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A study of activation antigens involved in the pathological mechanisms and pathways of cutaneous malignant disease with particular emphasis on cutaneous T cell lymphoma and malignant melanoma.

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A study of activation antigens involved in the pathological mechanisms and pathways of cutaneous malignant disease with particular emphasis on cutaneous T cell lymphoma and malignant melanoma

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CHAPTER

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Abstract

The investigation of dermatological conditions embraces the concept of a clinico-pathological correlation. The studies into cutaneous T cell lymphoma (CTCL) and malignant melanoma (MM), presented here involved predominantly immunocytochemical procedures and relate to the investigations into AP-1 protein expression in CTCL and melanocyte activation antigens in MM.

Results: Findings indicate that expression of AP-1 proteins differs not only according to type of CTCL but also according to stages of tumour progression. In MM activation antigen expression varies with tumour metastasis.

Consideration of the role of techniques in terms of sensitivity and specificity form a pivitol component in the evaluation of tumour antigen expression.

Key words: Cutaneous T cell lymphoma (CTCL), malignant melanoma (MM), immunocytochemistry (IMC).

Chapter 1   Introduction

With all dermatological conditions, there is a close link between the clinical manifestation of the disease and the histopathological appearance under the microscope. Within the field of dermatology, this clinico-pathological correlation has developed to such a high degree that it is now possible to identify stages of clinical disease progression with clearly defined histopathological observations. Examples of pathological conditions where this is possible are malignant disease such as cutaneous T cell lymphoma (CTCL) and also entities which do not necessarily exhibit all the characteristic features that are associated with malignant disease, either clinically or histologically; such as lymphomatoid papulosis (LyP).

The World Health Organisation (WHO) in conjunction with the European Organisation for Research and Treatment of Cancer (EORTC) have classified LyP as a low grade cutaneous T cell lymphoma (Willemze et al 2005). This lymphoma presents as recurrent, self healing, papulonecrotic or nodular skin lesion(s), which can present with histological features indicative of CD30 + malignant lymphoma or mycosis fungoides (MF), other forms of cutaneous T cell lymphoma. It is also a well-documented fact that there is a close association with patients who have LyP and other forms of CTCL not only histopathologically but also clinically. This suggests a common genetic link. Evidence for this comes from reports of patients exhibiting more than one form of cutaneous lymphoma contiguously. Thus patients may present with MF, Sezary syndrome (SS), or

Hodgkins disease and LyP, co- existing as clinically distinct cutaneous lymphomas (Beljaards and Willemze 1992). These appearances are not sequential, in other words patients may present with LyP first then MF or SS or CD30+ anaplastic large cell lymphoma, or conversely they may present with MF and then LyP or finally they may present with both conditions at the same time (Basarab et al 1998).

The fundamental observation with patients who present with LyP is that only up to 20% of them develop a second form of CTCL (Bekkenk et al 2000). The remaining 80-90% may have recurrent episodes of self healing lesions which may occur over many decades and may last for months or years and which often result in superficial hypopigmented scars, without any significant life threatening complications.

Histopathologically, LyP is classified under three distinct subtypes (types A, B and C). These are defined by the appearance of atypical population of T cells. Types A and C resemble the appearance of CD30+ anaplastic large cell lymphoma in that the cells are large with prominent nuclei and are CD30+. The distinction between the two types is based on the proportion of the atypical cells which exhibit a CD30+ immunophenotype. If the atypical cells are CD30+ then the lesion is type A; if a proportion are negative then the lesion is regarded as type C. In contrast, type B lesions present with smaller atypical lymphocytes which are CD30 negative. The immunophenotype of types A and C is equivalent to that of primary cutaneous anaplastic large cell lymphoma (CD4+, CD30+, TIA-

1+ (T-cell cytotoxic intracytoplasmic enzyme antigen) and granzyme B +). In contrast, type B cells exhibit an immunophenotype similar to MF in that the atypical cells are CD2+, CD3+, CD4+, CD8-, CD30-, TIA-1- and granzyme B -. MF is a CTCL which involves mature T-helper cells (CD4+) (Wain et al 2005). However, approximately 5% of cases have a cytotoxic (CD8+) immunophenotype (Wain et al 2003). Characteristically, whether the atypical cells are CD4 or CD8+, the cells are commonly epidermotropic and have nuclei which appear cerebriform in shape.

Clinically, MF is the most common CTCL with an annual incidence of 0.36 per 100 000. MF presents with early stage disease (a patch), followed by plaque and then nodular tumour stages. Like LyP, MF commonly occurs in mature adults, although just as in LyP this is not exclusively so, as young adults and children can also be affected. Atypical immunophenotypic profiles have recently led to the identification of specific subtypes of cases of MF and LyP (Bekkenk et al 2001 and Flann et al 2006), the clinical significance of which remains unclear due to the rarity of their incidence.

Evaluations of LyP and MF at the molecular level indicate that in 60-70% of LyP cases, it is possible to detect a clonal T cell receptor (TCR) gene rearrangement (TCR). In the case of MF a rearranged TCR is detected in the majority of lesions (Bekkenk et al 2001). This indicates close synergy between these cutaneous lymphomas. Currently there is significant interest in determining the proteins involved in activation pathways for CTCL, much of which has focussed on

activator protein 1 (AP-1), a dimeric transcription factor containing several members of proteins from the JUN (c-JUN, JUNB and JUND), FOS (c-Fos, FosB, Fra-1 and Fra-2), activating transcription factor [(ATF): ATF2, LRF1/ATF3, B-ATF, JDP1, JDP2] and musculoaponeurotic fibrosarcoma [(MAF): c-MAF, MAFB, MAFA, MAFG/F/K and NRL] protein families. Part of the research detailed here concerns evaluation of the degree of expression of some of these key molecules to determine which are expressed to a lesser or greater degree in relation to CTCL disease progression (Mao et al 2003).

A similarity with the studies highlighted above on cutaneous lymphoma, this thesis also aims to evaluate activation antigens in malignant melanoma (MM), with particular relevance to their use in a diagnostic setting, to assist in demonstration of the myriad of histological appearances of MM. The study of activation antigen expression for both cutaneous lymphomas and MM forms the basis of this thesis.

According to recent figures published by Cancer Research UK there are over 92,000 new cases of skin cancer reported each year in the UK alone (2006) (Orchard 2006 and Anon Cancer Stats 2006). Non-melanoma cancer (NMC) accounts for around 81,600 cases with malignant melanoma (MM) accounting for 10,400 cases. Skin cancer is now one of the most common cancer types reported in the UK. In terms of mortality rates MM represents the most significant skin cancer with over 2,000 deaths a year reported in the UK alone.

In most cases, clinical features of MM are quite characteristic, and although many MMs arise from an existing mole, the majority arise spontaneously. The primary trigger is ultra violet light, particularly UVB at a wavelength of 280-315 nm. Classical histological features of MM are well documented (Clark et al 1969 and Ackermann and David 1986), however atypical forms of these tumours can present with a myriad of features making diagnosis problematic. Many of these atypical forms of MM do not produce melanin and are broadly categorised under the heading of 'amelanotic MM'.

In such cases the role of immunocytochemical investigations is often essential to confirm the cell type of the tumour cells. In this regard the use of a host of melanocyte selective antibodies is employed, examples of which include HMB 45, Melan A and tyrosinase. All three of these antibodies recognise antigens associated with melanin synthesis, which are indicative of melanocyte differentiation. HMB 45 recognises an antigen termed gp100 and was first described for use in a diagnostic setting by (Gown et al in 1986) . The Melan A protein is a product of the *MART-1* gene, Chen et al (1996) and the antibody recognises a 22kDa doublet in melan-A mRNA positive melanomas (Jager et al 1996). Tyrosinase is a cytoplasmic protein involved in melanocyte differentiation and is a key enzyme in melanin synthesis (Kauffmann 1998). Whilst these antibodies are effective for labelling conventionally histologically typed MM, with the majority of cases labelling positively (Orchard 2000) amelanotic variants of the tumour have variable to negative labelling profiles with these antibodies, making their diagnosis more problematic (Orchard and Wilson Jones 1994).

Immunocytochemistry is also vitally important in detection of metastatic MM deposits in lymph node material, and more significantly in sentinel lymph nodes where there may be only low numbers of cells to detect (Shani et al 2005). A sentinel lymph node is the first draining lymph node away from the primary site of the tumour. Successful detection of such deposits is currently a key prognostic indicator and one of the most effective means of staging MM. Although these markers are highly selective for melanocytes, none of them is specific marker for malignancy. They all label non malignant transformed melanocytes and thus can give positive results in normal moles or naevi (Blessing et al 1998). Application of a panel of antibodies is therefore advocated in the majority of cases and a diagnostic cocktail of these antibodies has also been introduced and evaluated (Orchard 2002), which has shown improved sensitivity for the detection of tumour cells in sentinel lymph nodes (Orchard 2003). In addition some cases of MM will present with extensive dermal melanosis making normal immunocytochemical detection methods highly problematic. In such cases confirming the nature of the atypical cells as either melanocytes or melanophages (melanin containing macrophages) can also be difficult (Orchard 1999 and 2007). The advent of a MM specific antibody which labels malignant melanoma cells only is still awaited for use on routinely fixed paraffin wax embedded tissue blocks.

The studies documented here represent a significant body of research into mechanisms and pathogenesis of cutaneous malignant disease focussing on

cutaneous T cell lymphoma (CTCL) and MM. It encompasses investigations employing immunocytochemistry, real- time reverse transcriptase-polymerase chain reaction (RT-PCR), and fluorescence in situ hybridization (FISH). The research objectives primarily focus on assessment of activation signal proteins involved with both tumour types, with particular reference to the quantifiable expression of activation molecules in relation to tumour stage. Consideration is given to unusual variants of these tumours with specific regard to immunophenotypic profiles and how this has enabled a more expansive understanding of classification of both tumour types. Possible implications of such research, in terms of patient management and prognostic outcome, are discussed. Emphasis is placed on laboratory-based problem solving and the review of scientific procedures and methodologies to improve practical assays and investigations. Through such activity a more tailored approach to evaluation of these tumours has been proposed and developed. The objective of this thesis is to employ the techniques and methods outlined here to meet two goals.

1. To determine whether the antibodies employed can be used to increase accuracy of diagnosis.

2. To evaluate whether any of these markers may increase our understanding of activation pathways of both MM and CTCL.

Chapter 2   Method Development

St. John's Institute of Dermatology is an internationally renowned reference centre for the study of cutaneous disease. The range of sometimes quite rare variations of tumour types has provided the opportunity to expand research potential and associated applications of diagnostic techniques. Use of such rare cases has also provided the opportunity for more in depth analysis and potential to develop a detailed understanding of disease progression and prognostic significance.

Immunocytochemistry: Procedures and development

The procedures used in this study represent two distinct technical processes, developed to better study cutaneous malignant disease. Firstly, application of antibody markers previously untested on formalin fixed paraffin wax embedded tissue and secondly, development of tailored methods to enable dual antigen demonstration (double labelling) on the same section. In the case of the AP-1 protein families in particular, there has been little previous work on use of specific antibodies to these proteins on formalin fixed paraffin wax embedded tissue sections. The studies performed here have enabled generation of standardised working procedures, including full evaluation of dilution factors and incubation times for the primary antibodies and viability studies for use of each antibody. Evaluation of antigen retrieval procedures was also performed to establish optimum methodology and appropriate control material for each antibody tested.

For antigen demonstration it is generally accepted that frozen section material offers the best opportunity to demonstrate the widest range of antigens, as the tissue is unfixed and antigenic proteins are largely undenatured. However, frozen tissue has the disadvantage of reduced morphological clarity coupled with tissue section deterioration. Preservation of section integrity is enhanced by fixation, but inadequate fixation leads to loss of antigenicity through the diffusion of antigens into surrounding tissue as a result of cell lysis and degradation in frozen sections.

Fixation should ideally:-

- Preserve the morphological appearance

- Preserve the immunoreactivity of antigens

- Prevent extraction of antigens during the immunohistochemical procedures

- Not interfere with the subsequent antigen-antibody interactions

- Enable antigenic epitopes to survive subsequent antigen retrieval

The most widely used fixative in cell pathology is formalin. Modern day formulation of formalin is often 10% neutral buffered (NBF). Formalin remains the most popular fixative for the one simple reason that it provides acceptable standards of fixation whilst allowing the full scope of cellular pathology investigations to be undertaken, including retrospective molecular analysis on nearly all types of tissue samples. Post- fixation three-dimensional structure of

cell proteins is determined in great part by survival against protein denaturation, brought about by covalent bond formation between formaldehyde and amino groups present in the tissue. Formalin-induced cross-linking of amino acid side chains via methylene bridge formation also occurs, with subsequent changes in protein folding and structure. While some antigens remain unaffected by this process, others may be more susceptible to fixation and the overall result is loss of immunoreactivity due to masking of epitopes (Shi et al 2001). In general, because degree of cross-linking is proportional to time spent in fixative, tissue that has received prolonged fixation requires longer or more vigorous unmasking protocols. The issue of neutral pH for fixatives is important because acidic mixtures may induce undesirable changes to antigenic binding sites and therefore result in decreased sensitivity of any IMC procedure.

All the tissues used in this thesis were fixed for 24 hours in NBF at room temperature.

Following fixation, tissues were processed through to paraffin wax. Having optimised fixation, the next variable investigated was alcohol dehydration in tissue processing. It is now well documented that tissue processing temperatures and duration of dehydration and wax infiltration will all affect antigen preservation on tissue samples (Williams et al 1997). It is important to ensure that tissues are not processed at temperatures any higher than 60 º C, as higher temperatures not only affect preservation of antigenic epitopes, but may also result in destruction of tissue architecture. Antigen retrieval techniques rely on heat above 60º C for very short periods of time therefore reducing detrimental effects.

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Due to the many variables affecting extent of fixation-related cross-linking, it is necessary to find optimum treatment time and technique for each antigen. In this thesis this was achieved by considering a number of approaches involving unmasking antigen binding sites using either enzyme digestion methods employing trypsin or heat mediated antigen retrieval using a citrate buffer system at pH 6.0 and microwave oven heating to approximately 100º C. In the case of double labelling both methods were employed to demonstrate two different antigens on the same tissue section using two different coloured chromogens (Shani et al 2005). The IMC labelling technique employed throughout this thesis involved use of either a streptavidin biotin three step system (Dako- ChemMate HRP system) or in the case of double labelling, a dextran polymer system (Dako-Envision).

Two of the publications submitted for consideration with this thesis include additional supportive data generated by others, using specialist techniques not routinely available in dermatopathology laboratories. These publications are listed below and the author duly acknowledges their contribution with thanks:

1.   Mao, X., Orchard GE*., Vonderheid EC., Nowell PC., Bagot M., Bensussan A., Russell-Jones R., Young BD., Whittaker SJ. (2006). Heterogenous abnormalities of CCND1 and RB1 in primary cutaneous T-cell lymphomas suggesting impaired cell cycle control in disease pathogenesis. *Journal Investigative Dermatology*, **126**, pp. 1388-1395.

* indicates joint first author

2. Mao X, Orchard G*, Mitchell JT, Oyama N, Russell-Jones R, Vermeer MH., Willemze R., van Doom R., Tensen CP., Young BD., Whittaker SJ. (2008). A genomic and expression study of AP-1 in primary cutaneous T-cell lymphoma: evidence for dysregulated expression of JUNB and JUND in MF and SS. *Journal of Cutaneous Pathology*, **35**, pp. 899-910.

* indicates joint first author

Chapter 3   Summary of research

For the purpose of this thesis data compiled from the submitted publications covers four distinct but related areas of research:

1.   **MF and LyP with CD56 expression**

2.   **AP-1 CTCL findings**

3.   **IMC techniques and developments**

4.   **MM IMC with double labelling**

1.   **Cell lineage markers in MF and LyP**

Two of the selected publications included with this thesis comprise this section:

1.   Wain EM, Orchard GE*, Mayou S, Atherton DJ, Misch KJ, Russell-Jones R. (2005). Mycosis fungoides with a CD56 positive immunophenotype, *Journal of the American Academy of Dermatology,* **53**, pp.158-63.

2.   Flann S, Orchard GE*, Wain EM, Russell-Jones R (2006). Three cases of lymphomatoid papulosis with a CD56 positive immunophenotype, *Journal of the American Academy of Dermatology,* **55**, pp. 903-906.

*   Indicates joint first author

These publications report expression of CD56 (neuronal cell adhesion molecule), an antigen which is expressed by natural killer cells and cytotoxic T cells in three

cases each of MF and LyP. A compilation of the IMC findings from both publications is summarised below:-

CASE	CD2	CD3	CD4	CD8	CD30	Beta F1	Granzyme B	TIA-1**	LMP-1*	CD56
1. LyP 1	+	+	-	+	-	N/A	N/A	+	N/A	+
2. LyP 2	+	+	-	+	+	+	+	+	N/A	+
3. LyP 3	+	+	-	+	+	+	+	+	N/A	+
4. MF 1	+	+	-	+	-	N/A	-	+	-	+
5. MF 2	+	+	-	-	-	N/A	+	+	-	+
6. MF 3#	+	+	-	+	-/+	N/A	+	+	-	+

Table 1:   Summary of IMC findings for independently assessed 3 cases of LyP and 3 cases of MF:

*LMP-1 = Epstein-Barr virus latent membrane protein, **TIA-1 = T cell intracellular antigen, N/A = lack of material for assessment. # Case MF3 had two lesions - one poikiloderma, the other tumour stage. Poikilodermas are a heterogenous group of dermatoses which are characterized clinically, by erythema, mottled pigmentation and, at a later stage, epidermal atrophy. In this context they represent a stage in evolution of early MF. The designation -/+ for CD-30 expression in this case indicates poikiloderma lesion negative, tumour lesion positive

The expression profiles indicate pan T cell marking with CD2 and CD3, cytotoxic CD8 labelling for 5/6 cases, with conversely negative findings for CD4. Cytotoxic granules labelled in all cases with TIA-1 and in 4/5 cases with Granzyme B. The Beta F1 labels the beta chain of alpha/ beta chain of the T cell receptor (TCR) and was positive in the two cases of LyP examined. Material was not available for

all cases to be studied. However, beta F1 expression is commonly seen in cases of the majority of CTCL. The alternative expression of a gamma/delta TCR is extremely rare. Expression of CD30 was positive in LyP type A lesions (cases 2 and 3) and in tumour stage (case 3) MF lesion. CD30 expression in this context indicates lymphocyte activation. In cases of tumour stage MF positive CD30 expression can be indicative of systemic spread of CTCL from skin to lymph nodes. LMP-1 was negative in all cases of MF examined. All cases examined expressed CD56, although incidence of CD56 expression in the literature in cases of MF and LyP is rare. These data suggest that there may be a subset of CD56+ MF and LyP.

2.   AP-1 CTCL findings

This section is comprised of a series of four publications concerning expression of AP-1 proteins in CTCL (MF and SS), CD30 positive cutaneous large cell lymphoma and B cell lymphoma.

1.   Mao, X., Orchard GE*., Vonderheid EC., Nowell PC., Bagot M., Bensussan A., Russell-Jones R., Young BD., Whittaker SJ. (2006). Heterogenous abnormalities of CCND1 and RB1 in primary cutaneous T-cell lymphomas suggesting impaired cell cycle control in disease pathogenesis. *Journal of Investigative Dermatology,* **126**, pp. 1388-1395.

*   Indicates joint first author

2.   Mao, X., Orchard G., Mitchell JT., Oyama N., Russell-Jones R., Vermeer MH., Willemze R., van Doom R., Tensen CP., Young BD., Whittaker SJ..

(2008). A genomic and expression study of AP-1 in primary cutaneous T-cell lymphoma: evidence for dysregulated expression of JUNB and JUND in MF and SS. *Journal of Cutaneous Pathology,* **35**, pp. 899-910.

3. Mao, X and Orchard, G*. (2008). Abnormal AP-1 expression in primary cutaneous B-cell lymphomas. *British Journal of Dermatology,* **159**, pp. 145-51.

4. Mao, X.,Orchard GE*, Russell-Jones R.,Whittaker S.. (2007). Abnormal activator protein 1 transcription factor expression in CD30-positive cutaneous large-cell lymphomas. *British Journal of Dermatology,* **157**, pp. 914-21.

*   Indicates joint first author

These studies set out to attempt to establish which AP-1 proteins were significantly expressed in these groups of lymphomas, and thus indicate which may have a role in lymphoma pathogenesis (Mao et al 2006,2007, 2008a and Mao and Orchard 2008b). The table below indicated overall findings from this series of papers.

Signalling molecules and transcription antibodies	CD30 POSITIVE			CTCL		B-CELL LYMPHOMA				
	C-ALCL % POS	LyP A % POS	TMF % POS	SS % POS	MF % POS	HL % POS	PCMZL % POS	PCFC % POS	PCLBCL-T % POS	PCBCL % Pos
C-JUN	10	2.7	38	9	22	50				
JUNB	44	11	50	55	9	100				
JUND	100	48	75	21	70	100	56	86	83	50
C-FOS	22	10	12.5	6	30	29			17	
RAF-1	33	2.7			4.3				33	
P44/42 MAPK	90	2.7	89	84		100				
PAK 1							22	14	50	
CD30				3	13					
CCND1/BCL1	70			50	45		44	57	67	100
C-JUN							11	14		
ERK1/2 MAPK				24						

Table 2   Summary of immunohistochemical data on AP-1 protein expression in CD30 positive lymphomas, CTCL and B cell lymphomas.

C-ALCL=Cutaneous anaplastic large cell lymphoma, LyP A = Lyphomatoid papulosis type A, TMF = Transformed MF, SS = Sezary Syndrome, MF = Mycosis fungoides, HL = Hodgkin's lymphoma, PCMZL = Primary cutaneous marginal zone B cell lymphoma, PCFCL = Primary cutaneous follicular cell lymphoma, PCLBCL-LT = Primary cutaneous large B cell lymphoma- leg type, PCBCL = Primary cutaneous B cell lymphoma. Samples were considered positive when the antigen in question was expressed by 50 -100% of tumour cells, indicating expression of these antigens by the atypical population of cells within the tumour areas.

The cumulative data indicate that dysregulated expression of both JUNB and JUND occurs in CTCL. with JUNB over expressed to a greater extent in SS compared to MF and conversely JUND over expressed to a greater extent in MF compared to SS. In the case of CD30 positive lymphomas the findings are similar to the majority of lymphomas over expressing JUND and to a lesser extent JUNB. In contrast, AP-1 protein expression in B cell lymphomas indicates upregulation of JUND, CCND1 and PAK1 with down regulation of JUNB in PCBCL. These findings would suggest that the AP-1 activation mechanisms, between CTCL and B cell lymphomas have differences, the significance of which remains to be full elucidated.

3. Immunocytochemical techniques and developments

This section of results reflects the technical and practical input of acquired knowledge gained throughout the preparation for this thesis. The two publications comprising this section were invited reviews.

1) Orchard, G. (2006). Immunocytochemical techniques and advances in dermatopathology. *Current Diagnostic Pathology*, **12**, pp. 292-302.

2) Orchard, G. (2008). Detecting antigenic epitopes using immunocytochemistry in tumour pathology – is it really all about sensitivity? *Immunocytochemistry*, **6**, (3), pp.116-118.

The first was intended to inform of the scope of immunocytochemical techniques applicable within the field of dermatopathology (Orchard 2006). It reflects the author's acquisition of knowledge across the length and breath of investigative IMC techniques applicable to the field of dermatopathology. This encompassed signal amplification dextran polymer based labelling systems, immunogold methodologies for demonstration of antigenic epitopes at the ultrastructural level, immunoflourescence applications in evaluation of autoimmune blistering diseases and increasing importance of fully automated IMC staining procedures. The second publication was based on the need to inform on the issue of IMC assay sensitivity (Orchard 2008). Whilst preparing this thesis and dealing with tumour pathology it became apparent that antigenic demonstration in tumour pathology was not always uniform, but could be variable. This is commonly seen with MM where many activation antigens are strongly expressed at the dermal/epidermal junction but are much more variably expressed in dermal deposits of cutaneous disease. This labelling profile is not always governed by sensitivity of the assay but may also reflect lack of protein expression at light microscope level. In a wider perspective and as an assessor for UKNEQAS immunocytochemistry for some years, it was apparent that many scientists have a 'blinkered' view of this concept. The publication was intended to be informative and to encourage debate.

4.   Malignant melanoma immunocytochemistry using double labelling

Of the two publications relevant to this section, one involved collaborative work with St. John's Photobiology Department, assessing effects of cutaneous photodamage.

1)   Orchard, G. (2004). 'Dying for a tan'-immunocytochemistry in the study of cutaneous photodamage and malignant melanoma. *Immunocytochemistry*, **3**, pp. 45-46.

Studies of early signs of damage to epidermal keratinocytes caused by ultra violet light exposure can be measured by use of immunocytochemical markers for specific antigenic epitopes (Orchard 2005). Assessments involved using volunteers of skin type 1 and 2 (most susceptible to sun damage). There is an internationally recognised classification of six skin types:

Skin Types

Type 1   White skin that never tans, but always burns

Type 2   White skin that burns initially but tans with difficulty

Type 3   White skin that tans easily and rarely burns

Type 4   White skin that never burns and always tans (generally Mediterranean or oriental skin type)

Type 5   Brown skin (Asian skin type)

Type 6   Black skin (African and Caribbean skin type)

The normal process of skin aging is a chronological effect that happens to all individuals. These chronological changes are produced as a result of a multitude of factors including environment, diet, social activity, smoking for example. In order to determine the effects of ultra-violet light on photo sensitive skin types (types 1 and 2) it is important to study these effects on skin that is normally protected from the sun - buttock skin being the most commonly evaluated. The evaluations can then also be performed on sun exposed skin, generally forearm skin. These two sites can then be compared following controlled ultra-violet exposure.

Each individual had a different dose of UV that produced an erythema - reddening of the skin surface. In effect this represents mild sunburn. In order to determined minimal erythema dose (MED), the 24 hour perceptible MED for each individual used was determined by a geometric exposure series. Biopsies were then performed on the volunteers immediately after exposure to 0. 0.5, 1, 2 and 3 times this MED and then was repeated again at 12 and 24 hour intervals following UVR administration. Immunocytochemistry was then performed using antibodies to p53, thymine –dimers, Bcl-2, MIB-1 and CD1a. These antibodies then allowed key evaluations of measuring effects of photodamage. Specifically: p53 and thymine dimers are indicative of DNA damage in affected keratinocytes (Young et al 2007). The Bcl-2 proto-oncogene product inhibits apoptosis induced by various stimuli and is constitutively expressed in basal keratinocytes of normal skin epidermis, but has been shown to be reduced in rat skin following UV exposure (Iwasaki et al 1996). MIB-1 enables assessment of keratinocyte proliferation levels following photodamage and CD1a is a marker of Langerhans

cells. The Langerhans cell is the immuno-competent cell of the epidermis and is known to be highly photophobic (Young et al 2007). Using image analysis, quantifiable cell counts of positively stained cells could be determined.

The data revealed statistically significant increase in p53 expression, thymine dimer formation and MIB1 expression directly following administration of UVR but no significant change in BCL-2 expression was noted. This is explained by the fact that immediately following the administration of UVR there is DNA damage as demonstrated with p53 and thymine dimmer formation, fairly quickly after this, damaged keratinocytes are then driven into the cell cycle to replenish the epidermal compartment with viable replacement cells, this is demonstrated by increased expression of MIB1. CD1a data revealed significant reduction in Langerhans cell counts within the epidermis following UVR exposure. Within 24 hours of UVR exposure levels of p53, thymine dimer, MIB1, proliferation levels were reduced and Langerhans cells gradually returned to repopulate the epidermis (Orchard G, 2004).

The second paper submitted here concerns development and application of a novel procedure for characterisation of MM.

2)   Sahni, D. Robson A, Orchard G, Szydlo R, Evans AV, Russell-Jones. R. (2005). The use of LYVE-1 antibody for detecting lymphatic involvement in patients with malignant melanoma of known sentinel node status. *Journal of Clinical Pathology*, **58**, pp. 715-721.

The objective was to double label lymphatic vessels and MM cells in primary cutaneous MM cases for which a positive sentinel lymph node (SLN) assessment had been established from routine diagnostic investigations (Shani et al 2005). This could then be compared to the same number of SLN negative assessments as a control group. The aim was to establish whether tumour cells identified within lymphatics could be used as a replacement for SLN status. In the SLN negative group, tumour cells were only identified in 1 out of 18 cases compared to 5 of 18 in the SLN positive group, there was however no significant difference in lymphatic counts between SLN negative and SLN positive cases. These data would seem to Indicate that the use of LYVE-1 in conjunction with S100 protein, can help detect melanoma cells within lymphatics, but as yet is unreliable in predicting melanoma metastasis.

Chapter 4   Discussion

Skin cancer is a clinically defined disease where assessments of skin lesions can be highly detailed and in some respects is therefore almost unique when compared to other organ specific pathologies, which generally rely on sophisticated equipment for clinical assessment. Clinical appraisal by the human eye and the option to touch and feel such lesions has significant advantages. This concept is so well defined in dermatology that clinically defined stages of cancer progression can be attributed to specific microscopic findings, for example the clinical and pathological criteria of patch, plaque and tumour stages of MF. This is the case for both CTCL and MM, where very clearly defined clinical appearances guide microscopic differential diagnosis of a benign, dysplastic or malignant melanocytic lesion (Willemze et al 2005 and Ackermann and David 1986).

Why then in the case of CTCL do some patients simultaneously present with more than one type of lymphoma? There is clearly a link between LyP and other forms of CTCL with LyP representing an entity which could provide the key to this question. There is evidence from a wide range of sources to support the notion that LyP is a true lymphoma. At light microscope level, LyP demonstrates cytomorphological features of atypia as demonstrated by type A, B and C cells (Basarab et al 1998). In addition, there are accompanying IMC findings such as persistent CD30 positivity, which although not an absolute marker for malignancy,

in the context of CTCL is quite often an associated finding (Bekkenk et al 2000).

TCR studies have revealed rearrangements in 60- 70% of cases (Willemze et al

2005 and Weiss et al 1986). DNA analysis by flow cytometry has shown

hyperdiploidy, tetraploidy and hypertetraploidy (Lange Wantzin et al 1983). There

is also evidence of an abnormal karyotype in cytogenetic studies of skin biopsies

(Espinoza et al 1985) and evidence of metastatic potential within LyP cases has

been suggested by studies on down regulation of transforming growth factor beta

(TGF-β) receptors on LyP atypical cells (Kadin et al 2001). Collectively all these

data supports the notion that LyP is a lymphoma. Evidence from the studies

presented in this thesis highlights that some cases of LyP and MF express CD56,

suggesting a potential link with a cytotoxic CD8 positive immunophenotype in

rare cases, since nearly all CD56 positive cases were also CD8 positive (Wain et

al 2005 and Flann et al 2006). This finding may have a bearing on prognostic

outcome since evidence from data on CD56 positive subcutaneous pannicultis-

like T cell lymphoma and blastic natural killer cell lymphoma indicates worsening

prognosis (Jaffe et al 2001). These papers supported our earlier findings of CD8

positive cases of juvenile MF(Wain et al 2003). MF is generally a disease of

older patients, but occasionally occurs in children, with over 95 % of such cases

having immuno phenotype of CD4 (T/helper) positive. Cases of MF in children

are unusual. This study arose because of an initial case presenting in a child who

was CD8 positive a rare phenotype for MF. Molecular data assessing

rearrangement of the TCR and using PCR to detect single stranded

polymorphism, indicated that the child did indeed have a gene rearrangement

confirming the malignant nature of the disease, with further subsequent skin lesions confirming the original findings. This prompted a review of all the childhood cases of MF seen at St. John's, which covered decades of data. Evaluation of 34 cases of juvenile MF was performed. The study revealed several additional cases of CD8 positive MF (38%) of those cases examined. Occurrence of CD8 positive phenotype in juvenile cases of MF seemed far more prevalent than the overall data would seem to suggest. The patient's subsequent management was reviewed to determine whether these patients performed any better or worse prognostically (Wain et al 2003). Overall disease free survival rates were similar to adult onset disease. However, although this particular study was the biggest by far to be assessed, actual numbers were still quite low. However the data indicated that the finding of CD8 positive expression in juvenile cases of MF was far more prevalent than had been previously suggested. Future studies with detailed follow up data may enable more specific treatment and patient management regimes to be established earlier in the disease pathway.

In the case of LyP 80-90% of patients present with papulonodular lesions which are either singular or multiple, which self heal leaving a scar. The notion of spontaneous resolution of malignant lesions is not unique in cutaneous pathology. Cases of MM which resolve spontaneously due to a potent host response are well documented in the literature (Shai et al 1994). It is also the case that CTCL can run a relatively benign course with several stages of dormancy throughout the clinical progression of the disease. It could be the case that LyP may, in fact, be an indicator of immune dysfunction, which then allows a

similar but independent lymphoproliferative disease to breach the immune surveillance mechanisms.

In the case of MM the use of markers of proliferation is widely employed to help distinguish dysplastic from malignant melanocytic lesions. Malignant lesions in both CTCL and MM have higher proliferative cell counts. This is particularly useful in MM since proliferation rates of over 10% of the melanocytic cell population within any given lesion is more indicative of malignant transformation over a stable, benign state. For this purpose the antigen Ki67 and its antibody MIB-1 are often employed and the use of antibodies raised against the AP-1 family of proteins, may be complementary in this setting. It is well documented that this family of proteins is implicated in regulatory pathways of proliferation, differentiation, cell maturation, apoptosis, cell migration and transformation (Vesely et al 2009). What is also clear from the studies documented in this thesis, at light microscope level, Affymetrix expression microarray, real-time reverse transcriptase-polymerase chain reaction (RT-PCR) and fluorescence in situ hybridization (FISH) studies, is that certain proteins of this family are expressed at high levels. The data accumulated indicated that JUNB and JUND are over expressed in the majority of cases of Sezary syndrome and mycosis fungoides (Mao et al 2003, and 2008). Similarly, both phosphorylated and total extracellular signal-regulated kinase (ERK) 1and 2 mitogen-activated protein kinase (MAPK) proteins were increased (Mao et al 2008). This suggests that deregulation of AP-1 expression in cutaneous T cell lymphoma is characterized by aberrant expression of JUNB, and to a lesser extent JUND. These data suggest that JUNB is a significant transcriptional factor in the development of

CTCL. Possibly resulting from genomic amplification and subsequent activation

of ERK 1 and 2 MAPK in cutaneous T cell lymphoma (Mao et al 2008).

Immunocytochemical data from this study also demonstrated that JUND

expression can be seen in non tumour cell lymphocytes, whereas JUNB

expression is much less in such cells. This would seem to suggest that JUND is

mainly involved in lymphocyte proliferation, whereas JUNB is involved in

lymphocyte transformation and lymphoma progression or that they are mutated in

some way. In studies of CD30 positive CTCL no direct correlation was seen

between JUNB expression and CD30 expression. In LyP it was observed that

although JUND was expressed widely irrespective of the histological sub type of

LyP (A,B or C), JUNB was not expressed in the majority of skin lesions. However

it was expressed at high levels in cutaneous anaplastic large cell CD30

lymphomas (Mao et al 2007).

In comparison, primary cutaneous B cell lymphoma (PCBCL) revealed loss of

expression of JUNB, and increased expression of JUND in the majority of lesions

studied. In addition it was possible to demonstrate increase in cyclin D1 (CCND1)

expression and also PAK 1 which promotes dis-assembly of stress fibres and

focal adhesions and functions as a dominant oncogene, expressed in a variety of

cancers other than cutaneous B cell lymphoma (Mao et al 2008). There is

association between CCDN1 and PAK1, in that expression of both proteins is

upregulated in liver cancer (Parekh and Rao 2007) and the PAK1 gene maps to

human chromosome 11q13q14 (Bekri 1997), which is close to the location of

CCDN1 (11q13). It may well be that increased expression of PAK1 and CCDN1

protein expression relates to a copy number gain of chromosome 11 which is documented in cutaneous B cell lymphoma (Wiesner et al 2005). Taken collectively, these data would indicate differing activation pathways between CTCL and PCBCL.

It seems likely that in de-regulation of AP-1, expression occurs in CTCL as a result of specific chromosomal amplification of JUNB, translating into an over expression of JUNB at the protein level. It is known that JUNB has a specific role in T cell activation, binding to the IL-4 promotor and promoting Th2 differentiation and expression of Th2 cytokines (Li et al 1999 and Voice et al 2004). It seems likely therefore that the Th2 cytokine expression profile seen in Sezary syndrome, namely IL-4 and IL-10, may be a direct consequence of JUNB over expression. (Voice et al 2004)

As a result of the observations made in the authors' publications submitted for this thesis, coupled with a growing interest and involvement in application of immunocytochemistry in the pathology setting, the author has been approached and assigned as an assessor with UKNEQAS for immunocytochemistry (UKNEQAS ICC). UKNEQAS ICC represents the recognised quality control body for use of immunocytochemistry nationally. What became apparent in this role was the misunderstanding of why antigens associated with certain tumours were not universally expressed in all cases. What was being perceived as a lack of sensitivity for detection of certain antigens was in fact more concerned with reduced expression of such antigens at light microscope level. This issue

becomes more apparent when dealing with metastatic deposits, especially if the antigens concerned are related to activation antigens. These observations relate to molecular and cell protein expression of any given tumour and do not always relate to substandard immunocytochemistry procedures or more specifically decreased sensitivity. The use of appropriate positive controls is paramount to assess quality, since it represents the known and established level of sensitivity for a given antigen in a given assay.

There were two over-arching aims to the studies included in this thesis, the first of which was to establish the value and application of a small panel of immunocytochemical antibodies against activation antigens, for the study of CTCL and MM. The majority of the antibodies discussed within this thesis had not previously been tested for use on formalin fixed paraffin embedded tissue sections and, following extensive development work to ensure viability of the antibodies for use, the data compiled represents novel and incisive findings. The second aim concerns development of an understanding of the significance of the expression of certain activation antigens in terms of the tumour pathway in CTCL and MM.

The papers and data presented here have formed the basis of two streams of further continuing investigation.

1)   From the data compiled on activation antigen expression in MM,

the author, in conjunction with UKNEQAS ICC, is compiling results of an

international trial to evaluate use of HMB 45 and Melan A for MM

diagnosis. MM cases of primary cutaneous, secondary cutaneous and nodal

metastatic MM have been selected with a total of four assessments performed

over a one year period. For each assessment a different type of MM was chosen

from the cases above. These cases have been circulated to over 800

laboratories for each assessment throughout the UK and abroad with the request

that they perform staining for either HMB45 or Melan A, the two most common

monoclonal antibodies used for MM diagnosis (Gown et al 1986 and Orchard

1998). On the return of these slides to UKNEQAS ICC, the author along with a

panel of experts has scored all these returns in terms of sensitivity and specificity

of staining. All participants have provided detailed information on the techniques

performed. From these data, analysis of methodology and performance will be

extrapolated. These data will allow objective assessment of the best clones to

use and also the best techniques to employ to maximise sensitivity of the assay.

Inclusion of metastatic cases which are known to be variable in terms of

expression of these activation antigens will allow the author very good insight

into the nature of expression of activation antigens across the length and breadth

of histological presentations of MM.

2)   Extending from the CTCL AP-1 expression studies, the author is currently

pursuing studies to determine whether use of JUNB will be more beneficial in

helping establish final stage of transformation in cases of MF. Transformation is

the stage at which the patient's skin disease becomes systemic and represents

the patient's final stage before they succumb to it. Currently MF is negative for the expression of CD30 until it reaches end stage disease at which point the tumour cells often express CD30. This is a useful observation used to suggest imminent likelihood of transformation, but it is not however absolute. Evaluation of known cutaneous end stage disease cases of MF for expression of JUNB may prove useful in conjunction with CD30 expression, and clinical outcome observations for prediction of transformation in MF patients. If this theory is proved correct, JUNB may well be a useful prognostic tool within this area of pathology.

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Appendix- Supportive publications for the thesis

1. Orchard GE (2004) 'Dying for a tan'-Immunocytochemistry in the study of cutaneous photodamage and malignant melanoma. *Immunocytochemistry.* **3**, pp. 45-46.

2. Shani D, Robson A, Orchard G, Szydlo R, Evans AV, Russell-Jones R. (2005) The use of Lyve-1 antibody for detecting lymphatic involvement in patients of known sentinel node status. *Journal of Clinical Pathology.* **58**, pp. 715-21.

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