoma, 96% were treated within 2 weeks of consultation, the majority being excised immediately (76%).

Other advantages of PLCs that are not immediately apparent from this study are the non-quantifiable benefits including the patient satisfaction from the ease of access, GPs satisfaction from the ease and rapidity of referral and the excellent teaching opportunity for both basic and higher surgical trainees, who can examine patients under the supervision of the consultant. The clinic also provided an invaluable opportunity for data collection, patient education and eases the burden on other clinics. Disadvantages of the PLC include a potentially heavy workload and an increase in the number of benign lesions excised. The number of patients attending each week was also unpredictable, although seasonal variation is encountered, with more patients attending in the summer months (Grover *et al.,* 1996) as described above. Since the study period there has been a steady increase in attendance of the PLC in parallel with increasing public awareness of melanoma and the damaging effects of sunlight, such that now an attendance of between 50 and 90 patients is experienced, the maximum number attending over late summer.

3.6   CONCLUSION

This study has shown a significant improvement in survival in those patients diagnosed with melanoma via the PLC. This same group also had a longer disease-free interval before any recurrences developed. These results support the use of pigmented lesion clinics as a means of improving the outcome for patients with melanoma and incorporate the requirements of the NHS Cancer Plan. In addition the PLC confers a benefit on the population it serves and to GPs by providing rapid access to experts with an interest in melanoma.

CHAPTER 4

USE OF TISSUE MICROARRAY TECHNOLOGY TO DISCOVER PROGNOSTIC MARKERS FOR MELANOMA

4.1   INTRODUCTION

4.1.1   Rationale

With the development of novel adjuvant therapies that may alter the long-term survival of melanoma patients (Kirkwood *et al.,* 2001), the need for accurate prognostic markers for the disease is greater than ever. Currently the establishment of prognosis in primary melanomas relies on several clinicopathological variables including Breslow thickness, Clark's level, presence of ulceration, patient age, tumour site and patient sex (Balch 1992; Chang *et al.,* 1998; Smolinski *et al.,* 2001; Balch *et al.,* 2001a & b). Once the cancer has metastasised, the most important determinant of prognosis is the nodal status of the patient. Sentinel lymph node biopsy (Morton *et al.,* 1992) is now widely practiced as a means of accurately staging patients and predicting outcome (Balch *et al.,* 2001) but is associated with significant morbidity (Hettiaratchy *et al.,* 2000; Jakub *et al.,* 2003) and has not yet been shown to have a bearing on overall patient survival. Non-invasive strategies, if able to provide similar information, would provide a desirable alternative for estimating prognosis.

Normal epidermal melanocytes are found at the dermal-epidermal border amongst the keratinocytes. Their proliferation leads to nests of mature melanocytes, known as acquired melanocytic naevi. With the development of a melanoma, their growth starts in a radial growth phase, before progressing to a vertical, invasive phase where they acquire the potential to metastasise (Clark *et al.,* 1989). The underlying mechanisms of this process are complex and poorly understood; some patients with thin melanomas may suffer from early metastasis and death, whilst other patients with thick melanomas at the time of surgery are cured (Fearfield *et al.,* 2001; Salti *et al.,* 2002; Kalady *et al.,* 2003). Establishing an accurate prognosis is most problematical in the intermediate thickness (1.01-4.0mm) melanomas. If high-risk patients could be more accurately identified at presentation, adjuvant therapy could be justified and patient outcome improved by treatment prior to the onset of metastatic disease.

4.1.2   Metastasis formation

The molecular basis of human melanoma progression and the changes associated with the transition of melanoma cells from the radial growth phase to the vertical growth phase remain unclear.   The production of metastases depends on the completion of a complex, multistep process that involves the survival and growth of a unique subpopulation of cells with metastatic properties (Fidler *et al.*, 1990): tumour progression and metastasis involves the separation of the tumour cells from the primary tumour mass, migration through the extracellular matrix, entry and survival within the vasculature, extravasation into a foreign environment and the establishment of a new focus of growth.   At the core of this process lie the changing adhesive properties of the tumour cells, which dictate their interactions with the surrounding extracellular matrix and neighbouring cells.

4.1.3   Cell adhesion molecules

Cell adhesion molecules are cell surface molecules which bind specifically to other cell surface molecules (ligands) on other cells or extracellular matrix components.   Cell-cell adhesion can influence cell growth and survival either indirectly through signalling cascades or through a direct interaction with growth factor receptors.   Structurally, many different kinds of molecules can mediate intercellular adhesion, but the majority of cell adhesion molecules known to date fall into three gene families: the integrins, the cadherins and the immunoglobulin superfamily (Chothia *et al.*, 1997).   Cell adhesion molecules do not simply function as a molecular glue organising cells into static structural entities, but also support and direct the exchange of information between cells (Klymkowsky *et al.*, 1995).   Adhesion molecules themselves actively participate in transducing signals into the cells.   Their prime role seems to be in organogenesis and tissue remodelling as well as the maintenance of tissue organisation and the direction of leukocyte migration throughout the body (Johnson 1999).

Alterations in cell adhesion molecule activity in tumour cells can lead to a disruption of normal cell-cell and cell-matrix contacts and to the development of new interactions, both of which are important aspects of metastasis formation (Johnson *et al.*, 1999).   Adhesion molecules mediate these interactions and modulation of adhesion molecules has been associated with metastatic spread in melanoma (Xie *et al.*, 1997; Hsu *et al.*, 2000).

4.1.4　Integrins

Integrins are heterodimeric cell surface receptors formed by non-covalent association of an alpha chain with a beta chain (Hynes 1992). Integrins mediate cation-dependent adhesion to extracellular matrix molecules and to cell surface ligands. The ligand specificity is determined by the combination of alpha and beta chains and since there are a large number of variations of each, many diverse cell-cell and cell-matrix adhesion molecules can be expressed by a given cell. Effective ligand binding by integrins requires "activation", a change in the avidity of the receptor due to conformational changes. These are induced by an "inside-out signal" delivered from other receptors on the same cell (Hynes 1992; Hughes & Pfaff 1998). Compared with benign melanocytic lesions, melanomas demonstrate loss of expression of the laminin receptor α6β1 (Natali *et al.*, 1991) as well as an increase in the expression of α4β1 (Schadendorf *et al.*, 1995) and the vitronectin receptor αvβ3 (van Belle *et al.*, 1999). These integrins seem to be functionally important since changes in their expression correlate with prognostic parameters and with clinical outcome in melanoma patients (Natali *et al.*, 1991; Schadendorf *et al.*, 1995; van Belle *et al.*, 1999).

4.1.5　Cadherins

Cadherins are a family of calcium-dependent surface glycoproteins that mediate cell-cell adhesion. Their extracellular domains connect neighbouring cells, and their cytoplasmic tails are linked non-covalently to the actin cytoskeleton via catenins (Johnson 1999). E- (epithelial), N- (neural) and P- (placental) cadherins are expressed in a cell-, tissue- and development-specific manner. E-cadherin is the major cadherin in the human epidermis and the major adhesion link between epidermal melanocytes and keratinocytes, whereas fibroblasts and endothelial cells (but not keratinocytes) or melanocytes express N-cadherin.

There appears to be a shift in the cadherin expression profile of melanoma cells during melanoma progression (Hsu *et al.*, 1996). Progression of melanoma in the radial growth phase is intimately linked with loss of E-cadherin, triggering release of cancer cells from the primary cell focus. Normally, keratinocytes control cell growth, expression of melanoma-associated cell surface molecules and other functions of melanocytes. However, melanoma cells are refractory to keratinocyte-mediated regulation, and therefore also to decreasing motility, proliferation and invasive potential. This loss of keratinocyte dominance has been shown to occur in parallel with

downregulation of E-cadherin (Hsu *et al.*, 1996). Once E-cadherin has been lost the melanocytic cells have the ability to escape from neighbouring keratinocytes and invade the dermis (Guilford 1999). Disruption of E-cadherin-mediated cell adhesion is thought to facilitate tumour invasion, whereas restoration of E-cadherin expression results in growth retardation and inhibition of the invasive and metastatic behaviour of melanoma cells (Christofori & Semb 1999). The loss of E-cadherin disrupts protein traffic and cellular architecture which both have been suggested to be primary defects resulting in loss of growth regulation and tumour development (Peifer 2000). The signalling of E-cadherin is mediated through β-catenin and subsequent activation of target genes (Gruss & Herlyn 2001). Loss of cadherin frees up β-catenin for intracellular interactions. The loss of E-cadherin expression which is observed in the earliest identifiable malignant lesions, i.e. radial growth phase melanoma, is a critical event in the development of metastatic potential, leading not only to the expression of new adhesion molecules but also to broad changes in gene expression which contribute to or even constitute the biological behaviour of melanomas (Klymkowsky *et al.*, 1995). Melanoma cells express high levels of N-cadherin that may interact with N-cadherin-positive fibroblasts and endothelial cells (Hsu *et al.*, 1996). The N-cadherin-mediated gap junctions between melanoma cells allow the transfer of cell signals essential for tissue function and homeostasis.

P-cadherin has not been extensively investigated in melanoma. Experimental studies have linked P-cadherin expression to the progression of rat tongue carcinogenesis with the possibility that it may activate mechanisms responsible for cell proliferation (Sakaki *et al.*, 2003). P-cadherin has also been implicated in the higher proliferation rates of aggressive breast carcinomas (Paredes *et al.*, 2002). Regarding melanomas, in an immunohistochemical study by Sanders and colleagues, loss of P-cadherin was demonstrated in the most aggressive vertical growth phase melanomas compared with thinner melanomas (Sanders *et al.*, 1999).

4.1.6   Immunoglobulin adhesion molecules

Melanomas express a diverse group of cell adhesion molecules of the immunoglobulin superfamily. These include: neural adhesion molecules (NCAM), the endothelial cell adhesion molecule MCAM (also known as MUC18, Mel-CAM, CD-146) as well as adhesion molecules such as ICAM-1 (CD54) that mediate interaction with leukocytes and CEACAM1.

4.1.6.1 MCAM

MCAM (also known as MUC18, Mel-CAM, CD146) is a predominant cell adhesion molecule of human melanomas that also mediates homotypic aggregation of melanoma cells through heterophilic adhesion to an as yet unidentified ligand present on melanoma cells (Shih *et al.*, 1997; Shih *et al.*, 1999; Johnson 1999). MCAM may be stained using immunohistochemical techniques (Shih et al. 1998). There is evidence to suggest that changes in the adhesive properties of melanocytes and melanomas are connected with the upregulation of MCAM expression (Grimm *et al.*, 1995). MCAM appears to be strongly upregulated in melanomas compared with benign lesions and is expressed by more than 80% of metastatic lesions (Lehmann *et al.*, 1987). Most work on MCAM has been done using animal models, where its expression seems to play a role in the initiation of new foci of growth of melanoma (Luca *et al.*, 1993; Xie *et al.*, 1997). MCAM expression has also been correlated with the ability of human melanoma cell lines to form metastases in nude mice (Luca *et al.*, 1993). More directly, Xie and colleagues studied non-metastasising melanoma cell lines (Xie *et al.*, 1997). MCAM expression was enforced by transfection on the non-metastatic MCAM-negative primary cutaneous melanoma cells, which were then injected into athymic nude mice. The enforced expression rendered them highly tumorigenic and increased their metastatic potential in the mice compared with parental and control transfected cells. Anti-MCAM monoclonal antibody reversed these effects in the transfected cells. Similarly, anti-MCAM antibodies have been shown to suppress tumour growth and formation of metastases in nude mice by Mills and colleagues (Mills *et al.*, 2002). These data provide indirect evidence that the development of metastatic capacity in melanoma may be associated with an increase in MCAM expression and that MCAM expression may play a role in the initiation of new foci of growth of melanoma.

4.1.6.2 CEACAM

CEACAM, also known as CD66a, is a member of the carcinoembryonic antigen family, also belonging to the immunoglobulin superfamily (Thompson *et al.*, 1991). It is known to mediate both homophilic and heterophilic adhesion (Thompson *et al.*, 1991) and although expressed in a number of human tissues, its expression has not been reported in normal melanocytes (Prall *et al.*, 1996). CEACAM is down-regulated in malignant tissues derived from breast (Riethdorf *et al.*, 1997), colon (Neumaier *et al.*, 1992) and prostate (Luo *et al.*, 1999). However, in an immunohistochemical study of CEACAM1 in cutaneous melanoma, Thies *et al.* found expression to correlate with metastatic disease. Therefore, CEACAM appears to play an alternative role in

melanoma compared with other cancers (Thies *et al.*, 2002). These findings are in a agreement with the earlier suggestion that CEACAM1 has a role at the invading front of melanoma, facilitating invasion of blood vessels and lymphatics as well as migration to distant sites (Brummer et al., 2001).

4.1.7　Other adhesion molecules

4.1.7.1　CD44

CD44 is a widely distributed transmembrane glycoprotein, originally described as a homing receptor for lymphocytes (Stoolman, 1989). However it has since been found to be involved in such processes as cell-cell adhesion and cell-matrix interaction (Underhill 1992). Multiple isoforms of CD44 are generated by alternative splicing of transcripts of this gene (Screaton *et al.*, 1992). CD44 molecules containing the v3 domain are able to bind growth factors and might play a role in the control of cell proliferation (Bennett *et al.*, 1995). There is increasing evidence that CD44 may contribute to malignant progression and metastasis formation (Bartolazzi *et al.*, 1994). For example, the extent of CD44 variant 6 (v6) correlates with the stage of tumour progression in human colorectal adenocarcinomas (Heider *et al.*, 1993), bladder carcinoma (Nemec *et al.*, 1987) and non-Hodgkin's lymphoma (Horst et al., 1990). The data suggest that the mechanism involved may be largely dependent on the ability of CD44 to mediate cell attachment to hyaluronate, an extracellular polysaccharide (Stamenkovic *et al.*, 1991). The interaction of CD44 with hyaluronate contributes to tumour cell proliferation, migration, invasion and formation of metastatic tumour emboli (Catterall *et al.*, 1999).

In the case of melanoma cells, CD44 overexpression enhances both tumorigenicity and the potential for experimental metastasis formation (Birch *et al.*, 1991). One investigation examining the expression of several CD44 variants demonstrated upregulation of CD44H in benign lesions, dysplastic lesions and melanoma *in situ*, but expression within melanoma diminished with increasing invasiveness, rather than Breslow thickness (Harwood *et al.*, 1996). In this study the CD44 splice variants, including v3 were not detected in any lesions. These results suggest a possible role for downregulation of CD44H in modulating the biological behaviour of melanoma. The results are supported by data from other larger studies (Karjalainen *et al.*, 2000) that demonstrated downregulation of CD44 with melanoma progression. Another study also found no CD44v3 expression in a series of 57 melanoma specimens (Ranuncolo *et al.*, 2002). Seiter and

co-workers, however, did find splice variants v5, v7, v8 and v10 expressed in melanomas to varying degrees (Seiter *et al.*, 1996). CD44v10 was upregulated in thick melanomas and v5 was upregulated in lymph node metastases again suggesting that melanoma progression is accompanied by upregulation of distinct isoforms of CD44. Furthermore, Seiter et al. found CD44v5 expression at high levels in cell lines known to metastasise. Thus, there is no consensus view as to the expression of CD44 splice variants in melanoma as yet, particularly the involvement of CD44v3.

4.1.7.2 Nm23

*nm*23 was identified as a gene that is strongly up-regulated in K-1735 melanoma cell lines with low metastatic potential compared with variants of this cell line with high metastatic potential (Steeg *et al.*, 1988). Follow-up studies supported the hypothesis that the Nm23 protein may act as a metastasis suppressor *in vitro* and *in vivo* (Leone *et al.*, 1991) and reduced expression was found in patients with advanced hepatocellular carcinoma (Nakayama *et al.*, 1992). However, other studies demonstrated high levels of Nm23 in tumours such as squamous cell lung carcinoma (Huwer *et al.*, 1994). Reichrath *et al.* compared benign naevi and melanomas and did not find a correlation with *nm*23 expression challenging the hypothesis that *nm*23 is a tumour suppressor gene (Reichrath *et al.*, 1997). Döme *et al.* found *nm*23 expression in melanoma to become "highly heterogeneous" with increased tumour thickness (Döme *et al.*, 2000). This study found that primary tumours characterised by lymphatic progression had the lowest expression levels of Nm23. However, this did not correlate with survival. Holmes and MacKie also did not find a correlation between *nm*23 expression and the survival of melanoma patients (Holmes & MacKie 1996). Thus, no clear overall role for *nm*23 expression in melanoma progression has been established.

4.1.7.3 Bcl-2

Abnormal expression of *bcl*-2, an anti-apoptotic regulator gene, has been shown to be involved in the pathogenesis of cancers (Manning & Patierno 1996) and might be an important factor for tumour progression. Studies conducted on murine melanoma cells have shown that the down-regulation of the *bcl*-2 gene is associated with nitric oxide-mediated apoptosis and that cells transfected with the *bcl*-2 gene are more resistant to apoptosis (Xie *et al.*, 1997b). Moreover, *bcl*-2 antisense oligonucleotide treatment of human melanoma resulted in significantly reduced levels of

Bcl-2 and increased the cellular sensitivity to chemotherapeutic drugs (Jansen *et al.*, 1998). Gene expression of *bcl-2* appears to be increased with tumour progression of melanoma, which may reflect an increased malignant potential caused by inhibition of apoptosis and a growth advantage for metastatic melanoma cells (Leiter *et al.*, 2000). High expression of *bcl-2* in metastatic melanoma compared with primary melanoma has also been found, supporting the idea that *bcl-2* expression is associated with advanced melanoma and contributes to the malignant phenotype (Utikal *et al.*, 2002).

4.1.7.4   Ki-67

The proliferation rate of transformed cells is a putative prognostic indicator, correlating with metastatic dissemination and long-term survival in various neoplasms (Tubiana & Courdi 1989; Balch *et al.*, 2001a & b). One of the most reliable methods of assessing the degree of proliferative activity is the Ki-67 index (Gerdes *et al.*, 1984). Ki-67 is not expressed in resting cells but is expressed from the mid-G1 phase of the cell cycle through to S, G2 and M phases (Gerdes *et al.* 1984; Guillaud *et al.*, 1989). It is therefore considered to be a potential marker of proliferation and hence possibly a useful prognostic marker. The level of proliferative activity, as measured by Ki-67 antigen expression, has been shown to correlate with tumour grade and patient survival in a variety of human malignancies (Jochum *et al.*, 1996; Wakimoto *et al.*, 1996). Correlations between Ki-67 expression and tumour thickness are well documented for melanoma (Korabiowska *et al.*, 1994; Ramsay *et al.*, 1995; Vogt *et al.*, 1997; Sparrow *et al.*, 1998; Niezabitowski *et al.*, 1999; Hazan *et al.*, 2002) and a link with patient survival has been established for thick tumours (Ramsay *et al.*, 1995; Böni *et al.*, 1996; Henrique *et al.*, 2000). However, there is no agreement as to its use as a prognostic marker for thin tumours or for predicting outcome in melanoma patient cohorts of a wide range of depths (Böni *et al.*, 1996; Sparrow *et al.*, 1998). Therefore the prognostic value of proliferative activity in cutaneous melanoma, as measured by Ki-67 expression, is still a conflicting issue.

4.2   TISSUE MICROARRAY

The study of biological markers using conventional whole-specimen sectioning and immunochemical staining can be time-consuming and laborious. In 1998 Kononen and colleagues (Kononen *et al.,* 1998) described a method for examining several hundred individual specimens simultaneously by precisely arraying them into a paraffin block. The investigators designed a robot to punch cylinders (0.6mm wide, 3-4mm high) from 1000 individual tumour biopsies embedded in paraffin (see Figure 4.2). The selection of cylinders with 0.6mm diameters allowed the preservation of histological information with minimal damage to the original blocks. Punch biopsies were then arrayed (at positions with precise coordinates) in a 45 x 20mm paraffin block. Up to 200 consecutive tissue sections (4-8μm) were cut from each block array. Serial sectioning allowed rapid, parallel analysis of the arrayed tumour punches by immunohistochemistry, fluorescence *in situ* hybridisation (FISH) and RNA/RNA *in situ* hybridisation. Kononen and colleagues tested their method by analysing 645 breast tumours for eight markers and the results obtained, using the high-throughput microarray method, were in good agreement with the literature. The authors demonstrated that the tissue microarray provided a high-throughput technique for the molecular profiling of tissue specimens by various methods, including immunohistochemistry. The technique greatly facilitates comprehensive molecular profiling of cancer specimens with minimal tissue requirements. A further advantage of the technique is that all specimens are processed at the same time using identical conditions. It also markedly reduces the amount of archival tissue required, preserving sufficient tissue for other research or diagnostic needs (Mills *et al.,* 1995).

4.2.1   Examples of different tissue microarray types

Typical tissue microarrays constructed in the literature include multi-tumour arrays, progression and prognosis arrays.
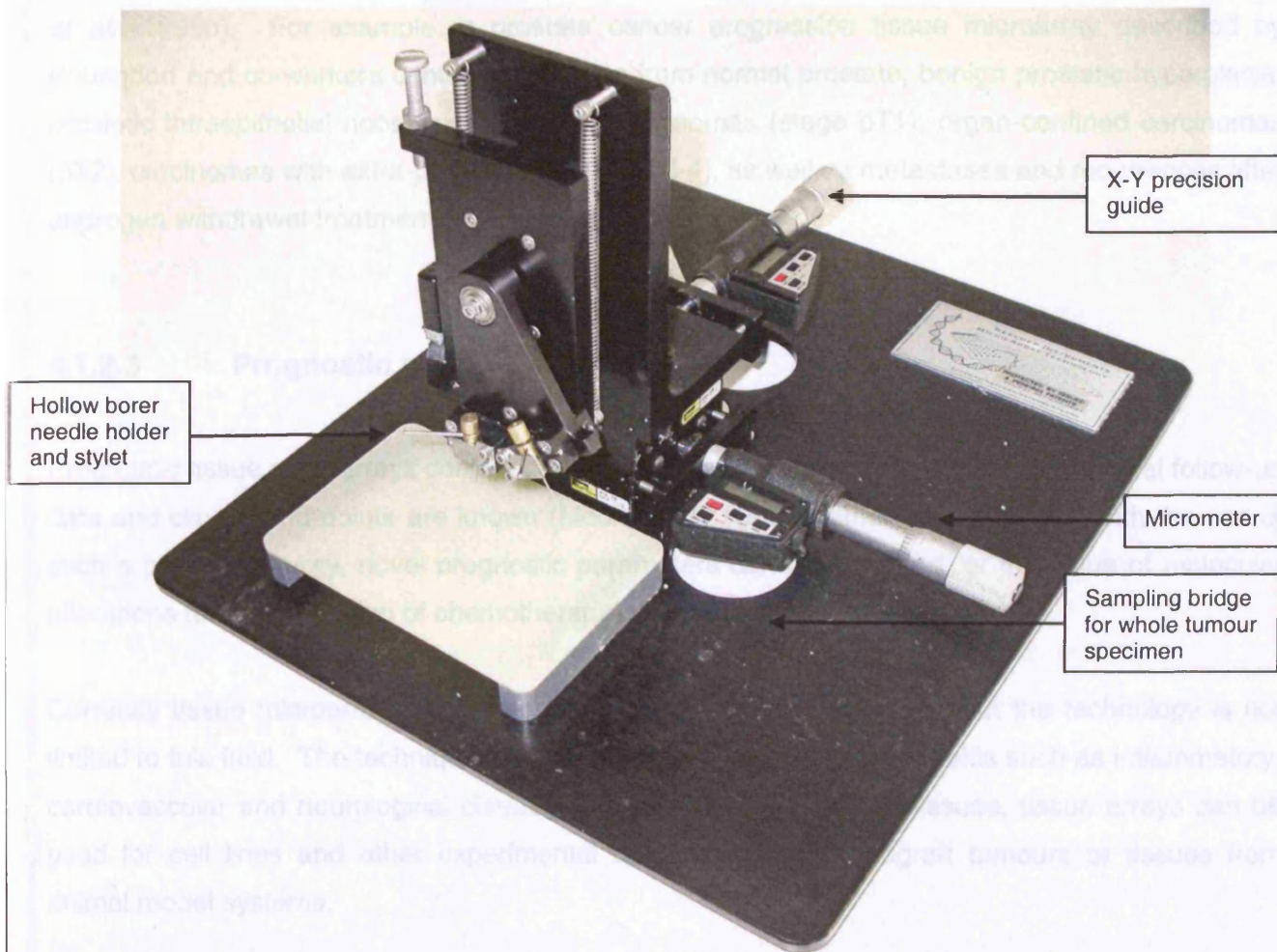
Figure 4.2 Tissue Microarrayer™

4.2.1.1   Multi-tumour arrays

Multi-tumour tissue microarrays are composed of samples from multiple tumour types. These arrays are used to screen different tumour types for molecular alterations of interest. The first example of a multi-tumour tissue array contained 397 samples from 17 different tumour types (Schraml *et al.,* 1999).

4.2.1.2   Progression arrays

These have been used to study molecular alterations in different stages of one particular tumour, such as breast, urinary, bladder, kidney and prostate cancer (Bubendorf *et al.,* 1999a; Bubendorf

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et al., 1999b). For example, a prostate cancer progression tissue microarray described by Bubendorf and co-workers contained samples from normal prostate, benign prostatic hyperplasia, prostatic intraepithelial neoplasia, incidental carcinomas (stage pT1), organ-confined carcinomas (pT2), carcinomas with extra-prostatic growth (pT3-4), as well as metastases and recurrences after androgen withdrawal treatment.

4.1.2.3 Prognostic arrays

Prognostic tissue microarrays contain samples of tumours from patients for whom clinical follow-up data and clinical end-points are known (Moch *et al.*, 1999; Richter *et al.*, 2000). With the use of such a prognosis array, novel prognostic parameters can be identified, or the value of molecular alterations for the prediction of chemotherapy response can be tested.

Currently tissue microarrays have only been used in cancer research, but the technology is not limited to this field. The technique may be adapted and used in other fields such as inflammatory, cardiovascular and neurological diseases. Similarly, as for patient tissues, tissue arrays can be used for cell lines and other experimental tissues such as xenograft tumours or tissues from animal model systems.

4.2.2 The problem of tumour heterogeneity

Because of the small size of tissue cores (0.6mm) taken from paraffin-embedded tumour specimens heterogeneous expression patterns of investigated markers could lead to significant differences in results from tissue arrays compared with conventional immunohistochemical analysis (Schraml *et al.*, 1999). The number of tissue cores per tumour specimen required on an array to reduce the error rate attributable to tissue heterogeneity and to maintain efficient processing of tissue has been determined for a variety of tumour types. Hoos *et al.* studied a group of 59 fibroblastic tumours with variable protein expression patterns by immunohistochemistry for Ki-67, p53 and the retinoblastoma protein (pRB) (Hoos *et al.*, 2001). Data from conventional full tissue sections were compared with the results from one, two and three 0.6mm core biopsies on an array. Concordance for Ki-67 and p53 staining between tissue arrays with triplicate cores per tumour and full sections were 96 and 98%, respectively. For pRB staining concordance was 91%. The use of three cores per tumour resulted in lower numbers of lost cases

and lower non-concordance with standard full sections as compared to one or two cores per tumour. Correlations between phenotypes and clinical outcome were not significantly different between full sections versus array-based analysis. Camp et al. examined the number of cores required to adequately represent the expression of three common antigens in invasive breast carcinoma in 38 cases (Camp *et al.*, 2000). They compared the staining of 2 to 10 microarray cores and the whole tissue sections from which they were derived. The results showed that analysis of two cores was comparable to analysis of a whole tissue section in more than 95% of cases. Other validation studies using other tumours have shown that three cores are needed for colorectal carcinoma (Jourdan *et al.*, 2003) and three to four for prostate carcinoma (Rubin *et al.*, 2002). No validation study to date has examined melanoma tissue microarrays.

It has been suggested, however, that tissue array technology has been developed to examine tumour populations and not to survey individual tumours. Results from tumour population validation studies have borne this out. For example, Mucci *et al.*, compared results from standard slides with tissue array in 50 primary and metastatic prostate tumours, without assessing the number of cores needed to achieve high concordance with individual specimens (Mucci *et al.*, 2000). The data showed that focal neuroendocrine expression levels were similar between the two groups, supporting the use of high-density tissue arrays to screen for protein expression, even when expression is focal. Similar studies were performed on breast cancer specimens (Gillett *et al.*, 2000), on peripheral T cell lymphomas (Rassidakis *et al.*, 2002) and on lymphoid neoplasms (Hedvat *et al.*, 2002), all showing high concordance between the tumour populations in the tissue arrays and the whole specimens.

The work in this chapter was done by me apart from the sectioning and staining of the tissue microarray and whole tissue specimens.

4.3   AIMS

The aims of this chapter were:

- To validate the use of tissue array as an investigative tool for melanoma immunohistochemical research
- Assessment of a variety of melanoma associated proteins for use in predicting prognosis

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4.4   MATERIALS AND METHODS

The methodology of the construction and analysis of the tissue microarray are detailed in section 2.10. The following constitutes a summary:

A database of 222 primary melanoma patients, with a minimum of seven year follow-up, was created and relevant paraffin-embedded specimens retrieved. The specimens were sectioned and stained with Haematoxylin and Eosin (H&E) and reviewed for quality and presence of melanoma. Four core biopsies were randomly obtained from the paraffin-embedded tissue within the previously identified and marked area and transferred to a recipient paraffin block using the technique described by Kononen et al. (Kononen et al. 1998) (figure 4.2). An Excel file (Microsoft, USA) was simultaneously constructed with the coordinates of the specimen biopsies during the construction of the array.

The master block was sectioned and slides of 5μm thickness produced, which were stained using the Dako Envision method (DAKO Ltd., Cambridgeshire, UK). The slides were scored by two independent observers without prior knowledge of the clinical outcome for staining positivity and stain intensity. Cores were scored as positive if any degree of staining was evident within the melanoma cells. The tissue array was then scanned and a semi-quantitative scoring system was constructed for staining intensity based on the variation across the specimens (see figure 4.4.5). This consisted of being classified as either negative, weak, moderate or strongly staining, as compared with a positive control. The stain intensity score was taken as the strongest staining sample from the four biopsies that represented each tumour.
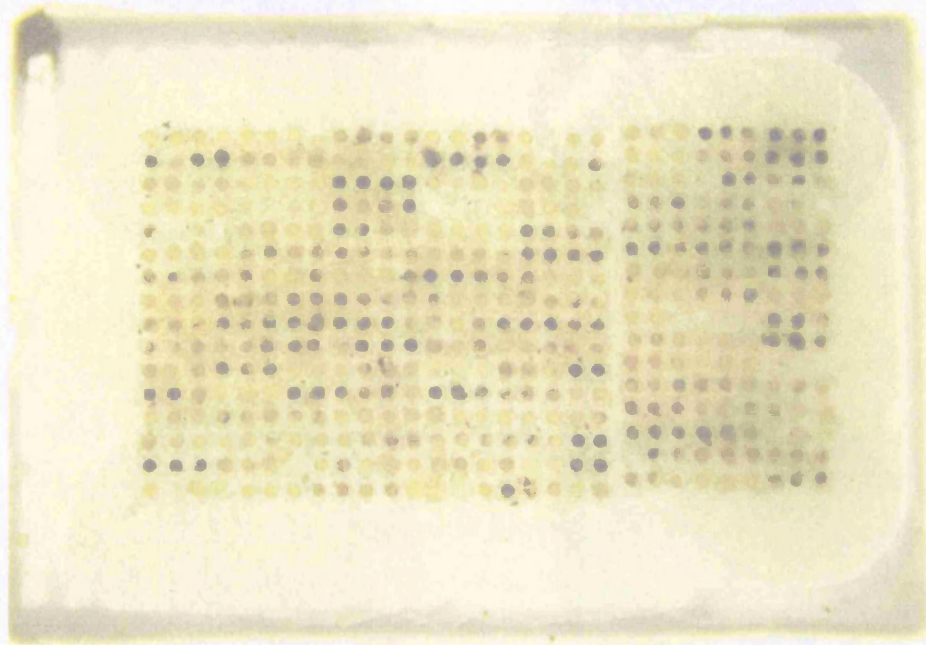
Figure 4.3 An example of a completed tissue array master block.

Assessment of association between markers and clinicopathological features was carried out using Chi-squared analysis. Univariate analysis was undertaken using the Kaplan Meier method with the JMP® statistical package (Version 4.0, SAS Institute, USA). Log Rank tests were performed on the data, which were then analysed using Chi-squared tests to obtain p values. Cox multivariate analysis was performed using SPSS (SPSS Inc., Chicago, Illinois, USA). The level of statistical significance accepted was p<0.05.

Figure 4.4.5 Four cores from the melanoma tissue array, stained in this example with LHM2, parent monoclonal antibody of the RAFT scFvs (magnification x5). The staining scores were assessed as being (in a clockwise direction from the top right) strong, negative, moderate and weak.

4.5 VALIDATION OF THE MELANOMA TISSUE MICROARRAY

4.5.1   Background

It is strongly suggested that tumours with prominent intra-tumour heterogeneity need verification of data generated by tissue microarray analysis (Kononen *et al.,* 1998). The issue of the relation between the immunohistochemical data derived from tissue microarray relative to traditional full section immunohistochemistry has not yet been clearly determined for all tumours. Currently no published data exist on the validation of tissue microarray for melanoma. The number of tissue cores per tumour specimen required on an array to reduce the error rate attributable to tissue heterogeneity and to maintain efficient processing of tissue has previously been determined for various tumours including breast carcinoma (Camp *et al.,* 2000) and human fibroblastic tumours (Hoos *et al.,* 2001). The use of a higher number of cores per tumour results in lower numbers of lost cases and lower non-concordance with full sections as compared with one or two cores. Most standard immunohistochemistry stains result in readings that distinguish between positive and negative categories, whereas others have a higher degree of complexity requiring the distinction between intensity of staining.

In an effort to validate the use of tissue microarray for melanoma immunohistochemical investigation, a study was conducted to define the concordance of 0.6mm core biopsies in quadruplicate on tissue microarray in comparison to full section analysis for expression and intensity of expression of MCAM. Although some histological detail is retained in microarray disks, it can be difficult to distinguish certain characteristics compared with examination of a whole specimen. To reduce the chances of inappropriate areas being included in the array, areas of invasive melanoma distinct from other tissue were carefully outlined. The thirty whole specimens used for comparison were chosen at random from the 120 specimens that were deemed suitable for inclusion into the final tissue microarray (see section 4.6.1). The whole specimens were sectioned and stained under the same conditions as the tissue microarray (see section 2.10) and the staining then compared between the whole sections and the array.

4.5.2 Results and discussion

All paraffin-embedded specimens were successfully sectioned and stained with H&E. Suitable areas of tumour were identified and the tissue array was constructed. The master block suffered no loss of tissue cores during processing and only one sampling error was obtained, which was excluded from further analysis. Details of the melanoma specimens included in the study and the results of their analysis are shown in Table 4.5.2.1. An example of a part of the tissue array is shown in Figure 4.5.2.1 and the corresponding whole section from which it was obtained is shown in Figure 4.2.5.2. Concordance between the tissue array analysis and whole specimen analysis is summarised in Figures 4.5.2.3. and 4.5.2.4. The results showed a significant improvement in concordance as the number of cores increase up to 4. For example, a concordance of 80% or greater for both staining positivity and staining intensity is found for a melanoma tissue array when three cores per specimen are used.
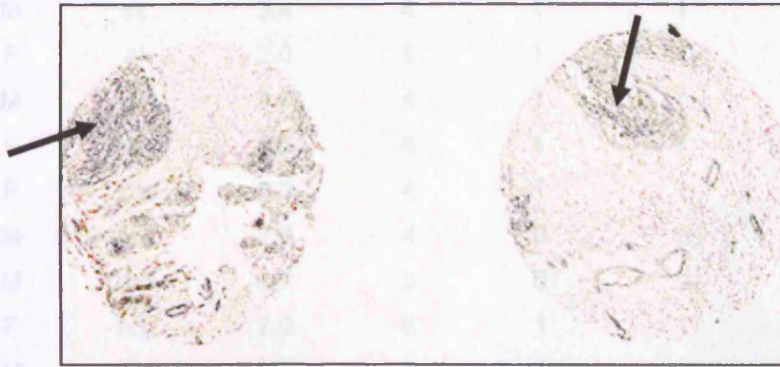
Figure 4.5.2.1 Two cores from the tissue array staining strongly for MCAM (arrows) (magnification x 5.0).
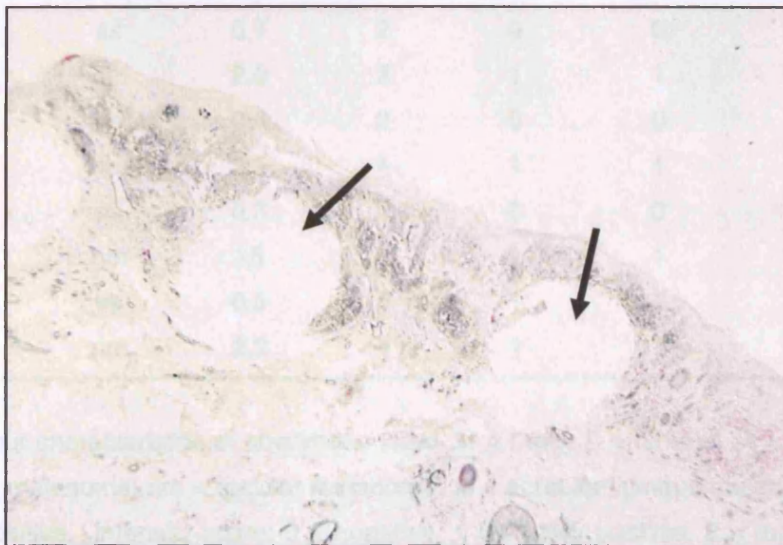
Figure 4.5.2.2 Whole section from which core biopsies in Figure 4.5.2.1 were obtained. Arrows mark the areas from which core biopsies were obtained (magnification x 2.5).

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Patient number	Sex of patient	Tumour subtype	Breslow thickness (mm)	Clark's level	Positivity score		Intensity score	
					Full section	Array (4 cores)	Full section	Array (4 cores)
1	F	ss	2.3	4	1	1	2	2
2	F	ss	1.2	4	1	0	1	0
3	M	ss	1.6	4	1	1	2	2
4	F	ss	2.0	4	1	1	3	3
5	M	lm	1.7	4	1	1	1	1
6	M	lm	1.0	4	1	1	2	2
7	M	lm	7.7	4	1	1	3	3
8	F	lm	0.7	2	1	1	1	1
9	F	lm	0.5	2	0	0	0	0
10	F	lm	3.6	4	1	1	3	3
11	M	lm	1.9	4	1	1	2	2
12	M	ss	3.4	4	1	1	2	2
13	F	al	2.0	4	1	1	2	2
14	M	al	3.4	4	1	1	1	1
15	F	nm	3.6	4	1	1	1	1
16	F	nm	2.3	4	1	1	1	1
17	M	nm	2.8	4	0	0	0	0
18	M	nm	0.7	3	0	0	0	0
19	F	nm	7.0	5	1	1	3	3
20	M	nm	3.7	4	0	0	0	0
21	F	nm	6.2	5	1	1	2	2
22	F	nm	20	5	1	1	3	3
23	F	ss	0.7	2	0	0	0	0
24	F	nm	2.0	3	1	1	3	1
25	F	ss	0.4	2	0	0	0	0
26	F	nm	2.2	4	1	1	2	2
27	F	ss	0.3	2	0	0	0	0
28	M	nm	15	5	1	1	3	3
29	M	ss	0.5	2	1	1	1	1
30	F	nm	2.2	4	1	1	2	2

Table 4.5.2.1 Tumour characteristics of specimens used. M = male, F = female; ss = superficial spreading, lm = lentigo maligna melanoma, nm = nodular melanoma, al = acral lentiginous melanoma. Positivity score: 0 = negative, 1 = positive. Intensity score: 0 = negative, 1 = weakly positive, 2 = moderately positive, 3 = strongly positive.
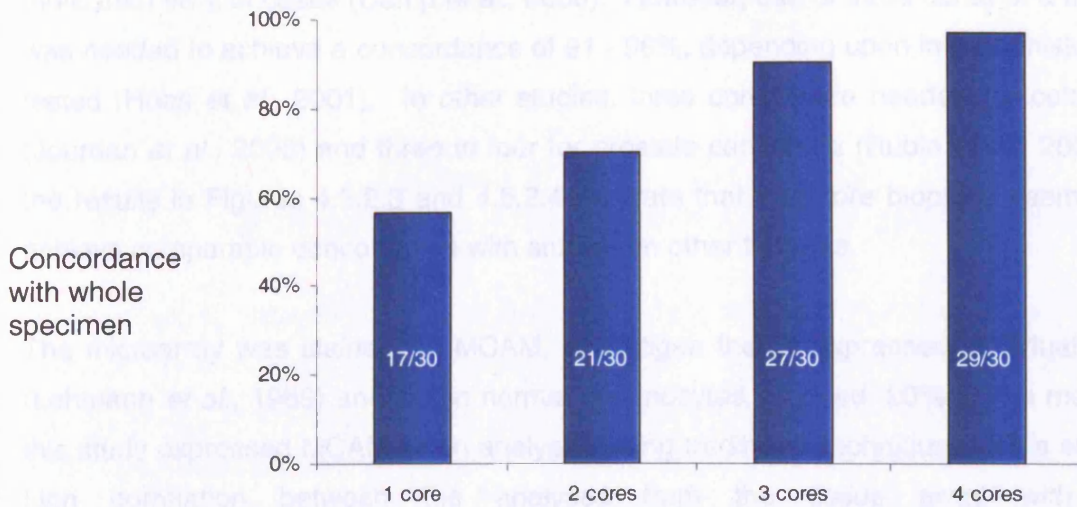
Figure 4.5.2.3   Concordance between tissue array analysis and whole specimen analysis for MCAM expression positivity (actual numbers shown in columns).

Concordance with whole specimen

(1 core: 17/30, 2 cores: 21/30, 3 cores: 27/30, 4 cores: 29/30)
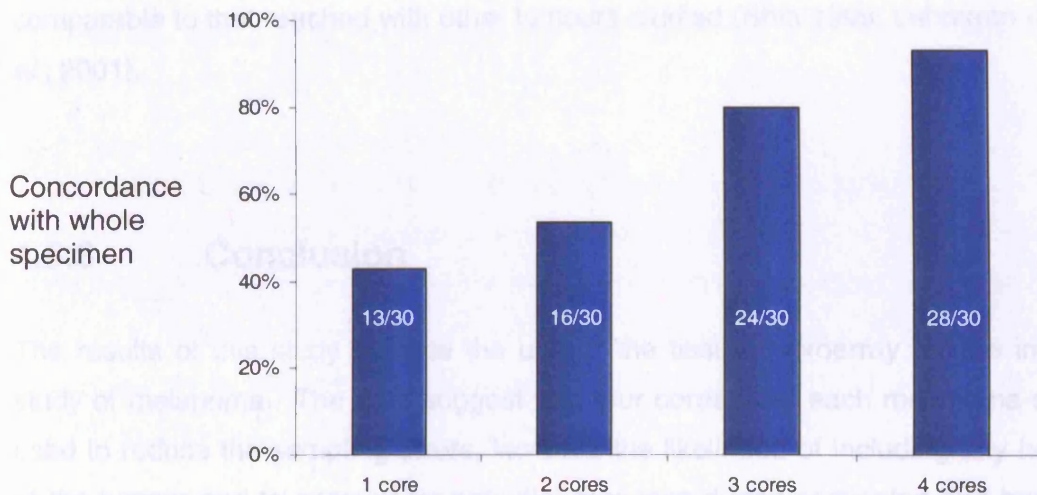
Figure 4.5.2.4   Concordance between tissue array analysis and whole specimen analysis for MCAM expression intensity (actual numbers shown in columns).

Concordance with whole specimen

(1 core: 13/30, 2 cores: 16/30, 3 cores: 24/30, 4 cores: 28/30)

One of the problems envisaged with the use of tissue array for the examination of melanoma relates to the variability in depth of melanoma specimens. Theoretically, this may not only result in technical difficulties in the construction of the array, but also in loss of cores from the array upon sectioning. No such difficulties, however, were encountered in this study. Clearly though, the depth of melanomas included in the array master block will limit the number of sections that are subsequently cut and which include all melanomas in the original array.   A study on breast

carcinoma found that use of two cores was comparable to analysis of a whole tissue section in more than 95% of cases (Camp *et al.*, 2000). However, use of three cores of a fibroblastic tumour was needed to achieve a concordance of 91 - 96%, depending upon immunohistochemical marker tested (Hoos *et al.*, 2001). In other studies, three cores were needed for colorectal carcinoma (Jourdan *et al.*, 2003) and three to four for prostate carcinoma (Rubin *et al.*, 2002). In this study the results in Figures 4.5.2.3 and 4.5.2.4 illustrate that four core biopsies seem to be needed to achieve comparable concordance with studies on other tumours.

The microarray was stained for MCAM, an antigen that is expressed in virtually all melanomas (Lehmann *et al.*, 1989) and not in normal melanocytes. Indeed, 90% of the melanomas used in this study expressed MCAM when analysed using traditional techniques. This study has shown a high correlation between the analyses from the tissue array with the traditional immunohistochemical staining techniques. This was highest, as would be expected, when MCAM positivity alone was studied, with a concordance of 96.6%. The correlation when the intensity of staining was taken into account was still high, achieving a value of 93%. These values were achieved using four core biopsies from each paraffin-embedded melanoma. The concordance is comparable to that reached with other tumours studied (Shih 1999; Lehmann *et al.*, 1989; Moch *et al.*, 2001).

4.5.3   Conclusion

The results of this study validate the use of the tissue microarray for the immunohistochemical study of melanoma. The data suggest that four cores from each melanoma specimen should be used to reduce the sampling errors, increase the likelihood of including any heterogeneous areas of the tumour and to account for potential core loss during sectioning and handling of the master block.

4.6 RESULTS OF SECTIONING AND SCORING TISSUE ARRAY

4.6.1 Clinicopathological features of array specimens

Although all 222 paraffin-embedded specimens were successfully sectioned and stained with H&E only 120 had enough archival material available for the present study and were therefore implanted into the recipient block. Suitable areas of tumour were identified and the tissue array was constructed. H&E staining of the array revealed a further 13% of specimens unsuitable for analysis due to either lack of melanoma tissue (sampling error) or loss from the block. This figure compares with two previous studies using array technology that found 10-17% unusable cores (Schraml et al., 1999; Mucci et al., 2000). This meant that 76 specimens were assessed from 4 cores, 33 from 3 cores and 8 from 2 specimens and in 3 specimens no cores were quantifiable. Detailed analysis was performed only on the 76 melanomas from which 4 cores were available, as this number was shown to be accurately representative of the whole melanoma specimen (see section 4.5).

Table 4.6.1 illustrates details of all the patients and the specimens included in the tissue microarray. Notably the median tumour thickness is considerably lower than the mean tumour thickness, which was skewed by six very thick tumours (ranging between 10mm and 35mm in thickness). Without those tumours the mean tumour thickness was calculated as 1.96mm. The majority of tumours included in the array were less than 1mm in thickness. The inter-observer disagreement when analysing the stained microarray was 5.6%.

Variable	Tissue array
Number of patients in microarray	120
Mean age at diagnosis	59.86 (range 21 – 97)
♀: ♂	1.35:1
Site of melanoma (%):	
Head & neck	23 (19%)
Trunk	26 (22%)
Upper limb	24 (20%)
Lower limb	47 (39%)
Tumour subtypes represented (%):	
Superficial spreading	67 (55.8%)
Lentigo maligna melanoma	11 (9.2%)
Nodular melanoma	35 (29.2%)
Acral lentiginous	5 (4.2%)
Other	2 (1.7%)
Mean Clark's level	3 (range 1-5)
Mean tumour thickness	2.93mm (range 0.1-35mm)
Median tumour thickness	1.6mm
Number of ulcerated melanoma (%)	26/120 (21.7%)
Number developing regional recurrence (%)	42/120 (35%)
Number of patients surviving in cohort (%)	73/120 (60.8%)

Table 4.6.1 Details of patients and melanomas included in the final tissue microarray.

4.7 MCAM AND CEACAM: THE IMMUNOGLOBULIN SUPERFAMILY

4.7.1 MCAM: expression and correlation with clinicopathological features

Figure 4.7.1 illustrates examples of cores from the array. There was a high correlation between MCAM intensity and both Clark's level and Breslow thickness (Spearman correlation p < 0.001 for both; Figures 4.7.2 & 4.7.3). Strongly staining core biopsies (intensity of 3) were all Clark's levels 3 to 5. In addition, the proportion of Clark's level 2 and 3 tumours within each intensity group decreased with intensity. This pattern was similar when compared with Breslow depth. All strongly staining tumours were over 1.01mm thick and the proportion of thick tumours increased within each intensity set. A summary of all correlations is shown in Table 4.7.1. Regarding the site of the tumour, tumours on the head and neck and the trunk were more likely to be strongly staining for MCAM.
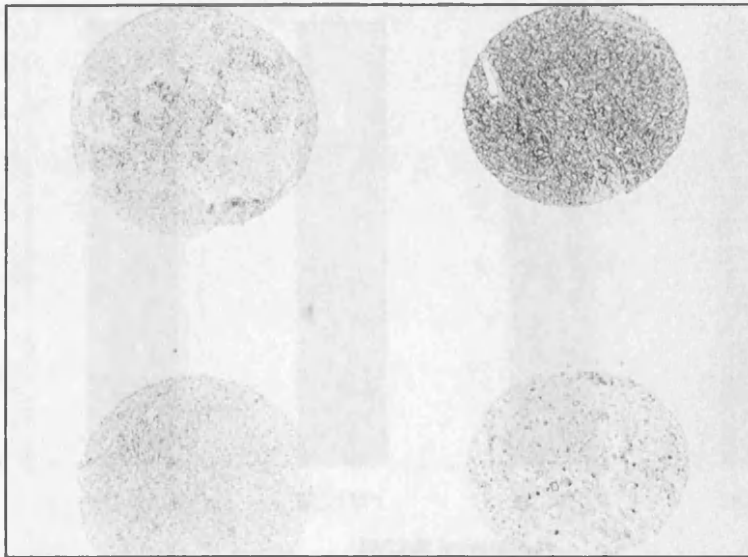
Figure 4.7.1 Core biopsy melanoma specimens stained for MCAM expression on tissue array slide (magnification x3.5). The cores were scored (in a clockwise direction starting from top right) as strong, weak, weak and moderate intensity.

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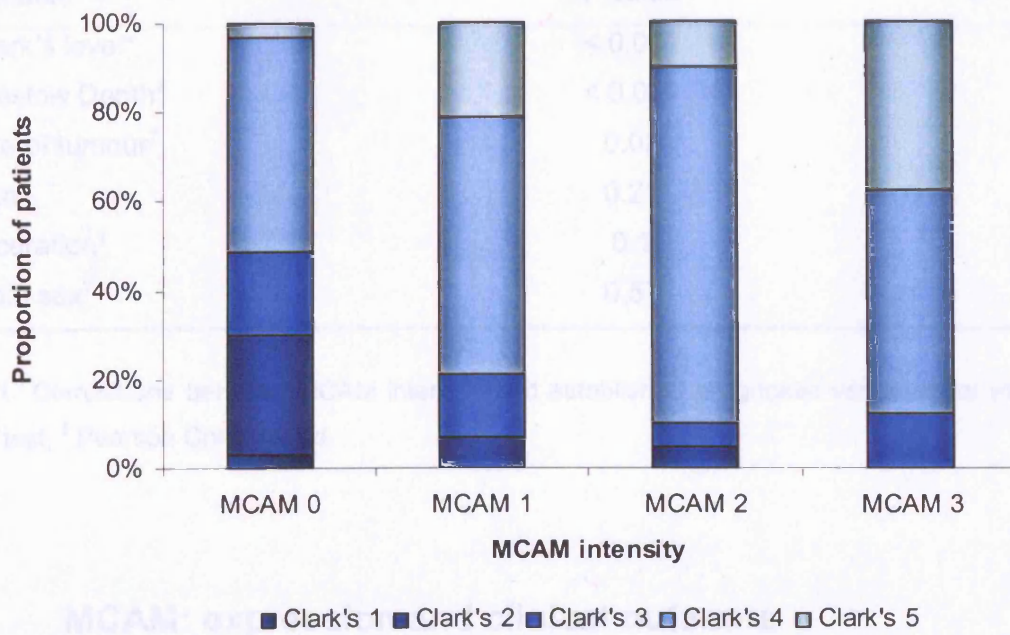
Fig 4.7.2 Figure illustrating the association of the intensity of MCAM expression with Clark's levels (Spearman's correlation p< 0.001). Cores were scored from negative (0) to strong (3).
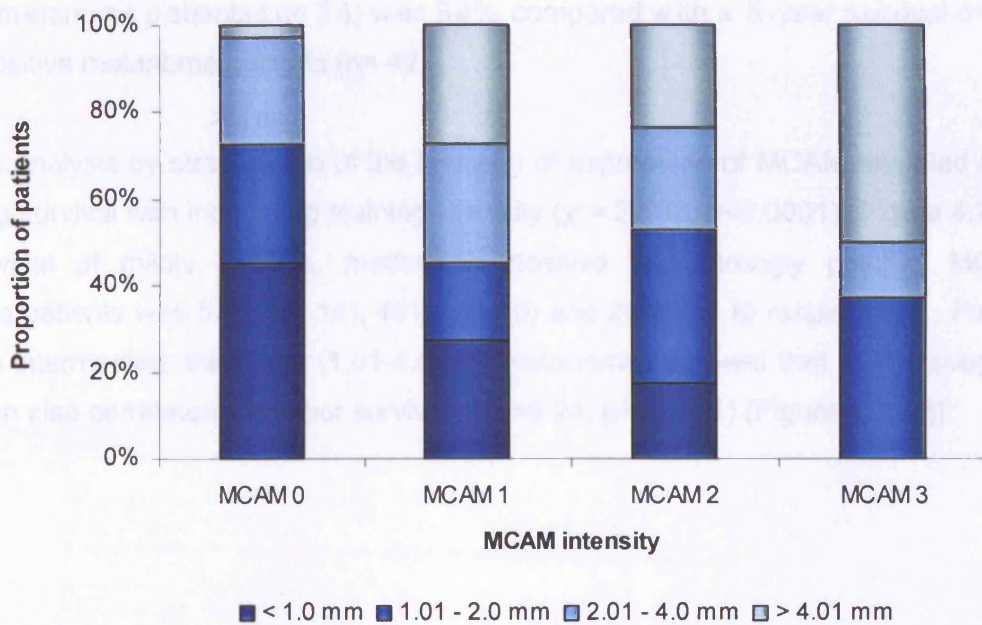
Figure 4.7.3 Figure illustrating the association between the intensity of MCAM expression and Breslow depth (Spearman's correlation p< 0.001). Breslow depth was grouped according to the "T" staging from the AJCC staging classification of melanoma. Cores were scored from negative (0) to strong (3).

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Variable	P value
Clark's level*	< 0.001
Breslow Depth*	< 0.001
Site of tumour[t]	0.03
Age[t]	0.21
Ulceration[t]	0.1
Male sex[t]	0.57

Table 4.7.1 Correlations between MCAM intensity and established prognostic variables for melanoma. * Spearman test, [t] Pearson Chi-squared.

4.7.2   MCAM: expression and clinical outcome

Figure 4.7.2.1 shows univariate survival curves in relation to MCAM expression. The log rank test of significance between Kaplan-Meier estimates of survival revealed that those patients whose tumours were MCAM positive had a significantly higher probability of poor outcome than those whose tumours were scored negative (χ^2= 18.0082, p<0.0001). The 5-year survival for the MCAM negative melanoma patients (n= 34) was 94% compared with a 5-year survival of 46% for the MCAM positive melanoma patients (n= 42).

Subgroup analysis by stratification of the intensity of expression of MCAM revealed a sequentially worsening survival with increasing staining intensity (χ^2= 22.33, p<0.0001) (Figure 4.7.2.2). The 5-year survival of mildly positive, moderately positive and strongly positive MCAM staining melanoma patients was 57% (n= 14), 46% (n= 20) and 29% (n= 8) respectively. Further analysis within the intermediate thickness (1.01-4.0mm) melanomas showed that in this subgroup, MCAM expression also correlated with poor survival (χ^2 =5.24, p=0.0221) (Figure 4.7.2.3).
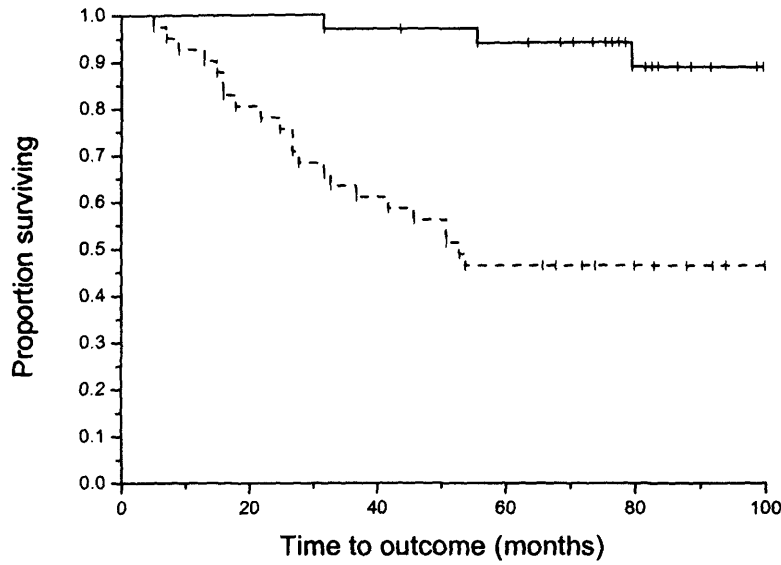
Figure 4.7.2.1 Survival curves comparing MCAM positive and MCAM negative melanomas. Log rank χ^2 = 18.0082, p<0.0001. ——— MCAM negative, ------- MCAM positive.
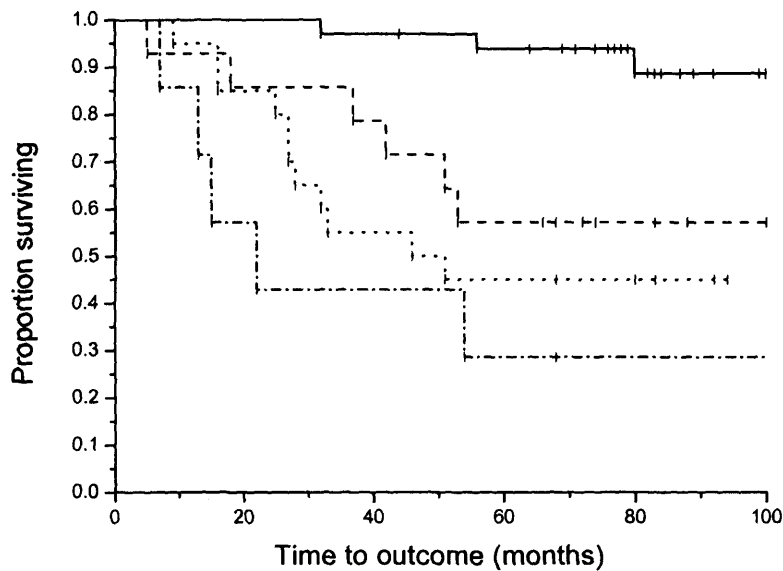
Figure 4.7.2.2 Survival curves comparing staining intensity of MCAM in primary melanomas. Log rank χ^2 = 22.3268, p<0.0001. ——— MCAM negative, ------- MCAM 1+ positive, ········ MCAM 2+ positive, . .

. MCAM 3+ positive. — — —

Proportion surviving

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0

0   20   40   60   80   100

Time to outcome (months)
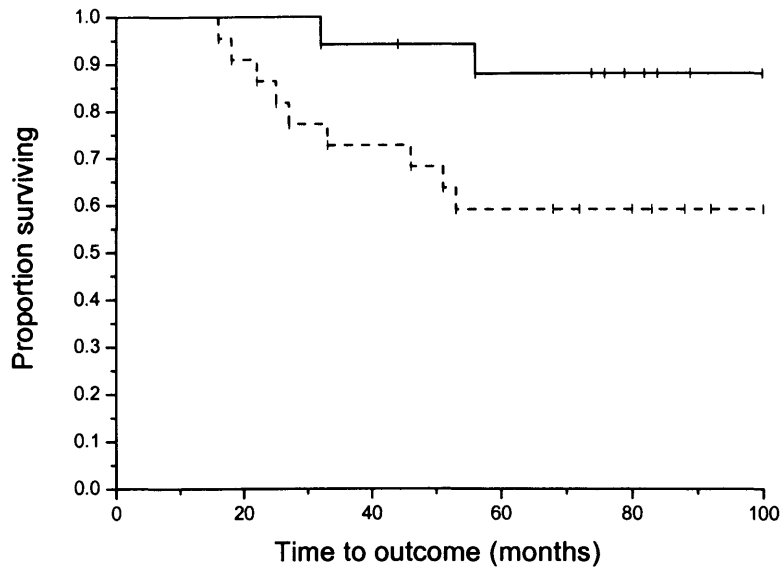
Figure 4.7.2.3 Comparison of MCAM expression in primary melanomas 1.01-4.0mm thick. Log rank χ^2 = 5.2413, p = 0.0221. ———— MCAM negative, ——————— MCAM positive.

Cox multivariate analysis showed both MCAM expression positivity and intensity were independently predictive of poor outcome (both p <0.001). In both cases this was over and above established m arkers o f prognosis, such a s B reslow t hickness a nd C lark's l evel. F or p atients whose melanomas expressed MCAM, the risk of poor outcome was 16-fold higher compared with those patients whose melanomas did not express this cell adhesion molecule (Table 4.7.1).

		95% CI for the Hazard Risk		
Variable	Hazard risk	Lower	Upper	P-value
MCAM expression	16.335	3.797	70.280	<0.001
Site	0.524	0.342	0.801	0.003
Clark's level	3.686	1.330	10.219	0.012
Breslow thickness	1.119	1.006	1.246	0.039
Age	1.020	0.993	1.049	0.154
Ulceration	1.479	0.571	3.829	0.420
Male sex	0.773	0.311	1.918	0.579

Table 4.7.1 Cox multivariate analysis including MCAM expression and other established markers of prognosis. MCAM expression positivity can be seen to be independently predictive of poor prognosis.

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4.7.3   Discussion

Conventional immunohistochemistry has shown that certain cancer phenotypes correlate with patient outcome (Drobnjak *et al.,* 1994; Heslin *et al.,* 1998; Thies *et al.,* 2002). With the introduction of novel therapies to be used in high-risk patients such as interferon alpha (Kirkwood *et al.,* 2001), the prediction of patient outcome is essential in the management of melanoma. In addition to providing the clinician with information useful in determining management, it facilitates appropriate patient follow-up regimes and is of incalculable psychological benefit for the patient.

Previous work *in vitro* and *in vivo* suggested the potential of MCAM as a prognostic marker in melanoma (section 4.1.6.1). The present study was therefore designed to investigate the expression of MCAM in melanoma and to correlate its expression positivity and intensity with patient outcome. The results demonstrated that expression of MCAM in primary cutaneous melanoma was significantly associated with poor outcome ($p < 0.0001$). Multivariate Cox regression analysis showed that MCAM expression is an independent predictor of adverse outcome ($p < 0.0001$). Patients with primary cutaneous melanoma who did not express MCAM had a significantly lower risk of dying from the disease than patients that were MCAM positive (RR = 16; table 4.7.1). Thus, analysis of survival including MCAM expression may improve risk estimation of survival in melanoma patients.

The considerable predictive power of MCAM expression is highlighted by incorporating other known prognostic factors including Clark's level, Breslow thickness, presence of ulceration, sex and age of the patient (Balch *et al.,* 2001a) in a Cox multivariate analysis. When this tool is used, only MCAM, Clark's level, Breslow thickness and site remained as independent predictors for outcome in our study. The reason for Clark's level remaining significant is likely to be the high proportion of tumours included in the array that were less than 1 mm thick (34%) (Balch *et al.,* 2001a&b).

Furthermore, this investigation has demonstrated that not only MCAM expression, but also MCAM intensity, was correlated with survival. This suggests that the degree of MCAM upregulation is associated with the disease course. This was revealed in the log rank tests on the Kaplan Meier estimates analysed by Chi-squared tests and was corroborated by Cox multivariate analysis. In addition, univariate analysis showed that the survival of those patients with tumours of intermediate thickness could be predicted by MCAM expression positivity (Figure 4.7.2.3). Tumour thickness alone showed no statistically significant correlation with survival within this subgroup,

suggesting that MCAM expression positivity is a more powerful tool for the prediction of prognosis for intermediate thickness melanomas.

Currently tumour thickness and the presence of ulceration have been shown to be the most powerful predictors of outcome in patients with primary cutaneous melanoma, apart from patients with tumours less than 1mm thick, in which the Clark's level becomes the most important factor (Balch *et al.*, 2001a & b). Some patients with thin tumours at the time of surgery may develop terminal metastatic disease, whereas other patients with thick melanomas survive (Fearfield *et al.*, 2001; Salti *et al.*, 2002). This emphasises the need to improve the accuracy of risk estimation. A variety of immunohistochemical markers have been identified as adjunctive prognostic parameters in primary human melanomas, including loss of melastatin expression (Duncan *et al.*, 2001), expression of $ß_3$ integrin (Hieken *et al.*, 1996) and CEACAM1 expression (Thies *et al.*, 2002). These studies are comparable to the current study as their cohorts include at least 100 patients with follow-up periods of 5 to 10 years and multivariate analysis was used to establish the independent prognostic significance of these markers.

Determination of MCAM expression status is not only of prognostic interest but may also provide insights into the mechanisms of tumour cell progression and metastasis formation in melanoma. No previous study has used a Cox multivariate model in addition to univariate analysis to demonstrate that upregulation of MCAM expression in a tumour is associated with poor outcome. MCAM has previously been tested on a large cohort of melanoma specimens (n= 386) in a study by Ostemeier *et al.*, yet despite showing a correlation with survival in univariate analysis, the study failed to show prognostic significance when multivariate analysis was used (Ostmeier *et al.*, 2001). This study was performed using melanoma biopsies on cryopreserved specimens. Obtaining frozen sections of primary melanomas is unlikely to be practical and the authors acknowledged that studies with paraffin-embedded material had to be performed. In addition, only one biopsy was taken from each tumour specimen leaving the possibility that non-representative parts of the biopsies may have been tested. The anti-MCAM antibody used in Ostemeier's study was not the commercially available antibody used in the present study. MCAM epitopes can be differentially expressed (Kraus *et al.*, 1997) and this may further explain differences in outcomes between the two studies.

MCAM is a predominant cell adhesion molecule of human melanomas that also mediates homotypic aggregation of melanoma cells (Johnson 1999). During progression of melanocytic lesions from benign to malignant forms, there is a dynamic shift in expression of adhesion molecules. This shift reflects the aggressive properties of melanoma cells as they invade the

dermis from the epidermis (Johnson 1999). Cell-cell adhesion molecules show similar changes during melanoma progression. For example, among the cadherins there is a shift from E-cadherins to N-cadherin (Hsu *et al.*, 2000). *In vitro* studies have shown that human melanoma cell lines adhere to immobilised MCAM (Shih *et al.*, 1997) by expressing an as yet unidentified ligand. Animal studies have revealed correlations between MCAM expression and the ability of human melanoma cell lines to form metastases (Luca *et al.*, 1993). In one study, enforced MCAM expression on non-metastatic MCAM-negative primary cutaneous melanoma cells resulted in an increased metastatic capacity in nude mice (Xie *et al.*, 1997). This property was reversible by treatment of mice with anti-MCAM antibody.

On the basis of these findings, MCAM, as the major adhesion molecule for melanoma-melanoma cell adhesion, has been suggested to play a role in melanoma progression, including the establishment of close cell-cell communication that stimulates growth, survival and invasion. However, despite many assumptions in the literature, MCAM expression positivity and intensity had not been shown to correlate with outcome in patient studies.

This study showed that MCAM expression correlates significantly with patient outcome. MCAM expression was upregulated in those patients with a poor prognosis compared with others. This was still the case in patients with intermediate thickness tumours in whom tumour thickness alone could not predict outcome.

4.7.4 CEACAM1: expression and correlation with clinicopathological features

Breslow depth was the only clinicopathological feature that was significantly correlated with CEACAM1. A higher proportion of thick tumours expressed CEACAM1 compared with thin tumours (Pearson Chi-squared 0.013, p = 0.012) (Figure 4.74). Thin tumours (< 2.0mm) were mostly negative for this marker. Interestingly, there was no relationship between CEACAM1 expression and other prognostic indicators tested including Clark's level.
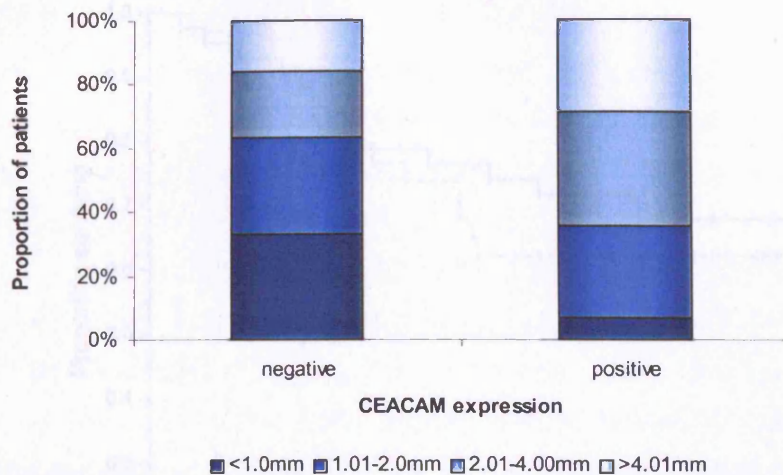
Figure 4.7.4 Figure illustrating the association between the expression of CEACAM1 and Breslow depth (Spearman's correlation p = 0.012). Breslow depth was grouped according to the "T" staging of the AJCC staging classification of melanoma.

4.7.5   CEACAM1: expression and clinical outcome

There was a trend of patients with CEACAM1 negative tumours (n = 36) to do better than those with positive tumours (n = 40), however CEACAM1 expression failed to show a statistically significant correlation with patient outcome in this series of patients (Log rank χ^2 = 0.2858, p = 0.59) (Figure 4.7.5.1). Cox multivariate analysis also failed to show CEACAM1 as an independent predictor of prognosis (p = 0.151) (Table 4.7.5.1). Breslow thickness and Clark's level were the only two of the other established clinicopathological markers of prognosis that retained significance in the Cox model used.

Figure 4.7.5.1 Survival curves comparing CEACAM1 positive and CEACAM1 negative melanomas. Log rank χ^2 = 0.2858, p = 0.59. ———— CEACAM1 negative, -------- CEACAM1 positive.

| Variable | Hazard risk | 95% CI for the Hazard Risk | | P-value |
		Lower	Upper	
Breslow thickness	1.159	1.044	1.287	0.006
Clark's level	3.189	1.356	7.503	0.008
CEACAM1	1.899	0.792	4.553	0.151
Age	1.013	0.986	1.040	0.362
Site	0.833	0.586	1.184	0.309
Male sex	0.756	0.309	1.850	0.540
Ulceration	1.247	0.508	3.066	0.630

Table 4.7.5.1 Cox multivariate analysis including CEACAM1 expression and other established markers of prognosis. CEACAM1 expression is not independently predictive of prognosis.

4.7.6   Discussion

Having established MCAM as an independent marker that may be used to predict prognosis, CEACAM1 was studied using the tissue array. CEACAM1 or CD66a, formerly known as biliary glycoprotein I, is a member of the carcinoembryonic antigen family and also, like MCAM, of the immunoglobulin superfamily (Thompson *et al.*, 1991). This study of CEACAM1 expression on melanoma, using a tissue microarray, did not show a statistically significant correlation between CEACAM1 expression and outcome, despite demonstrating a statistically significant relationship between tumour thickness and marker expression. Using the log rank tests on the Kaplan Meier estimates analysed by Chi-squared tests a trend was apparent, suggesting that patients with tumours not expressing CEACAM1 fared better than those with tumours that were positive. This finding is supported by the results of the only published series examining CEACAM1 expression and its use as a possible prognostic marker. Thies et al. used traditional whole specimen staining of 100 primary cutaneous melanomas with a 10-year follow up (Thies *et al.*, 2002). A total of 28 of 40 patients with CEACAM1-positive primary melanomas developed metastatic disease, compared with only six of 60 patients with CEACAM1-negative melanomas. In their study, Kaplan Meier analysis revealed a highly significant association between CEACAM expression and survival (p <0.0001). Furthermore, multivariate Cox regression analysis, including CEACAM1 expression adjusted for tumour thickness, presence of ulceration and mitotic rate showed CEACAM1 to be an independent predictor of prognosis. However, in the study by Thies and colleagues, other established variables for predicting prognosis were not included in the Cox model and no comment is made in the discussion as to how this would have affected the result. It is possible that had the present study included a larger cohort of patients the trend suggested by the univariate survival curves would have reached significance, mirroring the results of the study by Thies *et al.*

CEACAM1 is down-regulated in malignant tumours originating from glandular tumours, particularly in colorectal (Neumaier *et al.*, 1993) and prostate cancer (Luo *et al.*, 1999), where its loss of expression is related to the loss of glandular differentiation (Busch *et al.*, 2002). This is especially apparent in prostate cancer, where a sharp decline of CEACAM1 expression is noted when glands fuse (Busch *et al.*, 2002).

The only ligands of the extracellular domain of CEACAM1 identified so far are CEACAM1 itself and CEACAM6 (Oikawa *et al.*, 1992). Thies *et al.* suggested that both homophilic and heterophilic interactions of CEACAM1 may be relevant during glandular differentiation (Thies *et al.*, 2002). The role of heterophilic interactions is apparent in endothelial cells, where CEACAM1 expression is

observed on the albuminal side of the microvessels (Ergün *et al.,* 2000), suggesting interaction with an unidentified ligand in the surrounding basal lamina. That these interactions with the basal lamina are of physiological relevance is indicated by the fact that the cytoplasmic domain of CEACAM1 interacts with the cytoskeleton (Sadekova *et al.,* 2000) and may therefore be an important mediator of cell-matrix interactions. In the study by Thies *et al.* CEACAM1 expression was shown to be particularly upregulated at the invading front, where cell-matrix interactions play an important role, thus implying heterotypic interaction of CEACAM1 with matrix molecules (Thies *et al.,* 2002). This is in accordance with findings that expression of CEACAM1 and β_3 integrin, which has an established role in the invasion and metastasis of melanoma (Hsu *et al.,* 1998), is found localised at the invading front (Brummer *et al.,* 2001). A potential function of CEACAM1 in invasion is corroborated by the findings that CEACAM1 is strongly expressed by the intermediate trophoblast at the implantation site, as well as the extravillous trophoblast cells with an invasive phenotype in primary culture, suggesting that CEACAM1 may be functionally involved in the motility or invasiveness of cells (Bamberger *et al.,* 2000). Thus, CEACAM1 may play a role in melanoma metastasis formation by facilitating the invasion of blood vessels and lymphatics as well as migration to distant sites.

In conclusion, this study demonstrated a correlation between CEACAM1 expression and tumour thickness and suggested that a link between CEACAM1 and outcome exists, supporting the findings of Thies *et al.* (2002). However, this was not statistically significant and shows that tissue array analysis is not necessarily superior to traditional immunohistochemistry. Further studies are needed on larger cohorts of patients to determine whether adding power to the study would result in statistically significant results.

4.8   CADHERINS

4.8.1 P- and E-cadherin: correlation with clinicopathological features

There were no correlations between P-cadherin expression and any of the established variables for melanoma p rognosis. The s ame w as f ound for E -cadherin i n a ll e xcept for s ite o f t umour, which had a highly significant relationship (Pearson Chi-squared 0.003, p = 0.002). Most melanomas strongly-staining for E-cadherin were situated on the legs, whilst most melanomas that were negative for E-cadherin were situated on the arms and head and neck regions.

4.8.2 P- and E-cadherin: expression and clinical outcome

Expression o f P -cadherin s howed a h ighly s tatistically s ignificant c orrelation w ith o utcome (Log rank χ^2 = 10.1336, p = 0.0015) (Figure 4.8.2.1). Patients with tumours that were negative for P-cadherin correlated with poorer outcomes than those whose tumours expressed P-cadherin. The 5-year survival for P-cadherin negative melanoma patients was 40% (n=24) compared with a 5-year survival of 75% for the P-cadherin positive patients (n=54).

On further stratification of P-cadherin positive tumours into weak and strong staining, a statistically significant difference in staining intensity with outcome was revealed (Log rank χ^2 = 14.19, p = 0.0008) (Figure 4.8.2.2). Interestingly, patients with weakly positive P-cadherin expression had a better prognosis than patients with high P-cadherin expression (see discussion, section 4.8.3).
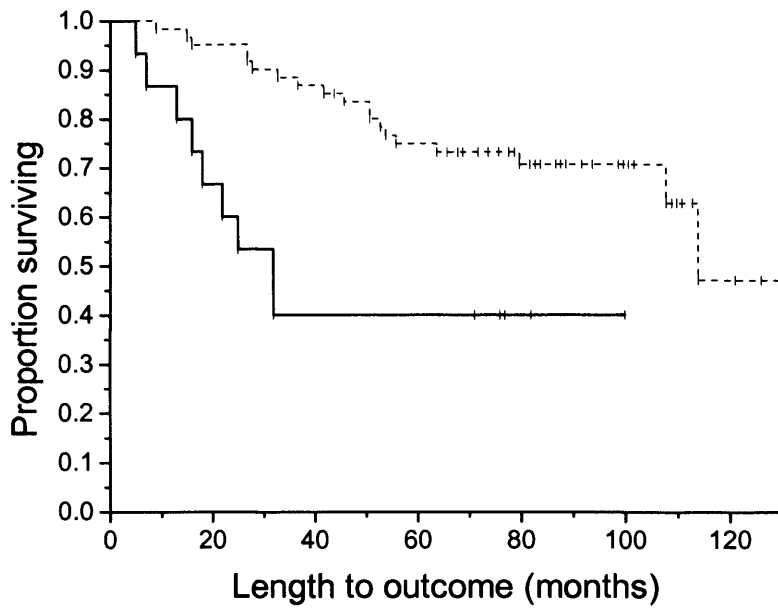
Figure 4.8.2.1 Survival curves comparing P-cadherin positive and P-cadherin negative melanomas. Log rank χ^2 = 10.1336, p = 0.0015. ——— P-cadherin negative, ------- P-cadherin positive.
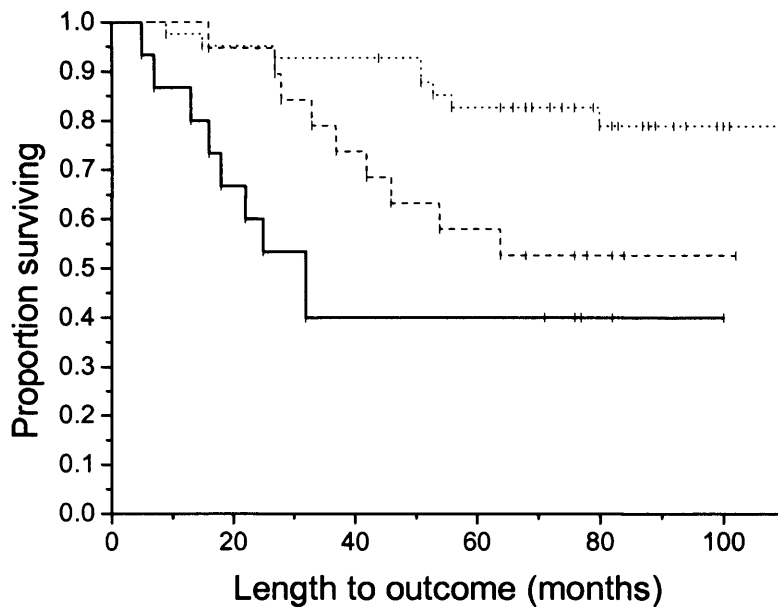
Figure 4.8.2.2 Survival curves comparing staining intensity of P-Cadherin in primary melanomas. Log rank χ^2 = 14.19, p = 0.0008. ——— P-cadherin negative, ·······P-cadherin 1+ positive, ------- P-cadherin 2+ positive.

Cox multivariate analysis on the data showed that P-cadherin expression did not quite reach statistical significance as an independent marker of prognosis. In this model, only Breslow thickness and Clark's level retained independence, over and above other established clinicopathological variables (Table 4.8.2).

| Variable | Hazard risk | 95% CI for the Hazard Risk | | P-value |
		Lower	Upper	
Clark's level	3.001	1.303	6.910	0.010
Breslow thickness	1.153	1.034	1.285	0.011
P-cadherin	0.440	0.172	1.125	0.087
Site	0.813	0.574	1.151	0.242
Age	1.013	0.986	1.040	0.359
Male sex	0.809	0.337	1.945	0.636
Ulceration	1.170	0.466	2.939	0.738

Table 4.8.2 Cox multivariate analysis including P-cadherin expression and other established markers of prognosis. P-cadherin expression was not shown to be independently predictive of poor prognosis.

Loss of E-cadherin expression, similar to P-cadherin, was correlated with a poorer outcome (Figure 4.8.2.3). However, this result did not reach significance in the log rank test of significance between Kaplan-Meier estimates of survival (Log rank χ^2 = 0.7807, p = 0.3769).
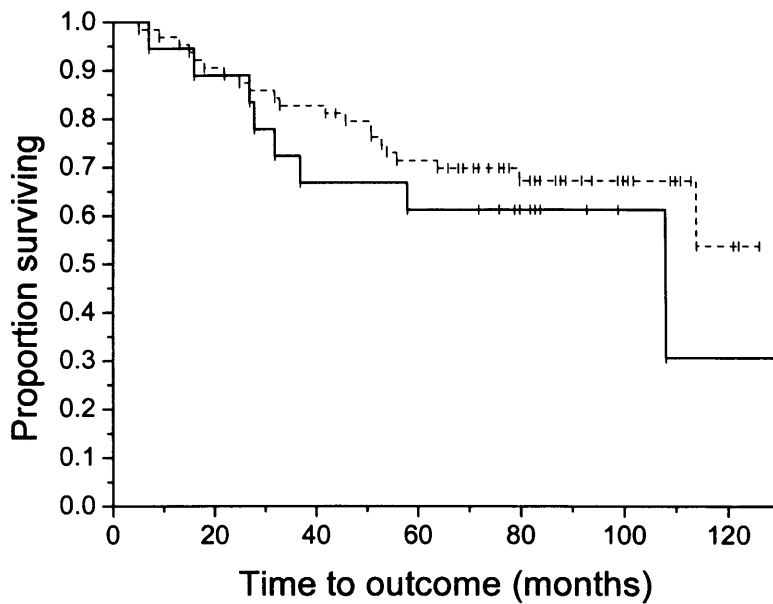
Figure 4.8.2.3 Survival curves comparing E-cadherin positive and E-cadherin negative melanomas. Log rank χ^2 = 0.7807, p = 0.377. ———— E-cadherin negative, -------- E-cadherin positive.

4.8.3   Discussion

The results of this study examining the relationship between P- and E-cadherin and melanoma patient survival have demonstrated that loss of expression of both markers appears to be associated with poor prognosis. This is especially true of P-cadherin, where a highly statistically significant difference in outcome between patients whose tumours expressed P-cadherin and those whose tumours did not, was revealed (χ^2 = 10.1336, p = 0.0015). Stratification of P-cadherin positive tumours into strong and weak staining again revealed a statistically significant relationship between the intensity of P-cadherin expression and outcome. Interestingly, patients with the weak-staining tumours fared best, those with strong-staining tumours did badly, but patients with P-cadherin negative tumours had the poorest outcome; this will be further discussed below. P-cadherin expression did not retain significance as an independent marker of prognosis when analysed in a Cox regression analysis with other established prognostic variables (p = 0.087).

Previous studies on E-cadherin expression had used cell culture, or compared benign with malignant lesions. This suggested a link between loss of E-cadherin expression and melanoma progression. However, no study to date had examined the relationship between E-cadherin

expression and melanoma patient outcome. In this study, loss of E-cadherin expression tended to be associated with poor outcome. However, this did not reach statistical significance ($\chi^2 = 0.7807$, $p = 0.3769$) (Figure 4.8.2.3). This result may have been explained by the difference in patient numbers in each group (E-cadherin positive n = 19 and negative n = 57).

Loss of E-cadherin is associated with high-grade morphology of tumours and there is a significant inverse relationship between E-cadherin expression and survival in some tumour patients, including colorectal cancer (Dorudi *et al.*, 1993), breast cancer (Moll *et al.*, 1993; Siitonen *et al.*, 1996) and head and neck cancers (Schipper *et al.*, 1991). Normal cultured human melanocytes express both E-cadherin and P-cadherin, but it is E-cadherin that is primarily responsible for adhesion of melanocytes to keratinocytes (Tang *et al.*, 1994, Hsu *et al.*, 1996). Danen *et al.* found E-cadherin to b e e xpressed o n c ultured n ormal melanocytes a nd n aevus c ells, w hilst t wo n on-invasive, non-metastatic melanoma cell lines showed low expression and four invasive, metastatic melanoma cell lines did not express E-cadherin (Danen *et al.*, 1996). Moreover, melanoma cells transduced with E-cadherin lost their invasive capacity (Hsu *et al.*, 2000). Thus a role for the loss of E-cadherin expression in melanoma metastasis has been proposed. Loss of E-cadherin appears to be one of the critical steps in progression of melanoma, because it may trigger the release of cancer cells from the primary growth focus (Christifori & Semb 1999). This process is not only due to loss of physical adhesion but also to multiple events that lead to uncontrolled proliferation and progressive i nvasion (Guilford 1999). After the loss of E-cadherin expression, melanocytic cells can escape from neighbouring keratinocytes and invade the dermis.

Conflicting data have been published on cadherin expression in primary melanoma. Seline *et al.* found that t he expression of both E- and P-cadherin varied inversely with disease progression (Seline *et al.*, 1996) and proposed that loss of cadherin expression is an inevitable feature of tumour progression. Sanders and colleagues used immunohistochemistry and Western blot after tissue fractionation of surgically excised dysplastic naevi and melanoma specimens to determine cadherin expression (Sanders *et al.*, 1999). Loss of E-cadherin was seen in a small number of vertical growth phase melanomas only when metastasis occurred and E-cadherin was retained in all radial growth phase tumours. There was, however, a "dramatic" loss of P-cadherin expression in vertical growth phase melanomas with metastasis formation. These results suggest that shifts in cadherin profiles occurred late in the biological progression of melanocytic tumours and were similar to a study by Danen *et al.* who found higher E-cadherin expression in advanced primary melanomas than in early primaries (Danen *et al.*, 1996). The study by Danen and colleagues, however, used only small sample sizes: 7 early primary melanomas and 20 "advanced primary" (>1.5mm), of which only 4 advanced primary melanomas stained positively. Conversely, Silye *et*

al. showed E-cadherin expression to be absent in melanomas in their radial growth phase and in Clark's level II and III lesions, whereas E-cadherin was expressed in a high proportion of melanomas in the vertical growth phase, in Clark's level IV and V lesions and in metastasising melanomas (Silye *et al.,* 1998). These results were in agreement with a study by Cowley *et al.* who found that E-cadherin expression tended to be higher in melanomas than in naevi (Cowley *et al.,* 1996), but again this study used relatively small sample sizes (11 benign lesions and 14 melanomas).

In the present study the association of poor outcome with loss of E-cadherin expression did not reach significance. It has been proposed that although E-cadherin expression in melanomas implies potential preservation of adhesive function, normal cadherin expression does not always equate to normal function, because cadherin function can be modified by both the specificity and amount of different cadherins on cell surfaces (Steinberg & Takeichi 1994) and cadherin glycosylation (Yoshimura *et al.,* 1996). This may explain why retention of E-cadherin expression did not confer a survival advantage to patients whose tumours expressed this marker in the present study. The conflicting evidence presented as to the shift in cadherin profiles with melanoma progression may be explained by a loss of cadherin in early melanoma disease progression, followed by re-expression in more advanced forms of the disease, (Seline *et al.,* 1996; Danen *et al.,* 1996; Silye *et al.,* 1998; Sanders *et al.,* 1999). This theory proposes that melanoma cells initially escape keratinocyte control or dominance with the loss of cadherin expression. Once the melanoma cells have developed a metastatic phenotype the cadherins may be re-expressed enabling the cells to reattach and form new foci of growth. This model would also explain the results of the investigation examining the intensity of expression of P-cadherin and patient outcome. Patients with tumours with the weakest P-cadherin expression fared best, those whose tumours had complete loss of P-cadherin fared worst and the outcome of those with strong P-cadherin expression was in-between.

4.9 CD44V3 AND NM23

4.9.1 CD44v3 and Nm23: expression and correlation with clinicopathological features

The expression of CD44v3 was highly correlated with Clark's level, Breslow thickness and patient age (Table 4.9.1.1). The strong correlation between CD44v3 intensity and both Clark's level and Breslow thickness (Spearman correlation $p < 0.001$ for both) is illustrated in Figures 4.9.1.1 & 4.9.1.2. Melanomas with the strongest CD44v3 staining intensity were all less than 4.0 mm in depth. Similarly, the proportion of Clark's level 5 tumours was highest in the negatively stained group, and decreased in proportion with increasing intensity of staining. The observed staining pattern is consistent with a role of CD44v3 expression early in melanoma progression.

Variable	P value
Clark's level*	< 0.001
Breslow Depth*	< 0.001
Age[t]	< 0.001
Ulceration[t]	0.113
Site of tumour[t]	0.31
Male sex[t]	0.833

Table 4.9.1.1 Correlations between CD44v3 staining intensity and established prognostic variables for melanoma. * Spearman test, [t] Pearson Chi-squared.

■ Clark's 1 ■ Clark's 2 ■ Clark's 3 ▣ Clark's 4 ☐ Clark's 5

Figure 4.9.1.1 Figure illustrating the association of the intensity of CD44v3 expression with Clark's levels (Spearman's correlation p< 0.001). The staining intensity was scored as negative (0), weak (1), moderate (2) or strong (3).
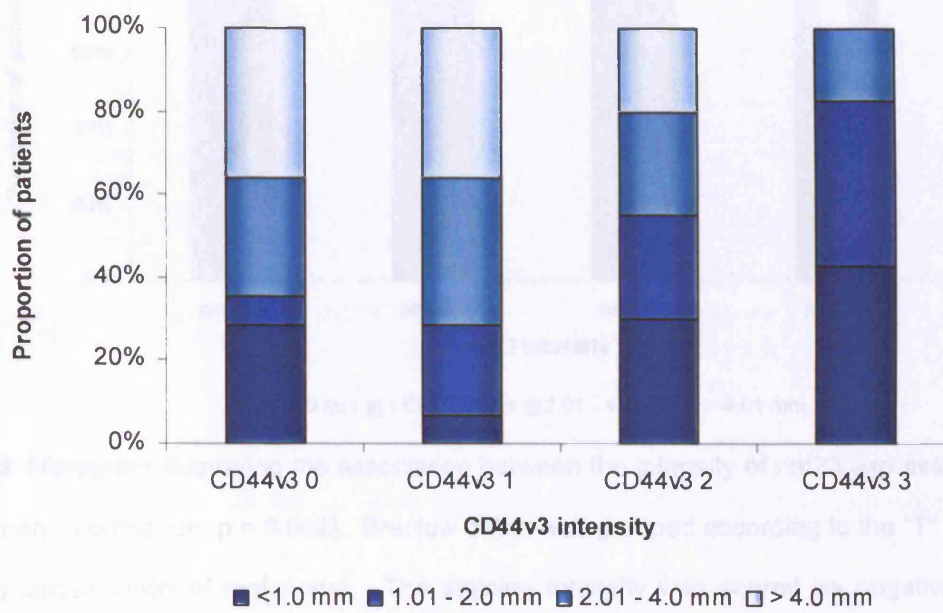
■ <1.0 mm ■ 1.01 - 2.0 mm ■ 2.01 - 4.0 mm ☐ > 4.0 mm

Figure 4.9.1.2 Figure illustrating the association between the intensity of CD44v3 expression and Breslow depth (Spearman's correlation p< 0.001). Breslow depth was grouped according to the "T" staging of the AJCC staging classification of melanoma. The staining intensity was scored as negative (0), weak (1), moderate (2) or strong (3).

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Nm23 expression was strongly correlated with Clark's level, Breslow depth and patient age, whilst a trend towards an association with tumour ulceration was also observed (table 4.9.1.2)

Variable	P value
Clark's level*	<0.001
Breslow Depth*	0.002
Age[†]	0.014
Ulceration[†]	0.069
Site of tumour[†]	0.551
Male sex[†]	0.934

Table 4.9.1.2 Correlations between nm23 intensity and established prognostic variables for melanoma. * Spearman test, [†] Pearson Chi-squared.
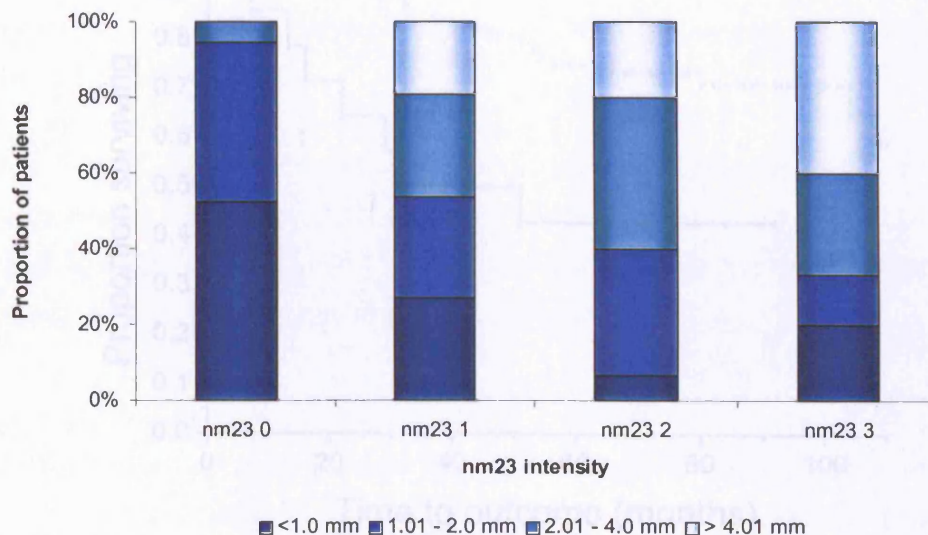
Figure 4.9.1.3 Histogram illustrating the association between the intensity of nm23 expression and Breslow depth (Spearman's correlation p = 0.002). Breslow depth was grouped according to the "T" staging from the AJCC staging classification of melanoma. The staining intensity was scored as negative (0), weak (1), moderate (2) or strong (3).

Figure 4.9.1.3 illustrates that no negatively staining tumours were over 4.01mm and most were less than 2.01mm in depth. A clear positive correlation of staining intensity with Breslow thickness and Clark's level was observed (Figure 4.9.1.3 and data not shown).

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4.9.2 CD44v3 and nm23: expression and clinical outcome

Figure 4.9.2.1 shows univariate survival curves in relation to CD44v3 expression. The log rank test of significance between Kaplan-Meier estimates of survival showed that those patients whose tumours were CD44v3 negative had a significantly higher probability of poor outcome than those whose tumours were CD44v3 positive (χ^2= 7.2219, p = 0.0072). The 5-year survival for the CD44v3 negative melanoma patients (n= 20) was 43% compared with a 5-year survival of 75% for the CD44v3 positive melanoma patients (n= 57).
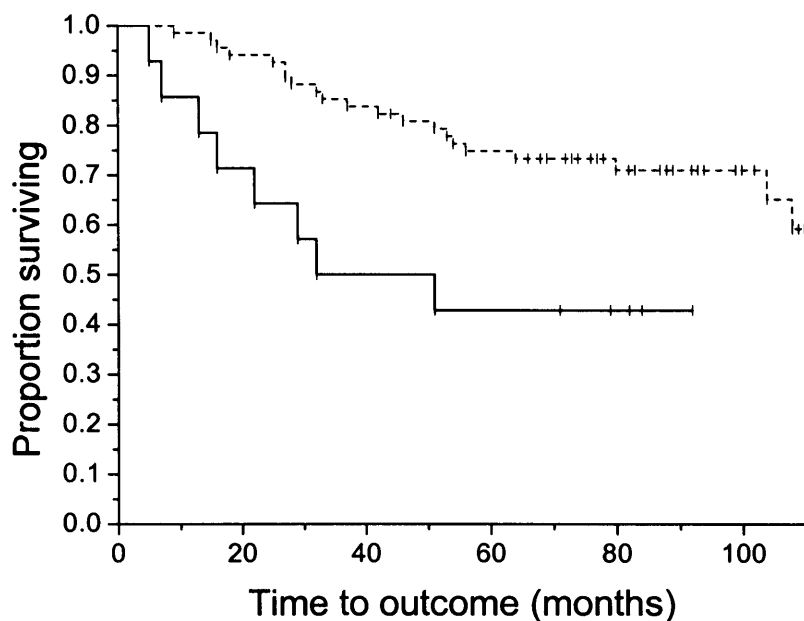
Figure 4.9.2.1 Survival curves comparing CD44v3 positive and CD44v3 negative melanomas. Log rank χ^2 = 7.2219, p = 0.0072. ———— CD44v3 negative, -------- CD44v3 positive.
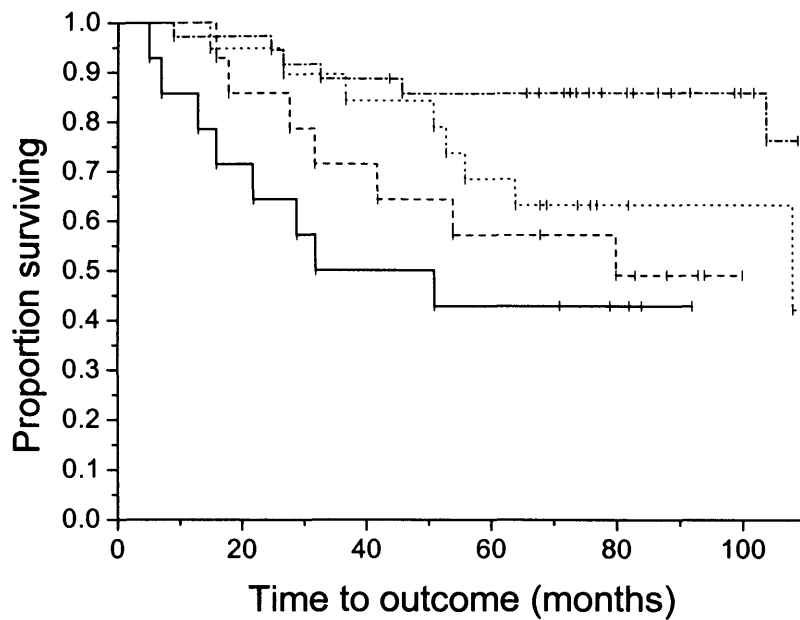
Figure 4.9.2.2 Survival curves comparing staining intensity of CD44v3in primary melanomas. Log rank χ^2 = 12.5162, p = 0.0058. ———— CD44v3 negative, ------- CD44v3 1+ positive, ········· CD44v3 2+ positive, . _ . _ . _ CD44v3 3+ positive

As with MCAM (Figure 4.7.2.2), the CD44v3 positive tumours could be stratified according to the intensity o f e xpression (Figure 4 .9.2.2). This d emonstrated t hat the s tronger t he e xpression o f CD44v3 in the tumour, the better the patient outcome. The correlation with outcome was statistically highly significant (χ^2 = 12.5162, p = 0.0058).

By contrast, the expression of nm23 was negatively associated with a favourable patient outcome. Figure 4.9.2.3 illustrates that the 5-year survival of patients with an nm23-expressing tumour was 60% (n= 47), whereas patients with tumours that did not express this marker had a 5-year survival rate of 90% (n= 29). Furthermore, an even stronger correlation with outcome was found when patients were stratified according to staining intensity (Figure 4.9.2.4). However, neither CD44v3 nor nm23 expression reached statistical significance when analysed in Cox multivariate models, i.e. n either marker w as shown t o b e i ndependently p redictive o f p rognosis w hen a nalysed w ith established clinicopathological markers of prognosis.
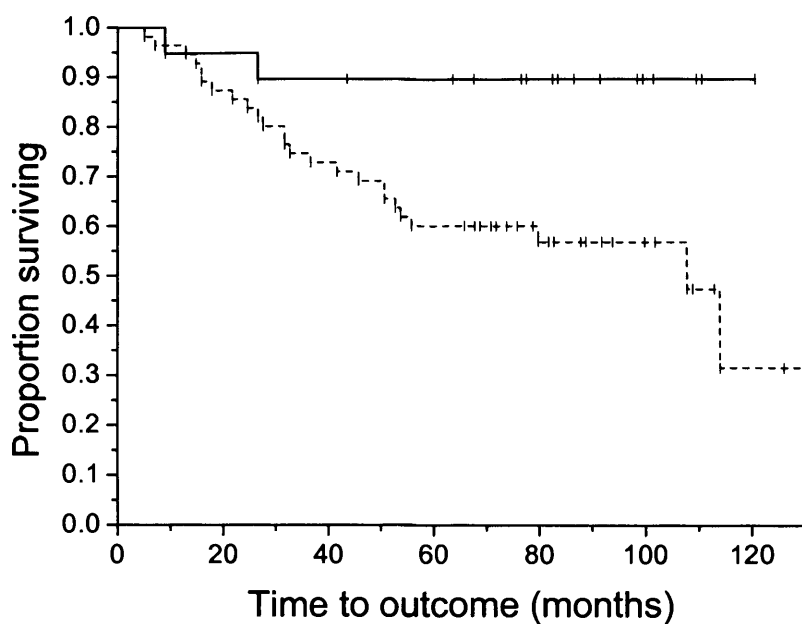
Figure 4.9.2.3 Survival curves comparing nm23 positive and nm23 negative melanomas. Log rank χ^2 = 5.9571, p = 0.0147. ———— nm23 negative, ------- nm23 positive.
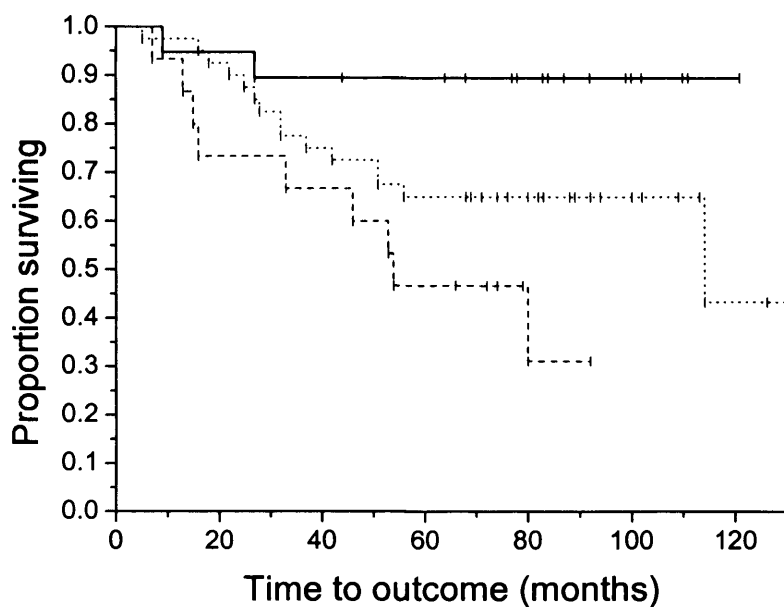
Figure 4.9.2.4 Survival curves comparing staining intensity of nm23 in primary melanomas. Log rank χ^2 = 11.3281, p = 0.0035. ———— nm23 negative, ········ nm23 1+ positive, ------- nm23 2+ positive.

| Variable | Hazard risk | 95% CI for the Hazard Risk | | P-value |
		Lower	Upper	
Clark's level	3.173	1.444	6.971	0.004
Breslow thickness	1.147	1.032	1.274	0.011
CD44v3	0.528	0.191	1.465	0.220
Site	0.822	0.582	1.160	0.264
Age	1.008	0.981	1.036	0.573
Sex	0.802	0.351	1.835	0.601
Ulceration	1.082	0.442	2.646	0.863

Table 4.9.2 Cox multivariate analysis including CD44v3 expression and other established markers of prognosis. CD44v3 expression is shown not to be independently predictive of poor prognosis.

| Variable | Hazard risk | 95% CI for the Hazard Risk | | P-value |
		Lower	Upper	
Breslow thickness	1.166	1.048	1.296	0.005
Clark's level	2.665	1.186	5.988	0.018
Nm23	2.262	0.502	10.185	0.288
Male sex	0.638	0.259	1.571	0.328
Site	0.839	0.587	1.200	0.336
Age	1.014	0.985	1.044	0.343
Ulceration	1.223	0.493	3.032	0.664

Table 4.9.3 Cox multivariate analysis including nm23 expression and other established markers of prognosis. Nm23 expression is shown not to be independently predictive of poor prognosis.

4.9.3 Discussion

Patients with melanomas that did not express CD44v3 had a significantly poorer outcome compared with those whose tumours expressed this marker. Conversely, patients whose tumours did not express nm23 did much better than those whose tumours did express nm23. Subgroup analysis showed that marker expression of both CD44v3 and nm23 retained significance when stratified a ccording t o t he i ntensity o f e xpression o f the p rotein. F or C D44v3, t he s tronger t he

staining intensity, the more favourable the patient outcome, whereas for nm23, the stronger the stain, the poorer the patient outcome.

4.9.3.1 CD44v3

CD44 is a widely distributed transmembrane glycoprotein. Originally described as a homing receptor for lymphocytes (Stoolman 1989), it also appears to function in such diverse physiological processes as lymphocyte activation, cell-cell adhesion, cell-matrix interaction and cell motility (Underhill 1992). Interest has focused on its possible role as an adhesion molecule involved in the malignant progression and metastatic potential of certain tumours (Gunthert et al., 1995; Harwood et al., 1996; Saegusa et al., 1999; Pirinen et al., 2000; Setala et al., 2001; Hefler et al., 2002; Suzuki & Yamashiro 2002; Bankfalvi et al., 2002). Characterisation of the human CD44 gene demonstrated that it has at least 20 exons and is located on chromosome 11. Multiple isoforms are generated by alternative splicing of transcripts of this gene (Screaton et al., 1992). The larger splice variants (CD44v1-10) contain additional amino acids positioned in the extracellular domain of the molecule. They have a more restricted pattern of expression and appear to be preferentially associated with epithelial cell subpopulations. The functions of these isoforms remain relatively unclear (Stamenkovic et al., 1991), however CD44 molecules containing the v3 domain are able to bind growth factors and might play a role in the control of cellular proliferation (Bennett et al., 1995).

There is increasing evidence to suggest that CD44 may contribute to malignant progression and metastasis formation (Bartolazzi et al., 1994). As referred to in the introduction to this chapter, CD44 expression correlates with the tumour stage in human colorectal adenocarcinomas (Heider et al., 1993), bladder carcinoma (Nemec et al., 1987) and non-Hodgkin's lymphoma (Horst et al., 1990). The metastatic capacity of B16 mouse melanoma cells (Hart et al., 1991) and human melanoma cell lines (Birch et al., 1991) is enhanced by CD44H expression and this capacity can be blocked by anti-CD44 antibodies (Guo et al., 1994).

The evidence relating to splice variants is conflicting, with different studies detecting widely varying expression levels of splice variants encoded by the metastasis-associated exons v5 and v6. Harwood et al. explored the immunohistochemical pattern of CD44 expression in a range of melanocytic lesions using a panel of monoclonal antibodies raised to CD44H and the variants v3-v6 and v8/9 (Harwood et al., 1996). Skin biopsies of 106 lesions from 100 patients were assessed and included benign and dysplastic naevi, melanoma in situ, melanomas in the horizontal and

vertical growth phase and cutaneous and lymph node metastases. The study found that the expression of CD44H diminished with increasing invasiveness and the pattern of expression observed correlated significantly with the growth phase of the lesion, rather than its Breslow thickness. These results were confirmed in a later study on 292 stage I melanomas by Karjalainen et al. (Karjalainen et al., 2000). In the present study CD44v3 expression was significantly correlated with both Clark's level and Breslow thickness. In Harwood's study, CD44 splice variants were not detected in any of the lesions using their antibodies. Ranuncolo and co-workers also failed to detect expression of CD44v3 in their series of 57 primary cutaneous melanomas (Ranuncolo et al., 2002), as did Seelentag et al. in 28 melanomas (Seelentag et al., 1997). Both of these groups used anti-CD44v3 antibodies supplied by different manufacturers compared with the antibody used in the present study. Differences in the methods of antigen retrieval and detection may further contribute to conflicting results in immunohistology studies.

Döme et al. analysed 46 primary melanomas with a minimum follow-up of 61 months and were the first to demonstrate the expression of the v3 splice variant by immunohistochemistry in melanoma (Döme et al., 2001). CD44v3 expression was only observed in tumours thicker than 1.0mm. The expression of CD44v3 was not detected in the non-metastatic group of patients. The marker was detected in only one lymph node-metastatic tumour; but was prevalent in the organ-metastatic group. The 5-year survival of patients with CD44v3 positive tumours was lower than those who did not express this marker. These results are wholly at variance with those found in the present study. Döme's group analysed a relatively small number of tumours (46) and detected CD44v3 using a different antibody compared with this study, which may have contributed to the discrepancy in results.

This study showed a clear correlation between CD44v3 staining intensity and outcome, with the strongest staining being correlated with the most favourable outcome. Although loss of CD44v3 expression has not previously been shown to correlate with poor outcome in melanoma, it has been shown in several other cancers. Decreased CD44v3 expression in oral squamous cell carcinoma was significantly correlated with unfavourable tumour-free and overall survival (Bankfalvi et al., 2002). Suzuki and Yamashiro analysed CD44v3 expression in 93 Japanese lung adenocarcinoma patients by immunostaining to study the relationship between marker expression and invasion in lung adenocarcinoma (Suzuki & Yamashiro 2002). A significantly reduced expression of CD44v3 was observed in invasive lesions compared with non-invasive lesions, suggesting that reduced expression of CD44v3 is associated with invasion in lung adenocarcinoma. This observation supported an earlier study, which found reduced expression of CD44v3 to be associated with poor survival in non-small cell carcinoma of the lung (Pirinen et al.,

2000). Loss of CD44v3 has also been correlated with poor survival of ovarian cancer patients (Saegusa *et al.*, 1999). By contrast, no relationship between CD44v3 expression and survival was found in vulvar carcinoma (Hefler *et al.*, 2002) or gastric cancer (Setala *et al.*, 2001).

The role of CD44v3 in tumour progression remains to be determined. The v3 splice variant is the only CD44 isoform able to bind heparin-binding growth factors and cytokines through its heparin sulphate side chains (Bennett *et al.*, 1995; Van der Voort *et al.*, 1999). Cells expressing the v3 variant may therefore accumulate matrix-bound growth factors. This could improve the survival of cells remaining in their original environment and relieve the selective pressure to progress towards a metastasis-forming phenotype. This would explain the findings of this study, in which the stronger the expression of CD44v3, the better the prognosis of the patient.

4.9.3.2 Nm23

The nm23 gene was originally identified by differential colony hybridisation experiments on related K-1735 murine melanoma cell lines of low and high metastatic potential (Steeg *et al.*, 1988). These experiments identified a gene expressed at ten-fold higher mRNA levels in two cell lines of low metastatic potential than in five cell lines of high metastatic potential. Transfection of the murine nm23 cDNA into highly metastatic K-1735 murine melanoma cells reduced its metastatic potential, independent of the effect on tumour cell growth rate (Leone *et al.*, 1991). Similarly, transfection of the *nm*23 gene into B16F10 melanoma cells also resulted in significant suppression of invasiveness and metastatic ability and also enhanced survival of tumour-bearing mice (Parhar *et al.*, 1995).

Nm23 protein is present in many human tissues and is temporarily up-regulated during lactation and intestinal epithelial regeneration (Gumbiner *et al.*, 1992). A major biological role of the Nm23 protein is its nucleoside diphosphate kinase activity that transfers a phosphate group between nucleoside di- and tri-phosphates. This is not considered to be associated with a potential anti-metastatic activity. Other functions attributed to the nm23 gene product include a role in the activation of small G-proteins during the initial steps of signal transduction (Kiura *et al.*, 1990), the modulation of TGFß1-mediated cell adhesion (Urano *et al.*, 1993; Hsu *et al.*, 1994) and c-*myc* transcription (Postel *et al.*, 1993). The exact relationship between the various functions of nm23 and its role in tumour progression and metastasis remains unclear.

However, average mRNA levels of cell lines derived from primary and metastatic melanomas shown no significant difference (Easty *et al.,* 1996). Previous studies of nm23 mRNA levels in metastatic melanomas have shown significantly lower levels in patients developing metastases within 2 years of diagnosis than in those with less aggressive disease (Florenes *et al.,* 1992) and longer survival for patients with higher levels of mRNA (Xerri *et al.,* 1994).

Nm23 expression in other cancers showed varying results: low levels of nm23 mRNA have been found to correlate with the development of metastases in breast cancer (Bevilacqua *et al.,* 1989), stomach cancer (Nakayama *et al.,* 1993) and liver cancer (Nakayama *et al.,* 1992), whilst in human neuroblastoma (Hailat *et al.,* 1991), breast carcinoma (Sastre-Garau *et al.,* 1992), thyroid carcinoma (Zou *et al.,* 1992), colon carcinoma (Haut *et al.,* 1991), cutaneous squamous cell carcinoma and squamous carcinoma of the lung (Engel *et al.,* 1993) high levels of nm23 mRNA have been demonstrated in more advanced and aggressive lesions.

Recent studies evaluating immunohistochemical staining for nm23 expression in melanocytic lesions do not appear to mirror the findings of earlier mRNA studies with respect to prognosis. Although Lee et al. demonstrated and inverse correlation between staining and tumour thickness (Lee *et al.,* 1996), other studies have found no correlation between nm23 expression and the development of nodal metastases (Easty *et al.,* 1996; Saitoh *et al.,* 1996) or survival outcome (Holmes & MacKie 1996; Reichrath *et al.,* 1997; van den Oord *et al.,* 1997). McDermott *et al.* have studied the largest series of melanomas to date for nm23 expression using immunohistochemical techniques (n=145) (McDermott *et al.,* 2000). This study found a strong positive correlation of staining intensity with survival, in keeping with previous mRNA studies and the findings of Lee et al. (Lee *et al.,* 1996). These results reached statistical significance in univariate analysis, but did not retain significance in multivariate analysis.

The findings of the present study are at variance with those of McDermott and co-workers but in keeping with those of Saitoh et al. (Saitoh *et al.,* 1996). Saitoh *et al.* found a correlation between nm23 expression and early local recurrence, Clark's level and Breslow thickness, but not with the presence of lymph node metastases using paired primary and metastatic lesions from the same patient. In this study a correlation between nm23 expression, Clark's level, Breslow thickness, age and poor outcome was found. Furthermore, the degree of positivity of nm23 staining correlated with outcome, such that stronger staining predicted a worse outcome. It has been proposed that melanocytes do not require a high level of nm23, either because of their differential status or because nm23 is not relevant to melanoma – a theory that does not seem plausible, bearing in mind the results of this study. The anti-Nm23 antibody used in other studies may not be specific

for an epitope associated with metastatic or aggressive potential. Therefore, immunohistochemistry results may depend on the specificity and selectivity of antibodies and methods employed. The conflicting findings of this study and others on nm23 expression and the progression of melanoma and other tumours most likely reflect the multiple functions and complex role of this marker in tumour biology.

4.10   OTHER MARKERS INVESTIGATED

A variety of other markers were investigated using the melanoma tissue microarray. Of the remaining markers (AP-2, β-catenin, bcl-2, ki-67 and MG50), only the intensity of expression of bcl-2 was shown to correlate with prognosis in this cohort of melanoma patients (p= 0.05). A summary of the results is shown in Table 4.9.

Bcl-2 is a proto-oncogene and part of a regulatory pathway that prevents programmed cell death. It is involved in the 14:18 chromosome translocation observed in some follicular lymphomas, which leads to increased expression of the gene (Pezzella et al., 1990). Bcl-2 is thought to exert its effect on preventing apoptosis by protecting the cell from the oxidative stress that results from free radical accumulation (Hockenbery et al., 1990). Previously, Grover and Wilson investigated bcl-2 expression in a series of melanoma lymph node metastases using flow cytometry and found a correlation of high expression with a shorter survival (Grover & Wilson 1996).

Marker	Expression (univariate)		Intensity (univariate)		Multivariate analysis	
	Log Rank (χ^2)	P-value	Log Rank (χ^2)	P-value	Hazard Risk	P-value
AP-2	1.498	0.221	4.83	0.185	0.854	0.817
β-catenin	0.727	0.394	2.53	0.469	1.54	0.383
bcl-2	0.452	0.5	7.75	0.05	0.566	0.442
ki-67	2.62	0.106	2.89	0.409	1.21	0.743
MG50	0.361	0.548	2.31	0.511	9236	0.986

Table 4.10.1 Table summarising the results of other immunohistochemical markers examined with the melanoma tissue microarray. None of these markers demonstrated correlations with melanoma patient outcome.

More recently, Vlaykova et al. used immunohistochemistry to investigate 60 metastatic melanomas and found that the presence of Bcl-2 immunoreactivity was associated with a longer disease-free survival. However, the study also found that focal bcl-2 expression was strongly associated with a worse prognosis compared to a diffuse expression or lack of Bcl-2 staining (Vlaykova et al., 2002). In the present study increased bcl-2 expression correlated with poor outcome, in agreement with the results of Grover and Wilson (Grover & Wilson 1996). However, no correlation was found between positivity alone and outcome. The relationship with the pattern of staining and patient outcome in the study by Vlaykova et al. may explain why, in the present study, no correlation was found between the positivity of bcl-2 and survival, whereas one was between the intensity of expression and survival were associated (Vlaykova et al., 2002). It is possible that the technique of tissue microarray is not suitable for the assessment of bcl-2 or other markers, in which the pattern of staining is more significant than the presence or absence of staining itself.

Of the remaining markers investigated, only the staining of Ki-67 was shown to correlate with the clinicopathological variables analysed. Table 4.9.2 shows that ki-67 expression is strongly correlated with Breslow thickness, melanoma ulceration and Clark's level. Despite this, no relationship with patient outcome was found in either univariate or multivariate analysis. Figure 4.9.2 illustrates the relationship between Ki-67 expression level and Clark's levels. It can be seen that staining intensity increased with increasing Clark's level, such that no Clark's level 1 tumours were stained more than weakly and most Clark's level 5 tumours were stained moderately or strongly.

Variable	P value
Breslow Depth*	0.001
Ulceration[t]	0.005
Clark's level*	0.024
Age[t]	0.247
Male sex[t]	0.688
Site of tumour[t]	0.945

Table 4.10.2   Correlations between ki-67 expression level and established prognostic variables for melanoma. * Spearman test, [t] Pearson Chi-squared.
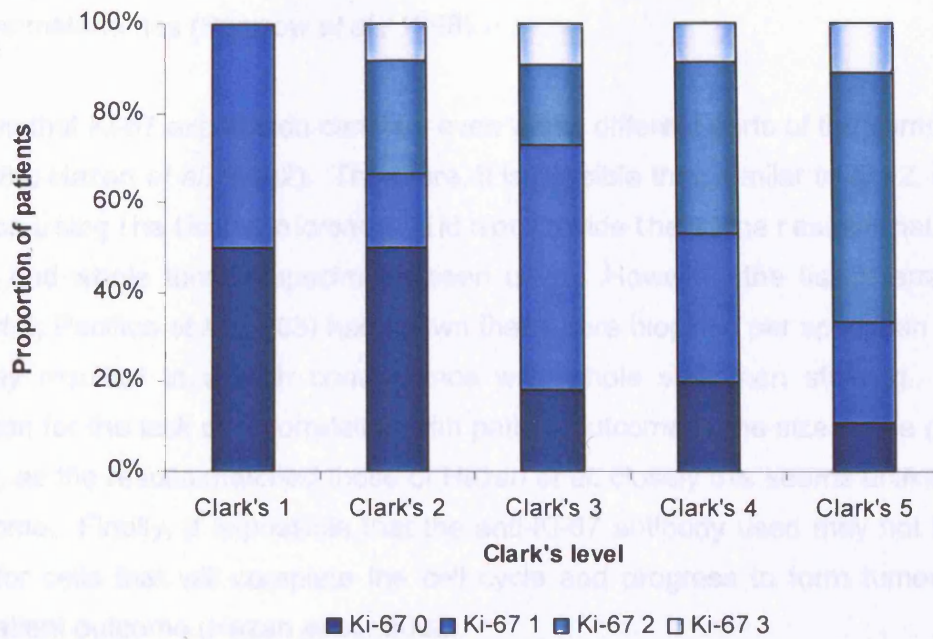
Figure 4.10.2 Figure illustrating the association of the intensity of *ki*-67 expression with Clark's levels (Spearman's correlation p = 0.024). The expression of *ki*-67 was scored as negative (0), weak (1), moderate (2) or strong (3).

Because Ki-67 is not expressed in resting cells but is expressed from the mid-G1 phase of the cell cycle through to the S, G2 and M phases, it is considered to be a reliable marker of proliferation and hence possibly a useful prognostic marker. Ki-67 has previously been studied for correlations of expression with melanoma clinicopathological variables as well as outcome. In the largest series of melanoma specimens studied so far (n= 137), Hazan and colleagues showed a highly significant correlation of Ki-67 expression and tumour thickness, results that mirror those of this study (Hazan *et al.*, 2002). Hazan *et al.* also found a correlation between ulceration and Ki-67 staining was found, although this did not reach significance (p = 0.09) and no statistically significant correlation between patient outcome and marker expression was found. These results are therefore comparable with those of the present study. Other studies have also found correlations between tumour thickness, Clark's level and Ki-67 expression (Ramsay *et al.*, 1995; Böni *et al.*, 1996; Henrique *et al.*, 2000). Other immunohistochemical studies have shown a correlation between Ki-67 expression and melanoma patient outcome (Korabiowska *et al.*, 1994; Ramsay *et al.*, 1995; Vogt *et al.*, 1997; Niezabitowski *et al.*, 1999; Straume *et al.*, 2000; Ostmeier *et al.*, 2001). However, most of these studies evaluated cohorts consisting of mainly thicker tumours (Korabiowska *et al.*, 1994; Ramsay *et al.*, 1995; Vogt *et al.*, 1997; Niezabitowski *et al.*, 1999; Henrique *et al.*, 2000). Indeed, Böni *et al.* found that *ki*-67 expression only correlated with survival in patients whose tumours were more than 1.5mm in depth (Böni *et al.*, 1996). Similarly,

Sparrow *et al.* found no association between Ki-67 expression and thin metastasising primary cutaneous melanomas (Sparrow *et al.*, 1998).

It is known that Ki-67 expression can vary even within different parts of the same tumour (Ostmeier *et al.*, 1989; Hazan *et al.*, 2002). Therefore, it is possible that, similar to *bcl*-2, evaluation of Ki-67 expression u sing t he t issue m icroarray d id n ot provide t he s ame r esults t hat w ould h ave b een obtained had whole tumour specimens been used. However, the tissue array validation study (section 4.5; Pacifico *et al.* 2003) had shown that 4 core biopsies per specimen used for the tissue microarray resulted in a high concordance with whole specimen staining. Another potential explanation for the lack of a correlation with patient outcome is the size of the cohort investigated. However, as the results matched those of Hazan *et al.* closely this seems unlikely to have affected the outcome. Finally, it is possible that the anti-Ki-67 antibody used may not have been specific enough for cells that will complete the cell cycle and progress to form tumours that result in a poorer patient outcome (Hazan *et al.*, 2002).

4.11 RELATIONSHIPS BETWEEN MARKERS INVESTIGATED

The final section of this chapter investigates the relationships between the markers studied. Table 4.10 summarises the relationships revealed between pairs of markers. Highly statistically significant relationships are observed between various pairs of markers. Of note is the relationship between P-cadherin and E-cadherin and between the cadherins and ß-catenin. Relationships revealed between the various adhesion molecules may not mean that their function is related but may have arisen due to various changes in expression occurring simultaneously. However, some relationships observed are supported by evidence in the literature, as discussed below.

Cell a dhesion m olecules d o n ot s imply function a s a molecular glue o rganising c ells i nto static structural entities, but also support and direct the exchange of information between cells (Klymkowsky & Parr 1995). This is supported by the nature of the relationship between certain adhesion molecules and patient outcome revealed in the present study. Adhesion molecules themselves actively participate in transducing signals into the cells both through interaction of their cytoplasmic regions with kinases such as focal adhesion kinase (Schlaepfer & Hunter 1998) as well as through interaction with growth factor receptors mediated by extracellular regions (Johnson

1999). Adhesive interactions mediated by one adhesion molecule can also influence the expression or activity of a second adhesion molecule.

Disruption of E-cadherin-mediated cell adhesion facilitates tumour invasion, whereas restoration of E-cadherin expression results in growth retardation and inhibition of the invasive and metastatic phenotype in carcinoma cells (Christifori & Semb 1999). It has been shown that E-cadherin-mediated adhesion with keratinocytes can modulate MCAM expression (Shih *et al.,* 1994). Melanocytes cultured in the absence of keratinocytes express MCAM and this can be reversed by the addition of keratinocytes. Melanoma cells are generally resistant to the influence of keratinocyte co-culture and this has been shown to be associated with the lack of E-cadherin expression by the melanoma cells (Hsu *et al.,* 1996). In the present series no relationship between E-cadherin and MCAM was found, although as expected, a close relationship between E- and P-cadherin was observed. Loss of P- and E-cadherin expression has been previously shown in the earliest identifiable malignant lesions (Christifori & Semb 1999). It is a critical event in the development of metastatic potential, leading not only to the expression of new adhesion molecules but also to broad changes in gene expression which contribute to or even constitute the biological behaviour of melanomas.

ß-catenin was one of the markers in which no correlation with patient outcome was revealed; yet a highly significant relationship with both P- and E-cadherin was found (as well as with CD44v3, AP2 and nm23). Formation of the cadherin - ß-catenin complex is essential for E-cadherin-mediated cell adhesion (Ozawa *et al.,* 1990), but the role of ß-catenin is far more than that of a structural link to the cytoskeleton. ß-catenin is a key regulatory molecule linking adhesion to the control of gene transcription (Klymkowsky & Parr 1995). ß-catenin is a multi-functional protein, binding to several different partners in addition to the classical cadherins. Free ß- catenin can bind to the cellular proteins glycogen synthetase kinase 3 (GSK-3ß) and adenomatous polyposis coli (APC), which target it for destruction. Pathways triggered by mutations in the APC, mutations in ß-catenin or loss of E-cadherin expression all lead to an increase in the pool of free ß-catenin. It has been speculated that these pathways are associated with the transition of cells to more migratory mesenchymal phenotypes in carcinomas (Johnson 1999). Precursors of epidermal melanocytes arise during fetal development from the neural crest and migrate throughout the embryo to take up residence in a complex process, which has much in common with the metastatic process (Erickson 1993). The biological changes set in motion during melanoma development may trigger a reversion to this melanoblast phenotype and one of the critical events could be disruption of keratinocyte-melanocyte interaction. Disturbances in cadherin expression or function, one of the

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earliest steps in the development of metastases, may also be one of the earliest steps in the development of cutaneous melanoma.

Marker	S	M-CAM	CD44v3	AP-2	P-cadherin	E-cadherin	β-catenin	MG50	Ki-67	Nm23	Bcl-2
Bcl-2	CC	0.371**	-0.150	0.069	-0.042	0.126	0.175	.208	.191	0.470**	1.000
	σ	0.001	0.195	0.566	0.723	0.271	0.133	.077	.099	0.000	.
	N	74	76	72	75	78	75	73	76	74	80
Nm23	CC	0.397**	-0.146	0.417**	0.131	0.097	0.346**	0.454**	0.450**	1.000	
	σ	0.000	0.210	0.000	0.263	0.410	0.002	0.000	0.000	0.000	
	N	76	76	74	75	74	76	73	75	76	
KI-67	CC	0.098	-0.065	0.452**	-0.077	-0.092	0.141	0.475**	1.000		
	σ	0.402	0.576	0.000	0.509	0.428	0.228	0.000	0.000		
	N	75	76	73	75	76	75	74	79		
MG50	CC	0.031	0.043	0.388**	0.036	0.057	0.312**	1.000			
	σ	0.797	0.713	0.001	0.760	0.629	0.007	0.000			
	N	73	77	71	75	75	73	79			
β-catenin	CC	0.105	0.292*	0.287*	0.509**	0.428**	1.000				
	σ	0.368	0.011	0.013	0.000	0.000	0.000				
	N	76	76	74	75	75	77				
E-cadherin	CC	0.066	0.180	0.072	0.356**	1.000					
	σ	0.577	0.114	0.547	0.002	0.000					
	N	74	78	72	75	83					
P-cadherin	CC	0.099	0.212	0.133	1.000						
	σ	0.398	0.066	0.262	0.000						
	N	75	76	73	77						
AP-2	CC	0.101	0.016	1.000							
	σ	0.393	0.891	0.000							
	N	74	74	74							
CD44v3	CC	-0.112	1.000								
	σ	0.333	0.000								
	N	76	84								
M-CAM	CC	1.000									
	σ	0.000									
	N	76									

Table 4.11 Summary of statistically significant relationships between markers investigated (** $p < 0.001$, * $p < 0.05$). S = Spearman Rho, CC = correlation coefficient, σ = sigma(2-tailed), N = patient number evaluated.

4.11   SUMMARY

This chapter has demonstrated the suitability of using tissue microarray technology for the assessment of the prognostic potential of melanoma-associated proteins. When using four core biopsies from e ach m elanoma represented, a h igh c oncordance w ith r esults a chieved from the whole specimen were found. A number of melanoma-related cellular adhesion molecules were studied and significant relationships with patient outcome was found for MCAM, P-cadherin, CD44v3, N m23 a nd B cl-2 (see F igures 4 .11 a nd 4 .12 for s ummaries o f r esults). F or s ome o f these markers, such as MCAM, although predicted to be correlated with tumour progression and patient outcome from laboratory studies, no patient outcome studies have previously been undertaken. The results show that tissue array can bridge the gap from laboratory work to clinical use. Implementation of markers in the routine staining and assessment of melanomas excised from patients will add to the currently available armoury of tests that are routinely used and improve the accuracy of the prediction of prognosis

By further investigating the relationship between the markers, insights into the biology of melanoma development and progression may be gained. Most studies in this complex field are use cell lines or melanoma models. By studying melanoma-associated proteins expressed in humans, further advances in this area will be made.

4.12   CONCLUSION

The tissue array provides an ideal method for investigating immunohistochemical markers on a large n umber o f p athological s pecimens s imultaneously. I t i s n ot o nly o f b enefit i n the field o f cancer research, but promises to provide a high-throughput strategy of analysing other tissue specimens relevant to plastic surgery, such as Dupuytren's tissue, tendon samples or burn scar tissue. This study identified a number of melanoma-associated proteins correlated with patient outcome. These are valuable prognostic markers for predicting outcome in melanoma patients and potential targets for therapeutic manipulation.
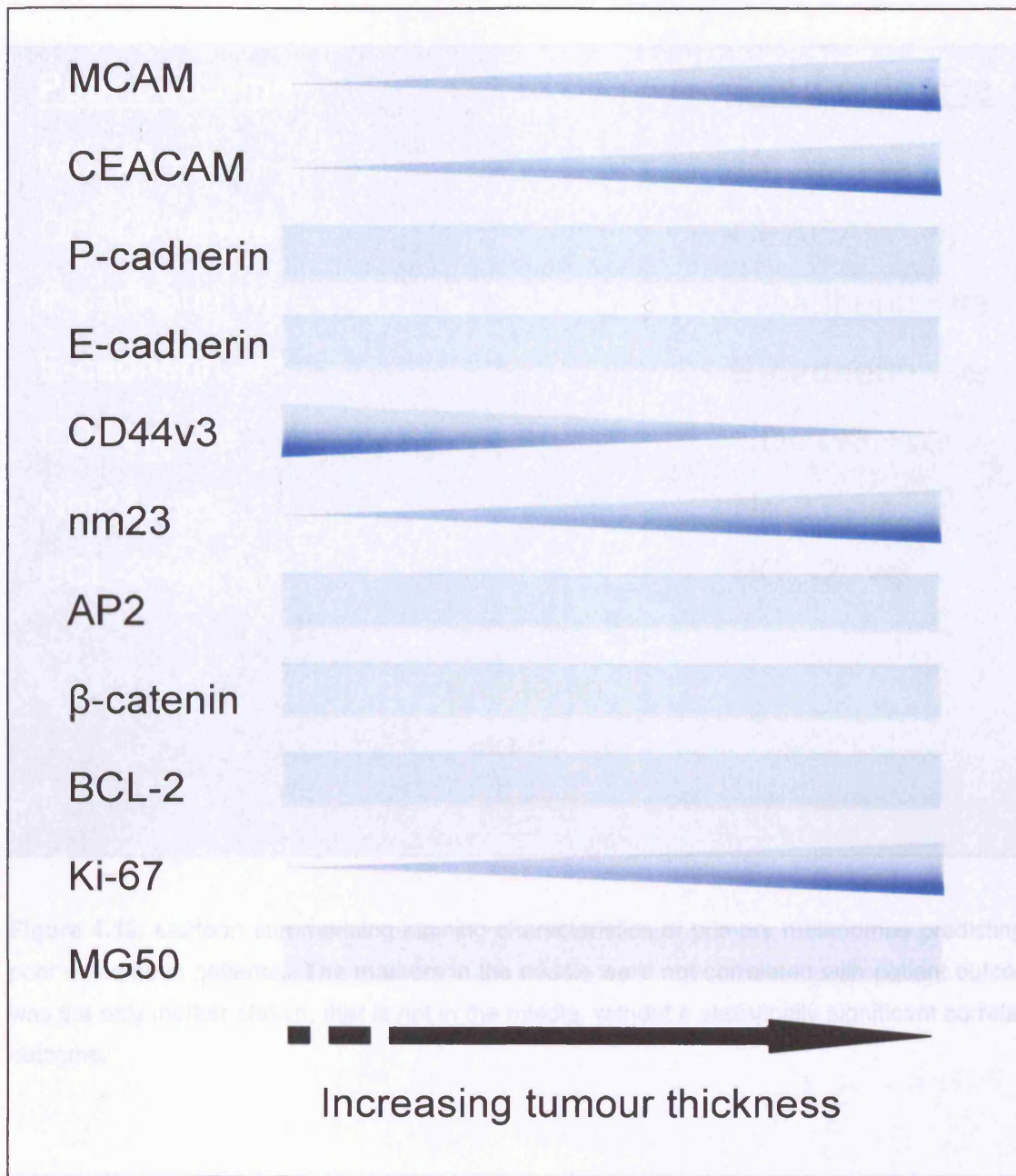
Figure 4.11 Cartoon summarising the relationship of each of the markers studied with tumour depth. The variation in the breadth of the shape alongside each of the markers represents the staining intensity according to Breslow depth.

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Poor outcome	Good outcome
MCAM +ve	MCAM -ve
CEACAM +ve	CEACAM -ve
P-cadherin -ve	P-cadherin +ve
E-cadherin -ve	E-cadherin +ve
CD44v3 -ve	CD44v3 +ve
Nm23 +ve	Nm23 -ve
BCL-2 +ve	BCL-2 -ve

AP-2

β-catenin

ki-67

MG50

Figure 4.12 Cartoon summarising staining characteristics of primary melanomas predicting either good or poor outcome in patients. The markers in the middle were not correlated with patient outcome. E-cadherin was the only marker shown, that is not in the middle, without a statistically significant correlation with patient outcome.

CHAPTER 5

SCFV COCKTAILS

5.1   INTRODUCTION

Many melanoma patients first present with metastases, which are often clinically undetectable (Weiss *et al.*, 1995; Hofmann *et al.*, 2002). With current diagnostic methods, detection of metastases lacks sensitivity and specificity (Shirkhoda *et al.*, 1987; Takahashi *et al.*, 1992; Holder *et al.*, 1998; Terhune *et al.*, 1998; Hofmann *et al.*, 2002; Voit *et al.*, 2002) (for review, see section 1.6). Earlier detection of metastases would provide opportunities for early treatment, which may result in cure, not currently achievable. As discussed in chapter 1, radioimmunoscintigraphy (RIS) promises to offer accurate detection of occult metastases with optimal sensitivity and specificity. Ideally it would be used around the time of first presentation, in order to screen for metastases, after which appropriate management could be undertaken.

When used for *in vivo* tumour targeting whole monoclonal antibodies have shown limitations due to their high molecular weight. T heir large size leads to long circulatory h alf-lives and prolonged antibody retention in the blood (Adams 1998c). It hinders their diffusion into extra-vascular tumour sites and results in poor tumour penetration (Juweid *et al.*, 1992). Clearance from normal tissues is slower, especially in blood-rich organs such as the liver, lung, spleen or kidneys, reducing the contrast obtained between tumour and normal tissue (Yokota *et al.*, 1992). Therefore, there is a considerable delay in reaching high tumour to normal tissue ratios. Mouse IgG has been shown to bind to human Fc receptors (McCool *et al.*, 1985). Therefore, binding of IgG to Fc receptors present on circulating immune cells also contributes to non-specific accumulation of whole monoclonal antibodies and reduced blood clearance (Herlyn *et al.*, 1983; Buchegger *et al.*, 1986; Herlyn *et al.*, 1986).

Antibody fragments have therefore formed the focal point for improving the tumour to normal tissue contrast in melanoma antibody localisation studies (Kang *et al.*, 1999; Kang *et al.*, 2000; Pacifico *et al.*, 2003). Single chain (Fv) antibody fragments (scFvs) are one-sixth the size of whole antibodies (27 kD) and represent the minimal portion of an antibody that can still retain full antigen binding. ScFvs exhibit improved biodistribution and blood clearance compared with whole monoclonal antibodies (Colcher *et al.*, 1999). Another advantage of scFvs is improved tumour penetration (Plückthun *et al.*, 1997; Adams *et al.*, 1998a; Colcher *et al.*, 1999). The short residence time of scFvs in the circulation and in normal tissues results in good tumour to normal tissue ratios by reducing the background accumulation thereby improving tumour targeting (Colcher *et al.*, 1990; Colcher *et al.*, 1998). However, this simultaneously limits the maximal amount of scFv that can accumulate in the tumour. The lower molecular weight of scFvs allows

rapid plasma clearance and improved tumour penetration, resulting in higher tumour-to-normal tissue ratios at early time points *in vivo* (Begent *et al.*, 1996). The reasons for the faster dynamics are not clearly understood but most probably relate both to the smaller physical size of the molecule and to the fact that they lack the Fc portion of the antibody, which promotes uptake and metabolism of whole monoclonal antibodies by phagocytic cells (Holliger *et al.*, 1997).

Despite considerable progress in the production of antibodies and their fragments recognising human melanoma-associated antigens specifically and in targeting methodology, the amount of radioactivity that accumulates specifically in melanoma lesions following injection of radiolabelled anti-MAA antibody fragments remains low. Strategies to improve this critical parameter are being investigated to increase the efficacy of immunoscintigraphy (Adams *et al.* 1999a).

Increasing the dose of monoclonal antibody or scFv does not enhance the contrast achieved with immunoscintigraphy. Wahl *et al.* have shown that increasing the dose of radiolabelled anti-HMW-MAA monoclonal antibody injected into human melanoma-bearing nude mice from 6.25 to 1,875µg did not markedly change the percentage of relative radioactivity uptake in melanoma compared with normal tissues (Wahl *et al.*, 1986). Rogers *et al.* have obtained similar results with anti-CEA monoclonal antibody: an increase of the dose of radiolabelled monoclonal antibody from 16 to 500µg/mouse resulted in a linear increase in the absolute concentration of radioactivity found in the transplanted tumour with no detectable change in the percentage of radioactivity uptake relative to normal organs (Rogers *et al.*, 1986). Furthermore, increasing the dose resulted in decreased tumour to blood ratios. Similarly Pimm and Baldwin found that an increase in the amount of radioactivity localised to a human osteosarcoma xenograft was associated with an increase in the dose of radiolabelled antibody injected up to 500µg per nude mouse (Pimm & Baldwin 1984). When the dose was greater than 500µg/mouse, a reduction in the fractional accumulation of radioactivity in transplanted tumour was observed. In clinical studies, dose escalation has been reported to result in no difference in biodistribution or the quality of tumour images (Carrasquillo *et al.*, 1988a; Taylor *et al.*, 1987) or in an increase in detection sensitivity of malignant lesions (Halpern *et al.*, 1985; Taylor *et al.*, 1987; Carrasquillo *et al.*, 1988b; Murray *et al.*, 1988). Furthermore, blood-pool clearance was prolonged (Halpern *et al.*, 1985; Taylor *et al.*, 1987) and an increase in fractional accumulation of radioactivity in some non-tumour tissues was observed (Taylor *et al.*, 1987; Carrasquillo *et al.*, 1988b). Thus alternatives to increasing the dose of antibody administered are required to optimise tumour imaging.

Other methods that have been employed to optimise tumour targeting have ranged from changes in the peri-tumoral environment to alterations in the targeting molecules themselves. There have

been attempts to increase antibody uptake in tumours by using cytokines that increase blood flow within the tumour. Interleukin 2 conjugated to a monoclonal antibody has been shown to increase tumour blood flow, which has in turn lead to an increase in antibody uptake of up to four-fold (LeBerthon *et al.*, 1991). As an alternative strategy, upregulation of target antigen expression with interferon administration has been used to improve targeting (Krasagakis *et al.*, 1991). Methods that would ensure that the antibodies remain in the circulation would also allow higher tumour accumulation to occur - Adams *et al.* have shown that scFv uptake is very high in mice after they were subjected to nephrectomy (Adams *et al.*, 1998b). Furthermore, modifications to the scFv themselves, such as making scFvs multimeric and thermally stable also improves tumour uptake and retention (Willuda *et al.*, 1999; Kupsch, personal communication).

Pre-targeting of the tumour involves two or three step strategies to improve targeting. The first step is followed by administration of a second reagent in a form, which will be captured or activated by the bound antibody. The high affinity and tetravalency of streptavidin for biotin has been exploited to this end. Streptavidin has been conjugated to antibody and targeted with ^{111}In-labelled biotin for imaging (Kalofonos *et al.*, 1990). Others have imaged a pre-targeted biotinylated antibody with an avidin chase followed by a final step of radiolabelled biotin (Paganelli *et al.*, 1991).

5.1.1   RAFT scFvs

Three scFvs (B3. B4 and RAFT3) have previously been developed against distinct and very melanoma-specific cell surface antigens (Kang, MD thesis 1997; Kupsch *et al.*, 1999; Kang *et al.*, 1999; Kang *et al.*, 2000; Hamilton, MD thesis 2001; Odili, MD thesis 2002). However, relatively low absolute amounts of scFv were found to localise to the tumour. In addition, remaining non-specific background accumulation was observed in the kidney. As described in previous studies above (Rogers *et al.*, 1986; Wahl *et al.*, 1986; Pimm & Baldwin 1984; Carrasquillo *et al.*, 1988a; Taylor *et al.*, 1987), increasing the dose of scFv administered did not improve contrast as background accumulation increased correspondingly.

5.1.2 Heterogeneity and underlying mechanisms

Tumour cell populations are heterogeneous in various biochemical and biological properties, including their antigenic profile (Byers & Johnston 1977; Burchiel *et al.*, 1982; Heppner 1984; Natali *et al.*, 1983; Natali *et al.*, 1985). The degree to which melanoma cells within a tumour express specific antigens is not only of considerable theoretical interest but also of practical importance. Heterogeneous expression of melanoma-associated antigens could be a limiting factor in the usefulness of antibodies as diagnostic or therapeutic agents.

In vitro analysis of human tumour cell lines with antibodies to histocompatability antigens and to tumour-associated antigens has detected three types of heterogeneity: among cell lines derived from different patients (Burchiel *et al.*, 1982), among cell lines originating from different metastases of the same patient (Albino *et al.*, 1981) and among cells within a cell line (Burchiel *et al.*, 1982). Albino *et al.*, using cell lines derived from three metastatic masses from the same patient, reported a variation in reactivity with a panel of monoclonal antibodies (Albino *et al.*, 1981). Immunohistochemical studies of cultured human melanoma cells by Wilson and colleagues demonstrated that antibodies reactive with antigens of similar molecular weight were recognising a heterogeneous group of antigenic determinants on melanoma cells (Wilson *et al.*, 1982). Burchiel and colleagues studied panels of cell lines from different patients with monoclonal antibodies and flow cytometry and found considerable heterogeneity with regard to antigen expression (Burchiel *et al.*, 1982). They found that the expression of melanoma-associated antigens changed as a function of the cell cycle and that the change correlated with increasing cell size. This implies that tumour cell populations with low levels of antigens may not bind sufficient amounts of antibodies and therefore escape from therapy. However, using *in vitro* experiments it is not possible to conclude whether these differences reflected distinct cell populations *in vivo* or were due to variations related to adaptation to tissue culture: expression of antigens by established cell lines may not reflect that of the tumour cells from which they originated. Indeed, a study by Zhang and co-workers on colorectal cancer cells revealed only an approximately 50% overlap of genes expressed by cell lines and those *in situ*, i.e. *in vivo* (Zhang *et al.*, 1997). This therefore means that 50% of genes expressed in cell lines are "false positives" and 50% of genes expressed *in vivo* are "false negatives" when tested on cultured cell lines. Therefore, only one third of genes are expressed in cell culture as they are expressed *in vivo*.

The expression heterogeneity observed *in vitro* is, however, supported by *in vivo* studies. Natali *et al.* compared the antigenic profile of surgically removed primary melanomas with that of autologous metastases as well as multiple autologous metastases (Natali *et al.*, 1983). In spite of

an homogenous morphological appearance, multiple lesions removed from the same patient differed significantly in their reactivity with a panel of monoclonal antibodies in direct immunofluorescence. The authors went on to investigate antigen expression for correlations with tumour characteristics. The extent of the antigenic heterogeneity did not correlate with melanin synthesis, site of origin of the primary tumour, sites of metastatic foci or treatment. They did, however, notice that the variation was less marked in patients carrying the primary tumour simultaneously with their metastases. A follow-up study was undertaken, published in 1985, in which the extent of antigenic heterogeneity of primary and metastatic lesions from patients with melanoma was characterised using a semi-quantitative method (Natali *et al.*, 1985). It showed for the first time heterogeneity in the distribution of distinct antigenic determinants of HMW-MAA in autologous lesions.

In order to overcome the limitations of assessing antigen heterogeneity using cell lines Berd *et al.* analysed melanoma cells dissociated from metastatic masses using flow cytometry as a quantitative assay (Berd *et al.*, 1989). They found that the most consistently expressed antigen in the 53 metastases studied was HMW-MAA. However, the expression showed considerable variability, far more than that observed with cell lines. They also found a reasonable concordance in antigen expression on different tumour masses from the same patient, contrary to results previously reported with cell lines isolated from different metastases of the same patient (Albino *et al.*, 1981).

As most previous work had been carried out on nodular melanomas, Kageshita *et al.* compared the differential expression of melanoma-associated antigens in primary and metastatic acral lentiginous and nodular melanoma (Kageshita *et al.*, 1991). Primary acral lentiginous lesions expressed significantly lower levels of HMW-MAA than nodular lesions. Similar to a previous study (Natali *et al.*, 1983), no correlation between heterogeneity and clinicopathological features was found. Thus it is apparent that antigen or epitope heterogeneity will have consequences for the efficacy of targeting tumours with epitope-specific antibodies. Indeed, Siccardi found a correlation between false negative radioimmunoscintigraphic images and low expression of HMW-MAA in biopsies form those patients (Siccardi, 1990).

5.1.2.1   Mechanisms of antigenic heterogeneity

Various mechanisms exist that result in antigenic heterogeneity. At the genomic level point mutations, multiple genetic loci and multiple alleles at a single locus lead to the appearance of distinct gene products. At the transcriptional level, differential splicing of specific mRNA species

may lead to protein heterogeneity (Schwarzbauer et al., 1983). Cell cycle-related events may also contribute to this, because the expression of histocompatability antigens and tumour-associated antigens has been found to change on cultured melanoma cells moving through the growth cycle (Burchiel et al., 1982). In addition, altered expression of transcription factors and changes in methylation may play a role (Weetman, 1988; Hietala et al., 1997). Genetic deletions, translocations and amplification (sometimes leading to multidrug resistance) and chromosomal alterations have all been shown to play a part in changing antigen expression (Hayashi et al., 1990; Sedlak et al., 1997; Hulkkonen et al., 2002). Co-translational and post-translational modifications of a protein may occur generating antigens with an identical primary structures but varying degrees of processing. Examples include modulation and immunoselection, which have been thought to play a role if patients mount an immune response to antigens expressed by melanoma cells (Ferrone & Pellegrino, 1978).

The possibility that the antigenic phenotype of the cell changes continuously is supported by the susceptibility of antigens to modulation by various agents, such as interferons (Giacomini et al., 1984b) and cell cycle-related events (Burchiel et al., 1982) as well as by reports of quantitative changes of melanoma-associated antigens during the serial passage of melanoma cells in culture (Zehngebot et al., 1983). Furthermore, it has been shown in murine melanoma that the stability of phenotypes in a malignant lesion may be influenced by interactions among clonal cell subpopulations (Hill et al., 1984). Heterogeneity has also been noted in other tumour types, such as among tumour cells in primary or metastatic lesions surgically removed from patients with breast carcinoma (Schlom et al., 1980).

The antigenic heterogeneity of tumours thus introduces variability in tumour localisation of antibodies. A given tumour contains a large variety of cells expressing different antigens, which prevents antibodies with a single specificity from completely coating all the tumour cells within a lesion. Antigenic modulation sometimes occurs between primary and secondary tumour (Natali et al., 1985) and the degree of detection is related to the number of antigen-positive cells (Siccardi, 1990). This represents a potentially major limitation in the development of immunodiagnostic and immunotherapeutic approaches to melanoma. Indeed Siccardi observed a down-regulation of HMW-MAA in stage IV melanoma patients resulting in sub-optimal antibody images but no down-regulation for patients in earlier stages (Siccardi, 1990). Since the use of radioimmunoscintigraphy is intended for the detection of metastatic disease, the antigenic heterogeneity of HMW-MAA and other MAAs is of paramount importance.

5.1.2.2 Loss of MHC Class I and mechanisms of immune escape

Major histocompatability complex (MHC) class I antigens are polymorphic transmembrane glycoproteins non-covalently associated with β_2-microglobulin (Ploegh et al., 1981). They play a key role in recognition of antigen-expressing cells by T cells because they display processed peptides with antigenic epitopes and expose them on the cell surface. T lymphocytes recognise a cell as antigenic only when MHC molecules and internally processed antigens provide targets for the immune system (Zinkernagel & Doherty 1979). Alterations in MHC expression related to normal tissues have been observed in several tissue types (Ruiter et al., 1982; Gutierrez et al., 1987) and malignant transformation of cells is frequently associated with reduction or loss of MHC class I expression (Tanaka et al., 1988). In one study loss of MHC class I has been observed in 15% of colon carcinomas, 8% of gastric carcinomas and 11% of melanomas (Lopez Nevot et al., 1989). The alterations or loss of MHC class I can be associated with tumour growth characteristics, suggesting the influence of cellular immunity on the host-tumour interaction (Festenstein & Garrido 1986). Furthermore, MHC class I expression exerts an effect on the immune response. The underlying mechanisms are similar to those described above for antigenic heterogeneity. In addition, defects in antigen processing and transport of MHC molecules to the cell surface contribute to loss of MHC expression. Loss of MHC class I molecules results in escape from T cells, but concomitantly increases susceptibility to lysis mediated by natural killer cells (Maio et al., 1991).

In comparison with the primary tumours, a decrease in HLA antigen expression has been seen in visceral metastases of melanomas and is generally associated with a poor prognosis (Bröcker et al., 1985). Whether the reduction of MHC class I expression is a critical event in the progression to cancer or rather a phenomenon that results from the genetic instability associated with malignancy is unclear. Nevertheless the crucial role played by MHC class I antigens in the lysis of target cells by T cells has raised the possibility that reduction or heterogeneity of MHC class I expression by tumour cells may facilitate their escape from immune destruction by cytotoxic T lymphocytes. This has been suggested to underlie the significant association between low levels of MHC class I antigen expression in primary cutaneous melanomas and poor prognosis (Gattoni-Celli et al., 1993). These effects may be partially antagonised by the use of immunomodulators such as interferon (Geersten et al., 1999). This escape from immunosurveillance is the result of several molecular events occurring at different levels of the complex interaction of melanoma cells with reactive lymphocytes.

5.1.3 Antibody cocktails

In order to overcome the problem of antigenic heterogeneity it has been proposed that a cocktail of antibodies targeting different antigens, or different determinants on the same antigen, could be used to improve tumour targeting (Burchiel *et al.*, 1982; Natali *et al.*, 1983; Natali *et al.*, 1985). By using this strategy, not only should the number of false negative radioimmunodetection scans be reduced, but targeting may also be improved, by an amplification of the signal as a result of simultaneously coating more of the tumour cells with different radiolabelled antibodies. The hypothesis that targeting may be improved by amplifying the signal with the use of a cocktail of anti-tumour scFvs is shown schematically in Figure 5.1.3.

Other potential advantages of using a cocktail include combinations of Mabs as carriers of distinct components of a therapeutic system, which have to be delivered in close proximity at the cell surface, so that they may assemble and function in selected areas of high antigen expression. For example, one antibody could mediate antibody-dependent cytotoxicity and the other complement-dependent cytotoxicity, therefore making escape less likely. A similar approach has been investigated using bispecific antibody fragments. Holliger *et al.* used bispecific antibody fragments to recruit the whole spectrum of antibody functions by retargeting serum immunoglobulin in combination with a T cell-activating diabody against the same tumour antigen (Holliger *et al.*, 1997). The bispecific diabodies were able to recruit complement, induce mononuclear phagocyte respiratory burst and phagocytosis mediated by one and T cell activation by the other diabodies. Furthermore, the cocktail was shown to promote synergistic cytotoxicity towards colon carcinoma cells in conjunction with CD8+ T-cells at concentrations that were too low to achieve therapeutic effect with the diabodies when used in isolation.

More recently Kipriyanov *et al.* constructed a bispecific diabody against human CD19 (lymphoma) and CD16 (natural killer cells) and combined it in a cocktail with another bispecific diabody targeting CD19 and CD3 (T cells) (Kipriyanov *et al.*, 2002). Co-stimulation by using both diabodies significantly increased the lysis of tumour cells. Treatment of tumour-bearing mice with the cocktail resulted in complete resolution, whilst treatment with either diabody alone showed only partial tumour regression. It is possible that a similar synergy might be observed with a cocktail of monospecific scFvs targeting different epitopes. Indeed, it has been shown that using a cocktail of monospecific scFvs carrying immunotoxins against B-cell lymphoma resulted in complete regression of tumours, whereas each component when used individually did not (Flavell *et al.*, 1997).

The cocktail principle

Once 'R3'-targeted epitopes are saturated, increasing the dose of R3 will not improve the contrast, but will only increase the background levels of radiation.

Adding 'B4', targeting a different epitope, should increase the contrast achieved without increasing the background radiation

Similarly, adding a further scFv targeting another epitope (B3) should further improve the contrast achieved

Figure 5.1.3 Schematic representation of the "cocktail principle", illustrated with the three scFvs (R3, B3 and B4) used in this chapter. The cocktail could increase contrast if used at saturating antibody dose (estimated as 125µg per mouse for the HER2 antigen, which is expressed at levels similar to HMW-MAA (Adams *et al.*, 1998b)) provided that tumour accumulation is stable.

5.1.3.1   *In vitro* studies using antibody cocktails

Krizan and colleagues studied both melanoma cell lines and fresh tumour biopsies with a panel of monoclonal antibodies by flow cytometry (Krizan *et al.,* 1985). The percentage of labelled cells and relative fluorescence intensity when stained with individual monoclonal antibodies varied with different cell lines and biopsy samples under saturating conditions. Use of a cocktail of monoclonal antibodies that recognized different melanoma-associated antigens reduced these variations. In cell lines both the percentage of labelled cells and relative fluorescence intensity increased substantially when the cocktail prepared from five monoclonal antibodies rather than an individual monoclonal antibody was used for staining. The cocktail of monoclonal antibodies also increased the percentage of labelled tumour biopsy cells and relative fluorescence intensity. These d ata s how t hat a c ocktail o f monoclonal a ntibodies c an b e m ore e ffective t han a s ingle monoclonal antibody for melanoma cell labelling *in vitro* and might be more effective for tumour imaging and therapy. In a study on breast carcinoma, Tagliabue and colleagues demonstrated significantly improved immunodetection in serous effusions from breast carcinoma by using up to nine monoclonal antibodies directed against different epitopes (Tagliabue *et al.,* 1986). Ziai *et al.* used a cocktail of antibodies and increased the staining intensity of cultured melanoma cells, without an increase in the number of cells stained, whereas with surgically removed specimens, the staining pattern became homogeneous compared with heterogeneous when a single antibody was used alone and with no loss of specificity (Ziai *et al.,* 1987). Similarly, Matzku *et al.* observed an increase in binding of antibodies to melanoma cells when two or three were used in combination (Matzku *et al.,* 1989). These results suggest that use of a combination of monoclonal antibodies recognising distinct determinants of HMW-MAA may increase the sensitivity of immunoscintigraphy.

Another application of a cocktail of antibodies in autologous stem cell transplantation has been investigated by a German group (Hempel *et al.,* 2000). Following chemotherapy, stem cells from twelve breast cancer patients were collected and incubated with antibody-coated magnetic beads to remove contaminating tumour cells. Prepared stem cell grafts were transplanted 24 hours later and patients were administered with the monoclonal antibody 17-1A, an immunotherapeutic agent that exerts its effect through antibody dependent cellular cytotoxicity (ADCC), induction of an idiotypic network cascade and possibly also by complement activation (Frodin *et al.,* 2002). Tumour cells were found in five of the 12 patients prior to chemotherapy. However, following the treatment regime a consistent reduction in tumour cells in four of the five patients was observed. These four patients subsequently faired significantly better than an untreated control group in

terms of tumour load. Use of a cocktail of antibodies both with the magnetic beads and also as immunotherapeutic agents may considerably further improve patients' outcome using this technique. Other potential *in vitro* applications of antibody cocktails include the more sensitive detection of tumour cells in biopsies (Taha *et al.*, 1989). This would be especially useful in sentinel node biopsy in which it could reduce the numbers of false negative results obtained. Cocktails of antibodies (polyclonal antibodies) are already used as more sensitive secondary layers in Western blot, ELISA and FACS for exactly the same reason.

5.1.3.2 Previous use of antibody cocktails *in vivo*

Previous attempts to use cocktails of antibodies to improve tumour targeting have used monoclonal antibodies. Gaffar *et al.* first used a combination of two anti-colorectal cancer monoclonal antibodies in human xenograft-bearing hamsters and found that "the presence of [the additional] antibody seems to potentiate the tumour accretion of the CEA antibody, suggesting that the antibody mixture is more effective for tumour radioimmunodetection than either antibody given alone" (Gaffar *et al.*, 1981). However, the authors did not state the dose of the antibody cocktail used compared with the individual components, nor did they demonstrate that the antibodies used bound different epitopes. Munz and colleagues found that adding equal amounts of iodinated $F(ab')_2$ fragments targeting different epitopes resulted in an increased maximum amount of bound labelled protein as compared with either component alone demonstrating an additive binding effect (Munz *et al.*, 1986). The biologic half-life of the antibody mixture in the tumour was significantly greater than that of either monoclonal antibody alone. Matzku *et al.* aimed to overcome the limited sensitivity of single monoclonal antibodies by using a cocktail of three monoclonal antibodies, each targeting a distinct epitope of HMW-MAA (Matzku *et al.*, 1989). In this study, neither the combination of the three radiolabelled monoclonal antibodies nor increasing the dose of individual antibodies increased the amount of radioactivity specifically localized to the human melanoma xenografts in nude mice. However, Sardi *et al.* found that a mixture of monoclonal antibodies improved tumour targeting in a study in nude mice (Sardi *et al.*, 1989). In this study a hand-held gamma probe was used to detect radiolabelled antibody in tumour. In addition, the individual organs were excised and the radioactivity measured at the end of the experiment and higher counts were detected in the tumour, whilst the counts in the normal organs remained relatively stable.

Wahl et al. argued that if saturating doses of a cocktail are given, the unbound excess antibody will result in a very high background (Wahl et al., 1990). Therefore the authors gave low doses of anti-

HMW-MAA antibody to patients, proposing that improved T:NT ratios would be achieved with sub-saturating doses. Two antibodies were administered intra-lymphatically to 17 patients. Gamma camera images were taken, lymph nodes removed and the presence of tumour was compared with radioactivity counts. Good contrast was achieved in the lymph node-positive patients with little background activity. However, the results were not compared with those achieved with the individual components of the cocktail. Volpe *et al.* showed an improvement in the detection of colorectal metastases in patients using a cocktail of monoclonal antibodies when compared with the individual antibodies (Volpe et al., 1998). Antibody cocktails have also been used in radioimmunoguided surgery (de Nardi et al., 1997). De Nardi et al. observed an improved sensitivity using a cocktail of monoclonal antibodies for intra-operative radioimmunodetection of colorectal tumours compared with a single antibody. No study has yet investigated the use of scFvs in a cocktail to improve targeting and increase the sensitivity of immunodetection. Theoretically using scFvs as a cocktail, with their inherent advantages over whole monoclonal antibodies, could further enhance tumour targeting.

The work in this chapter was done by me apart from: historical control studies (work done by my predecessors), PCR cloning and genetic sequencing (both by Dr Jörg Kupsch).

5.2   AIMS

The purpose of the present study was to improve *in vivo* tumour targeting by using a cocktail of scFvs that targets three different epitopes on melanoma cells.

5.3   METHODS

5.3.1   Production and Purification

Three scFv clones were used for the study: RAFT3 (Kang *et al.*, 2000), B3 (Kupsch *et al.*, 1999) and B4 (Kang *et al.*, 1999; Kupsch *et al.*, 1999). The production and purification of the scFvs was undertaken as described in chapter 2, section 2.2. Optimal conditions for induction of individual scFvs in large-scale bacterial culture have been determined empirically (Kang, MD thesis 1997). Briefly, scFvs were expressed in *E. coli* TOP10 and made from a standard 1-litre induction. A 10 ml culture in LB_{GA} (10 ml Luria Broth + 0.5ml 40% D-glucose + 100μg/ml ampicillin), inoculated with a pipette tip stabbed into a bacterial glycerol stock was grown at 30°C overnight in a rotary shaker at 250 rpm (see chapter 2). In the morning overnight cultures were diluted 1:100 up to 1 litre in LB_{GA} and shaken at 250 rpm for 6 hours at 30°C (to an OD_{600} of approximately 1.5). After approximately 6 hours, the bacteria were pelleted and the supernatant poured off. The bacteria were resuspended in 1 litre of LB containing 100 μg/ml ampicillin and 100 μM IPTG. Cultures were then shaken overnight (for 16-18 hours) at 30°C and 250 rpm.

All scFvs were purified by IMAC purification on High-Trap chelating sepharose columns (Amersham Pharmacia Biotech, chapter 2.2.6). After elution, scFvs were concentrated to 1 ml in IMAC elution buffer and aliquoted before storage at -80°C. For the purpose of these experiments, the scFvs were not desalted. They were analysed by Western blot (section 2.3.4), SDS-PAGE (using Coomassie staining; section 2.3.3) and by cell ELISA on melanoma cells (section 2.4.3). Further analysis of the scFvs was by low-pressure gel filtration (section 2.2.7) on HiPrep™ Superdex 75 XK 16/60 columns (Amersham Pharmacia Biotech). For gel filtration, two millilitre aliquots were collected and tested for presence of scFv by Western blot.

5.3.2   Production of the cocktail

Three separate preparations of the scFvs RAFT3, B3 and B4 were produced. RAFT3 is a mouse hybridoma-derived scFv (Kupsch *et al.*, 1995; Kang *et al.*, 2000), whilst the two other scFvs are human phage library-derived (Kupsch *et al.*, 1999). After appropriate testing and analysis of the preparations, the cocktail was made by combining equal amounts of each of the scFvs.

5.3.3 Competition ELISA

In o rder to d emonstrate t hat t he t hree scFv clones b ound d ifferent e pitopes, a competition c ell ELISA was set up, essentially as described by Stahli (Stahli *et al.*, 1983). Melanoma cells were grown in 96 well ELISA plates (see section 2.4.2). All the ELISA plate wells were initially loaded with 500ng/50µl of LHM2 IgG (parent mouse monoclonal antibody) per well and serial dilutions of all scFvs (RAFT3, B4 and B4) were carried out across the plate from 500 to 1ng/50µl per well. LHM2 IgG binding was detected using rabbit anti-mouse IgG-HRP conjugate (Dako), which does not cross-react with scFv. The ELISA was completed as previously described (Section 2.4.3) and the plate read at 490 nm. A similar technique has been described by Giacomini *et al.* in which the binding of radiolabelled monoclonal antibody was compared with cold antibody allowing the affinity to be determined (Giacomini *et al.*, 1985).

5.3.4 Addition ELISA

Using the method described by Friguet *et al.* "addition" ELISAs were performed to further confirm the differential binding of the three scFvs to different epitopes (Friguet *et al.*, 1983). ScFvs were added to the first column of wells at saturating concentrations, as determined from previous cell ELISAs. An additional scFv was then added to the first column of wells, also at saturating concentrations, and the combination of pairs of scFvs titred as described in section 2.4.3. The maximum OD obtained was compared with those of the individual scFvs that were titred in parallel on the same ELISA plate. Differential epitope binding was demonstrated if the maximal OD observed was similar to that expected from the sum of the ODs of the individual components at saturating concentrations.

5.3.5 *In vivo* methods

The scFv cocktail was examined for its *in vivo* targeting properties (sections 2.7 & 2.8) and compared with those of its individual components RAFT3, B3 and B4. As described above, combining equal amounts of each of the component antibodies produced the cocktail. Each mouse received 0.5µg of scFv cocktail, i.e. 0.167µg of each component scFv. The individual scFvs were also administered at 0.5µg per mouse.

5.3.6   Radiolabelling

Radiolabelling was carried out using the Iodogen™ method (Pierce) and ^{125}I as the radiolabel (Section 2.5). Immunoreactivity after labelling was assessed by comparing equivalent amounts of labelled and unlabelled scFv in cell ELISA (Section 2.4.3).

5.3.7   Biodistribution

Biodistribution data were obtained in Balb/c nu mice bearing human melanoma xenografts (Sections 2.7 & 2.8). Half a microgram of ^{125}I-labelled scFv cocktail in 100μl PBS was injected via the tail vein and the mice culled at appropriate time points. Three to five mice were used for each time point. Mice were sacrificed at 1, 3, 6 and 18 hours after injection. A blood sample was obtained by cardiac puncture and organs removed for weighing and gamma counting. Radioactivity in the tumour, blood, both quadriceps, both femurs, spleen, liver, kidneys and lungs was counted. Results were expressed in terms of percentage of the injected dose per gram of tissue (%ID/g) and from this tumour-to-normal tissue ratios (T:NT) were calculated. T:NT values were calculated for each mouse individually and expressed as the average for each group and time point. It should be noted that when the organs weigh less than 1g the %ID/g can exceed 100%.

5.4   *IN VITRO* RESULTS

5.4.1   Purification of scFvs

RAFT3, B3 and B4 scFvs were made with sufficiently good purity and yield. Figure 5.4.1 shows an example of a Western Blot and SDS-PAGE of the scFvs individually and mixed in equal amounts as a cocktail. Note the increased yield achieved for the RAFT3 scFv shown by the SDS-PAGE. The yields for the three scFvs were 100µg/ml, 300µg/ml and 1000µg/ml for B3, B4 and RAFT3 respectively, per litre induction.

Binding affinities have previously been determined for the scFvs studied and are shown in Table 5.4.1 along with their respective antigen densities (Kang, thesis 1997). The affinities ranged from 4.96×10^{-8} to 6.31×10^{-8} M^{-1} and the antigen densities ranged from 4.3×10^5 to 2.8×10^6. All three clones showed comparable resistance to serum proteases and thermal stability when incubated with 50% mouse serum in PBS for 24 hours at $37^{\circ}C$ (Odili, MD thesis 2001). No degradation or loss of antigen binding was observed under these conditions. In addition, all 3 scFvs are expressed as monomers (Odili, MD thesis 2002).

KM B3 B4 R3 Cocktail   KM   B3   B4   R3   Cocktail Ova 1 Ova 3 Ova 5
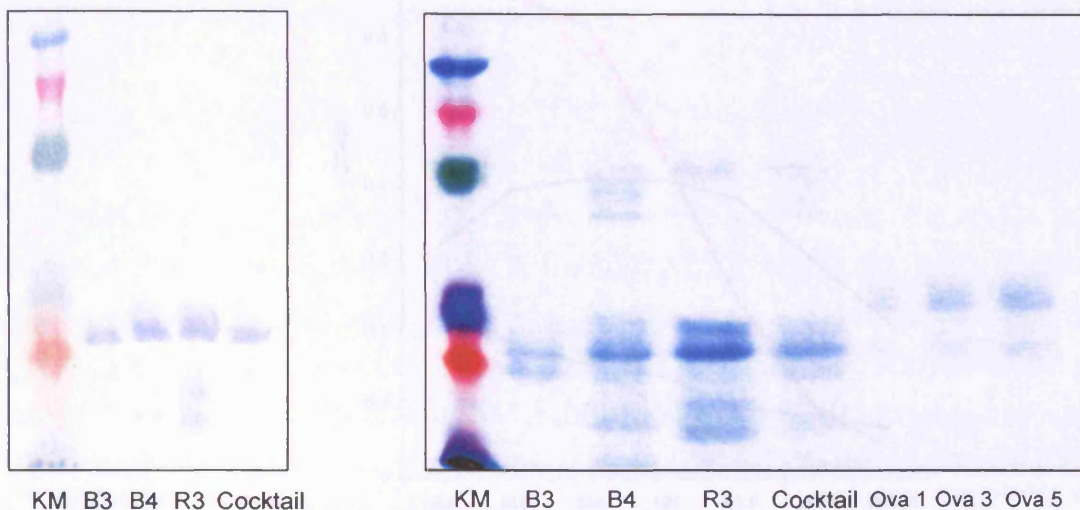
Figure 5.4.1.1 Western blot and SDS-PAGE showing the purity and yield of the individual scFvs and the cocktail, respectively. The yield was estimated by comparison with ovalbumin standards (1,2 and 5µg). 9E10 hybridoma supernatant was used to detect scFv in Western blot. KM: kaleidoscope molecular weight marker.

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Clone	K_D (M^{-1})	Antigen density (molecules per cell)
RAFT 3	4.96×10^{-8}	4.3×10^5
B3	6.31×10^{-8}	1.9×10^6
B4	6.05×10^{-8}	2.8×10^6

Table 5.4.1 Binding affinity (dissociation constant, K_D) of the scFvs studied and antigen density as determined by Scatchard plot (adapted from Kang, MD thesis 1997)

Binding to melanoma cells was tested in a cell ELISA. Figure 5.4.1.2 shows the binding of the individual components of the cocktail. In addition, it shows the predicted and observed binding of a cocktail of the three scFvs. The binding of the cocktail is similar to that predicted and is shown to be of increased potency at equivalent scFv concentrations.

B3 ——— R3 ——— B4 • expected cocktail ——— actual cocktail
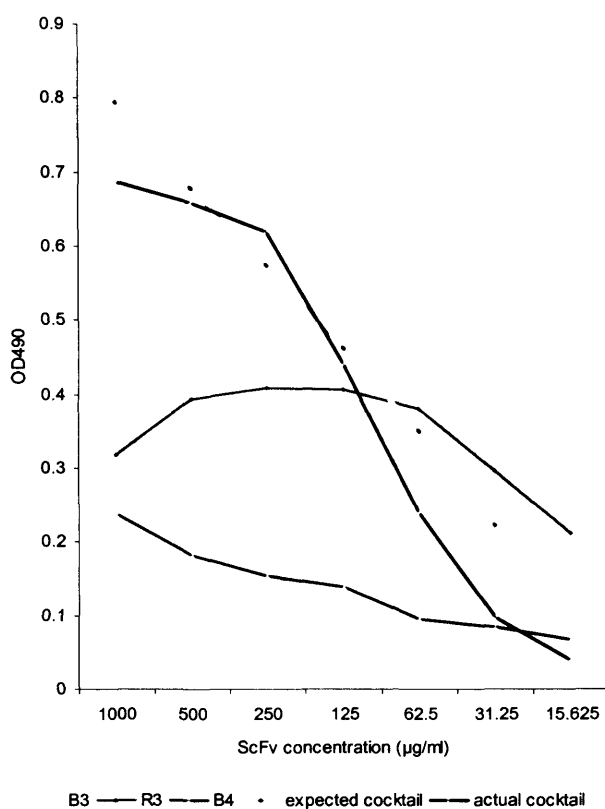
Figure 5.4.1.2 Cell ELISA showing binding of each individual component of the cocktail, the predicted and observed binding of the cocktail. Results are representative of 3 experiments. The predicted OD of the cocktail is calculated by the sum of the OD of the individual components at equivalent total scFv concentration.

5.4.2 Confirmation of epitopic specificity

Epitopic specificity was confirmed by both addition and competition ELISA assays. Table 5.4.2.1 shows an example of the results of an addition ELISA. The first row represents the individual scFvs at maximal OD. The other rows show the results of combining two combinations of pairs of scFvs. The OD reached for the combinations is approximately the sum of the maximal ODs for the individual components.

ADDED	B3	B4	R3
NONE	0.248	0.325	0.486
B3	-	0.599 (0.573)	0.714 (0.734)
B4	0.599 (0.573)	-	0.987 (0.811)
R3	0.714 (0.734)	0.987 (0.811)	-

Table 5.4.2.1. Table showing maximum ODs in cell ELISA using individual scFvs and combinations of two at saturating concentrations. Observed ODs are shown with those predicted from combining two shown in brackets. Results are representative of 3 cell ELISAs.

Inhibition of binding of LHM2 IgG by each of the scFvs was tested. Figure 5.4.2.2 illustrates the results of a competitive ELISA using B3, B4 and RAFT3. The OD of LHM2 (parent Mab of RAFT3) is seen to increase as the concentration of RAFT3 decreases, therefore demonstrating competition for binding to the same epitope. No such competition, however, was observed for B3 and B4, showing that the two scFvs bind to different epitopes. The results obtained with addition and competition ELISA were in agreement and showed that B3, B4 and RAFT3 bind distinct and non-overlapping epitopes on melanoma.

5.4.2.2a
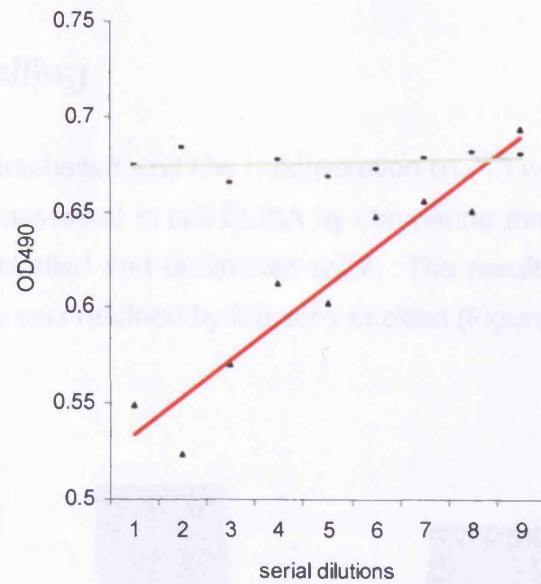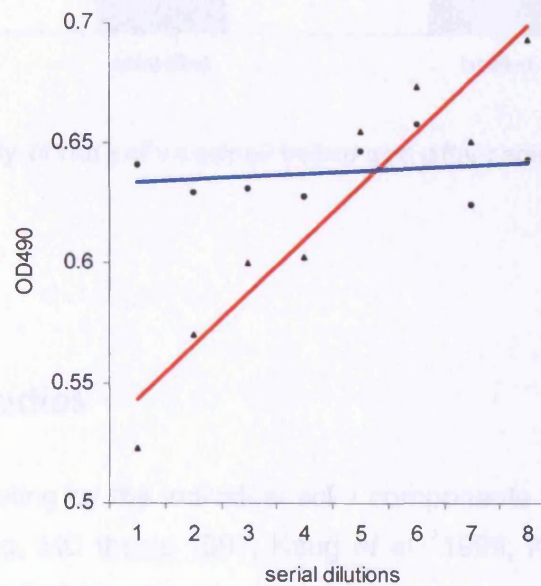
5.4.2.2b

Figure 5.4.2.2 Competition ELISA demonstrating shared epitope specificity for RAFT3 and LHM2 Mab but no inhibition with B3 (5.4.2.2a) and B4 (5.4.2.2b). Results are representative of 3 experiments.

5.5   *IN VIVO* RESULTS

5.4.3   Radiolabelling

Single c hain F vs were r adiolabelled a nd t he i ncorporation of ^{125}I w as c alculated a s 1 2%. T he effect of radiolabelling was assessed in cell ELISA by comparing the melanoma binding exhibited by equivalent amounts of labelled and unlabelled scFv. The results showed that approximately 88% of the immunoreactivity was retained by the scFv cocktail (Figure 5.4.3).
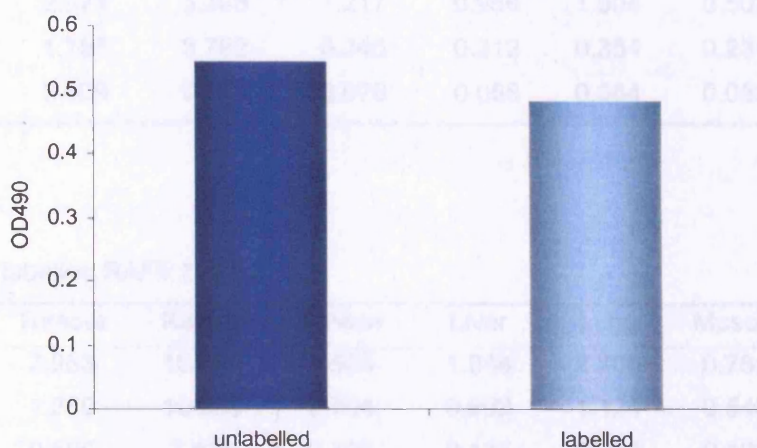
Figure 5.4.3 I mmunoreactivity of t he s cFv c ocktail before a nd a fter r adiolabelling a s d etermined b y c ell ELISA

5.4.4   *In vivo* studies

The *in vivo* melanoma targeting by the individual scFv components of the cocktail has previously been studied in detail (Kang, MD thesis 1997; Kang *et al.*, 1999; Kang *et al.*, 2000). The scFv cocktail was studied and the findings are shown here in comparison with archival data. Results of the percentage injected dose of ^{125}I-labelled scFv obtained for the individual scFvs and the cocktail are shown in Tables 5.4.4.1 - 4. Initial tumour uptake for B3 scFv was 1.355% at 1 hour post-administration compared with 2.854% for B4 and 2.263% for RAFT3.

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5.4.4.1 %ID/g for ^{125}I labelled B3 scFv

	Tumour	Kidney	Spleen	Liver	Lung	Muscle	Blood
1 hour	1.355	5.704	0.627	2.704	0.953	0.381	1.598
3 hours	1.066	3.341	0.355	2.325	0.477	0.318	0.387
6 hours	0.786	1.779	0.139	0.948	0.206	0.093	0.123
18 hours	0.506	0.357	0.068	0.391	0.095	0.059	0.079

5.4.4.2 %ID/g for ^{125}I labelled B4 scFv

	Tumour	Kidney	Spleen	Liver	Lung	Muscle	Blood
1 hour	2.854	11.409	1.584	2.044	3.328	0.678	4.414
3 hours	2.521	5.388	1.217	0.956	1.904	0.502	2.758
6 hours	1.795	3.792	0.346	0.312	0.354	0.231	0.421
18 hours	0.509	0.356	0.076	0.068	0.064	0.053	0.074

5.4.4.3 %ID/g for ^{125}I labelled RAFT 3 scFv

	Tumour	Kidney	Spleen	Liver	Lung	Muscle	Blood
1 hour	2.263	16.939	1.535	1.844	2.260	0.761	3.205
3 hours	1.280	10.603	0.764	0.992	1.124	0.548	1.021
6 hours	0.680	7.828	0.196	0.174	0.234	0.123	0.135
18 hours	0.420	2.329	0.073	0.085	0.064	0.054	0.061

5.4.4.4 %ID/g for ^{125}I labelled scFv cocktail

	Tumour	Kidney	Spleen	Liver	Lung	Muscle	Blood
1 hour	24.220	183.710	6.350	11.930	7.807	7.820	18.167
3 hours	26.730	150.060	5.750	12.760	6.490	7.657	21.777
6 hours	22.800	115.810	3.606	10.510	5.370	4.821	13.574
18 hours	5.146	6.260	1.213	1.677	1.343	0.763	3.932

Tables 5.4.4.1 - 4 Percentage injected dose of ^{125}I labelled scFvs per gram of tissue. Each data point represents the mean from 4-5 mice.

The accumulation of B3 scFv then declined steadily over the remaining time points, such that at 18 hours post-injection the %ID/g was 0.506%. The B4 scFv showed superior initial uptake, with a value of 2.854% at 1 hour post-administration. However, by 18 hours the value was similar to that of B3. Similarly, RAFT3 initially showed targeting superior to B3, but with time the decline in percentage injected dose was comparable with the other scFvs. The blood clearance of the three scFvs was similar. B3 showed an accumulation in blood of 1.598% after 1 hour, which dropped to 0.079% by 18 hours. B4 and RAFT3 both accumulated to a greater degree in the blood after 1 hour (4.414% and 3.205%, respectively) but then cleared efficiently to levels similar to B3 by 18 hours.

The percentage of injected dose per gram of tissue for the scFv cocktail is shown in Table 5.4.4.4 and Figure 5.4.4.4. A considerably higher amount is seen to accumulate in the tumour at 1 hour compared with any of the individual components. Similar to the individual scFvs, the values decline over time, such that at 18 hours post-injection 5.146%ID/g of cocktail is sequestered in the tumour. Likewise, the amount of cocktail in all other organs is noticeably higher at equivalent time-points compared with individual scFvs and declines in a comparable manner.

The tumour to normal tissue ratios of the individual scFvs and the cocktail are shown in Tables 5.4.4.5–8 and Figures 5.4.4.1-4. In all four experiments an improvement in the tumour to normal tissue ration is seen over time with each organ. The kidney consistently exhibited the lowest T:NT ratio and the muscle the highest. Despite the very much higher values of %ID/g observed with the scFv cocktail compared with the individual scFvs the T:NT ratio is comparable across all time points, as this figure is a ratio derived from the values calculated from the %ID/g. The cocktail did not improve the T:NT ratio observed for the kidney compared with the individual scFvs. A range of 0.180 to 1.429 was observed at the 18 hour time point as the maximal T:NT ratio for the kidneys. At 18 hours the individual scFvs had a range of 5.738 to 7.495 for the T:NT of the spleen. In contrast, the cocktail reached a T:NT ratio of 17.081 at 18 hours ($p < 0.05$). There was no significant difference in contrast in the liver, lung or blood between either of the individual scFvs or the cocktail. The T:NT values for the muscle are approximately the same for the individual scFvs. The cocktail however, showed a considerably higher value (15.982) for this tissue. In summary, therefore, the cocktail showed a very much-improved tumour to normal tissue ratios in the spleen and the muscle. The %ID/g varied between different experiments but the tumour to normal tissue ratio remained approximately the same, for the same experiment repeated twice, a phenomenon noted by others (Jackson et al., 1998) (see Discussion).

5.4.4.5 T:NT ratios for ^{125}I labelled B3 scFv

	Kidney	Spleen	Liver	Lung	Muscle	Blood
1 hour	0.238	2.162	0.501	1.422	3.562	0.848
3 hours	0.319	3.006	0.459	2.237	3.356	2.757
6 hours	0.442	5.644	0.830	3.818	8.494	6.415
18 hours	1.421	7.495	1.295	5.355	8.632	6.444

5.4.4.6 T:NT ratios for ^{125}I labelled B4 scFv

	Kidney	Spleen	Liver	Lung	Muscle	Blood
1 hour	0.250	1.802	1.397	0.858	4.208	0.647
3 hours	0.468	2.071	2.636	1.324	5.022	0.914
6 hours	0.473	5.189	5.755	5.072	7.773	4.265
18 hours	1.429	6.694	7.481	7.949	9.645	6.875

5.4.4.7 T:NT ratios for ^{125}I labelled RAFT 3 scFv

	Kidney	Spleen	Liver	Lung	Muscle	Blood
1 hour	0.134	1.474	1.227	1.001	2.973	0.706
3 hours	0.121	1.675	1.29	1.139	2.336	1.254
6 hours	0.087	3.468	3.906	2.905	5.526	5.035
18 hours	0.180	5.738	4.941	6.563	7.781	6.885

5.4.4.8 T:NT ratios for ^{125}I labelled scFv cocktail

	Kidney	Spleen	Liver	Lung	Muscle	Blood
1 hour	0.101	3.224	1.541	2.384	2.733	1.079
3 hours	0.355	5.249	2.12	4.231	4.647	1.618
6 hours	0.212	6.362	2.283	4.437	6.011	1.849
18 hours	0.983	17.081	3.235	5.493	15.982	4.949

Tables 5.4.4.5 - 8 Tumour to normal tissue ratios of ^{125}I labelled scFvs per gram of tissue. Each data point represents the mean of 4-5 mice.
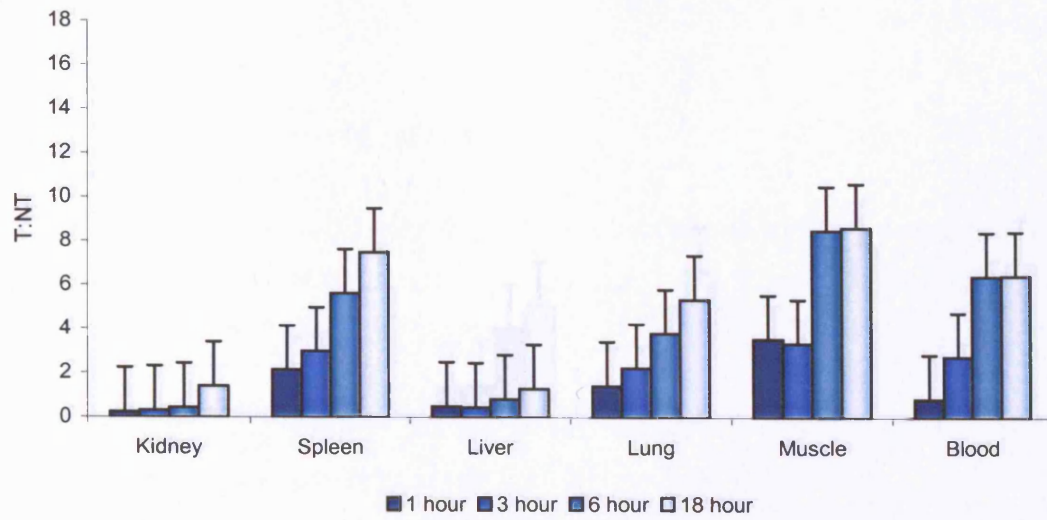
Figure 5.4.4.1 T umour t o n ormal t issue r atios (T:NT) o f t he B 3 s cFv. Error b ars r epresent 1 s tandard deviation from the mean.
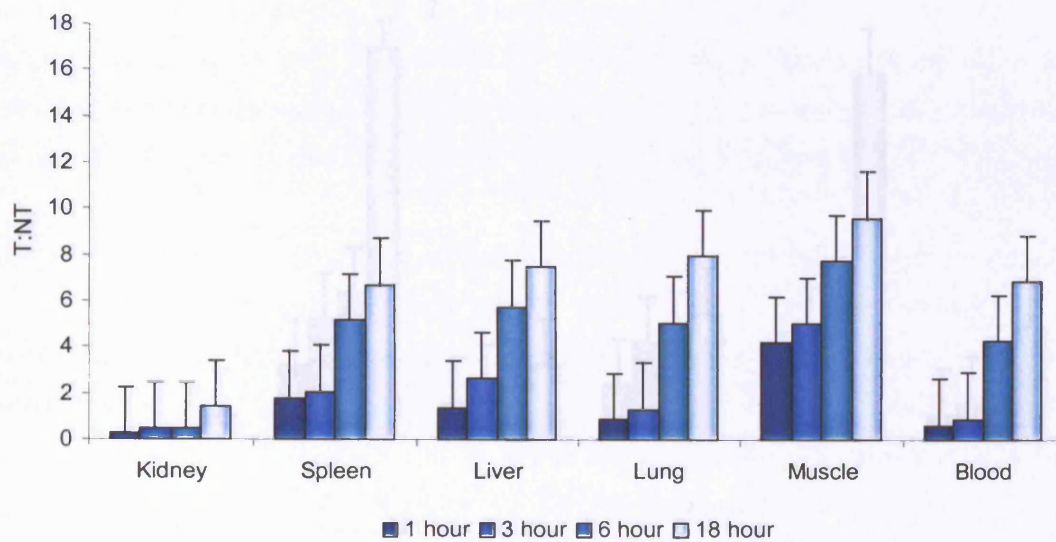
Figure 5.4.4.2 Tumour to normal tissue ratios (T:NT) of the B4 scFv.

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Figure 5.4.4.3 Tumour to normal tissue ratios (T:NT) of the RAFT3 scFv

Figure 5.4.4.4 Tumour to normal tissue ratios (T:NT) of the scFv cocktail
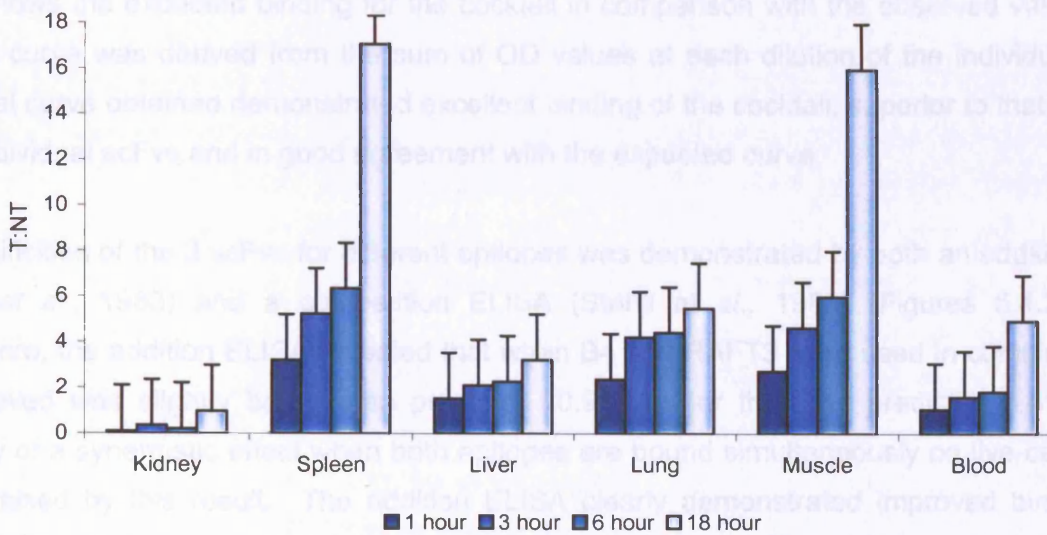
5.5   DISCUSSION

5.5.1   Analysis of *in vitro* results

The use of a cocktail of scFvs was investigated as a strategy to improve tumour localisation by overcoming potential antigen expression heterogeneity *in vivo* and reducing the background accumulation of radiation for imaging purposes.

The yield of the clones B3 and B4 was similar, but that for RAFT 3 was considerably higher (Figure 5.4.1.1). Nevertheless, it was possible to combine the proteins in equal amounts, such that each contributed to one third of the cocktail. SDS-PAGE and Western blot demonstrated the purity of the three clones and the cocktail. From previous experiments (Kang, MD thesis 1997; Odili, MD thesis 2001) it was known that the scFv clones were comparable in properties known to be important for the *in vivo* targeting by scFvs, such as affinity for the antigen (Adams *et al.*, 1998b) thermal stability and resistance to serum proteases. In addition, all three scFvs are expressed as pure monomers (Odili, MD thesis 2001) and all three clones bound well to melanoma cells in cell ELISA, as expected from the similar affinities and antigen expression levels (Table 5.4.1). Figure 5.4.1.2 shows the expected binding for the cocktail in comparison with the observed values. The expected curve was derived from the sum of OD values at each dilution of the individual scFvs. The actual curve obtained demonstrated excellent binding of the cocktail, superior to that observed for the individual scFvs and in good agreement with the expected curve.

The specificities of the 3 scFvs for different epitopes was demonstrated by both an addition ELISA (Friguet *et al.*, 1983) and a competition ELISA (Stahli *et al.*, 1983) (Figures 5.4.2.1 & 2). Furthermore, the addition ELISA revealed that when B4 and RAFT3 were used in combination the OD achieved was slightly better than predicted (0.987 rather than the predicted 0.811). The possibility of a synergistic effect when both epitopes are bound simultaneously on live cells (i.e. *in vivo*) is raised by this result. The addition ELISA clearly demonstrated improved binding of a combination of scFvs compared with the binding of individual scFv components at saturating concentrations (Fig. 5.4.1.2). The competition ELISA further confirmed that RAFT3 binds to the same epitope as its parent monoclonal antibody LHM2 and that the B3 and B4 scFvs bound to different epitopes (Fig. 5.4.2.2). The combination of B3 and B4 binding at saturating concentrations (Table 5.4.2.1) shows that the two epitopes are non-identical.

Mouse hybridoma-derived RAFT3 binds to HMW-MAA, as does its parent monoclonal antibody LHM2 (Kupsch *et al.*, 1995). Although the human phage library-derived B3 and B4 scFvs were shown to bind epitopes different to RAFT3, the molecular identity of the B3 and B4 antigens is unknown. B3 and B4 are nevertheless very melanoma specific (Kupsch *et al.*, 1999) and the possibility that they may target different epitopes on HMW-MAA or different antigens altogether remains. When previously tested on a panel of non-melanoma tumour sections, some tissue cross-reactivity between the three scFv clones was observed, although the reactivity patterns were not identical for the three clones. Using immunohistochemistry, it was shown that although both B3 and B4 stained basal cell carcinoma, B3 also stained breast carcinoma (Kupsch *et al.*, 1999). An attempt was also made to characterise the B3 and B4 antigens by Western blot and immunoprecipitation of melanoma cell extracts, however without success. The differences observed between B3, B4 and RAFT3 in tissue section staining patterns, cellular distribution of antigen and expression levels suggested that the antigens are not identical. Furthermore, the phage library from which B3 and B4 were derived, was selected on keratinocyte sub-populations that expressed HMW-MAA antigen without success, indicating that B3 and B4 are not directed against this antigen (Clarke & Kupsch, unpublished).

5.5.2 Analysis of *in vivo* results

The scFv cocktail retained most of its immunoreactivity after radiolabelling (Figure 5.4.3). In biodistribution studies in human melanoma xenograft-bearing Balb/c nude mice, both the individual scFvs and the cocktail exhibited rapid clearance. All the individual scFvs localised to the tumours, reaching maximum uptakes of approximately 3% at 60 minutes following intravenous injection. These results are comparable to tumour targeting studies of scFv reported by other laboratories (Colcher *et al.*, 1990; Milenic *et al.*, 1991; Whitlow *et al.*, 1993) which found peak values in the range of 3-7 %ID/g in xenografts 30-60 minutes after injection, decreasing to about 1 %ID/g at 24 hours. The value of 24% reached by the cocktail in the tumour was exceptional, but was paralleled by high values observed in the other tissues. The difference in %ID/g shown for the cocktail compared with its individual components and differences in %ID/g between different experiments (data not shown) are a phenomenon observed by other investigators (Jackson *et al.*, 1998). Nevertheless, the ratios of scFv distribution remained the same, resulting in reproducible tumour to normal tissue ratios. In agreement with published reports, these studies demonstrated that rapid tumour uptake and rapid clearance from blood and normal tissues resulted in high tumour to muscle ratios (Colcher *et al.*, 1998; Adams & Schier, 1999).

The tumour to normal tissue ratios were noticeably enhanced by the use of a cocktail for the spleen and muscle. Despite unambiguous evidence of improved binding by use of a cocktail of scFvs in vitro there was no improvement in T:NT ratios for other tissues nor with the blood. In addition, there was no improvement in renal accumulation of scFv. However, this would not be expected, as the individual scFvs were not modified (such as by charge or by size, see chapter 6).

The finding that the T:NT for muscle was considerably improved should not be underestimated. Clinically, initial melanoma recurrence usually occurs either locally, in transit or in the regional lymph nodes (neck, groin or axilla) (Balch et al., 2001a). Were a recurrence to occur at any of these sites, the background tissue would most often be muscle, especially at the periphery. Thus, greater contrast in radioimmunoscintigraphy at these sites is invaluable.

There are several possible explanations why an improvement in targeting was not observed for all organs. The most likely argument is that the doses used for the in vivo experiments were sub-saturating. The ELISAs that demonstrated the enhancement in binding by the cocktail were all performed at saturating concentrations, so that all possible binding sites on the melanoma cells were occupied. Due to the vast number of antigens in the xenograft tumours in comparison to the ELISA plates, it is unlikely that saturation of the binding sites could have occurred. Adams et al. showed that a dose of 125µg scFv per mouse is required to saturate tumour xenografts expressing the HER2 antigen at levels similar to those of HMW-MAA on melanoma (Adams et al., 1998b). On the other hand, if saturating doses were used, an increase in background accumulation would be a potential difficulty, one of the very problems this strategy aimed to overcome (Matzku et al., 1989; Wahl et al., 1990). Despite this, Wahl et al. achieved good binding using a cocktail at a low monoclonal antibody dose that the authors claim was sub-saturating (Wahl et al., 1990). Another reason for the lack of improved tumour to normal tissue ratios for most organs could be the limited heterogeneity on cells from a cell line compared with those found in patients (Ziai et al., 1987; Natali et al., 1985). The increased heterogeneity in patients would theoretically not only result in improved contrast due to less background, but also increased sensitivity, with fewer false negative scans. According to Chatal et al. an increase in the sensitivity of immunoscintigraphy in patients may be obtained by injecting monoclonal antibodies to two tumour-associated antigens (Chatal et al., 1984).

As presented in the methodology, the tumour to normal tissue ratios have been calculated by taking the mean %ID/g for the tumour at each time point and individually calculating the T:NT ratio for each mouse. The value presented is the mean of each group at each time point. There are arguments for and against using this methodology to calculate the T:NT ratios. The alternative

would be to calculate the T:NT ratio from the mean %ID/g across all mice in the group with the mean %ID/g of the tumour. The latter method may be less likely skewed by a single erroneous result in %ID/g but does not truly represent the T:NT achieved in each subject mouse. No established method for this calculation is apparent on review of the literature. Thus the former method was used as it was argued that this produced a more accurate representation of the contrast achieved in each mouse.

It is important to consider the differences between investigations performed with a cocktail of antibodies recognising distinct antigens from those that recognise distinct determinants on the same antigen as well as between investigations in patients and nude mice transplanted with human tumours. The approach used with antibodies to distinct antigens aims to overcome heterogeneity in the antigenic profile of tumour cells (Chatal *et al.*, 1984). On the other hand, the approach to use monoclonal antibodies to distinct determinants of the same tumour-associated antigen aims at increasing the number of antibody molecules targeted to a tumour cell and will therefore be effective only when monoclonal antibodies are used in saturating concentrations. Furthermore, in tumour-bearing patients anti-tumour antibodies react not only with malignant lesions, but also with any normal tissues expressing the same or cross-reacting antigens. In nude mice transplanted with human tumours the reactivity of anti-tumour antibodies with normal tissues is virtually absent, enabling clearer interpretation of results. In the present study it was not determined whether the B3 and B4 epitopes are on entirely different antigens or are part of the HMW-MAA. From previous results it seems likely that the three antigens are non-identical (Kupsch *et al.*, 1999).

5.5.3   Comparison with other cocktail studies

As referred to in the introduction, strategies using cocktails of antibodies have previously been studied in an attempt to improve tumour targeting and with varying degrees of success. No study has yet used scFvs in an antibody cocktail. Matzku *et al.* undertook a similar study using nude mice and whole monoclonal antibodies (Matzku *et al.*, 1989). In that study three monoclonal antibodies targeting distinct determinants on HMW-MAA were used. *In vitro* analysis showed that combining two or three monoclonal antibodies induced a two- to threefold increase in the amount of radioactivity bound to the melanoma cells, when each monoclonal antibody was used at saturating concentrations. However, only a "slight increase" was detected at non-saturating concentrations. *In vivo* experiments showed that increasing the amount of the individual

antibodies did not improve tumour targeting, nor did it improve with the use of a cocktail of all three antibodies. Data not shown, but discussed by the authors, demonstrated reduced expression of HMW-MAA on melanoma cells once transplanted into the mice, which may have explained or at least contributed towards the results (Siccardi *et al.*, 1990). They went on to perform dose escalation experiments in which increasing doses of monoclonal antibodies were injected into tumour-bearing mice that were then subjected to semi-quantitative autoradiography. A highly heterogeneous distribution was recorded at low doses that became more homogeneous with increased doses. This observation suggested that the increase in tumour uptake associated with the increase in the dose of monoclonal antibody injected resulted from enhanced monoclonal antibody binding in the central parts of the xenografts leading to saturation of the binding sites. They repeated their cocktail experiment at saturating concentrations and still found no improvement in binding and a reduction in the relative uptake of the monoclonal antibody due to an increase in background accumulation. These results suggest that increasing the dose of the cocktail would not improve the results of the present study, although they may have been improved had their antibodies targeted different antigens, rather than distinct determinants on the same antigen.

An investigation by Munz *et al.* using a mixture of two F(ab')$_2$ fragments recognising distinct tumour-associated antigens reported a significantly enhanced tumour contrast in nude mice bearing human colon carcinoma xenografts (Munz *et al.*, 1986). Radioimmunoimaging demonstrated the tumour contrast obtained with a mixture of F(ab')$_2$ fragments to be a "considerable" improvement over that using either component alone. Mice receiving the cocktail showed higher tumour to normal tissue contrast at equivalent doses to either F(ab')$_2$ fragment alone, results that reached statistical significance. Tumour contrast did not increase with increasing dose of monoclonal antibody fragments given alone; the highest contrast was obtained with the mixture of both antibodies as increasing the dose of one simultaneously increased the background radiation. Kawabata *et al.* using a similar model to Munz and co-workers found no significantly greater uptake of a mixture of F(ab')$_2$ fragments (Kawabata *et al.*, 1988). Chatal *et al.* found a considerable increase in the sensitivity of immunoscintigraphy in patients with colon carcinoma by injecting monoclonal antibodies to two tumour-associated antigens compared with using a single antibody for imaging (Chatal *et al.*, 1984). Naruki *et al.* used two F(ab')$_2$ fragments targeting CEA and CA 19-9 and demonstrated good tumour targeting in seven patients, but did not compare the results with those obtained with either antibody used alone, a limitation they acknowledged in the discussion (Naruki *et al.*, 1994). Ballantyne *et al.* used a similar combination in a study aimed at imaging both colorectal and pancreatic cancer metastases and found

preferential localisation to colorectal compared with pancreatic carcinoma deposits, possibly the result of an antigen expression level effect (Ballantyne *et al.*, 1988).

Like Matzku *et al.*, Wahl *et al.* used a cocktail of antibodies reactive with different epitopes of the same antigen HMW-MAA (Matzku *et al.*, 1989; Wahl *et al.*, 1990). T o avoid high background radiation each antibody was given at low protein dose to prevent tumour saturation as well as to possibly increase tumour to background ratios. Seventeen patients with stage I or II melanoma were studied using the antibody cocktail for lymphoscintigraphy. Very low background levels were observed in lymph node positive tumours, and no false positives occurred.

Although the results of the experiments with the cocktail of anti-melanoma scFvs have not produced *in vivo* results as good as those observed *in vitro*, the use of a combination of scFvs may have an advantage over individual scFvs in patients with melanoma because of the heterogeneity in the expression of distinct determinants of HMW-MAA and other antigens on melanoma cells within a lesion and among lesions at different anatomic sites (Ziai *et al.*, 1987; Natali *et al.*, 1985). Antibody cocktails have already been shown to be more efficacious in tumour therapy when used in radioimmunotherapy (Song *et al.*, 1998). The improvement in therapy may have been a result of overcoming heterogeneity and targeting m ore cells, or t he cocktail m ay have targeted m ore epitopes on individual tumour cells. Furthermore, combinations of scFvs to distinct melanoma determinants may be useful as carriers of distinct components of a therapeutic system (e.g. two chains of a toxin), which have to be delivered in close proximity at the cell surface, so that they may assemble and function in selected areas of high antigen expression. Zhang *et al.* tested four antibodies *in vitro* against different melanoma and other cancers cell-surface gangliosides alone and in combination (Zhang *et al.*, 1995). Increased tumour cell recognition and complement-dependent cytotoxicity resulting from the combination of three or four of the antibodies was found with all cell lines tested and this was most striking when each antibody was used at suboptimal concentration.

Strategies aimed at reducing immune escape are also likely to benefit from the use of antibody cocktails. Melanomas express complement-inactivating surface markers at high levels as a further means of immune escape (Gorter & Meri 1999). Therapeutic antibody cocktails may be able to overcome the resulting relatively high complement resistance. Melanomas also express high levels of MHC class II, but without the B7 or CTLA-4 (co-stimulatory molecules). Normally MHC class II plus either B7 or CTLA-4 activate circulating T cells. On the other hand expression of MHC class II only induces circulating T cell anergy. Transfection of melanoma with B7 or CTLA-4 has been shown to convert melanoma cells into good targets for T cells. If a cocktail could be

developed with, for example, B3 and B4 as well as another scFv fused to B7, this could overcome escape via MHC class II expression (Rohrbach *et al.,* 2000). Furthermore, scFvs fused to MHC class I have been used to confer melanoma an "allo-phenotype" *in vitro,* resulting in T cell-mediated lysis (Willemsen *et al.,* 2001). With both B7 and MHC class I fusions molecules, a scFv cocktail could be extremely efficient.

5.6 CONCLUSION

This study has shown an improvement in melanoma targeting by an antibody cocktail *in vitro.* If expression is more variable *in vivo* than *in vitro* this cannot be easily tested in mice. LHM2 monoclonal antibody and the scFvs used can only be detected using anti-mouse IgG immunoconjugates, which will subsequently result in high background in immunohistology. The data showed an enhancement in tumour targeting with the use of a cocktail of scFvs targeting different epitopes on human melanoma cells for both the spleen and muscle, when compared with the individual scFvs.

It is somewhat difficult to predict any improvement that may be expected with a similar study in patients as it is still unclear whether the three scFvs used actually target different antigens or different determinants on the same antigen. However, despite this a considerable improvement could be expected due to the substantially increased antigenic heterogeneity in humans compared with that in cell lines or human xenograft tumours. Unfortunately, use of human xenograft-bearing animals is the best model available for such experiments. Whether the results may have been improved with saturating doses is open to debate, as this would probably have concomitantly increased background levels of radioactivity (although this could be counteracted by the rapid clearance of scFvs). Nevertheless, as discussed above, a cocktail of scFvs is likely to be of benefit in immunotherapy, especially when two agents need to be delivered in close proximity to one another. In conclusion the results of this study revealed moderate improvements in tumour targeting with the use of a cocktail of scFvs, which may further be improved upon by using the same strategy in humans.

CHAPTER 6

SCFV MULTIMERS

6.1   INTRODUCTION

A variety of strategies have been adopted to improve the *in vivo* tumour targeting of scFvs. Increasing the valency and molecular weight have been identified as key features for optimising the design of antibody fragments for tumour targeting. ScFv multimers (60-120 kDa) are significantly larger than monomers (~30 kDa) and have the advantages of improved tumour targeting and rapid tumour penetration, but not the fast renal clearance time and high renal accumulation typical for scFv monomers (Hudson & Kortt 1999). Multimerisation leads to higher functional affinity (avidity) by increasing the number of binding sites (valency) of the antibody fragment. The molecular weight is also increased and has been thought by some authors to extend the serum half-life of the molecules in the circulation, as they would not be filtered through the glomeruli (Pack et al., 1992; King et al., 1994). The prolonged blood residence would allow higher tumour accumulation but with reduced contrast to normal organs. However, this theory is probably too simplistic as more recent studies have shown that scFv multimers as large as tetramers are still retained in the kidneys (Schultz et al., 2000). Previous studies have shown that multimers exhibit improved tumour targeting, as measured by tumour-to-normal tissue ratios, compared to monomeric scFvs (Adams et al. 1998a; Adams et al.1998b; Fitzgerald et al. 1997).

Monomeric scFvs are small monovalent molecules, which are rapidly cleared from the circulation (Colcher *et al.*, 1989; Begent et al., 1996). Unfortunately, the monovalent nature confers a disadvantage to scFvs, resulting in limited uptake and retention in tumour xenografts and this can render tumour images sub-optimal (Larson et al., 1997). In contrast, the whole monoclonal antibody is a bivalent molecule with relatively improved avidity when compared with monovalent Fab fragments and scFvs. A similar increase in avidity is therefore possible when scFv fragments are combined into larger multimers (i.e. dimers, trimers and tetramers) (Plückthun et al., 1997; Willuda et al., 2001). An inverse relationship between molecular weight and tumour penetration does exist, however, as seen with whole monoclonal antibodies and a compromise is therefore required between molecular weight, valency and tumour penetration. Also of importance in these multimeric molecules is the flexibility between antigen-binding sites allowing effective cross-linking of target antigen molecules (Wu et al., 1999).

The ideal multimer would exhibit slow blood clearance allowing adequate time for tumour uptake. Higher valency and avidity provide multiple antigen-binding sites, effectively reducing the off-rates, thereby allowing increased antibody retention by the tumour. A multivalent protein capable of binding more than one antigen molecule will bind with a greater free energy than one that can only

bind a single antigen site (Plückthun et al., 1997). Many scFv fragments have been reported to form dimers to some extent, where the V$_H$ domain of one chain is paired with the V$_L$ domain of another chain and vice versa (Essig et al., 1993; Holliger et al., 1993; Whitlow et al., 1993; Whitlow et al., 1994; Desplancq et al., 1994). Various strategies have been investigated to modify the size and valency of antibody fragments in attempts to improve tumour targeting. These designs have included alteration of scFv peptide linkers to form diabodies (Holliger et al., 1993; Kortt et al., 1997) and triabodies (Iliades et al., 1997), disulphide linkage of scFvs through C-terminal cysteine residues (Cumber et al., 1992; Adams et al., 1993; Kipriyanov et al., 1994) and covalent linkage of two scFvs in tandem through an additional peptide linker (Gruber et al., 1994; Mallender & Voss 1994; Kurucz et al., 1995; De Jonge et al., 1995; Mack et al., 1995; Schmidt et al., 1996).

6.2   MULTIMERISATION STRATEGIES

6.2.1   Shortening of the ScFv Peptide Linker

The scFv is composed of the heavy chain variable domain (V$_H$) and light chain variable domain (V$_L$) joined by a short polypeptide linker (typically 15 amino acids), which is designed to facilitate the association of the V$_H$ and the V$_L$ domains from the same chain. Multimers can be produced by altering the length of the peptide linker between the V domains of the scFv fragment. The linker region can be shortened to 5 amino acids or less to prevent refolding of the molecule into the normal, paired orientation of the V domains, thus forcing the spontaneous formation of stable dimers (so-called diabodies) with two antigen-binding sites via non-covalent bonds (Holliger et al., 1993). Reducing the linker length to less than 3 amino acid residues can force scFv association into trimers (triabodies, ~90 kDa) or tetramers (120 kDa) (Dolezal et al., 2000). This produces molecules with increased avidity as well as increased size. In theory this should create molecules with a size above the threshold for renal re-absorption (Rennke et al., 1978), thus reducing renal clearance and accumulation.

The fraction of dimer formed is dependent on linker length (with short linkers between five and ten amino acid residues forcing the almost quantitative formation of dimers), but also on external parameters such as ionic strength, pH and the presence or absence of antigen (Arndt et al. 1998).

Different scFv fragments with intermediate linker lengths also differ greatly in the amount of dimer formed, but the details of this phenomenon are yet to be fully understood (Kortt *et al.*, 1997). Diabodies can be constructed to be bivalent and either monospecific or bispecific. In the latter case these heterodimeric scFvs target two different antigens (Atwell *et al.* 1996) and have been shown to be effective in retargeting cytotoxic T cells to kill lymphoma cells (Holliger *et al.* 1996) or breast cancer cells (Zhu *et al.* 1996). In principle, any lymphocyte can be re-targeted to tumour cells using bivalent scFvs. Diabodies therefore possess considerable potential for immunotherapeutic applications.

6.2.2   Chemical cross-linking

ScFvs can be engineered with unpaired cysteine residues to allow dimer formation via a disulphide bond. Antibody fragments with unpaired cysteines have been produced in E. coli (Cumber et al. 1992; Rodrigues et al. 1993; Hu et al. 1996; Fitzgerald et al. 1997) by fusing these residues onto the C-terminus of the V_H chain (Kipriyanov et al. 1994). The presence of the sulfhydryl group can allow site-specific chemical cross-linking for dimer formation (King et al., 1992). Dimer formation can also occur spontaneously under some conditions, allowing not only the formation of mono-specific dimers for immunodetection, but facilitating the creation of bivalent dimers for immunotherapy (Norton et al., 2001).

Dimer formation usually occurs after secretion from bacteria into an oxidising environment. Whilst the formation of disulphide bonds is not normally spontaneous, an unpaired cysteine can be used to generate disulphide bonds during refolding and chemical cross-linking after scFv purification (Cumber et al. 1992). Such dimers can have improved thermal stability as well as enhanced tumour targeting (Cumber et al. 1992; FitzGerald et al. 1997). Good results have also been obtained with cysteines engineered at the V domain interphase of an Fv, which has resulted in the production of molecules with improved stability and yield (Glockshuber et al. 1990; Webber et al., 1995). However, other this strategy requires knowledge of the three dimensional structure of the recombinant protein, can have variable success (Fitzgerald et al. 1997) and can result in a reduction of expression yield (Plückthun et al. 1997; Waibel et al. 1999). Disulphide-linked dimers can also be made in non-E.coli expression systems including mammalian cells (Sibler et al., 2003) and the yeast, P. pastoris (Gukan et al., 2003).

6.2.3   Naturally Evolved Multimers

A simple alternative multimerisation strategy is to use a combination of the "rational" approach and "directed evolution" to isolate scFvs with a natural tendency to multimerisation.   The rational approach involves aligning the antibody sequence to well-expressing clones (Knappik & Plückthun 1995), together with exchanging exposed hydrophobic residues (Nieba et al.   1997) and the possibility of grafting complementarity-determining regions (CDRs) directly onto very stable and well expressed frameworks (Jung et al. 1997).   Alternatively, the evolutionary approach may be used, where selection pressures place a bias towards selection for improved expression and stability (Proba et al. 1998; Jung et al. 1999). Antibodies undergo cycles of genetic diversification, followed by selection and screening for a desired property such as yield.   The evolutionary approach can be adapted such that selection pressures place a bias towards selection for multimerisation.   Antibodies undergo cycles of genetic diversification followed by Darwinian selection for a desired property.   Selection for multimerisation could be one of these properties (Odili, thesis 2002; Kupsch, personal communication).

6.2.4   Other multimerisation strategies

Other methods of multimer formation have consisted of fusions of scFvs to additional protein domains such as streptavidin, Ig kappa constant regions and leucine zippers, which have been used to produce multimers by fusion to scFv fragments isolated from phage display libraries (Kostelny et al. 1992; Pack et al. 1992; McGregor et al. 1994; Dubel et al. 1995; de Kruif & Logtenberg 1996) with varying degrees of success. Other strategies employed have been: helix-turn-helix domains (Plückthun & Pack et al. 1997); the C_H1 and C_L domain of the Fab can be replaced with a heterodimeric-coiled coil, the so-called helix-stabilized Fv fragment (Arndt et al. 2001); the formation of disulphide bonds (Hu et al. 1996) and the fusion of self-associating peptides to the carboxyl terminus (Willuda et al. 2001). Disulfide-bonded scFvs and scFv dimers have been made with improved stability (Reiter et al. 1996). Some examples of multimerisation strategies are shown in Figure 6.1.
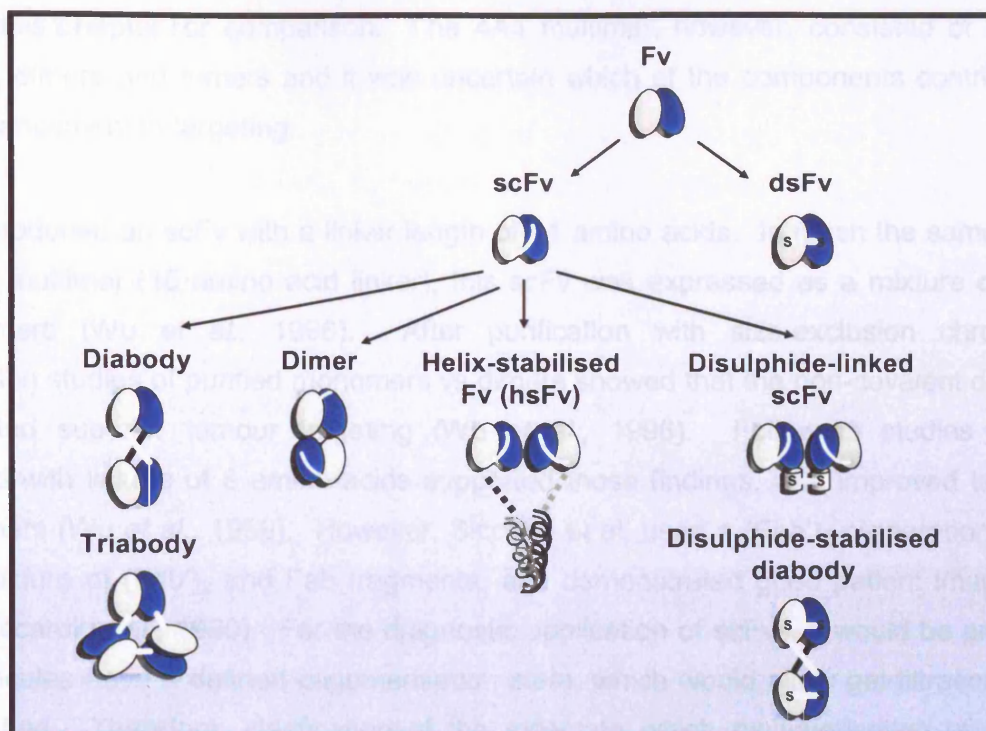
Figure 6.1 Diagram of Recombinant Antibody Fragments (see references in section 6.2.4)

It may be possible to combine these multimerisation strategies to produce a large array of multimers with differing properties for imaging and therapy. However, a risk of combining these strategies is of producing toxic "daisy-chains". These are large molecular assemblies of scFv molecules linked by alternating covalent and non-covalent bonds. Such large complexes cause "jamming" of the secretion apparatus (Hayhurst et al., 2000), expression toxicity and low protein yield. In the laboratory at RAFT this has been seen with non-covalent and disulphide-linked dimers (Kupsch, personal communication). Furthermore, to date there have been no published papers on the successful combination of these strategies.

6.2.5   RAFT multimers

Odili previously investigated the advantages of multimeric scFvs over monomeric scFvs (Odili, thesis 2002). A comparison was made between the RAFT3C4 monomer with the RAFT4A4 multimer. Significant improvements in tumour targeting were demonstrated with much improved tumour to normal tissue ratios for clone 4A4. The results of this work are shown in the results

section of this chapter for comparison. The 4A4 multimer, however, consisted of a mixture of monomers, dimers and trimers and it was uncertain which of the components contributed to the overall enhancement in targeting.

Wu et al. produced an scFv with a linker length of 14 amino acids. In much the same way as the RAFT 4A4 multimer (15 amino acid linker), this scFv was expressed as a mixture of monomers and multimers (Wu *et al.*, 1996). After purification with size-exclusion chromatography biodistribution studies of purified monomers vs dimers showed that the non-covalent dimers clearly demonstrated superior tumour targeting (Wu *et al.*, 1996). Follow-up studies with dimers constructed with linkers of 8 amino acids supported those findings, with improved targeting over the monomers (Wu *et al.*, 1999). However, Siccardi et al. used a $(Fab')_2$ preparation, that was in reality a mixture of $(Fab')_2$ and Fab fragments, and demonstrated good patient imaging with the mixture (Siccardi *et al.*, 1990). For the diagnostic application of scFvs, it would be preferable that these molecules have a defined oligomerisation state, which would allow gel filtration to be used for purification. Therefore, clarification of the extent to which multimerisation of RAFT scFvs improves tumour targeting is required.

6.2.6   The RAFT-3A11 scFv

The RAFT-3A11 clone used for the following experiments has been extensively studied as a monomer (Hamilton, thesis 2001). Affinity maturation by chain shuffling was used to produce 3A11 from the LHM2-derived parent scFv. The resulting scFv was then analysed in vitro and in vivo. Chain shuffling is the term for the replacement of heavy or light chains of a scFv molecule and has previously been used as a means to improve affinity for the target antigen (Clackson *et al.*, 1991; Ohlin *et al.*, 1996; Schier *et al.*, 1996; Park *et al.*, 2000). In the thesis of Hamilton, a combinatorial library of V_K segments was used to produce scFvs consisting of the parent mouse V_H and a large variety of human V_Ks (Hamilton, thesis 2001). This library was screened using antibody phage display techniques. Filamentous bacteriophage "displaying" the different scFvs on their surface were selected on melanoma cells to identify scFvs with increased affinity for the antigen. These scFvs were produced as recombinant protein in E.coli and then assessed. RAFT-3A11 was one of the best of approximately 10,000 different binders isolated.

SDS-PAGE demonstrated that around 0.5 – 1 mg of pure 3A11 scFv was produced per litre of bacterial supernatant. The epitopic specificity was the same as that of the parent monoclonal

antibody LHM2. The scFv showed rapid blood clearance with a $t_{1/2}\alpha$ of 7-9min and a $t_{1/2}\beta$ of 175-206 minutes. In biodistribution studies RAFT-3A11 scFv achieved 4.071%ID/g localising to tumour at 1 hour dropping to 1.186% at 18 hours and good tumour to normal tissue ratios were achieved (e.g. 55.236 for muscle and 46.694 for bone), considerably higher than those achieved by the parent scFv. The 3A11 results will be detailed further in the results section of this chapter for completeness and comparison.

The work in this chapter was done by me apart from: historical control studies (work done by my predecessors), PCR cloning and genetic sequencing (both by Dr Jörg Kupsch).

6.3   AIMS

The aim of this study was to investigate a possible improvement in tumour targeting with increasing multimerisation of scFv. To this end a series of 3A11 scFv pure diabodies and triabodies was constructed. The multimeric 3A11 scFvs were analysed *in vitro* and *in vivo* and compared with the properties of the 3A11 scFv monomer and the spontaneous multimer, clone 4A4.

6.4   *IN VITRO* METHODS

The multimerisation strategy used for 3A11 was to shorten the scFv peptide linker. However, using traditional techniques of splicing by overlay extension (SOE) (Horton *et al.*, 1989) and PCR resulted in contamination with the original scFv peptides, i.e. in re-isolation of the original scFv monomer clones, thought to be due to contamination with the PCR template (Kupsch, personal communication).

Therefore, an alternative tactic was adopted: domain swapping was used in order to prevent re-isolation of the original clone and guarantee formation of multimers with short linkers after PCR. The 3A11 diabody (DB) and triabody (TB) scFvs were made by PCR with appropriate primers. The diabody linker consisted of a 5 amino acid linker (alanine, serine, glycine, glycine, glycine) and the triabody had a 2 amino acid linker (alanine and serine). Both clones have the V_L-linker V_H domain orientation and were sequenced to ensure the correct scFvs had been produced.

3A11 d iabody (DB) a nd t riabody (TB) s cFvs w ere e xpressed i n E . c oli T OP10 a nd m ade from standard 1-litre inductions (Section 2.1). Briefly, a 10 ml culture (LB_{GA}) containing a pipette tip stabbed into bacterial glycerol stocks was grown at 30°C overnight in a rotary shaker (250 rpm). The following morning overnight cultures were made up to 1 litre in the same medium and grown with shaking at 250 rpm for 6 hours at 30°C to an OD_{600} of approximately 1.5. At 6 hours, the bacteria were pelleted and the supernatant poured off. The bacteria were resuspended in 1 litre of LB containing 100 μg/ml ampicillin and 100 μM IPTG. Optimal conditions for induction of individual scFvs in large-scale bacterial culture have been determined empirically (Kang, MD thesis 1997). Cultures were then shaken overnight (for 16-18 hours) at 30°C and 250 rpm and the supernatant harvested the following morning.

All scFvs were purified by IMAC purification on HiTrap chelating sepharose columns (Amersham Pharmacia Biotech; Section 2.2). ScFvs were concentrated to 1 ml in IMAC elution buffer and aliquoted before storage at -80°C. For the purpose of these experiments the scFv preparations were not desalted. The scFvs were analysed by Western blot (section 2.3) and by ELISA o n melanoma cells (section 2.4.3). Further analysis of the scFvs was by gel filtration (section 2.2.7) using the ÄKTA FPLC™ system (Amersham Pharmacia Biotech, Uppsala, Sweden). Two ml aliquots were collected and the samples corresponding to the peaks were subjected to Western blot analysis to confirm the presence of scFv. The molecular weights were then calculated based on a previously determined calibration curve.

6.5 *IN VIVO* METHODS

The scFv m ultimers w ere e xamined for t heir i n vivo t argeting p roperties. The monomeric 3 A11 scFv and 4A4 scFv (a mixture of monomers, dimers and trimers) have previously been examined in vivo (Hamilton, MD thesis 2001; Odili, thesis 2002). These results are included here for comparison.

6.4.1 Radiolabelling

Radiolabelling was carried out using the Iodogen™ method (Pierce) and ^{125}I as the radiolabel (Section 2.5). Immunoreactivity after labelling was assessed by comparing equivalent amounts of radiolabelled scFv to unlabelled scFv in cell ELISA. Ultrafiltration after radiolabelling was performed using VectaSpin Micro™ centrifuge tube filters (Whatman, Maidstone, Kent) to concentrate the radiolabelled scFv to the original volume used for radiolabelling.

6.4.2 Biodistribution and Pharmacokinetics

Biodistribution data were obtained in Balb/c nu mice bearing human melanoma xenografts (Section 2.7). Half a µg of ^{125}I labelled scFv in 100 µl PBS was injected via the tail vein and the mice culled at appropriate time points. Four to five mice were used for each time point. Mice were sacrificed at 1, 3, 6 and 18 hours after injection. A blood sample was obtained by cardiac puncture and organs removed for weighing and gamma counting. Radioactivity in the tumour, blood, both quadriceps, both femurs, spleen, liver, kidneys and lungs were counted. Results were expressed in terms of percentage of the injected dose per gram of tissue (%ID/g). From this the tumour-to-normal tissue ratios (T:NT) were calculated individually for each mouse. The average T:NT value for each organ and the time-point was then calculated.

6.6   *IN VITRO* RESULTS

As discussed in section 6.2.6, previous results for the 3A11 monomer showed that 0.5 – 1mg of pure scFv was produced per litre of bacterial supernatant. The epitopic specificity was confirmed as the same as the parent monoclonal antibody, LHM2, and the scFv showed good melanoma binding. Figure 6.6.1.1 shows a Western blot of the 3A11 preparation and comparing it with the 3C4 monomer and 4A4 multimer, both of which were studied extensively by Odili (Odili, thesis 2002) and will be further discussed below.

The 3A11 diabody and triabody were tested in Western blot and SDS-PAGE and then in cell ELISA to test for melanoma binding. Western blot analysis was performed under reducing and non-reducing conditions. The results revealed pure protein, which, under non-reducing conditions, migrated faster and produced a better signal, as the disulphide bonds of the protein are not disturbed. SDS-PAGE analysis showed both the diabody and triabody to be produced with good purity and yields of 1mg/ml (Figure 6.6.1.2). Figure 6.6.1.3 shows a Western blot including a 3A11 monobody. The different mobilities are due to linker lengths of 25 amino acids (monobody), 5 amino acids (diabody) and 2 amino acids (triabody). All the modified clones are in the V_L-V_H orientation and the original (15 amino acid linker) in a V_H-V_L orientation. The 3A11 diabody and triabody showed improved expression and binding over the monomer and monobody, the monobody binding melanoma only weakly in spite of reasonable expression. It is possible that the long linker in the monobody clones caused folding problems that affected binding but not expression. Both the 3A11 multimers bound melanoma strongly, as demonstrated in Figure 6.6.1.4, which shows the results of the cell ELISA.
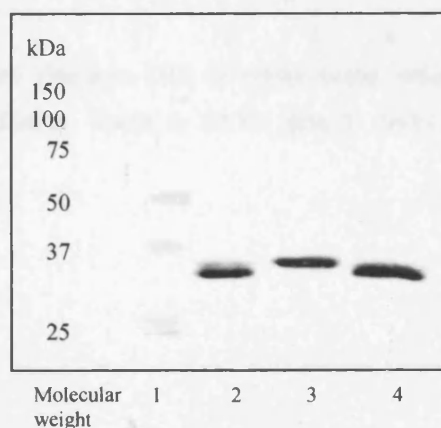
Figure 6.6.1.1 Western blot of scFv clones 4A4 (lane 2), 3C4 (lane 3) and 3A11 (lane 4) (Odili, thesis 2002). The molecular weight marker is shown in lane 1.
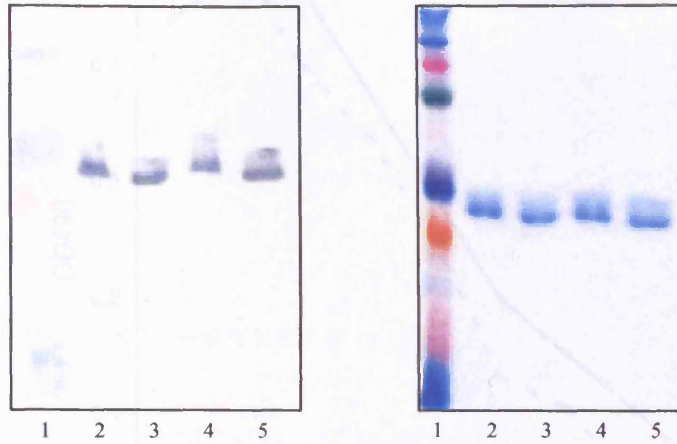
Figure 6.6.1.2 Western blot (left) and SDS-PAGE (right) demonstrating purity of the 3A11 diabody and triabody under non-reducing (lanes 3 & 5) and reducing (lanes 2 & 4) conditions. Lane 1: molecular weight marker, lanes 2 & 3: 3A11 triabody, lanes 4 & 5: 3A11 diabody.
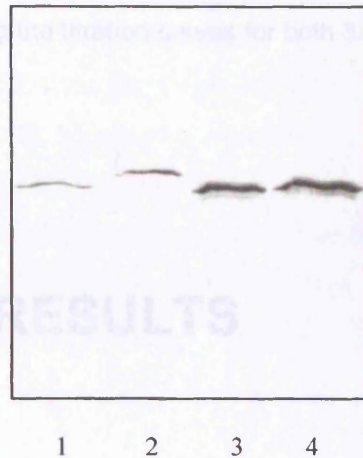
Figure 6.6.1.3 9E10-stained Western blot of small scale induction supernatants from clones in *E. coli* TOP10 under reducing conditions. Lane 1: 3A11, lane 2: 3A11 monobody, lane 3: 3A11 diabody, lane 4: 3A11 triabody.
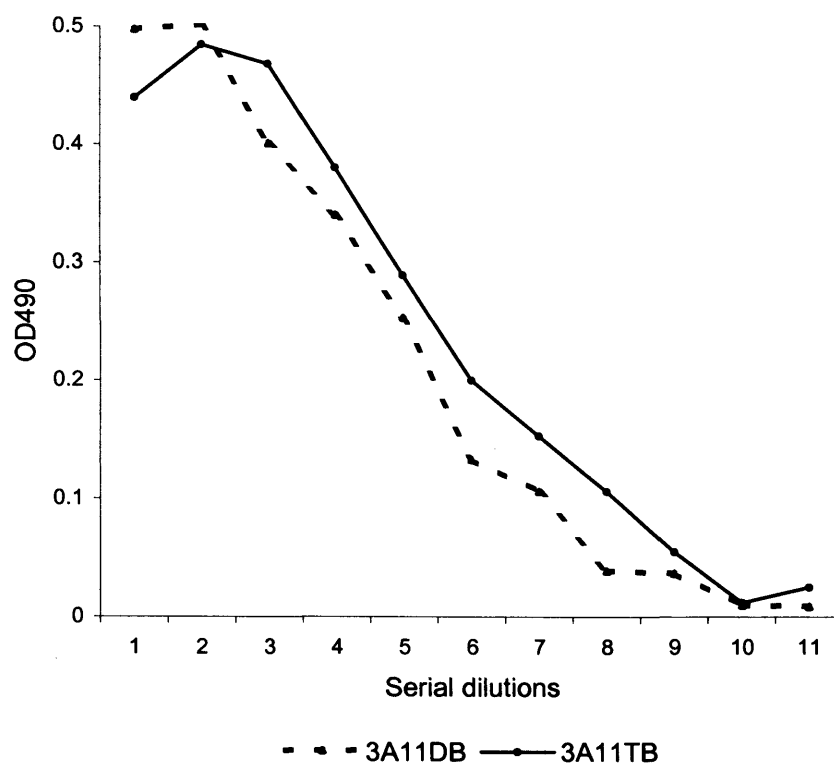
Figure 6.6.1.4 Cell ELISA showing the titration curves for both 3A11 diabody and triabody. Both curves are the mean of three experiments.

6.7   *IN VIVO* RESULTS

6.7.1   Radiolabelling

Incorporation of ^{125}I with the 3A11 diabody and triabody was 10.46% and 8.14%, respectively. The effect of radiolabelling on immunoreactivity was assessed in cell ELISA by comparing the melanoma binding exhibited by equivalent amounts of labelled and unlabelled scFv. The results indicated that approximately 90% of the immunoreactivity was retained by both scFvs (data not shown).

6.7.2   *In vivo* studies

Biodistribution results in tumour and normal organs are shown as %ID/g in Tables 6.7.2.1–3. Maximal tumour accumulation at the time-points measured was found at 1 hour for 3A11 monomer and diabody, whilst maximal tumour accumulation for 3A11 triabody was at 3 hours.

6.7.2.1   3A11 scFv (monomer)

RAFT3A11 monomer scFv achieved 4.071%ID/g localising to tumour at 1 hour, which was cleared steadily over time to 1.186% at 18 hours post-injection (Table 6.7.2.1). The highest %ID/g was found in the kidney at 1 hour (22.950%), which decreased to 2.894% at 18 hours. Values for other normal organs showed a range of 1.320% - 3.930% at 1 hour and decreased steadily with time. The 3A11 monomer showed tumour to normal tissue ratios rapidly increasing with time to values much in excess of one except for the kidney (Table 6.7.2.4). By 18 hours T:NT ratios ranging from 4.120 for liver to 55.236 for muscle were observed. It should be noted that the values for bone may have been subject to considerable experimental error since very small samples of bone were obtained and very low counts were measured, compounding any sampling error. In addition, the very high muscle value achieved was somewhat skewed by one mouse with a very low %ID/g in muscle (almost zero) which almost certainly represented a sampling error. If that mouse is excluded, the tumour to muscle ratio is slightly greater than 17 at this time-point.

6.7.2.2   3A11 diabody

The percentage of the injected dose of 3A11 diabody that localised to the tumour was 0.584% at 1 hour. Accumulation did not clear rapidly and the amount at 18 hours was still 0.489% ID/g (Table 6.7.2.2). The amount that localised to the normal organs at 1 hour ranged from 7.035% ID/g for the lung to 0.829% ID/g for the muscle. For the normal organs 3A11 diabody showed a general trend of steady decrease with time. However, a higher than expected amount was observed in many organs at the 6 hour time-point. The tumour to normal tissue ratios showed rapidly increasing values with time (Table 6.7.2.5). Excluding the kidney, these ranged from 7.005 for the spleen to 32.583 for the muscle. The kidney showed a maximal T:NT ratio of 0.390 at 18 hours.

6.7.2.3   3A11 triabody

Similar to the diabody, 3A11 triabody accumulation remained stable in the tumour, initially achieving a %ID/g of 0.919% at 1 hour and only decreasing slightly to 0.829% at 18 hours (Table 6.7.2.3). In the other organs %ID/g values from 0.253% in the muscle to 4.836% in the kidney were observed at 1 hour. In most organs a steady clearance of the radiolabelled scFv was observed with %ID/g values of less than 0.29% reached for all organs apart from the kidney at 18 hours. At this time point reasonable tumour to normal tissue ratios were achieved of up to 18.896 in the spleen and 14.686 in the lung (Table 6.7.2.6). As for the other scFvs in the series, poor contrast was observed for the kidney, even at 18 hours (0.432).

6.7.2.1   %ID/g for [125]I Labelled 3A11 scFv (monomer)

pi	Tumour	Kidney	Spleen	Liver	Lung	Muscle	Bone	Blood
1 hour	4.071	22.950	2.490	2.718	2.974	1.320	1.411	3.930
3 hours	3.153	9.147	1.012	1.186	1.203	0.547	0.461	1.419
6 hours	2.959	5.866	0.933	1.040	0.870	0.211	0.553	0.687
18 hours	1.186	2.894	0.168	0.288	0.060	0.066	0.026	0.112

6.7.2.2   %ID/g for [125]I Labelled 3A11 diabody

pi	Tumour	Kidney	Spleen	Liver	Lung	Muscle	Bone	Blood
1 hour	0.584	4.556	0.762	3.270	7.035	0.829	0.719	1.105
3 hours	0.094	1.230	0.478	0.726	0.375	0.061	0.148	0.211
6 hours	0.385	2.009	0.298	0.217	0.663	0.115	0.282	0.414
18 hours	0.489	1.406	0.110	0.448	0.178	0.017	0.101	0.086

6.7.2.3   %ID/g for [125]I Labelled 3A11 triabody

pi	Tumour	Kidney	Spleen	Liver	Lung	Muscle	Bone	Blood
1 hour	0.919	4.836	2.004	3.237	1.720	0.253	0.587	0.981
3 hours	1.274	6.276	1.053	1.812	1.375	0.272	0.376	1.352
6 hours	0.401	4.771	0.271	0.837	0.511	0.117	0.139	0.230
18 hours	0.829	2.025	0.066	0.283	0.065	0.088	0.130	0.073

Tables 6.7.2.1-3   Percentage injected d ose per g ram (%ID/g) v alues achieved with t he 3 A11 m onomer, diabody and triabody, respectively. pi: post-injection.

6.7.2.4   T:NT ratios for ^{125}I Labelled 3A11 scFv (monomer)

pi	Kidney	Spleen	Liver	Lung	Muscle	Bone	Blood
1 hour	0.184	1.677	1.536	1.405	3.164	2.961	1.063
3 hours	0.345	3.115	2.658	2.622	5.762	6.845	2.222
6 hours	0.571	3.372	2.982	3.712	16.401	5.350	5.437
18 hours	0.426	7.152	4.120	24.106	55.236	46.694	11.697

6.7.2.5   T:NT ratios for ^{125}I Labelled 3A11 diabody

pi	Kidney	Spleen	Liver	Lung	Muscle	Bone	Blood
1 hour	0.156	1.198	0.408	0.207	1.612	0.860	0.572
3 hours	0.141	1.588	1.105	0.739	5.604	1.012	1.389
6 hours	0.271	2.443	1.908	1.125	9.372	1.764	1.517
18 hours	0.390	7.005	8.181	7.735	32.583	18.594	7.114

6.7.2.6   T:NT ratios for ^{125}I Labelled 3A11 triabody

pi	Kidney	Spleen	Liver	Lung	Muscle	Bone	Blood
1 hour	0.255	0.634	0.305	0.801	3.986	1.745	0.990
3 hours	0.284	2.771	2.117	1.064	7.224	6.548	1.601
6 hours	0.087	3.841	2.286	0.981	8.470	6.906	3.330
18 hours	0.432	18.896	4.079	14.686	9.531	8.257	12.702

Tables 6.7.2.4-6   Tumour to normal tissue ratios (T:NT) achieved with the 3A11 monomer, diabody and triabody, respectively. pi: post-injection.

6.7.2.4   Summary and analysis of results

All three scFvs in the series exhibited good tumour targeting and good contrast for all organs apart from the kidney. Nevertheless, there was no improvement in tumour targeting with increasing multimerisation of the 3A11 scFv. The gene sequences of the scFv multimers confirmed that the diabody and triabody had the intended 5 and 2 amino acid linkers, respectively. Excellent binding of pure preparations of the multimers *in vitro* and good yields suggested that production problems were unlikely to explain these unexpected results. Therefore, the proteins were analysed further by gel filtration, in collaboration with researchers at The Royal Free Hospital.

6.7.3   Analysis of multimers by Gel Filtration

Since the results did not show the expected improvement in tumour targeting with increasing multimerisation, the scFv multimers were subjected to analysis by gel filtration (Section 2.2.7). Access to suitable gel filtration equipment was generously granted by Dr. Kerry Chester's team at The Royal Free Hospital. The results were strikingly different to those expected from the sequencing analysis. Figure 6.7.2.5 shows the results of the gel filtration for the 3A11 triabody at 3 different concentrations. All experiments generated artefact peaks at around 38 minutes, which are routinely observed with the equipment at The Royal Free Hospital (Lee, Y-C, personal communication). The reason for the far larger peak seen at 170µg/ml was the very sensitive detection settings required to visualise the specific peak. The gel filtration experiments revealed elution peaks at approximately 60 minutes for high concentration scFv (1mg/ml) and 69 minutes for lower concentrations (both run at a flow rate of 2ml/min). Comparison with molecular mass standards showed that the first peak corresponded to an apparent molecular mass of about 60kDa, while the second corresponded to about 28kDa. These were assigned as dimer and monomer, respectively. A small shoulder observed to the left of the main peak at high concentrations (52 minutes) corresponded to a molecular weight of around 110kDa, which may have represented some 3A11 tetramer formation as specific bands were observed when aliquots from this peak were tested in Western blot (see Figure 6.7.2.5 top). The same pattern of monomers and dimers was observed with the 3A11 diabody at the same protein concentration (data not shown). Thus, monomers and dimers were observed, but no trimers, even with the 3A11 "triabody". The results showed that multimerisation was concentration-dependent. At the concentrations tested no mixture of monomers and dimers formed.

3A11TB 1000µg/ml
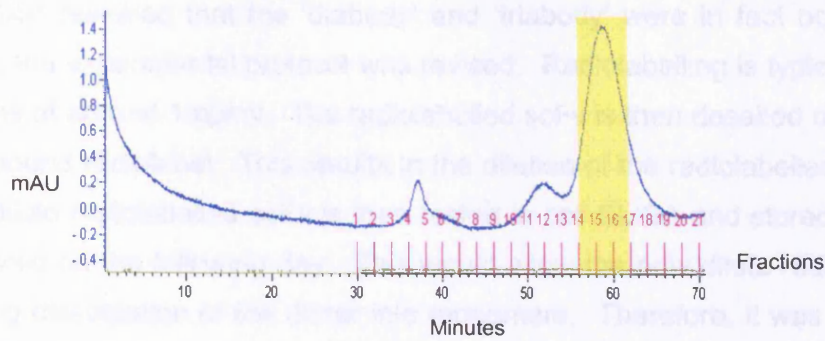
3A11 Triabody 330µg/ml
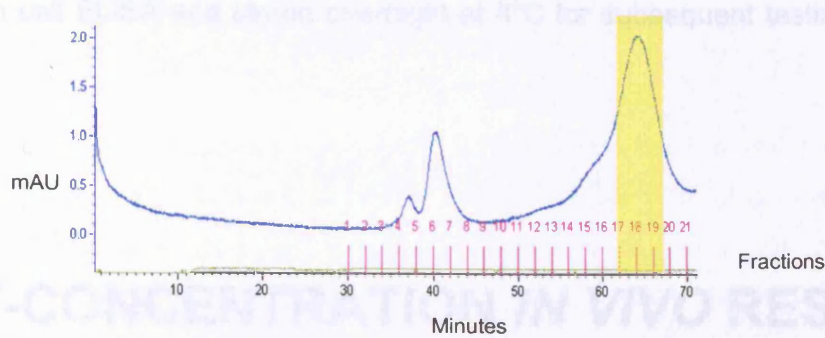
3A11 Triabody 170µg/ml
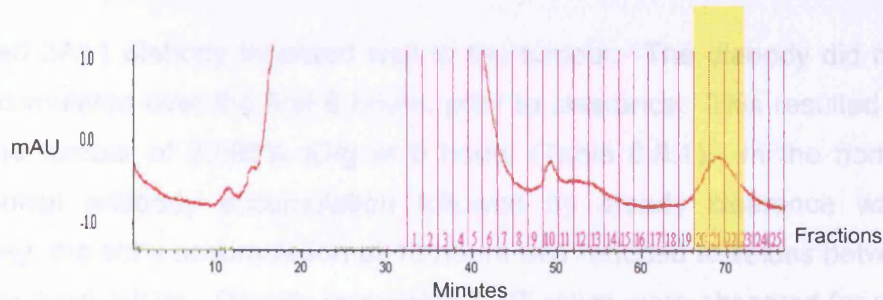
Figure 6.7.2.5 Examples of the results of gel filtration at different concentrations of 3A11 triabody. The pink lines correspond to the fractions collected and the yellow shaded areas correspond to those fractions that were positive for scFv in Western Blot. mAU: milli-absorbance units.

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6.7.4   Change of protocol

After the gel filtration revealed that the 'diabody' and 'triabody' were in fact both concentration-dependent dimers, the experimental protocol was revised. Radiolabelling is typically carried out at scFv concentrations of around 1mg/ml. The radiolabelled scFv is then desalted on a PD10 column to remove any unbound radiolabel. This results in the dilution of the radiolabelled scFv by nine- to twelve-fold. The dilute radiolabelled scFv is then tested in cell ELISA and stored overnight at 4°C prior to testing *in vivo* on the following day. This would allow the now dilute radiolabelled scFv to equilibrate, allowing dissociation of the dimer into monomers. Therefore, it was reasoned that by re-concentrating the radiolabelled scFv to 1mg/ml after desalting, the dimers would be prevented from dissociating. The radiolabelled scFv was reconcentrated by ultrafiltration to the original volume that it had prior to radiolabelling (section 2.5.3). The reconcentrated, radiolabelled scFv was then tested in cell ELISA and stored overnight at 4°C for subsequent testing in mice on the following day.

6.8 POST-CONCENTRATION *IN VIVO* RESULTS

6.8.1   Post-concentration 3A11 "diabody" scFv (dimer)

The re-concentrated 3A11 diabody localised well to the tumour. The diabody did not clear from the tumour but accumulated over the first 6 hours, prior to clearance. This resulted in a maximal accumulation in the tumour of 7.598% ID/g at 6 hours (Table 6.8.1). In the normal organs a pattern of high initial antibody accumulation followed by steady clearance was observed. Excluding the kidney, the scFv accumulation at 18 hours was reduced to values between 0.109 (in muscle) to 0.484 (in liver) %ID/g. Rapidly increasing T:NT ratios were observed for all organs and most of these reached high values (Table 6.8.5 and Figure 6.8.2). At 18 hours T:NT ratios of 69.770 were reached in muscle, 34.649 in bone, 29.644 in lung and 28.409 in liver. The high tumour to blood contrast of 23.787 at 18 hours is the most likely reason for the good T:NT values in the blood-rich organs analysed.

6.8.2   Post-concentration 3A11 "triabody" scFv (dimer)

The 3A11 triabody showed a very similar biodistribution pattern to that of the diabody, which would have been expected after the gel filtration revealed that they actually were both dimers. The scFv remained in the tumour for all the time-points, whilst in the normal organs initial accumulation was followed by clearance of the scFv. The %ID/g in the normal organs were not as high as those observed with the diabody, but the pattern observed was similar (Table 6.8.2). Despite this, excellent tumour to normal tissue ratios were reached at 18 hours (Table 6.8.6 and Figure 6.8.4). The highest T:NT value was observed for muscle (83.832), whilst in the bone 22.505, in the spleen 26.693 and in lung 20.771 were reached. The blood contrast at 18 hours was 16.941 similar to the diabody.

6.8.3   4A4 (multimer) results

For completion and further comparison, the results obtained with the 4A4 scFv are included here. 4A4 is a mixture of monomers, dimers and trimers and previously demonstrated improved tumour targeting compared with 3 scFv monomers tested, including 3A11 (Odili, thesis 2002). 4A4 scFv showed good biodistribution properties with high tumour uptake, which persisted for 6 hours (Table 6.8.3). Initial tumour uptake of 4A4 was 4.586 %ID/g at 1 hour following administration, stayed high at 6h (3.961 %ID/g) and the scFv remained in the tumour at relatively high levels even at 18 hours post-injection (2.608 %ID/g). 4A4 scFv localised very well to the tumour xenografts, and activity in normal organs and blood cleared rapidly, resulting in high tumour to normal tissue ratios at 18 hours (44.968 in muscle, 6.610 in blood) (Table 6.8.7 and Figure 6.8.3). The kidneys showed high initial activity, which cleared rapidly. Kidney activity reached peak levels at 1 hour following injection (24.337 %ID/g) and declined rapidly to 5.947 %ID/g at 3 hours and 0.517 %ID/g at 18 hours. Blood clearance was also rapid from 7.668 %ID/g at 1 hour to 0.398 %ID/g at 18 hours. The main differences between 4A4 and 3A11 diabody and triabody were its lower renal accumulation and slower blood clearance.

6.8.1   %ID/g for ^{125}I Labelled 3A11 "diabody" scFv (dimer) post-concentration

pi	Tumour	Kidney	Spleen	Liver	Lung	Muscle	Bone	Blood
1 hour	2.698	14.919	7.512	7.526	8.167	0.396	1.110	1.274
3 hours	0.780	14.042	4.914	4.696	4.937	0.660	0.798	0.584
6 hours	7.598	11.935	2.885	4.069	3.850	0.697	0.825	0.943
18 hours	6.127	4.929	0.422	0.484	0.383	0.109	0.322	0.345

6.8.2   %ID/g for ^{125}I Labelled 3A11 "triabody" scFv (dimer) post-concentration

pi	Tumour	Kidney	Spleen	Liver	Lung	Muscle	Bone	Blood
1 hour	1.679	17.406	0.768	1.414	0.851	0.430	1.342	2.805
3 hours	1.117	9.179	0.432	1.022	1.594	0.344	0.583	1.186
6 hours	2.741	7.908	0.844	1.194	0.720	0.248	0.618	0.924
18 hours	2.096	3.986	0.156	0.463	0.173	0.027	0.106	0.131

6.8.3   %ID/g for ^{125}I Labelled 4A4 multimer scFv (mixture of multimers & monomers)

pi	Tumour	Kidney	Spleen	Liver	Lung	Muscle	Bone	Blood
1 hour	4.586	24.337	3.259	2.976	3.885	0.867	1.732	7.668
3 hours	4.098	5.947	1.699	1.408	1.903	0.430	1.044	3.291
6 hours	3.961	1.967	0.887	0.921	1.045	0.204	0.415	1.701
18 hours	2.608	0.517	0.308	0.238	0.266	0.058	0.088	0.398

6.8.4   %ID/g for ^{125}I Labelled 3A11 scFv (monomer)

pi	Tumour	Kidney	Spleen	Liver	Lung	Muscle	Bone	Blood
1 hour	4.071	22.950	2.490	2.718	2.974	1.320	1.411	3.930
3 hours	3.153	9.147	1.012	1.186	1.203	0.547	0.461	1.419
6 hours	2.959	5.866	0.933	1.040	0.870	0.211	0.553	0.687
18 hours	1.186	2.894	0.168	0.288	0.060	0.066	0.026	0.112

Tables 6.8.1-4 Percentage injected dose per gram (%ID/g) values for the 3A11 diabody and triabody post concentration, in comparison with the (unconcentrated) 4A4 multimer and 3A11 monomer. pi: post-injection.

6.8.5 T:NT ratios for ^{125}I 3A11 "diabody" scFv (dimer) post-concentration

pi	Kidney	Spleen	Liver	Lung	Muscle	Bone	Blood
1 hour	0.190	1.136	0.952	1.341	7.877	2.770	3.556
3 hours	0.120	2.487	1.099	3.731	9.314	1.526	6.326
6 hours	1.045	4.000	6.031	5.028	18.379	10.487	12.026
18 hours	1.834	18.371	28.409	29.644	69.770	34.649	23.787

6.8.6 T:NT ratios for ^{125}I Labelled 3A11 "triabody" scFv (dimer) post-concentration

pi	Kidney	Spleen	Liver	Lung	Muscle	Bone	Blood
1 hour	0.193	2.762	1.268	3.138	6.972	2.155	0.861
3 hours	0.249	7.494	2.080	4.135	10.589	6.033	1.636
6 hours	0.379	5.564	2.997	5.197	15.005	5.685	4.500
18 hours	0.536	26.693	5.676	20.771	83.832	22.505	16.941

6.8.7 T:NT ratios for ^{125}I Labelled 4A4 multimer scFv (mixture of multimers and monomers)

pi	Kidney	Spleen	Liver	Lung	Muscle	Bone	Blood
1 hour	0.190	1.408	1.545	1.178	5.297	2.648	0.596
3 hours	0.689	2.412	2.911	2.153	9.530	3.925	1.245
6 hours	2.060	4.562	4.477	3.927	21.593	9.954	2.424
18 hours	5.069	8.735	11.023	9.837	44.968	30.081	6.610

6.8.8 T:NT ratios for ^{125}I Labelled 3A11 scFv (monomer)

pi	Kidney	Spleen	Liver	Lung	Muscle	Bone	Blood
1 hour	0.184	1.677	1.536	1.405	3.164	2.961	1.063
3 hours	0.345	3.115	2.658	2.622	5.762	6.845	2.222
6 hours	0.571	3.372	2.982	3.712	16.401	5.350	5.437
18 hours	0.426	7.152	4.120	24.106	55.236	46.694	11.697

Tables 6.8.5-8 Tumour to normal tissue ratios (T:NT) for the 3A11 diabody and triabody post-concentration, in comparison with the (unconcentrated) the 4A4 multimer and 3A11 monomer. pi: post-injection.

6.8.1 3A11 monomer
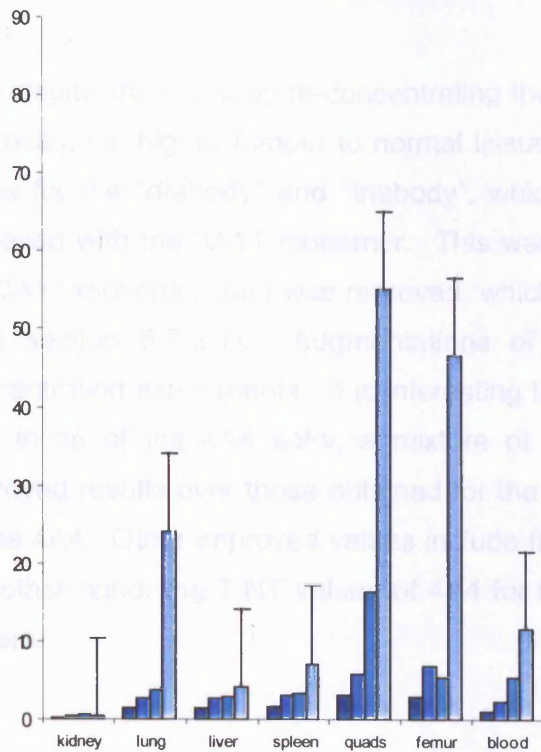
6.8.1 3A11 'DB'
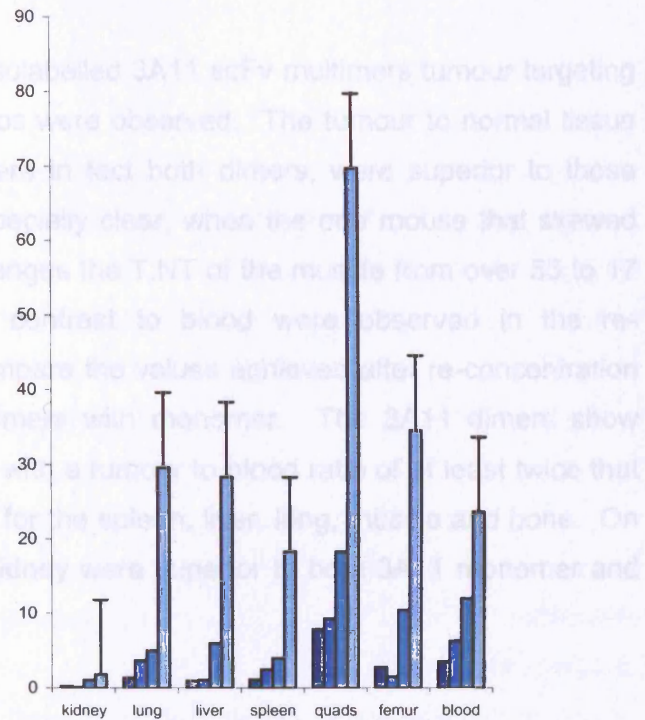
6.8.3 4A4
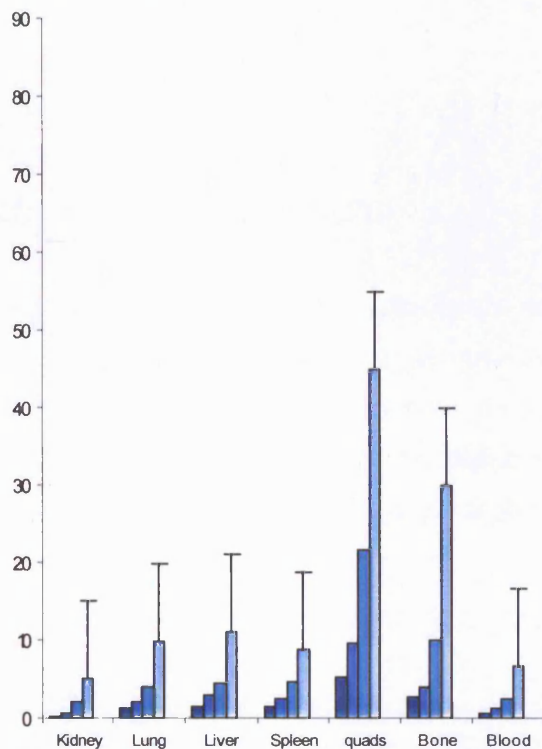
6.8.4 3A11'TB'
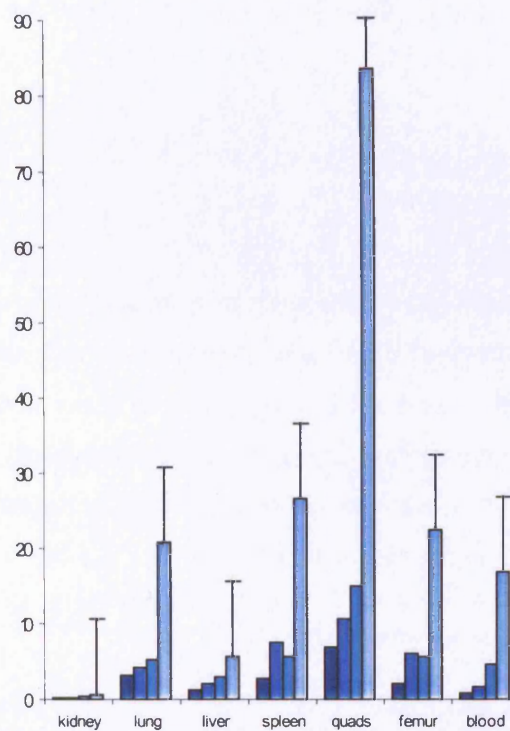
Figures 6.8.1-4 Graphical representations of tumour to normal tissue ratios of 3A11 monomer (1), 3A11 "diabody" post-concentration (2), 4A4 multimer (3) and 3A11 "triabody" post-concentration (4). Error bars represent 1 SD.

6.8.4 Summary and comparison of results

The results show that by re-concentrating the radiolabelled 3A11 scFv multimers tumour targeting improved, i.e. higher tumour to normal tissue ratios were observed. The tumour to normal tissue ratios for the "diabody" and "triabody", which were in fact both dimers, were superior to those achieved with the 3A11 monomer. This was especially clear, when the one mouse that skewed the 3A11 monomer data was removed, which changes the T:NT of the muscle from over 55 to 17 (see section 6.7.2.1). Augmentations of the contrast to blood were observed in the re-concentration experiments. It is interesting to compare the values achieved after re-concentration with those of the 4A4 scFv, a mixture of multimers with monomer. The 3A11 dimers show improved results over those obtained for the 4A4 with a tumour to blood ratio of at least twice that of the 4A4. Other improved values include those for the spleen, liver, lung, muscle and bone. On the other hand, the T:NT values of 4A4 for the kidney were superior to both 3A11 monomer and dimers.

6.9   DISCUSSION

The purpose of these experiments was to produce and assess, *in vitro* and *in vivo*, a series of pure anti-melanoma scFv multimers of varying size and valencies for their targeting efficiency of high molecular weight melanoma-associated antigen (HMW-MAA). Multimerisation strategies are used to improve tumour targeting, to reduce background accumulation (for imaging applications) and to reduce exposure of normal organs to toxic agents (for therapeutic applications). The experiments examined two clones derived from the 3A11 monomer that were initially considered to be a diabody and a triabody after sequence analysis.

In vitro experiments showed that the scFvs were produced with good purity and yields (around 1mg/ml). E xcellent m elanoma b inding w as d emonstrated i n cell E LISA f or b oth the multimeric clones. The expression yield and *in vitro* melanoma binding of the 3A11 multimers is superior to the 3A11 monomer whereas a similar series with the 4A4 scFv showed no improvement (Kupsch, personal communication). The 3A11 multimers were therefore chosen for testing in the mouse model.

6.9.1   *In vivo* results

Radioimmunopharmaceuticals typically display a biphasic pattern of clearance from the circulation (Milenic *et al.*, 1991). The primary phase represents the development of an equilibrium in all bodily tissues. This first phase has a half-life designated $t_{1/2}\alpha$ and is usually rapid (Adams, 1998b). The secondary phase is less rapid and reflects the gradual release of intact radiopharmaceuticals dissociating from the antigen and clearance of non-specifically accumulated scFv from normal tissues. The half-life of the secondary phase is termed $t_{1/2}\beta$. The data in the present study confirm the rapid pharmacokinetics of the RAFT 3A11 series of scFvs.

The in vivo results with the diabody and triabody in athymic mice bearing melanoma xenografts were initially disappointing. The expected improvement in tumour targeting and tumour to normal tissue ratios were not observed. Repeated experiments confirmed this to be the case. Subsequent analysis of the scFvs by gel filtration revealed unanticipated results. The 3A11 diabody and triabody both showed concentration-dependent multimerisation. Both proteins formed

dimers at high concentration and monomers at lower concentrations. In fact, their molecular weights were identical at equivalent concentrations, suggesting that notwithstanding their different genetic sequence, they were very similar scFvs. Further experiments were aimed at preventing the scFvs from reaching an equilibrium that favoured monomers at dilute concentrations after radiolabelling. The radiolabelled scFvs were re-concentrated to their original pre-radiolabelling concentrations and volume. Subsequent in vivo testing of the re-concentrated scFvs showed superior targeting with scFv dimers. Overall improvements in tumour targeting and tumour to normal tissue ratios were seen for the dimers compared with the monomer. The results observed were better than those achieved with the 4A4 clone, which is a mixture of monomer, dimer and trimer at concentrations of around 4mg/ml (Odili, MD thesis 2002). The tumour to blood ratios were extremely good as were the tumour to muscle ratios. These two values are of particular significance to melanoma targeting, as most initial recurrences are loco-regional recurrences around the site of the primary tumour or in the draining lymph node basin (Balch et al., 2001b). In humans, these s ites t end t o b e o n the l imbs for t he l ocal r ecurrences and a round s urrounding muscle and bone (such as the axilla or inguinal region) for the regional recurrences.

Despite the rapid clearance, all the scFvs localised to the tumours, reaching maximum uptakes of approximately 1.6 – 4.6%ID/g at 60 min following intravenous injection (Tables 6.8.1-4). These results are comparable to tumour targeting studies of scFv reported by other laboratories (Colcher et al., 1990; Milenic et al., 1991; Whitlow et al., 1993). The tumour accumulation of the 3A11 scFv of 1.186 %ID/g at 18 hours is similar to the values reported in other similar studies. For example, Adams et al. achieved a value of 1.42%ID/g at 24 hours (Adams et al., 1998a). The tumour accumulation of the reconcentrated 3A11 dimer was 2.096 – 6.127 %ID/g for the "triabody" and "diabody", respectively at 18 hours post-injection. Therefore both dimer preparations showed stable tumour retention to a greater extent than that reported for other dimers in the literature. For example, when Goel et al. tested scFv dimers in athymic mice bearing human colorectal cancer xenografts, accumulation declined rapidly from the tumour after 4 hours (Goel et al., 2001). Tetramers were stably retained in the tumour for 6 hours. Others have also found that although multimerisation into dimers or trimers improves initial tumour accumulation, the retention of antibody fragments did not remain stable in vivo (King et al., 1994; Viti et al., 1999). For example, King et al. tested a F(ab')$_2$ antibody fragment and observed a maximum tumour accumulation of approximately 3%ID/g at 6 hours post-injection, which then decreased to 1.5%ID/g and 0.25%ID/g at 24 and 72 hours, respectively. The tumour accumulation in the study by Viti et al. was 7.32%ID/g at 4 hours which decreased to 1.29%ID/g at 24 hours. In comparison, the %ID/g observed in the present study was 2.698%ID/g at 1 hour and 6.127%ID/g at 18 hours for the

diabody. For the triabody 1.679%ID/g at 1 hour and 2.096%ID/g at 18 hours for the triabody were observed.

The dimers had a rapid blood clearance. Several groups have observed rapid blood clearance of dimeric scFvs that was slightly prolonged compared with the monomer (Adams *et al.,* 1993; Wu *et al.,* 1996). The clearance of the dimer and monomer in the present study was similar and, combined with the stable tumour retention observed, resulted in good T:NT targeting values. These were superior to previous studies with antibody multimers that did not show stable tumour retention over time (see section 6.9.4 below).

6.9.2   scFv localisation to the kidney

Renal accumulation of small tumour-targeting molecules reduces the efficacy of both imaging and therapeutic studies. Significant renal accumulation is typical with antibody fragments, especially those small enough to be freely filtered in the kidney (below approximately 60kDa) (Silbernagl, 1988). Whilst renal localisation may represent only a minor nuisance in diagnostic imaging, in therapeutic studies nephrotoxicity becomes a possibility and may represent the dose-limiting factor (although other studies have suggested that the bone marrow would be the dose-limiting organ; Behr *et al.,* 1998). Indeed, whilst a great many imaging studies using antibody fragments have now been published, relatively few in vivo studies using therapeutic scFv constructs have been seen in the literature. Moreover, nephrotoxicity has been a serious concern in many of those published so far (Behr *et al.,* 1997; de Jong *et al.,* 1997; Reubi, 1997). Strategies to reduce renal accumulation are therefore of critical importance if the superior tumour to normal tissue ratios achieved using antibody fragments compared with whole immunoglobulins are to be exploited in cancer therapy.

The renal accumulation of antibodies and small peptide molecules is thought to be largely a function of the kidney's ability to filter and reabsorb peptides falling below a threshold of around 60kDa in size (Rennke *et al.,* 1978; Silbernagl, 1988). These molecules are filtered through the glomerular basement membrane and then actively reabsorbed in the proximal convoluted tubule by pinocytosis. Under physiological conditions, little or no protein or peptide material is lost in the urine. Evidence supporting this comes from studies such as that by Viti et al. who demonstrated improved tumour to kidney ratios with a dimer over a monomer (Viti *et al.,* 1999). However, in other studies multimerisation actually led to increased renal accumulation. King et al. found that renal accumulation was worse with a trimer than a dimer, which in turn was worse than the

monomer (King *et al.*, 1994). Furthermore, in the study by Goel *et al.*, the tetramer studied reached a maximal tumour to kidney ratio of only 3.4 (Goel *et al.*, 2001). This value is similar to the T:NT ratio of 5.069 at 18 hours observed with the 4A4 scFv (mixture of monomers and multimers; see Table 6.8.7), supporting the view that kidney retention is not determined by size alone.

The data from other studies, therefore, suggest that size alone does not determine renal accumulation. The renal accumulation of the 3A11 scFv series was considerable at early time-points and consistent with the renal accumulation typically seen with molecules of this size (Maack *et al.*, 1979; Silbernagl, 1988). No significant difference was seen between the clones studied. The slightly higher renal accumulation seen with the 3A11 scFv monomer at the 1-hour time-point was not evident at 18 hours. This is in contrast to what would have been predicted if size were the sole determinant of kidney accumulation (Maack *et al.*, 1979). If size alone were the determinant of kidney accumulation of the RAFT scFvs, the following order of renal clearance would be expected: 3A11DB/TB > 4A4 > 3A11 monomer. This order is not observed; in fact, the order is: 3A11DB/TB > 3A11 monomer > 4A4. The observed pattern is more consistent with size and charge both being important for renal accumulation.

The kidney is an organ rich in membranes with negative surface charge, which suggests the possibility of binding to the positively charged 3A11 (pI = 8.6). The 4A4 multimer demonstrated a faster renal clearance than the 3A11 dimers. It has a pI of 7.8, almost neutral, which supports a hypothesis that charge may be more important than size for renal clearance. Another clone, B3 (Kupsch *et al.*, 1999; J. Odili, unpublished data), a monomer with a pI of 6.7 also has a relatively low kidney accumulation (T:NT 0.442 at 6 hours and 1.42 at 18 hours – see Chapter 5) and like 4A4, a relatively slow blood clearance (tumour to blood ratio of 6.444 at 18 hours) suggesting that charge could play a role in kidney filtration or clearance.

As well as altering the charge of scFvs in an attempt to improve renal accumulation, other strategies have been suggested. These have included the co-administration of cationic amino acids such as lysine to block reabsorption of radiolabelled scFv by renal cells (Behr et al., 1998b; Hamilton et al., 2002). Another strategy is the interposition of a cleavable linker between the antibody fragment and the site of radiolabel, so that the radiolabel is excreted from the lysosomal compartments of renal cells into the urine after lysosomal proteolysis of the radiolabelled antibody fragment in the renal cells (Arano et al., 1999). However, the latter strategy was not tested in tumour-bearing mice and its effect on tumour targeting has yet to be shown.

The relatively high level of persisting renal accumulation observed in the present study (over 2%ID/g at 18 hours) might hinder therapeutic approaches using the 3A11 scFv dimers but would not prevent imaging studies.

6.9.3   ScFv Localisation to Other Organs

The distribution of the 3A11 dimer to other organs differed between the diabody and triabody preparations, although the tumour to normal tissue ratios were comparable.   The diabody preparation demonstrated better tumour localisation initially (Table 6.8.1), but by 18 hours the %ID/g for both preparations was very similar (Tables 6.8.1 & 6.8.2).   This was to be expected as both preparations consisted of 3A11 dimers at the time of injection.   The two preparations may still differ in their thermal stability or their tendency to dissociation into monomers in vivo (neither of which were tested).   Compared with the diabody, the tumour to liver ratios were lower for the triabody after concentration, which could be due to lower stability and possibly degradation in the liver.

The other discrepancies could have been due to differences in radiolabelling, differences in administration of the radiolabelled scFvs or possibly sampling errors.   The stability of the radiolabel in vivo for each scFv and the inherent resistance of the constructs to proteolysis were not studied specifically and could play a part in the overall targeting efficiency.   In agreement with published reports, the data demonstrated that rapid tumour uptake and rapid clearance from blood and normal tissues resulted in high tumour to muscle ratios.   In addition, the present study and that of Odili (Odili, thesis 2002) are the first to show a further improvement of in vivo targeting by stable tumour retention of scFv multimers.

6.9.4   Tumour to Normal Tissue Contrast

Tumour to normal tissue ratios are shown in Tables 6.7.2.4-6 and Tables 6.8.5-8.   A striking improvement in tumour to normal tissue (T:NT) contrast is seen with dimerisation.   This improvement should result in both increased imaging contrast and therapeutic efficiency.   Since the normal tissue accumulation remains similar to the 3A11 monomer, the increase in T:NT is almost entirely a function of increased tumour retention.   Adams et al. compared scFv monomers and dimers in scid mice bearing tumour xenografts and found slower clearance with the dimers,

leading to increased tumour retention (Adams et al., 1993). This appeared to be unrelated to the affinity of the original monomeric scFvs or the dimers themselves (Nielsen et al., 2000).

As noted previously, the tumour to muscle ratio for the 3A11 monomer at 18 hours (of 55.236) was skewed by a very low %ID/g value in one mouse. This potential error, however, demonstrates the effect of 2 methods of calculating T:NT values. The tumour to normal tissue ratios can either be calculated for each mouse individually and then the mean T:NT is derived for the group of mice. Alternatively, it can be calculated for each organ from the mean %ID/g of the group of mice. The latter method would be less likely to be skewed by a single erroneous result in %ID/g but does not truly represent the T:NT achieved in each subject mouse. A review of published papers using the nude or SCID mouse xenograft model revealed no detailed description of the exact methods employed for calculating T:NT ratios. It can be reasoned that the former method produces a more accurate representation of the contrast achieved mouse by mouse and, therefore, it was used in all the present studies. Although the mean %ID/g in tumour at the 18 hour time-point is 1.186 and in muscle 0.066, the accurate T:NT is 55.236 (taken as a mean of T:NTs) rather than a value of 17.9 which one might expect (i.e. 1.186/0.066). Excluding the apparently erroneous value obtained with one mouse gives a tumour to muscle ratio of almost 18 in this instance. Even with the potential vulnerability of this method to experimental error, it appears to present a more accurate reflection of the situation in vivo.

The results are most encouraging and demonstrate the improvement that can be achieved by dimerisation. In comparison to other published studies, the T:NT ratios achieved are highly promising. Results for the 3A11 dimers are superior to those obtained by King et al. using F(ab')$_2$ fragments (King et al., 1994) and those of Verhaar et al. using an anti-CEA scFv in mice (Verhaar et al., 1996) which was subsequently used to produce impressive patient images (Begent et al., 1996). Furthermore, the tumour to blood ratios in the present study are better than those achieved by Viti et al. (1999) (23.787 at 18 hours vs. 10.33 – see Table 6.8.5) who did not observe stable tumour retention.

The fact that the scFv dimers tended to persist in the tumours for a considerable period in these experiments is the main explanation for the improvement in tumour to normal tissue ratios. The increased tumour targeting and retention observed in the present study for the anti-HMW-MAA dimers is likely to be due to higher valency and avidity, rather than increased serum persistence. A comparison of the terminal half-lives of two versions of anti-CEA diabody with half-lives determined for monomeric scFvs in mice and humans, revealed that all exhibit terminal half lives within the range of about 3-5 hours (King et al., 1994; Begent et al., 1996; Wu et al., 1999).

Adams et al. have similarly observed prolonged xenograft retention (combined with rapid blood clearance) of an anti-HER2/neu diabody constructed with a five amino acid linker (Adams et al., 1998c).

6.9.5 Other explanations for improved results

While the major factor in the increase in tumour localisation is likely to be avidity for the antigen and hence prolonged tumour retention, other possible factors merit consideration. The studies in this chapter have attempted to standardise as many variables as possible, including target epitope, labelling technique and immunoreactivity. However, as discussed in section 6.9.2, molecular charge can play a part in the pharmacokinetics of targeting molecules and cannot be easily standardised in experiments of this type. Previous studies have demonstrated that increasing the pI by chemical cationisation of Fab' fragments can markedly increase renal excretion without affecting tumour localisation (Tarburton et al., 1990). Conversely, increasing the isoelectric point (pI) of a whole antibody from 8.9 to >9.5 has been shown to increase cellular uptake and reduce residence time in the systemic circulation (Pardridge et al., 1995). Interaction of the charged protein molecules with negatively charged surfaces of vascular endothelial cells and proximal renal tubular cells have been proposed as the explanation of these findings (Maack et al., 1979; Sumpio & Maack 1982). A Iteration of the isoelectric point may therefore be a strategy for tackling the problem of renal accumulation (Odili, MD thesis 2002) as the present study and others suggest that multimerisation strategies seem to be ineffective in achieving more than a modest reduction of kidney accumulation of antibody fragments. In previous studies, the primary effect of charge modification has been on renal clearance rather than cellular uptake (Tarburton et al., 1990; Pardridge et al., 1995).

6.9.6 Method of multimerisation - altered linker length

A number of research groups have shown that single chain Fv fragments, depending both on the V_H/V_L interface and the linker length, can spontaneously dimerise or form higher multimers (Essig et al., 1993; Whitlow et al., 1993; Holliger et al., 1993; Griffiths et al., 1993; Schodin et al., 1993; Odili, MD thesis 2002). A more systematic investigation of this phenomenon (Holliger et al., 1993; Whitlow et al., 1994; Desplancq et al., 1994) then demonstrated that the percentage of monomer is directly proportional to the linker length connecting the two variable domains. Molecular modelling and subsequently the determination of the crystal structure of a dimeric scFv (Perisic et al., 1994)

clearly showed that the V_H and V_L domains can dissociate and associate with a second scFv molecule, to create two V_H/V_L assemblies in one molecule, termed a diabody (Holliger *et al.*, 1993). In some cases, the monomers and dimers are in a concentration-dependent equilibrium (Kortt *et al.*, 1994; Lee *et al.*, 2002).

Alteration to the peptide linker is known to promote multimerisation. Hence it was used to construct the 3A11 multimers (Alfthan *et al.*, 1995). Shortening the linkers to less than 12 amino acids tends to promote multimer formation (Kortt *et al.*, 1997) whilst longer linkers (>17 amino acids) tend to result in the production of monomeric scFv (Whitlow *et al.*, 1993; Desplancq *et al.*, 1994; Wu *et al.*, 1996). However, linker engineering can have a number of drawbacks. The nature of the multimers formed seems rather unpredictable and a property of the combination of antibody and the linker used. In addition, reducing the linker length from 3 to 2 residues does not precisely dictate the transition from dimers to higher multimers. Instead, a mixture of multimers (dimers, trimers and tetramers) may form, and the tetramers may be unstable, reverting back to the dimeric or monomeric state (Dolezal *et al.*, 2000). Short linkers of length less than 12 residues tend to stabilise the diabody or triabody forms of the scFv molecule, as these prevent the association of V_H and V_L domains in monomeric scFv. The reversibility of dimerisation is dependent on buffer conditions and the scFv (Arndt *et al.*, 1998). HPLC gel filtration of the scFv 3A11 diabody and triabody showed peaks with the molecule existing as a monomer or dimer depending on the concentration. Both clones formed dimers at high concentration, despite differing linker lengths (2 amino acids for the triabody and 5 for the diabody). It is possible that by reducing the linker length to zero and/or changing the domain orientation form V_L-V_H to V_H-V_K, more stable (concentration-independent) dimers or higher multimers could be made (Plückthun & Pack, 1997).

The linker can also affect the affinity, stability and expression levels of scFvs (Alfthan *et al.*, 1995; Tang *et al.*, 1996), factors that are critically important for patient studies. Although simple and effective in principle, the diabody approach may not always produce molecules with properties suitable for patient studies. Various other strategies such as the fusion of leucine zippers or streptavidin have been used to produce scFv multimers but it is not uncommon that problems similar to those observed with diabodies are reported (Plückthun & Pack, 1997). This suggests that there may be no universal approach to multimer formation and the optimal strategy will have to be established for each antibody empirically.

Diabodies can possess some of the properties required for immunoscintigraphy and immunotherapy and can be constructed in either monospecific or bispecific formats. However, the two chains of the diabodies are capable of dissociating, as has been the case at low

concentrations in the present study. The stability of these moieties can be enhanced by the use of disulphide bonds at the V_H-V_L interface, covalently locking the two chains together (Fitzgerald *et al.*, 1997). This has been shown to improve thermal stability and could favour the correct pairing of the two different chains in bispecific diabodies. Previously published work has also indicated that the introduction of inter-chain disulphide bonds can reduce binding affinities and expression yields (Reiter *et al.*, 1994; Reiter *et al.*, 1996b; Plückthun & Pack, 1997) but this is not a universal finding (Fitzgerald *et al.*, 1997).

6.9.7   Concentration-dependent multimerisation

Based on the literature (Kortt et al., 2001), the intended multimerisation of 3A11 was initially assumed as a result of the genetic sequence. Multimer formation was subsequently characterised by submitting the 3A11 diabody and triabody to size-exclusion gel filtration chromatography. The results suggested a concentration-dependent multimerisation. High concentrations of protein favoured dimer (and possibly tetramer) formation, whilst lower concentrations favoured monomer formation.

Concentration of protein samples, typically to 1mg/ml, has previously been shown to increase multimer formation (Todorovska et al., 2001). Thus the monomer/dimer equilibrium in scFvs becomes biased towards dimers. Similarly, concentration can increase the proportion of triabodies and higher multimers in scFv diabodies (linkers between 3 and 12 residues) (Todorovska et al., 2001). This p henomenon s uggests t hat the V_H/V_L i nterface i n t he F v m olecule c an d issociate, enabling V-domains to shuffle and re-associate. The rate of Fv dissociation will be unique to each antibody. Several methods have been suggested to increase the stability of scFvs, including storage as antibody-antigen complex (Lawrence et al., 1998), the addition of buffer components, adjusting pH and ionic strength (Arndt et al., 1998), design of "knobs-into-holes" mutations (Zhu et al., 1997; Dall'Acqua & Carter 1998), or the introduction of inter-domain disulphide bonds (Brinkmann et al., 1997). All these methods have been shown to be effective for scFv multimer stabilisation in vitro.

Without any stability engineering it is possible that after injection into the mouse the initially concentrated scFv may reach a new monomer-dimer equilibrium in the circulation, resulting in dissociation into monomers. If this was the case, in vivo targeting could be further improved by

genetic engineering. However, it has been suggested that once the scFv had bound to antigen it could become trapped and unable to equilibrate further (Arndt et al., 1998).

6.9.8 Other factors influencing dimer to monomer transition

As discussed, the extent of dimerisation depends upon a number of factors including linker length, amino acid sequence, as well as other external factors such as pH and temperature.

The presence or absence of the antigen may also play a role in favouring monomeric or multimeric states. It has been suggested that a dynamic equilibrium exists between monomer and their dimers, which is influenced by external factors (Arndt et al., 1998). It has been shown for dimeric variants of streptavidin that monomers are the more thermodynamically stable forms and that in the absence of biotin, dimers can revert to the monomeric form (Sano et al., 1995; Sano et al., 1997). Ionic strength and pH have also been shown to influence the rate of dimer to monomer conversion. For some monomeric species this conversion is faster under physiological conditions (Arndt et al., 1998) and the conversion will depend on the V_L-V_H interface energy.

A range of dissociation constants for the V_L-V_H association has been observed for Fv fragments derived from antibodies and they can be as low as 10^{-7} M (Polymenis & Stollat, 1995; Mallender et al., 1996). Thus in some cases where the V_L-V_H interface is intrinsically stable, the re-equilibration of dimer to monomer may be incomplete or minimal, even in the absence of the antigen. Different scFvs may be trapped in the dimer state to different degrees. One study has demonstrated that human scFv fragments with high specificities of binding to human self-antigens selected from the same phage library consisted of a mixture of monomers and dimers (Griffiths et al., 1993). The binding kinetics were determined by surface plasmon resonance and whilst the kinetics of association were typical of known Ab-protein interactions, the kinetics of dissociation were relatively fast. The development of useful dimeric scFvs thus requires the understanding of the conditions more likely to favour dimerisation rather than monomeric states.

6.9.9 Size of scFv multimers and its effect on pharmacokinetics

ScFv multimers such as diabodies (~60kDa) and triabodies (~90kDa) are significantly larger than scFv monomers (~30kDa) and thereby can have an advantage for *in vivo* applications by

minimising the rapid clearance from the circulation (Colcher *et al.,* 1999). The advantage of increased size and moderate clearance rate is offset by decreased tumour penetration, since the distribution rate through the vascular network into the tumour is inversely correlated to the size of the protein *in vivo.* Indeed, some of the most recent *in vivo* data using radiolabelled diabodies in three different xenograft models show that diabodies have a significant advantage over monomeric scFvs (Wu *et al.,* 1996; Wu *et al.,* 1999; Adams *et al.,* 1998; Adams & Schier, 1999; Viti *et al.,* 1999). Similarly, the small size of the diabodies should have advantages for tumour penetration over the larger multimeric Fab chemical conjugates and intact whole Ig, although this remains to be demonstrated experimentally (Casey *et al.,* 1996). Previous investigations with anti-CEA diabodies (Wu *et al.,* 1999) and anti-HER2/*neu* diabodies (Adams *et al.,* 1998) reported tumour to blood ratios considerably higher than those achieved for F(ab')$_2$ (Pedley *et al.,* 1993 ; Casey *et al.,* 1999). This is the result of the rapid beta phase clearance from the blood.

The fast clearance of smaller molecules from the kidney reduces tumour accumulation (Dillman, 1989). S cFvs n ormally h ave a p ositive n et c harge t hat may a lso c ontribute to their h igh renal accumulation. Increasing their surface negative charge, or modifying the radionuclide chemistry can achieve improvements in the biodistribution and pharmacokinetics of these small recombinant antibody fragments, both strategies reducing the renal uptake (Arano *et al.,* 1999). Other strategies include pre-treatment or co-administration of cationic amino acids such as D-Lysine (Behr *et al.,* 1997; Carrasquillo *et al.,* 1998; Arano *et al.,* 1999; Hamilton *et al.,* 2002).

Interestingly, Adams *et al.* showed that diabodies had improved tumour targeting when compared with scFvs that had been dimerised via disulphide bonds and this might reflect higher *in vivo* stability of the diabodies (Adams *et al.,* 1998c). The linkers in diabodies and triabodies are relatively inaccessible to proteases compared with the long linkers required for monomeric scFv. Although covalent dimers continue to be produced (Beresford *et al.,* 1999; Pavlinkova *et al.,* 1999), diabodies (made by shortening the linker length) could be more stable than dimers. Since diabodies provide more rapid tumour penetration and clearance than (Fab')$_2$ (100kDa) they seem most promising as reagents for radioimaging using radionuclides with short half lives such as ^{99m}Tc.

6.10 CONCLUSIONS

The use of scFv molecules in a wide range of biological and medical applications will be affected by whether the scFv possesses a stable monomeric structure with a single antigen-binding site or a stable dimer structure with two antigen-binding sites.

If it is assumed that 3A11 dimer is applied to humans in 500µg aliquots, in a volume of around 3.5ml, it will be diluted to 0.0001mg/ml if the total adult blood volume is five litres. The same calculation in mice, assuming a blood volume of 2mls and 0.5µg scFv is injected, demonstrates a similar result. Local concentration effects may arise in tumours, where 3A11 will accumulate by binding to cell surface-expressed HMW-MAA, where this is abundant. However, once 3A11 has bound to HMW-MAA, it may be trapped in its monomer structure and be unable to form a dimer. A dimer or diabody structure would probably not therefore be expected to form in tumours even at high local concentrations of 3A11.

The present work has demonstrated the concentration-dependent nature of the RAFT 3A11 multimers and therefore the need to be wary of this when considering multimerisation strategies for future use in humans. Despite this, clearly improved targeting of the 3A11 dimer was revealed compared with not only the parent monomeric scFv, but also the mixture of monomers, dimers and trimers formed by the 4A4 clone. Thus dimerisation promises to be a useful strategy for improving radioimmunodetection of metastatic melanoma. Further improvements in melanoma targeting could be achieved by diabody engineering such as alteration of the V domain orientation, fine tuning of the linker length or the introduction of stabilising disulphide bonds. Diabody re-concentration proved a simple and effective strategy to improve in vivo targeting.

CHAPTER 7

SUMMARY AND FUTURE DIRECTIONS

7.1 SUMMARY

The incidence of melanoma is continuing to increase despite greater public awareness and advances in prevention and treatment (see Chapter 1 for review). This thesis has addressed three problems faced when dealing with this disease. It has examined secondary prevention (early detection), estimation of prognosis and the early detection of metastases. Advancements in any one of these areas will serve to improve the lot of the melanoma patient. The following paragraphs summarise the findings in this thesis and put forward suggestions for addressing questions raised.

7.2 PIGMENTED LESION CLINIC

Despite the existence of rapid-access pigmented lesion clinics for over a decade, no study has yet examined their impact on melanoma patient outcome. However, early detection has been shown to be an effective strategy for improving patient outcome in breast and colorectal cancer (Moss et al., 1994; Niv et al., 2002). Other PLC studies have examined the efficiency of the clinics, compared PLC patients with data from other studies (O'Connor & Barnes 1994) or looked at trends in melanoma patients presenting to PLCs over time (Bataille et al., 1999; Duff et al., 2001).

The work done on the PLC study in this thesis has shown that the strategy of early detection improves overall melanoma patient survival and improves disease-free interval in those patients that develop regional recurrences. Although it may be assumed that an efficiently run early-detection PLC will have the effect demonstrated in this thesis, there are pitfalls and dangers in making assumptions that have not been based on scientific analyses. An example of this is the very wide margins (>6cms) that were once used when resecting primary melanomas, which have since been shown to be unnecessary for local disease control (Veronesi et al., 1988). Therefore it was important that a study such as the present one was undertaken. This thesis has shown that the strategy of early detection, by means of reducing the inherent delays incurred in the system of GP referral and the bureaucracy of hospital administration, directly affects patient outcome. This is achieved without the need for additional publicity campaigns to run in conjunction with the PLC.

Furthermore, the pigmented lesion clinic allows rapid expert assessment of lesions that may otherwise be treated as unsuspicious by the GP. This has been shown to be a factor directly relating to the thickness of melanomas presenting (Ibbotson et al., 1995). The reduction in thickness of melanomas presenting to the PLC should also reduce the need for sentinel lymph node biopsy, a procedure with considerable associated morbidity and cost implications (Hettiaratchy et al., 2000; Jakub et al., 2003). The improvement in survival and disease-free interval conferred on patients by the PLC will also reduce the numbers of patients undergoing toxic (and currently ineffective) chemotherapeutic regimes. Finally, in the present healthcare system of budgetary constraints, the efficiency and effectiveness of a PLC does not only improve patient outcome, but considerably reduces the financial burden on the NHS.

7.2.1 Future directions

It is hoped that following publication of the results of the investigation into the PLC, other hospitals will be encouraged to establish similar clinics. By using this simple strategy nationally, the morbidity and mortality from melanoma may be significantly reduced. With the annual increases in incidence of melanoma, the importance of this cannot be overstated.

7.3 TISSUE ARRAY

Accurately predicting the prognosis of melanoma patients is crucial for making correct management decisions, the planning of follow-up regimes and for the psychological well being of melanoma patients. Currently it may be possible to inform a patient that they have an 80% chance of having no recurrence, but it is not possible to inform them if they will be within that 80%. With the discovery of novel prognostic markers, adding to the battery of established indicators of prognosis, the accuracy of the predictions made is improving. After relying on the Breslow thickness, Clark's level, presence of ulceration, patient age, tumour site and patient sex, the next most important determinant of prognosis is the nodal status of the patient. Sentinel lymph node biopsy (Morton et al., 1992) is now widely practiced as a means of accurately staging patients and

predicting outcome (Balch et al., 2001). However, it is associated with a degree of morbidity (Hettiaratchy et al., 2000; Jakub et al., 2003) and has not yet been shown to have a bearing on overall patient survival. Non-invasive approaches to predicting prognosis are therefore desirable.

The employment of high throughput tissue microarray analysis on 480 core biopsies form 120 melanoma patients has permitted the identification of several novel prognostic melanoma markers. These markers will be applicable to use alongside routine staining that is performed on lesions suspected to be melanomas, without incurring any further morbidity to the patient. In this thesis MCAM, P-cadherin, CD44v3 and nm23 were shown to be effective in predicting outcome in primary cutaneous melanoma. In addition, MCAM was shown to predict prognosis independent of other established clinicopathological variables.

7.3.1 Future directions

The use of tissue array as an efficient tool to study large numbers of specimens simultaneously has opened up the possibility of screening for many further potential markers. This applies not only to tumour specimens, but also to other tissues, such as connective tissue in the study of Dupuytren's disease, tendon research and burns scar tissue. Furthermore, improvements in the efficiency of tissue array analysis are continually being developed, including computer-aided analysis of array staining. The use of computerised image analysis to aid scoring of cores on a tissue array will increase the objectivity of the results.

In terms of melanoma research, many more avenues remain to be explored, even with the markers studied in this thesis. Tissue microarrays may be constructed purely of melanoma subtypes, such as acral lentiginous or nodular melanomas to determine any specific trends and peculiarities within melanoma subtypes. Arrays of intermediate depth melanomas, of which Breslow thickness does not predict outcome, could be made which would assist in the discovery of other markers that will improve the accuracy of the prediction of patient outcome. Another possibility is a tissue microarray of lymph node metastases. Indeed, if an array was constructed of lymph node metastases, it would be possible to study correlations between staining for certain markers and the effectiveness of chemotherapeutic regimes in clinical trials. In this way, it would

be possible to delineate sub-groups of patients who respond well to particular regimes, which may then be started at the earliest possible opportunity after sentinel lymph node biopsy. This could lead to improved response rates and possibly even remission from metastatic disease, not currently possible.

The tissue microarray also provides an opportunity to investigate other tumours. Of interest is the study of prognostic markers in relation to cutaneous squamous cell carcinoma (SCC). Cutaneous SCC is a tumour within the plastic surgeon's remit that has not been extensively studied, despite of having an age-adjusted incidence of 100-150 per 100,000 persons per year (Alam & Ratner 2001). High risk cutaneous SCCs are defined by their site, diameter, depth, histological differentiation, host immunosuppression and previous treatment modality (Motley et al., 2002). On the other hand, SCC of the head and neck has been studied for various prognostic markers, but these have not yet been studied in cutaneous SCC. Examples of markers with an association with head and neck SCC are: CD44 splice-variant isoforms, which may be down-regulated (Assimakopoulos et al., 2002); epidermal growth factor receptor; transforming growth factor alpha and cyclin D (Quon et al., 2001; Smith et al., 2001).

The identification of a cellular marker correlated with early metastasis and an aggressive disease course would be invaluable in the management of cutaneous SCC. If such SCCs were identifiable at the time of first excision, surgical management could be altered in an attempt to improve patient outcome. Currently it is recommended that for clinically well-defined low-risk tumours less than 2cm in diameter, surgical excision with a 4mm margin is appropriate, whereas in larger or higher risk tumours, margins of at least 6 mm are advised (Motley et al., 2002). Varying the width of excision margins is one example of how an aggressive SCC may be locally controlled at the earliest opportunity. If more accurate identification of high-risk tumours was possible, some patients may be spared potentially unnecessary aggressive surgical treatment.

7.4   RADIOIMMUNOSCINTIGRAPHY

Several melanoma-specific monoclonal antibodies and anti-HMW-MAA monoclonal antibodies in particular have been used successfully for radioimaging of melanoma patients (Herlyn et al., 1988; Siccardi et al., 1990). Most of the problems encountered with antibody radioimaging (human anti-mouse response, slow blood clearance, accumulation in normal organs) are caused by the relatively large size of the monoclonal antibodies. ScFv fragments are the smallest functional units of antibodies that contain the complete antigen-binding site. Their relatively small size, the ability to produce them in functional form in the bacterial periplasm, as well as their potential use in immunoscintigraphy and immunotherapy makes them an interesting tool for protein engineering. Whether cloned from hybridomas or obtained from phage libraries, scFvs can show poor production yields and exhibit low thermodynamic stability, thereby limiting their usefulness for in vivo applications even if they have excellent affinities and tumour specificity (Willuda *et al.*, 1999).

Limited stability remains one of the drawbacks, preventing the everyday use of scFvs in medical and biotechnological applications. Whilst the qualities required for in vitro stability are beginning to be elucidated, those for *in vivo* stability remain to be defined more clearly. In humans stability is not only influenced by thermal aggregation, but also by blood clearance, proteolysis and other effects. The optimal functional parameters of these molecules, such as the required stability, molecular weight and affinity are not fully understood and have to be optimised empirically depending on the diverse clinical requirements. It will therefore be important to understand the molecular basis of these factors to enable the routine use of scFvs in melanoma patients in the future.

7.5   ANTIBODY COCKTAIL

The use of a cocktail of single chain (Fv) antibody fragments directed against three different melanoma-specific antigens holds much promise for improving tumour targeting. The present study demonstrated improvements in melanoma binding in vitro and improvements in tumour to

normal tissue ratios in the spleen and the muscle in vivo. The use of a cocktail should overcome antigenic heterogeneity in patients. However, the in vivo results did not improve on those of the individual components of the cocktail as much as was hoped. This was most likely due to insufficient quantity of scFv used for the experiments, which did not allow saturation of the tumour binding sites. Nevertheless this strategy promises to be one that may prove useful for therapeutic applications.

7.5.1   Future directions

The cocktail strategy could be used for therapeutic applications in several ways. Combinations of scFvs to distinct determinants of HMW-MAA may be useful as carriers of distinct components of a therapeutic system (e.g. two chains of a toxin), which have to be delivered in close proximity at the cell surface, so that they may assemble and function in selected areas of high antigen expression. Another example would be the use of two different scFvs with appropriate molecules attached that would in-turn stimulate complement-mediated cytotoxicity and cell-mediated cytotoxicity and/or an immune response.

Other advantageous properties of scFvs also need to be further exploited. Increasing the yield of RAFT scFvs in an attempt to produce saturating quantities could be achieved by the use of large fermenter-based production systems. Recently, improved yields have been reported using heat-inducible vectors, which may further enhance scFv expression (Power & Hudson 2000). Other authors have described the successful employment of yeast cells for markedly increased protein production (Freyre et al., 2000).

7.6   SCFV MULTIMERS

ScFv multimers have been used as a strategy to improve tumour targeting by means of improving tumour retention and blood clearance as well as a means of reducing kidney accumulation. The study undertaken for this thesis has shown them to be effective for the former, but not the latter. The bi-valency of the dimers resulted in excellent tumour accumulation resulting in good tumour to

normal tissue contrast. However, the problem of renal accumulation remained. From this work and the work of others, it seems likely that size alone can therefore not be the only reason for the high kidney accumulation of scFvs.

7.6.1 Future directions

Alternative strategies aimed at reducing renal accumulation were discussed in chapter 6. One of the most promising strategies is to alter the electric charge of the scFvs. By creating a series of scFvs with similar properties, such as thermal stability and size, but with different isoelectric points, it will be possible to determine the influence of charge on renal accumulation. It is possible that the "ideal scFv" is a multivalent molecule of low isoelectric point that will exhibit excellent tumour retention, rapid blood clearance and little kidney accumulation. This might open the way for the development of more therapeutic constructs, which should cause little nephrotoxicity due to its short kidney residence time.

Most scFvs are positively charged and are difficult to convert into negatively charged constructs, although Ronda et al. have demonstrated that it can be done (Onda et al., 2001). However, Onda et al. did not look at biodistribution, only at toxicity in mice. Goel et al. also used a negatively charged scFv with a c-terminal $(glu)_5$ tail and did look at biodistribution but this scFv did not have reduced renal accumulation. Nevertheless, these effects merit further study in the context of the RAFT scFvs.

Bispecific multimeric scFv molecules capable of recruiting T-cell or complement responses have been developed in other tumour models (Holliger et al., 1993; Kipriyanov et al., 1998). These molecules are relatively simple to design and could be applied in the setting of melanoma. Finally, one group has recently reported successful tumour eradication in a colon carcinoma mouse model by the combination of an anti-vascular agent and an iodine-131 labelled antibody (Pedley et al., 2001). Such combinatorial treatments may represent the way forward in antibody-based tumour therapy. It is clear that many potential therapeutic approaches are available and the versatility of scFvs is a particular strength in this regard.

7.6.2   Pretargeting Strategies

Another approach would involve pretargeting strategies in which an unlabelled bispecific scFv (with one binding site targeting melanoma and the other binding site reacting with a small hapten) is administered first (Goodwin & Meares 1997). At a later point when the circulating unbound antibody levels are low and tumour targeting is optimal, a radiolabelled hapten is administered which localises to the antibody bound to the tumour, thereby reducing the radiation dose to normal tissues. This pretargeting approach can be modified further by conjugating streptavidin or biotin to the pre-administered antibody, followed by the injection of radiolabelled biotin or streptavidin, respectively (Paganelli et al., 1995). More recently a three-step regime has been developed, where biotinylated antibody is given first, followed by an avidin and/or streptavidin chase (2nd step), and finally by radiolabelled biotin (3rd step). This multistep approach has yielded impressive results in pre-clinical studies in animal models, when compared to conventional one-step radioimmunotherapy, and has been tested in clinical trials (Paganelli et al., 1999). Radioimmunotherapy would be of great value for melanoma patients with a high risk of developing distant metastases.

7.6.3   PEGylation of ScFvs

It has been shown that chemical modification of scFvs with PEG (PEGylation) can result in molecules with properties similar to Mabs that allow delivery of much higher doses to tumours (Lee et al., 1999). mPEG (Shearwater Polymers) has a molecular weight of 20-40 kDa and can be cross-linked to scFvs (King et al., 1994). This strategy should prolong the serum residence time and thus increase the initial tumour dose, with only a moderate increase in the antibody fragment size. As a result it would be expected that the %ID/g in the tumour is increased. PEGylation in combination with tumour pretargeting with the biotin/streptavidin system could therefore confer a therapeutic potential to scFvs.

7.6.4   Immunotherapy

Antibody fragment-based therapy for melanoma is still in its infancy. Several animal studies in other tumour models have been highly promising. For example, combining scFv with immuno-active agents such as interleukin-2 (Melani et al., 1998) or radiotherapeutic nuclides such as iodine-131 (Goel et al., 2001) has been encouraging. The potential of gene therapy in cancer has received much attention recently yet the problem of efficient targeted delivery has still to be solved. RAFT scFvs have been used to target retroviral vectors to melanoma cells *in vitro* (Martin et al., 1999) and *in vivo* (Chowdhury *et al.*, 2004).

The project is now working on a clinical imaging trial, using radiolabelled 3A11 monomer. A pilot trial will initially be conducted on 20 patients with stage III melanoma. Radioimmunoscintigraphic scans will be compared with CT scans and other imaging modalities to determine the effectiveness of radioimmunoscintigraphy with RAFT scFvs as an imaging mode to be introduced into routine practice.

7.7   CONCLUSION

In conclusion, this thesis has shown that secondary prevention is a successful strategy for improving melanoma outcome, several novel markers of melanoma progression have been identified and the work on scFvs has enhanced the targeting of melanoma in the animal model and brought the clinical trial significantly closer. Further improvements in targeting and reduced renal accumulation will hopefully unlock the potential of these exciting molecules for the therapy of metastatic melanoma.

ABBREVIATIONS

ADCC	Antibody Directed Cell Mediated Cytotoxicity
AJCC	American Joint Committee on Cancer
ATCC	American Tissue Culture Collection
CDR	Complementarity-Determining Region
Cps	counts per second
Cpm	counts per minute
EDTA	Ethylene diaminetetra-acetic acid
ELISA	Enzyme Linked Immunosorbent Assay
ELND	Elective Lymph Node Dissection
$F(ab')_2$	100 kDa antibody fragment
F(ab)	50 kDa antibody fragment
Fc	Constant region fragment
Fv	Variable region fragment
(His) 6	6 Histidine amino acid residues at the C-terminus of scFv for IMAC
HMW-MAA	High Molecular Weight Melanoma-Associated Antigen (proteoglycan)
HPLC	High Pressure Liquid Chromatography
Ig	Immunoglobulin
IgG1	Immunoglobulin (isotype G Class 1)
IMAC	Immobilised Metal-Ion Affinity Chromatography
ISG	Immunoscintigraphy
K_A	Association constant for equilibrium binding of antibodies
K_D	Dissociation constant for equilibrium binding of antibodies
KDa	Kilodalton
KeV	Kilo-electron Volt
MAA	Melanoma Associated Antigen
Mab	Monoclonal Antibody
PCR	Polymerase Chain Reaction
PEG	Poly-Ethylene Glycol 6000
PET	Positron Emission Tomography
pI	Isoelectric point

rPL	Recombinant Protein-L column
rpm	Revolutions per minute
RAFT	Restoration of Appearance and Function Trust
RIS	Radioimmunoscintigraphy
RIT	Radioimmunotherapy
RT	Room Temperature
ScFv	Single-chain Fv Antibody Fragment
SDS-PAGE	Sodium Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis
SLNB	Sentinel Lymph Node Biopsy
SPECT	Single Photon Emission Computerised Tomography
Tris	Tris(hydroxymethyl)aminomethane
Tween 20	Polyoxyethylene-sorbitan monolaurate
$t_{1/2}\alpha$	Distribution half-life
$t_{1/2}\beta$	Elimination half-life
V_H	Heavy chain variable region
V_K	Kappa chain variable region
V_L	Light chain variable region

PAPERS PUBLISHED FROM WORK IN THIS THESIS

1. *Effect of imidazole on the solubility of his-tagged antibody fragments.* S Hamilton, J Odili, **M Pacifico**, GD Wilson, J-M Kupsch. Hybridoma and Hybridomics. 2003 Dec; 22 (6): 347-55

2. *Use of an early-detection strategy to improve disease control in melanoma patients.* **MD Pacifico**, R Grover, R Sanders. BJPS 2004 Feb; 57 (2): 105-111

3. *Validation of tissue microarray for immunohistochemical profiling of melanoma.* **MD Pacifico**, R Grover, P Richman, F Daley, GD Wilson. Melanoma Res. 2004 Feb;14(1):39-42

4. *Development of a tissue array for primary melanoma with long-term follow-up: discovering MCAM as an important prognostic marker.* **MD Pacifico**, R Grover, P Richman, F Daley, F Buffa, GD Wilson. Accepted for publication by Plastic & Reconstructive Surgery

5. *Improving survival in melanoma: Long-term evaluation of an early detection clinic reviewing 4399 patients.* **MD Pacifico**, R Grover, R Sanders. Accepted for publication by The European Journal of Plastic Surgery

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