1995-10-01

Development of an In Vitro Assay to Predict Patient Response to Radiotheraphy

Mary Sheridan
Technological University Dublin

DEVELOPMENT OF AN *IN VITRO* ASSAY TO PREDICT PATIENT RESPONSE TO RADIOTHERAPY.

Mary T. Sheridan

A thesis submitted for the degree of Philosophiae Doctor to Dublin University, Trinity College Dublin.

Department of Biochemistry,
Trinity College Dublin.

October 1995

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ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Carmel Mothersill, for giving me the opportunity to carry out this work. The past three years has been a wonderful learning experience in many respects and for this I am very grateful.

I thank Professor K. Tipton for his guidance with this thesis.

Thanks to all my colleagues in the new 'Radiation Science Centre' - Maria for the many reassuring discussions; Sergio for all the gossip, true or false; Helen for all the informative discussions; Joe for assistance with cell culture; James and Robert for, em - always being there (well some of the time) !

Thanks to Fiona for doing the electron microscopy shown in this thesis.

I would like to thank Dr. Colin Seymour for irradiating my cultures in St Luke's Hospital.

Thanks to all my family and friends for their continual support and encouragement. Special thanks to Luke.

Thanks to my brother, Seán, for unlimited use of his computer.

I would like to thank Mr. T. O'Dwyer, of the Mater Misericordiae Hospital, Dublin, for supplying me with the head and neck tumours used in this study, and Mr. T. Walsh, of St. James's Hospital, Dublin, for the oesophageal biopsy material.

I would like to thank Dr. Philippe Lambin for supplying the three cells lines of varying radio-sensitivities used in the p53 experiments.

I dedicate this work to my mother and father.

Declaration

I declare that, except where otherwise stated in the acknowledgements, this thesis is entirely my own work and it has not been submitted to this or any other university as an exercise for a degree. I give permission to the library to lend or copy this thesis.

Mary Sheridan.

Summary

At the present time, the treatment plan for a patient with cancer is usually based on parameters such as tumour site, histological type, tumour stage and performance status. However, it is well known that the radiosensitivity of human cancers varies widely from one patient to another (Fertil et al., 1981), and that even within these broad categories some tumours will show greater response to radiotherapy than others. If those patients unlikely to be cured by radiotherapy could be identified prior to commencement of treatment, alternative or more aggressive therapies might be selected which may give a better chance of cure than the standard therapy.

The aim of this study was to develop an *in vitro* assay, the Mothersill outgrowth assay, to predict the individual patient response to chemo- and, in particular radiotherapy. This assay involves culturing explants cut from a tumour and then treating them in situ with clinical equivalent doses of chemo- and radiotherapy. Two types of cancer were examined, oesophageal carcinoma and head and neck squamous cell carcinoma. The sensitivity of the tumour to treatment was evaluated in terms of the % growth inhibition in the treated cultures relative to the untreated control. The nature of this assay allowed other parameters to be examined immunocytochemically as potential indicators of tumour radiosensitivity. These parameters have previously been shown to be of prognostic significance in a range of cancers, they include; % Ki67 positive cells: expression of EGFr, c-Myc and Bcl-2: co-expression of c-Myc and Bcl-2: expression of the non-functional protein product of p53.

The Mothersill outgrowth assay provided a system in which multiple parameters could be examined from limited tumour material. Due to the small patient number conclusive

results could not be obtained regarding the parameter, % growth inhibition, as an indicator of patient response to treatment. To obtain these results a larger time scale would be required to allow for the accumulation of a large number of patients and a long follow up time. The results obtained indicated that % Ki67 positive cells measured after irradiation was indicative of patient *in vitro* radiosensitivity; expression of Ki67 correlated positively with radiosensitivity. EGFr expression measured before and after treatment differentiated the radio- sensitive and resistant tumours. High expression of EGFr correlated with radio-resistance. Expression of c-Myc and Bcl-2 taken alone did not indicate tumour sensitivity, however tumours positive for c-Myc and negative for Bcl-2 were radiosensitive. Expression of p53 protein did not significantly differentiate the radio- sensitive and resistant head and neck squamous cell carcinoma tumours, although when examined in cell lines derived from colorectal and bladder tumours, a significant correlation was found between radiosensitivity and p53 protein expression; high % of p53 positive cells correlated with radio-resistance.

In conclusion, using the Mothersill assay it was possible to evaluate several parameters from a limited amount of tissue. Of these parameters, % Ki67, EGFr, c-Myc and Bcl-2 appear to have potential as indicators of tumour radiosensitivity. An increase in the number of head and neck squamous cell carcinoma patients and a longer follow up time will be required before any conclusions can be made regarding the potential of the parameter, % growth inhibition, to predict patient response to treatment.

ABBREVIATIONS

AJCC - American Joint Commitee on Cancer

Cisplatin - cis-diaminedichloroplatinum

CAM assay - cell adhesive matrix assay

CFE - colony forming efficiency

5FU - 5-Flurouracil

ELISA - enzyme-linked immunosorbent assay

PBS - phosphate buffered saline

SF_2 - surviving fraction after treatment with a single dose of 2 Gy

%GI - percent growth inhibition in a treated culture relative to an untreated control

SCC - squamous cell carcinoma

UICC - Union Internationale Contre le Cancer

Chapter 1

Introduction

1.1 What is Cancer?

Cancer is a multi step event which has a wide range of clinical manifestations: tumours arise at different sites, grow at different rates and may be benign or malignant. However the underlying factor that is characteristic of all tumours is the abnormal regulation of cell proliferation.

The highest human cancer incidence is generally but not exclusively found in organs with labile cell populations. Cells in labile and stable tissues die physiologically by apoptosis or loss from the external or internal surface without any inflammatory reaction. New cells are created to replace them. The highest incidence of cancer in western countries is found in the breast of women and in the prostrate for men, i.e., in organs under hormonal control with cyclic changes in epithelial cell proliferation, cells that belong to the labile cell populations (Iversen, 1992). The incidence figures are followed by colon cancers; tumours of the lung, stomach, and urinary bladder; followed again by melanomas, lymphomas, and cancer of the pancreas (Langmark, 1990). Most of these organs have a relatively high frequency of cell division (Preston-Martin, 1990). Of course, in most cases cell proliferation is strictly regulated so that the appropriate balance is maintained at all times. Such balance arises form the interplay of negative regulatory mechanisms such as the positive action of growth factors for each specific tissue and contact inhibition; a mechanism through which cells detect their neighbours and stop dividing when a certain density is reached . Proto-oncogenes such as c-myc and bcl2 regulate the cell cycle towards mitosis or apoptosis (Sachs et al., 1993). The tumour supressor gene, p53, is linked to G_1 arrest after DNA damage (Kastan et al., 1991), allowing repair of breaks in DNA strands and reducing the probability of

mutations, or if the damage is perceived to be too severe, p53 protects the genome by initiating apoptosis (Clarke et al., 1993). These proto-oncogenes can be altered or mutated to become oncogenes and thus lose their normal regulatory function. Oncogenes are defined as genes that initiate uncontrollable growth of mammalian cells (Bland et al., 1995). A cancer can then be defined as a population of cells multiplying in an unregulated manner independent of normal control mechanisms. If this occurs in an organ such as the liver, kidney, lung or brain then a solid mass results while uncontrolled multiplication of bone marrow stem cells gives rise to leukaemia in which the malignant progeny are detectable in the blood (Hall, 1990).

In becoming malignant cells often revert to a primitive cell type or de-differentiate. These cells multiply first into a small undetectable mass and eventually grow into a large primary tumour with it's own blood supply. In such a tumour the invasive region is confined to the periphery while the centre is often necrotic, Figure 1.1.

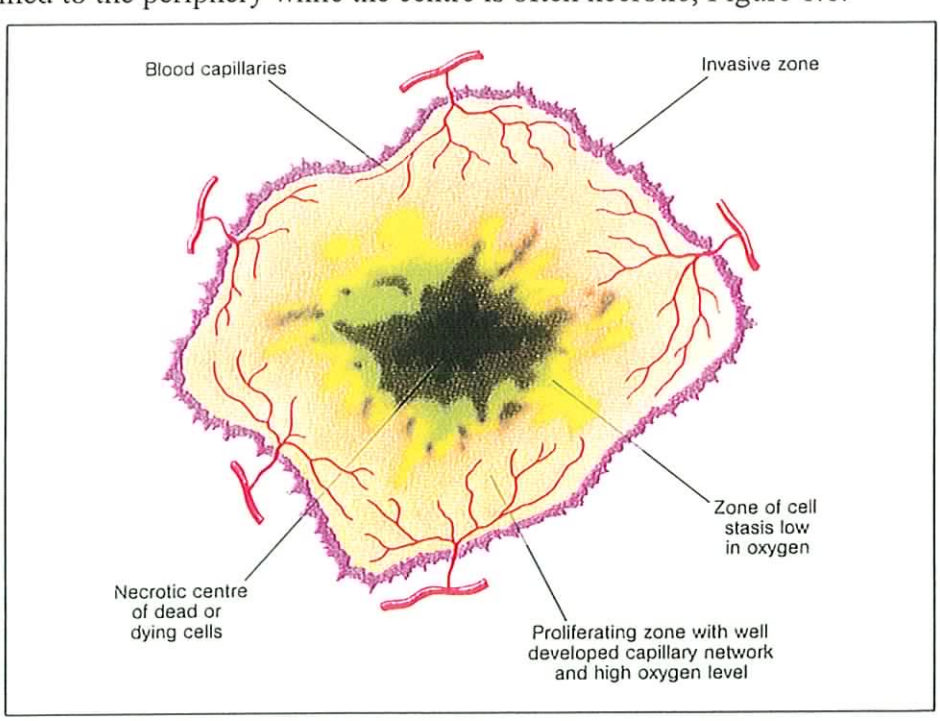

Figure 1.1. Diagrammatic representation of a slice through a large solid tumour (Beating Cancer; Hall).

1.2 Classification and Staging

The practice of dividing cancer patients into groups according to so-called stages arose from the fact that survival rates were higher for patients in whom the disease was localised than for those in whom the disease had extended beyond the organ of origin (Snow, 1989).

Staging of cancer is a method of gathering groups of classified patients for the purpose of analysis. Recording accurate information on the extent of disease for each site serves a number of related objectives: to aid the clinician in the planning of treatment, to give some indication of prognosis, to assist in evaluation of the results of treatment, to facilitate the exchange of information between treatment centre, and to contribute to the continuing investigation of human cancer (Snow, 1989).

The two major agencies concerned with the classification of malignant disease are the Union Contre le Cancer (International Union Against Cancer) (UICC 1987) and the American Joint Committee on Cancer (AJCC 1983). The rules of classification and stage grouping according to the UICC correspond exactly with those of the AJCC Manual for Staging of Cancer.

Classification

Meaningful classification depends on quantifying the extent of disease, this is based on three components:

T - extent of primary tumour

N - status of regional lymph nodes

M - presence or absence of distant metastases

T Categories: The criteria for categorising a primary tumour (T) is the apparent anatomic extent of the disease. Extent is commonly based on three features: depth of invasion, surface spread and size. The following is a general definition of the clinical basis for placement of a tumour in T1, T2, T3 or T4;

T0: No evidence of a primary lesion found grossly or microscopically. Evidence of malignant change without microinvasion and without a target lesion identifiable clinically.

T1: A lesion confined to the organ of origin. It is mobile, does not invade adjacent or surrounding structures or tissues, and is often superficial.

T2: A localised lesion characterised by deep extension in adjacent structures or tissues. Invasion is into capsulae, ligaments, intrinsic muscle and adjacent attached structures of similar tissue or function. There is some loss of tumour mobility, but it is not complete; therefore fixation is not present.

T3: An advanced lesion that is confined to the region rather than to the organ of origin, whether solid or hollow. The critical criterion is fixation, which indicates invasion into a fixed structure or past a boundary. These structures are most often bone and cartilage; but invasion of the extrinsic muscle walls , serosa and skin are also included.

T4: A massive lesion extending into another hollow organ causing a fistula, or into another solid organ causing a sinus. Invasions into major nerves, arteries and veins are placed in this category. Destruction of bone in addition to fixation is an advanced sign.

N Categories: The criteria for categorising lymph node (N) consist of size, firmness, capsular invasion, depth of invasion, mobility versus fixation, single versus multiple nodes, ipsilateral, contralateral and bilateral distribution, and distant nodes.

N0: No evidence of disease in lymph nodes.

N1: Palpable and moveable nodes limited to the first station. A distinction between an uninvolved and an involved palpable node needs to be made. This depends on the firmness and roundness of a node and its size, which is generally greater than 1 and more than 2 cm - usually up to 3 cm in size and solitary.

N2: Firm to hard nodes, palpable and partially moveable; they range from 3 - 5 cm in size. Such nodes show microscopic evidence of capsular invasion; clinically they may be matted together. Nodes can be contralateral or bilateral.

N3: Fixation is complete. Nodes beyond the capsule with complete fixation to the bone, to large blood vessels, to skin, or to nerves - usually greater than 6 cm in size.

N4: Nodes involved beyond the first station: they are in the second or distant stations. If the first two nodal stations are vertically arranged and both are involved, such double involvement is staged as N_4.

NX: Nodes inaccessible to clinical evaluation.

N- or N+ : Nodes evaluated by microscopic study and designated as negative or positive depending on findings (Rubin, 1983).

M Categories: The important feature in this category is the presence or absence of metastasis. The reason for this reflects the poor prognosis if metastases are present.

M0: No distant metastasis.

M1: Distant metastasis

MX: Presence of distant metastasis cannot be assessed.

Staging

After T, N, and M categories are assigned, they may be grouped into stages. Table 1.1. TNM classification and stage grouping, once established, must remain unchanged in the medical records. If there is doubt concerning the correct T, N, or M category to which a particular case should be allotted, then the lower, that is less advanced, category should be chosen (Snow, 1989).

Table 1.1. Stage grouping (Snow, 1989)

Stage	TNM Classifications
Stage 1	T1 N0 M0
Stage 2	T2 N0 M0
Stage 3	T3 N0 M0
	T1 or T2 or T3 N1 M0
Stage 4	T4 N0 or N1 M0
	any T N2 or N3 M0
	any T any N M1

The TNM system takes into account only the anatomic extent of the local invasion of the primary tumour, along with clinically detectable regional metastases or distant metastases. Other factors such as the growth rate of the tumour are not reflected in the TNM system (Snow, 1989). The TNM classification, although widely utilised in clinical practice is associated with a well recognised difficulty in predicting individual outcome. Not infrequently, tumours similarly staged on the basis of their origin, size, and metastatic node involvement will differ dramatically in their clinical course and response to therapy (Gapany et al., 1994).

1.3 Treatment Planning

The approach to treatment planning for cancer patients involves physicians from a wide range of specialities: surgery, irradiation and chemotherapy. This also extends to those who participated in the diagnosis: radiologist, pathologist and cytologist. This process is a multidisciplinary one and requires a group of physicians working as a team to define the methods of treatment. In practice this teamwork involves two stages.

The first stage consists of the development of a treatment protocol: critical study of the results of the medical team, comparative study of the results of this team and of other teams, therapeutic trials (phase II and randomised), development of new techniques, study of prognostic factors. The second stage consists of the review of each individual case, of each given patient, by a group of treating physicians. Within the general context of the protocol, this group of physicians must discuss the decisions specifically adapted to each patient (Cachin, 1989). At the present time, the treatment plan is usually based on parameters such as tumour site, histological type, tumour stage and performance status (Begg, 1993).

Surgery, radiotherapy and chemotherapy are the three modalities of treatment presently used for cancer. Surgery is in the majority of cases the primary form of treatment and it leads to good therapeutic results in a range of early non-metastatic tumours (Steel, 1993). Radiotherapy is used alone or in combination with surgery or chemotherapy. Chemotherapy is the most controversial of the treatment modalities and it's role in the treatment of cancer is presently being investigated with enthusiasm.

Squamous cell carcinoma of the head and neck, and oesophageal carcinoma are the two types of cancer examined in this thesis, they shall be discussed in the following sections.

1.4 Oesophageal Cancer

Cancer of the oesophagus accounts for about 2% of all malignant tumours. It is a common cancer in some regions of northern China and in a geographic belt that stretches from European Russia, north and east of the Caspian, through Turkey and Iran into central Asia (Sherman et al., 1994). Because of changes in the economic climate of many of these areas, the incidence of oesophageal cancer is changing as well. For, example, in China, the country with the highest mortality rate due to this disease, incidence rates have been decreasing since the 1970s, possibly reflecting increased consumption of food rich in protein, carotene, vitamins C and E, and riboflavin (Flood et al., 1995).

However in Ireland, the incidence of oesophageal cancer has increased by 50% over the past thirty years and in particular, Irish women over the age of seventy have had the highest increase in incidence, at 32%, in their European age-specific group (Cheng et al., 1992). The increase is due largely to the dramatic change in the histological presentation: the incidence of adenocarcinoma of the oesophagus has increased rapidly (Walsh, 1995).

The reason for these changes are unclear. Lifestyle factors such as cigarette smoking and a high alcohol intake are regarded as the chief risk factors for the development of squamous carcinoma. Little is known about risk factors for adenocarcinoma apart from the link with Barret's mucosa (Walsh, 1995).

Diagnosis

In almost all cases dysphagia is the symptom that leads to the diagnosis but, unfortunately, this is a late symptom, not developing until there is significant tumour growth. The magnitude of dysphagia is directly proportional to the obstruction of the lumen of the oesophagus (Sherman et al., 1994).

The following diagnostic procedures are used to confirm and document suspected cancer:

1. Endosonography

2. Fluoroscopy and barium swallow radiological examination

3. Endoscopy and accompanying biopsy

4. Exfolliative cytology by brush or lavage at time of endoscopy

5. Computed tomographic (CT) scanning of the thorax and upper abdomen.

Treatment

SURGERY

Resection of the oesophagus remains the mainstay of therapy for local and locoregional disease, and for patients with resectable disease, it is the standard treatment outside of a clinical trial (Flood et al., 1995). The 1, 2 and 5 year survival rates, as determined by Muller and colleagues, based on 76,911 patients, reported in 130 papers was no greater then 27, 12 and 10 percent respectively (Muller,1990). Although these survival rates are low they are considerably better than those provided by Earlam and Cunha-Melo's

meta analysis a decade earlier (Earlam and Cunha-Melo, 1980). The 1, 2 and 5 year survival rates based on 83,783 patients operated on between 1953 and 1978 were 18, 9 and 4. This increase in survival rates is mainly due to advances in technology, improvements in anaesthesia, decrease in post-operative mortality and morbidity rates (Zenone, 1992). Surgical resection provides excellent palliation; however the chance for cure with oesophagectomy alone is low, as shown by the survival rates above.

RADIOTHERAPY

In an attempt to improve these rates adjuvant therapy to surgery has been used, namely radiation. Five randomised trials (Gignoux, 1987; Huang, 1986; Luanois, 1981; Wang, 1989; Arnott, 1992) have evaluated the effect of preoperative radiotherapy and showed no significant survival improvement in the irradiated group. Another prospective randomised trial has evaluated the effects of postoperative radiotherapy on the survival rates and found similar results (Teniere, 1987).

CHEMOTHERAPY

Over 70 percent of patients with oesophageal cancer are considered to have systemic disease at diagnosis therefore systemic therapy is considered essential if cure is to be hoped for (Walsh, 1995). Since radiotherapy has produced no significant increase in the survival rates, chemotherapy is now being looked to for improving the survival of oesophageal carcinoma patients.

In a phase II trial of preoperative combined radiation and chemotherapy with cisplatin, vinblastine, and continuous infusion 5-flurouracil in 43 patients, carried out at the

University of Michigan Medical Centre, survival after oesophagectomy seemed considerably better than in the historical control subjects treated with oesophagectomy alone. The three year survival of the 43 patients was 46% compared with 23% of those who received only surgery. Considering only the 27% of those patients who had no cancer in their resected specimens, i.e., the complete responders, the 5-year survival was 70% (Orringer, 1993). In a recent study by Forastiere et al. (1994) patients were treated with infusional cisplatin, 5-FU and concurrent radiotherapy, followed by oesophagectomy. There was a 42% rate of pathologically complete response and within this group the estimated two year survival was 55%. These results indicate that there is a subgroup of patients for which multimodality therapy is particularly beneficial.

This presence of a sensitive subgroup was also commented on by Wilke et al. (1994) in their review, 'Current status and future directions in the treatment of localised oesophageal cancer'. In this review they said that results of combined modality treatment in potentially resectable oesophageal carcinomas have not shown that preoperative chemotherapy or chemo-radiotherapy is superior to surgery alone with respect to resectability, local tumour control and overall survival. However, chemotherapy-responders who subsequently underwent a complete tumour resection had a markedly improved long-term survival indicating that the inclusion of chemotherapy in the treatment of oesophageal carcinoma may improve prognosis. They conclude that, to date, there is sufficient evidence that preoperative treatment of oesophageal carcinoma may improve prognosis at least of subgroups of patients with this tumour.

If this subgroup of patients could be identified prior to commencement of treatment the survival rates for this group could be improved greatly, while the overall efficiency of

treatment planning would be enhanced. Administration of rigorous and costly treatment protocols to resistant patients could be avoided.

PALLIATIVE THERAPY

Since long-term survival in oesophageal cancer is so poor, treatment designed for palliation is necessary and is aimed primarily at enabling patients to continue eating. For patients with local recurrence, restoration of the swallowing function to allow handling of secretions and an optimal quality of life is crucial. Measures that are effective for the short term include oesophageal dilation, oesophageal prostheses (e.g. stents), bipolar electrocautery, and laser excision (Flood et al., 1995). The other palliative modalities primarily use radiation, usually external beam, either alone or in combination with systemic chemotherapy (Sherman et al., 1994).

Prognosis

The prognosis for patients with oesophageal carcinoma is poor due to frequent occurrence of both distant metastasis and local recurrence (Zenone et al., 1992). Despite more aggressive surgical and radiotherapy approaches, the 5-year survival rate has remained lower than 5% (Sherman et al., 1994).

Role of a Predictive Assay

Up to now, results of combined modality treatment in potentially resectable oesophageal carcinoma patients have not shown that preoperative chemotherapy or chemo-radiotherapy is superior to surgery alone with respect to local control and overall survival (Wilke et al., 1994). However, chemo-radiotherapy responders who subsequently underwent a complete tumour resection had a markedly improved long term survival. This was particularly evident in a phase II trial of preoperative combined radiation therapy and chemotherapy carried out at the University of Michigan (Orringer, 1993). These results showed that survival after oesophagectomy seemed considerably better than their historical control subjects treated with surgery alone, and that this improvement in survival was particularly evident in a subgroup of patients who responded completely to the preoperative treatment. The 5-year survival in this group was 70%.

These findings suggest a potential role of a predictive assay in treatment planning for oesophageal cancer. If this subgroup of patients could be identified using a predictive assay prior to commencement of treatment, they could be selected for combined modality treatment, while those patients unlikely to respond could be considered for alternative treatment.

1.5 Head and Neck Cancer

Head and neck cancer includes all carcinomas arising in squamous mucosal epithelium from the lip, oral and nasal cavities, pharynx to larynx and middle ear. Table 1.2 shows the mortality from cancers of the commonest sites (Souhami and Tobias, 1995). Head and neck cancer accounts for 5% of all cancers in the developed world (Silverberg et al., 1989). It is the fifth most common cancer after lung, breast, gastrointestinal, genito-urinary and, leukaemia and lymphoma. 90% of head and neck cancers develop from mucosa of the upper aerodigestive tract and are squamous cell carcinomas (Tupchong et al., 1994), the remainder originate from the salivary glands, skin, bone cartilage, vessels, nerves and soft tissue. Squamous cell carcinoma of the head and neck show a number of distinctive features, there is a male predominance (Tupchong et al., 1994), and the most common risk factors are tobacco and alcohol. The most common sites are oral cavity and larynx, however there is a wide variation for different countries and regions. Outcome following treatment depends on the site and extent of disease. The most important prognostic factor is the presence or absence of lymph nodes. Treatment failure occurs most commonly because of failure to control nodal disease.

One other important feature of head and neck squamous cell carcinoma is the risk of multiple primary tumours, be they synchronous or metachronous (occurring greater than 6 months after initial diagnosis). Multiple primary cancers occur in up to 15% of patients. Overall cure following treatment occurs in approximately 40% of patients, and survival has only marginally increased over the past 30 years.

Table 1.2. Annual mortality (England and Wales) from major sites of cancer of the head and neck (Souhami and Tobias, 1995).

Site	Males/yr	Females/yr
Lip	42	9
Tongue	206	141
Oral Cavity	188	106
Pharynx	422	293
Nose, middle ear and sinuses	143	113
Larynx	608	149

Diagnosis

Most cancers of the head and neck grow and present as malignant ulceration's of a surface mucosa. However symptoms and signs depend on location, e.g.;

Larynx: persistent hoarseness, pain, referred otalgia, dyspnea, and stridor.

Oral cavity: swelling or ulcer that fails to heal. Local pain is not always present.

A metastatic cervical node may be part of the clinical presentation of any of the tumours (Zagars and Norante, 1983). The following diagnostic procedures are used to confirm suspected cancer and evaluate extent of disease:

1. Biopsy of all suspicious areas; pan-endoscopy of the larynx, trachea and oral cavity
2. Chest X-ray
3. Computed tomography (CT) scan

4. Panorex film or bone scan for evaluation of the mandible

5. Magnetic resonance imaging (MRI) (used in vascular tumours or to asses soft tissue extent)

Treatment

Treatment for head and neck cancers consists of surgery, radiotherapy or a combination of both. In general, standard therapy consists of either surgery or radiation therapy for early lesions (T1 \ T2) and combined therapy for more advanced lesions (T3 \ T4) (Tupchong, 1994). Although both surgery and radiotherapy can be curative for localised squamous cancers of the head and neck (and even in some cases with lymph node involvement), the prognosis of patients with more advanced disease, especially with nodal involvement, is generally poor (Souhami, 1995). Metastatic disease is evident in only 5% of patients at initial presentation. Squamous cell carcinoma of the head and neck tend to be localised to the primary site or neck region, consequently cure rates tend to be relatively high.

Chemotherapy is rarely used and despite multiple trials its role is still experimental. Currently it is used in some centres as neo-adjuvant treatment for obstructive laryngeal tumours to decrease size prior to use of radio-therapy, this is done in an attempt to preserve the larynx. It is also used occasionally for recurrent tumours. The combination of cisplatin and 5-flurouracil is currently considered standard in the treatment of patients with recurrent or metastatic head and neck carcinoma (Merlano, 1994; Skoog, 1994).

SURGERY

Surgery has been the mainstay of therapy of head and neck cancer for decades. Early cancers can be excised without any deformity and/or loss of function. However more advanced (T3,T4) tumours require radical surgery, which is often mutilating, leaving soft tissue or bony defects. Since the mid 80's with the introduction of myocutaneous and free microvascular tissue transfer, all of these defects can be repaired satisfactorily and the patient discharged in 2 - 3 weeks following radical surgery.

RADIOTHERAPY

Over the last decade rapid advances have been made in the technology and sophistication of radiation therapy for the treatment of head and neck cancers. These include the use of high-energy linear accelerators, immobilisation devices, altered fractionation techniques and radiosensitising agents (various chemotherapeutic agents including 5-flurouracil (5-FU) and cisplatin) (Tupchong, 1994).

Irradiation can be used alone in small or medium-sized (T1, T2 and some T3) exophytic, superficial cancers without any severe alteration in mobility. Much progress has been made recently in the use of radiotherapy as the primary treatment for advanced carcinoma. Karim et al. (1987) in a study with radiotherapy as the primary treatment for loco-regionally advanced carcinoma of the larynx and salvage surgery in reserve, showed a high percentage of local control (67% at 3 years) and better quality of life due to organ preservation. Hyperfractionation regimens have also been shown to be superior to conventional fractionation in improving the local control rates for various head and neck carcinoma sites (Horiot et al., 1992 and Parsons et al., 1993). Accelerated

radiotherapy regimens are also being investigated. A phase II trial testing a continuous and accelerated regimen of radiotherapy in very advanced cancers has shown an improvement in the local control and the therapeutic ratio over the historical control group (Lusinchi et al., 1994).

A combination of surgery and radiotherapy is used for the treatment of more advanced disease. Radiotherapy can be given pre- or postoperatively.

CHEMOTHERAPY

Local recurrences are one of the main reasons of failure of therapy for locally advanced cancer of the head and neck, because of this the investigation into the use of chemotherapy to improve local control rates and survival continues. Traditionally, chemotherapy has been reserved for treatment of recurrent head and neck squamous cell carcinoma. Severe morbidities after surgery and radiation therapy, high mortality rates, and the poor outcome of chemotherapy for recurrent tumours have led to clinical investigations of many therapeutic variations that could be added to combined modality approaches. The approaches fall into three main categories:

(a) neoadjuvant or induction chemotherapy, in which a specific number of chemotherapy cycles are given before standard local and regional therapy is instituted. The goal is to decrease the size of the tumour and increase the chance of cure with subsequent surgery or radiation therapy.

(b) adjuvant chemotherapy which is administered following definitive standard treatment or sandwiched between surgery and radiation therapy, in an attempt to

eradicate the microscopic lesions presumed to remain after surgery, radiation therapy or both approaches

(c) concomitant chemotherapy and radiation therapy (usually used only in unresectable head and neck squamous cell carcinoma (HNSCC)) used in an attempt to eradicate systemic microscopic disease while simultaneously enhancing the cytotoxicity of radiation against macroscopic disease in the head and neck (Vokes et al., 1993).

The most active single agents in the treatment of head and neck cancer - cisplatin (CP), methotrexate, flurouracil (5-FU), and bleomycin - produce response rates of only 20% to 30%. Attempts have been made to try and improve these response rates by investigating combinations of the above drugs. A phase III randomised study comparing cisplatin and flurouracil as single agents and in combination for advanced squamous cell carcinoma of the head and neck, showed that although the response rate to the combination of CP and 5-FU was superior to that achieved with single agents, survival did not improve (Jacoobs et al., 1992).

Noeadjuvant

In two randomised trials on induction chemotherapy in advanced head and neck tumours, patients were randomised to receive local therapy, primary radiotherapy up to a dose of 55 Gy, with or without prior chemotherapy. The results of both trials showed: a significant reduction in the incidence of distant metastasis with chemotherapy: no significant difference in local and regional control between the patients treated with or without chemotherapy: no significant difference in survival of patients treated with or without chemotherapy (Jaulerry et al., 1992).

Adjuvant

A randomised clinical trial was conducted under the auspices of the Head and Neck Intergroup[1] to test the efficacy of sequential chemotherapy as an adjuvant to surgery and postoperative radiotherapy for patients with locally advanced but operable disease (Laramore et al., 1992). Eligible patients had completely resected tumours and were then randomised to receive either three cycles of cisplatin and 5-FU chemotherapy followed by postoperative radiotherapy, or postoperative radiotherapy alone. The results of the trial found no statistically significant differences between the two treatment groups in regards to local/regional control, survival or disease free survival.

In a review by Stupp and Vokes (1995) of the literature on induction and adjuvant chemotherapy they found the following; from four conclusive randomised trials no survival advantage has been shown: organ preservation can be achieved with induction chemotherapy followed by limited surgery and radiation in approximately two thirds of the patients with laryngeal carcinoma: patients achieving a complete response after induction chemotherapy have a better prognosis. The authors concluded that chemotherapy is indicated only in recurrent or metastatic disease and induction chemotherapy is limited to laryngeal carcinoma with organ preservation as intent.

Concomitant Chemo-radiotherapy

Concurrent radiotherapy and chemotherapy form the most promising primary chemotherapy approach to prolong survival of patients with locally advanced resectable

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[1]The Intergroup consisted of several groups in the United States of America: Radiation Therapy Oncology Group, Southwest Oncology Group, Eastern Oncology Group, Cancer and Leukaemia Group B, Northern California Oncology Group and Southeast Group.

and unresectable disease (Inuyama et al., 1995). Randomised studies have suggested that chemo-radiotherapy offers better local control and improved survival, although this is rarely shown (Stupp et al., 1995).

A prospective study carried out at the University of Texas M.D. Anderson Cancer Centre demonstrated that 5-flurouracil plus cisplatin followed by radiotherapy can induce a durable remission in a high proportion of patients with poor prognosis stage 4 nasopharyngeal carcinoma (Dimery et al., 1993). A prospective randomised trial, reported by Banchard et al. (1991), comparing postoperative radiotherapy versus cisplatin and radiotherapy showed statistically improved locoregional control, disease free survival, and overall survival at 2 years. Merlano et al. (1992) reported on the results of a randomised trial comparing standard radiotherapy to alternating chemotherapy of cisplatin and 5-FU with radiotherapy. They showed a statistically significant difference in the complete response rate, median survival, and overall survival at 3 years in favour of the combined approach.

The use of chemo-radiotherapy on regionally advanced disease offers the possibility of organ preservation.

Prognosis

The majority of recurrences in head and neck cancer develop within the first 2-3 years (90%), with the risk period extending to 5 years. As local control improves there is significant risk of increased second primary tumours occurring in the head and neck, lung and oesophagus (25% at 5 years), and more patients die of distant metastases (Tupchong et al., 1994). The approximate cure rates for head and neck cancer are related

to the site and stage of tumour and the presence of lymph node metastases. As an approximation, patients with T1/T2 lesions should achieve a 70% - 95% 5-year survival, and those with T3/T4 lesions, 20% - 40% cure rates. In general, patients with nodal metastases will have half the survival of those without such metastases, and prognosis worsens with increasing extent of nodal metastases (Tupchong et al., 1994)

Role of Predictive Assay

Al-Sarraf et al. (1995) observed that in those patients with a less than complete clinical response to chemotherapy, the previous response (partial response) predicted further response (complete response) to subsequent radiotherapy. Vokes et al. (1993) also noted that patients who have a complete response to chemotherapy have a better prognosis than patients who have no response. This observation, they suggested, does not prove that chemotherapy is responsible for the better outcome in patients who have a complete response: such a response might simply identify patients with more curable disease. These observations suggest that if one could identify, by means of a predictive assay or biomarkers, the subset of patients likely to respond to chemo-radiotherapy they could, in the case of those with advanced disease, be considered for less radical therapy that would lead to organ preservation, e.g. concomitant chemo-radiotherapy. As has been shown by the randomised trials this treatment does not always lead to improved survival, but improved local regional control and a lower incidence of distant metastases can be expected, and in the case of organ preservation a vastly improved quality of life.

1.6 Predictive Assays

The most important goal of a predictive assay is to obtain information that can be used to choose a treatment protocol, so that each individual patient will receive optimal treatment. As already stated, at the present time, the treatment plan is usually based on parameters such as tumour site, histological type, tumour stage and performance status (Begg, 1993). However, it is well known that the radiosensitivity of human cancers varies widely from one patient to another (Fertil et al., 1981), and even within these broad categories some tumours will show a greater response to radiotherapy than others. If those patients unlikely to be cured by radiotherapy could be identified prior to commencement of treatment, alternative or more aggressive therapies might be selected which may give a better chance of cure than the standard therapy.

Predictive assays should be simple, quick and reliable tests for parameters that will predict response to a particular treatment (Begg, 1993).

Assay Types

A wide variety of assay types are currently being investigated as potential predictors of individual tumour response to treatment. Some of these are listed in Table 1.2 . Assays of cytogenetic alterations and oncogene expression identify subgroups of patients who may potentially have a different prognosis and may also be indicative of potential tumour response to treatment. Other assays are purely designed to indicate treatment response; for instance, measurement of the number of DNA drug adducts in tumour cells after chemotherapy can be an indicator of the likelihood of response to treatment (

more adducts = greater response) (Begg, 1993). Some assays are direct measures of cell killing (e.g. colony forming assays) and are applicable to most forms of treatment, while others give an indirect measure of sensitivity (e.g. pO_2 and thiol content for radiation response, DNA adducts and p-glycoprotein for drug response) (Begg, 1993). The assays of particular interest here are those *in vitro* assays that measure intrinsic cell sensitivity.

Table 1.2. Potential predictors of patient response to treatment (Begg, 1993).

Parameter	Predictor of Treatment Response
Proliferation cell kinetics	+
Ploidy/ cytogenetics	?
Surface receptors/ antigens	?
Proto-oncogene expression	?
Intrinsic cell sensitivity	+
pO_2/ pH	+
O_2 and heat regulated proteins	+
p-glycoprotein	+
DNA-drug adducts	+
DNA repair enzymes	+
Tumour regression/ necrosis	+
Host cell infiltrates	+

Assays of Intrinsic Cell Sensitivity

The rationale for measuring the *in vitro* sensitivity of cells to cytotoxic agents is based upon the stem cell model for human tumours (Selby et al., 1983), which hypothesises that tumour stem cells are the target of therapy and that the *in vitro* sensitivity of the stem cells reflects their *in vivo* sensitivity (Brock et al., 1989). This notion is also supported, in the case of radiation biology, by the analyses of Fertil and Malaise (1981), who found that the radiosensitivity of cell lines derived from human tumours was characteristic of tumour type and that, on the average, tumour types that are more difficult to cure by radiotherapy produce cell lines that are more resistant to low doses of radiation. The survival curve parameter that they found best distinguished different tumour histological types was survival at 2.0 Gy of radiation (SF_2) and not parameters that describe the high dose part of the survival curve (Brock et al., 1989). Tucker et al. (1989) in a theoretical evaluation of the accuracy of predictive assays of tumour response to radiotherapy, found that predictive assays based on estimates of intrinsic radiosensitivity (cell survival at 2 Gy) are more likely to be correlated with clinical outcome than are assays based on proliferation kinetics, extent of tumour hypoxia, or tumour clonogen number, at least among tumours of the same size and histology.

Clonogenic Assays

Intrinsic sensitivity of tumour cells to drug or radiation treatment is widely measured using colony formation or clonogenic assays. The two most widely investigated colony assays are the Courtenay-Mills soft agar assay (Courtenay and Mills, 1978) and the cell adhesive matrix (CAM) assay (Baker et al., 1986).

West et al. (1993) has produced the most promising results for SF_2 as a predictor of *in vivo* radiosensitivty, using the Courtenay-Mills soft agar assay to measure the intrinsic radiosensitivity of cervical carcinoma. In this study West obtained SF_2 values for 88 tumour biopsies, taken from patients immediately prior to treatment with radiotherapy. A strong correlation was shown between SF_2 and failure to control local disease; patients alive and well at time of analysis had tumours with a mean SF_2 value of 0.38, which was significantly lower than the value from patients who failed locally (SF_2 = 0.54; $P < 0.01$). Patients with radio-resistant tumours ($SF_2 >$ 0.40, the median) had a significantly lower 3 year survival level than those with sensitive tumours ($SF_2 < 0.40$) ($P = 0.002$).

On the other hand two studies carried out by Grinsky (1994) and Brock (1990), using the CAM plate assay to determine *in vitro* parameters of biopsies from head and neck cancer patients, found that with respect to local control rates and overall survival, SF_2 values were not predictive of treatment outcome. However both authors stressed the need for a multivariate analysis, requiring international multicentric co-operation before any firm conclusions can be drawn.

Technical Difficulties

Many techniques have been described for detecting colony formation by tumour cells, almost all require first the production of single cell suspensions. This is not a simple matter for tumours vary widely in the ease with which they can be disaggregated (Steel, 1993). Often cell clumps left in the cell suspension after inefficient dissagregation may be seeded as single cells and lead to false positives when the final colony formation

count is being made. Poor colony forming efficiency (CFE) of human primary cells is another factor that contributes to the many technical difficulties involved in clonogenic assays. West et al. (1993) reported a mean CFE of 0.13 % for 88 cervical tumours cultured.

Fibroblast Radiosensitivity

The basis of the soft agar clonogenic assays, used for testing radiosensitivity of tumour cells, has been a selective growth of tumour cells. Some recent studies have shown results to the contrary. Stausbøl-Grøn et al. (1995) cultured 11 head and neck tumours using the Courtenay-Mills soft agar assay and using a new colony filter-technique quantified the number of tumour and fibroblast colonies in the primary agar cultures by use of immunohistochemical staining. For the 11 head and neck tumours, the majority of colonies were fibroblast positive, and the minority were epithelial tumour positive. Surviving fraction at 2 Gy based on colonies in agar alone, was found to be significantly correlated to SF_2 of fibroblasts, but not to SF_2 of tumour cells. These results suggest that SF_2 values estimated using the soft agar clonogenic assay may predominately be a measurement of fibroblast sensitivity. Even so, the results of West et al. (1993) on cervical cancer show this parameter to correlate significantly with patient survival rates.

Another role of fibroblast radiosensitivity being investigated is its role as a predictor of normal tissue response to radiotherapy. This would allow the dose to be limited or set according to the individual patient tolerance to radiotherapy and identify those patients with hypersensitivity to ionising radiation.

Studies by Burnet et al. (1992, 1994) have reported a significant correlation between SF_2 and both acute and late reactions of breast cancer patients treated with radiotherapy. Brock et al. (1995) also carried out a study to determine if the radiosensitivity of normal human skin fibroblasts is significantly correlated with the degree of acute or late normal skin damage. SF_2 values were evaluated for 22 patients with breast cancer, who went on to have radiotherapy, and results showed evidence for a correlation between fibroblast SF_2 and late skin reactions, but not for acute response.

The investigation of the potential of these clonogenic assays to measure intrinsic radiosensitivity continues. Meanwhile other assays are been developed as tools to measure likely tumour response to chemo- and radiotherapy. One of these assays is the Mothersill Outgrowth Assay.

1.7 Mothersill Outgrowth Assay

The Mothersill outgrowth assay was first developed as a rapid short term *in vitro* method for determining the differential radiation response of both normal and tumour tissue from the same patient (Mothersill et al., 1988). Radiation dose response curves form this study suggested that cell survival was in the range expected for mammalian cells but that, as is found clinically, tumour cells are far more resistant to radiation than normal cells. These results suggested a possible role for this system as a predictive assay but, further development of the system was needed.

The assay is based on explant culture and thus overcomes some of the technical problems experienced with the clonogenic assays, such as; obtaining a single cell suspension: poor colony forming efficiency: laborious technique requiring a certain amount of skill.

A single cell suspension is not required, the explants are semi-digested and then plated as pieces of tissue in Falcon 25 cm^2 flasks. One explant is plated per flask. Poor colony forming efficiency is avoided with this assay, as it does not concentrate solely on the clonogenic ability of the tumour cells but rather takes into account a heterogeneous tumour cell population, which should be truer to the *in vivo* situation.

The protocol for this assay is quick and easy. From time of receipt of a specimen, it takes approximately one hour to set up 50 cultures. No great technical skill is required. After approximately 72 hours in culture the explants have attached to the flask and cells begun to migrate from the central explant, after which time proliferation and growth rapidly occur. Intense mitotic activity is particularly apparent around the fringe of the explant at all times (Mothersill et al., 1988).

Obviously many factors operate *in vivo* to reduce cellular radiosensitivity, such as hypoxia, cell cycle effects favouring repair of potentially lethal damage, cell to cell contact effects, etc. (Peters & Brock, 1992). However, in this system cell to cell contacts stay partially in effect, as the cells grow out from a central initial population to form a confluent monolayer. They are in constant contact with neighbouring cells. In the clonogenic assay single cells are seeded and thus cell to cell contact is not an influencing factor in response to treatment.

The development of this system progressed such that cultures of tissue and tumours of epithelial origin from several sites could be established using this assay (Mothersill et al., 1988, 1990, 1994) . This system was also applied to the measurement of the effect of cytotoxic agents alone, and in combination with radiation, on cultures of normal and tumour tissue. The effect was measured in terms of growth inhibition for cultures of tumour and, in the case of normal tissue induced or upregulated protein expression was measured.

The results of a study on the effect of radiation and cytotoxic compounds, cis- and carboplatin, on the growth of normal and tumour bladder explant cultures (Mothersill et al., 1990), showed that when either drug or radiation was used singly, the tumour was resistant to treatment while the normal cells were severely effected. However, appropriate combinations of either drug with radiation reversed the unfavourable therapeutic ratio and resulted in higher tumour cell kill. While the assay was still at a very early stage of development, the author felt that with further development this system would merit validation by clinical trial.

This assay has also been applied to the identification of persons at risk for developing cancer due to environment carcinogen exposure, the main parameter examined was the

induction of the mutant form of the p53 oncosuppressor gene, which is associated with many tumours (Mothersill et al., 1994). The results of this study revealed considerable differences in the susceptibility of individual human tissue specimens to induction of mutant p53 following carcinogen exposure.

In the study to be presented in this thesis, the Mothersill outgrowth assay is applied to the establishment of cultures of oesophageal endoscopic biopsies (Sheridan et al., 1995), and head and neck tumours. Using these cultures, the individual response of tumours to treatment was measured *in vitro*, in terms of growth inhibition (Sheridan et al., 1995). Unlike the clonogenic assays, using this outgrowth assay it is possible to obtain information on different parameters from one culture e.g. protein expression measured immunocytochemically, before and after irradiation. This allows a whole panel of potential prognostic parameters to be examined from one simple assay.

1.8 Potential Prognostic Indicators

As stated previously the practice of dividing cancer patients into groups according to so-called stages arose from the fact that survival rates were higher for patients in whom disease was localised than for those in whom the disease had extended beyond the organ of origin. Thus the classification of a tumour gives some indication of prognosis and assists the clinician in the planning of treatment. However even within these groups there is, in reality, a huge variation in the prognosis, response to treatment and survival from patient to patient. Because of this, investigation into more definitive prognostic indicators has been ongoing.

Since one the common characteristics of cancer is uncontrolled cell proliferation, the assessment of the factors that control cell regulation of population are of particular interest. The development of a fully malignant tumour appears to involve the activation or altered expression of proto-oncogenes to oncogenes and the loss or inactivation of tumour suppressor genes the function of which is to control normal cellular activity (Preston-Martin et al., 1990). The cell cycle and cell growth are controlled by many factors, including signal transduction through epidermal growth factor receptors (EGFr) and regulation of cell death by oncogenes and tumour suppressor genes such as p53, c-myc and bcl2.

The expression or over expression of the protein product of these oncogenes has been shown to be of prognostic significance for some types of cancer, including head and neck and oesophageal carcinoma (Field, 1995; Mukaida, 1991; Volant, 1995). Expression of the protein product of these oncogenes can be assessed by immunocytochemical techniques employing antibodies which can identify the proteins, these include; Ki67 (DAKO) identifies proliferating cells: 2E9 (Monosan) recognises

the external binding domain of the EGF receptor: p53-240 (Novacastra) identifies the non-functional confirmation of the protein product of the p53 gene: 9E10 (obtained by kind permission from Dr. G. Evans, St Bartholomew's Hospital, London) binds to c-Myc, the protein product of the c-myc oncogene: bcl2 antibody (DAKO) recognises the protein product of the bcl2 oncogene.

1.8.1 Ki67

Ki67 is a murine monoclonal antibody, first described in 1983 by Gerdes and colleagues, who were attempting to raise monoclonal antibodies to antigens specific for Hodgkin and Reed-Sternberg cells (Gerdes et al., 1983). Ki67 stood out from other antibodies produced because it only reacted with cells which were proliferating. The name is derived from it's place of origin, Kiel in Germany, and the clone producing the antibody, which grew in the 67th well of the tissue culture plate (Gerdes, 1983). The Ki67 clone recognises a labile epitope on a nuclear antigen which was expressed in cycling cells and absent from quiescent cells. A further study by Gerdes et al. (1984) showed a very tight association between Ki67 immunoreactivity and the cell cycle, with expression appearing in mid to late G_1 , rising through S phase to G_2 to reach a maximum in mitosis, Figure 1.3. After mitosis the antigen is rapidly degraded, so that cells in the G_0 phase (non-cycling cells) consistently lack the Ki67 antigen. It is evident that the expression of Ki67 in cells indicates that they are proliferating, but it has also been shown that the quantification of the proportion of Ki67 expressing cells equates with the growth fraction of that population (i.e. number of cells proliferating).

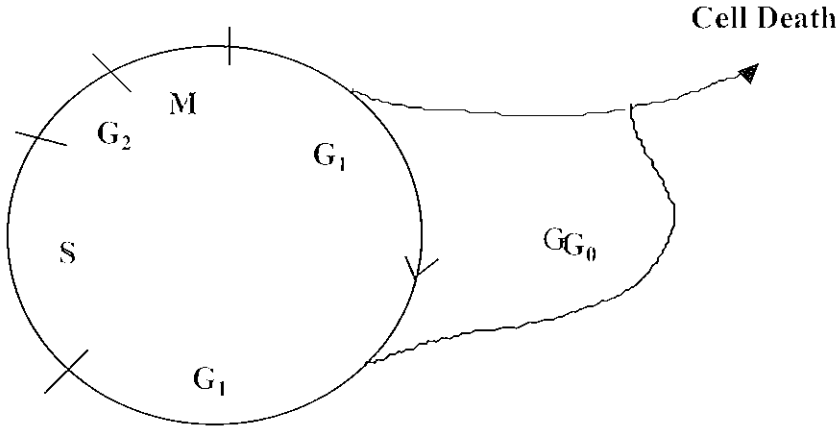


Figure 1.2. Schematic diagram of the stages of the cell cycle. Cells can exit from this, either permanently or temporarily. $G_{0,1,2}$ = $Gap_{0,1,2}$; S = synthesis; M = mitosis. Ki67 is expressed in all phases of the cell cycle except G_0.

Prognostic Value of Ki67

Information on the growth fraction of tumours may be used in the assessment of tumour grade, and in all tumours which have been studied by Ki67 staining, a highly significant correlation between Ki67 staining and the degree of malignancy has been reported (Ross et al., 1995).

Hall et al. (1988) evaluated the prognostic significance of Ki67 immunostaining in non-Hodgkins lymphoma and found a very strong correlation between a low Ki67 index and low grade histology and a high Ki67 index and high grade histology. When survival was analysed it was found that those patients with low grade lymphoma and high Ki67 index had a worse survival than those with a low index. In contrast, there was a trend for those patients with high grade lymphoma and a very high Ki67 index (>80%) to have a better survival than those with a lower index. A correlation between expression of Ki67 and tumour grade was also found by Sullivan et al. (1993) in a study

on cell proliferation in breast tumours, but not by Vittorio et al. (1993) in a study of 111 patients with non small cell lung carcinoma (NSCLC). However in the study on NSCLC a correlation was found between expression of Ki67 and disease free survival, patients with a high Ki67 score (>25%) had a significantly lower disease free survival.

A recent study on squamous cell carcinoma of the head and neck aimed to determine whether immunohistochemical evaluation of the abatement of proliferating cells after a first course of radiotherapy could predict the final response to treatment in oral SCC (Valente et al., 1994). The results of the study showed that the percentage of Ki67 positive cells at diagnosis had no significant correlation with the final therapeutic result of radiotherapy, but the difference in the number of proliferating cells after 10 Gy of radiotherapy significantly differentiated responders from non-responders. Another study, also evaluated the changes in Ki67 expression measured before radiation therapy and at radiation doses of 10 and 20 Gy (Ogawa et al., 1992). The results of this study suggested that high levels of expression (>48%) prior to radiation therapy was indicative of local recurrence and that tumours with a rapidly decreased Ki67 expression (<3%) at 20 Gy was related to poor clinical outcome.

There are several parameters for evaluating the proliferative index of a tumour other than expression of Ki67, these include; the number interphase nucleolar organiser regions (NOR), evaluated using a silver stain method: 5-bromodeoxyuridine (BrdUrd) incorporation: expression of proliferating cell nuclear antigen (PCNA) . Many studies have compared these different parameters to expression of Ki67 in order to determine their relative values as prognostic indicators.

A study of the proliferative activity of urothelial neoplasms compared three methods by which proliferative index can be measured; BrdU incorporation, Ki67 expression, and

nucleolar organiser regions. The results of the study found the proliferative indices evaluated using BrdU and Ki67 staining to be comparable, and that proliferative indices of over 10% were strongly associated with the presence of invasion (Limas et al., 1993). Another study compared the expression of Ki67 and PCNA in breast tumours, the results showed that the expression of PCNA correlated poorly with Ki67 expression and not with mitotic count (Sullivan et al., 1993). The authors concluded that while the Ki67 index appears to be suitable as a means of proliferation measurement in breast tumours, the use of PCNA as a marker of proliferative activity, appeared to be limited.

Evaluation

Expression of Ki67 may be measured immunocytochemically by an indirect immunoperoxidase technique. Dako supply a monoclonal mouse anti-human Ki67 antigen which reacts with a nuclear antigen expressed on all human proliferating cells. In squamous epithelium DAKO-Ki67 preferentially labels nuclei of cells but may also react with the cytoplasm of the epithelial cells (DAKO specification sheet). This antibody is not suitable for use on formalin-fixed, paraffin-embedded sections, but may be used for labelling acetone:methanol-fixed cryostat sections or fixed cell cultures.

In general there is a good correlation between the Ki67 index and other measures of cell proliferation such as [^{3}H]thymidine and BrdUrd uptake, DNA flow cytometric analyses and interphase AgNOR scores (Crocker, 1994). Previous studies have shown a potential for Ki67 as an indicator of prognosis and patient outcome in several types of cancer, due to this it's expression is further investigated in this thesis in relation to head and neck, and oesophageal cancer.

1.8.2 Epidermal Growth Factor Receptor (EGFr)

Epidermal growth factor (EGF) is a polypeptide growth hormone (Cohen, 1962) with mitogenic activity that promotes the growth of a wide range of cell types (Carpenter et al., 1979). To respond to EGF a cell must express a receptor for EGF on its surface. The EGF receptor is a phosphoglycoprotein of 170 000 daltons and is composed of three domains: an internal domain with tyrosine kinase activity, an external ligand-binding domain, and a transmembrane domain (Cohen et al., 1982). When EGF binds to the external domain of the receptor it stimulates the tyrosine specific protein kinase activity associated with the internal domain of the receptor (Cohen, 1983). This results in the autophosphorylation of the EGF receptor and phosphorylation of several target proteins which, in turn stimulates growth and proliferation. Figure 1.4, (Carpenter et al., 1979). This internal domain has been shown to have an amino acid sequence homology with the protein encoded by the v-erb B oncogene (Downward et al., 1984). The expression of EGF and EGFr seems to play an important role in carcinogenesis, progression, and differentiation of tumour cells.

EGFr is widely distributed in human tissues, including endocrine glands, epithelial, liver, brain, placenta, and skin. Many studies have reported overexpression of EGFr in several human malignancies such as soft tissue tumours (Perosio et al., 1989), gastric cancer (Hirono et al., 1995), papillary renal cell carcinomas (Uhlman et al., 1995), human colon carcinoma (Radinsky et al., 1995) and breast cancer (Sainsbury et al., 1995). In many of these cases overexpression of EGFr related to metastatic potential (Iida et al., 1995; Uhlman et al., 1995; Radinsky et al., 1995) and survival (Uhlman et al., 1995; Hirona et al., 1995).

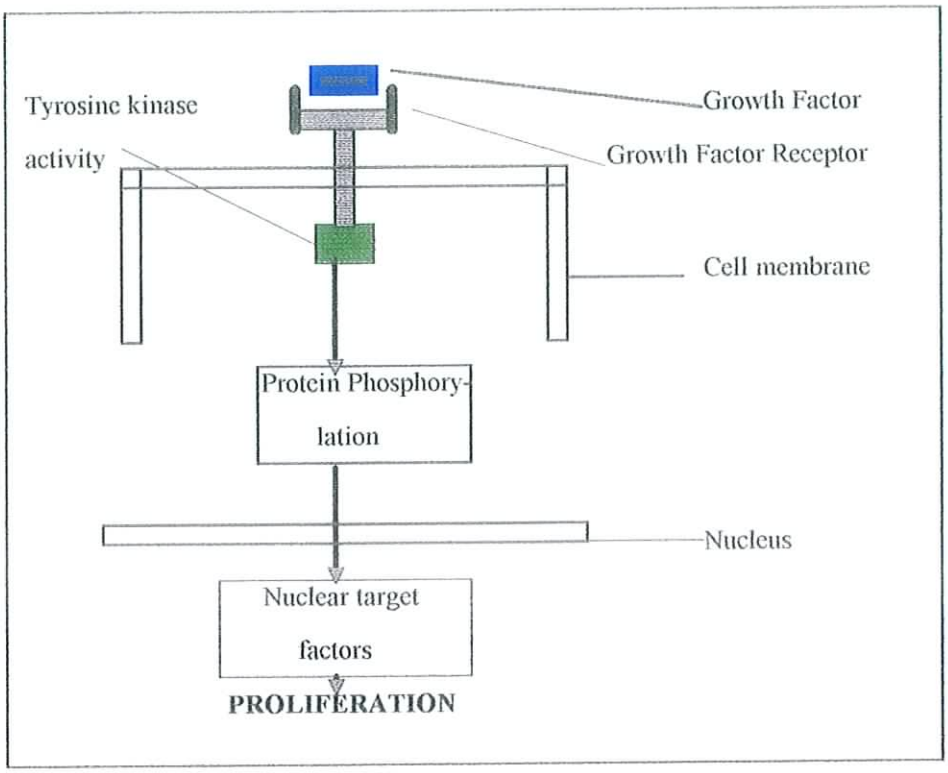


Figure 1.3. EGFr stimulated proliferation. When EGF binds to the external domain of the receptor it stimulates the tyrosine specific protein kinase activity. This results in the autophosphorylation of the EGF receptor and phosphorylation of several target proteins, which in turn stimulates proliferation.

Over-expression of the EGF receptor is a frequent, if not constant, step in the malignant transformation of squamous cells (Ozanne et al., 1986). In several studies reviewed by Ozanne et al. (1986) overexpression of EGFr was found to be a common property of squamous tumours. Studies on squamous cell carcinoma of the head and neck, and oesophagus have supported this finding. Weichselbaum et al. (1989) in a study on 11 early passage human head and neck squamous cell carcinoma cell lines found the EGFr gene to be amplified in 3 of the cell lines and over-expression of EGFr mRNA in 10 of 11 cell lines. Similarly, Ishitoya et al. (1989) observed EGFr gene amplification in 4 (19%) of 21 squamous cell carcinomas of the head and neck, while overexpression of the receptor was found in 8 (53%) of 15 SCC examined. These studies indicate that gene

amplification is not the only mechanism by which the level of EGFr receptor can be increased.

Over-expression of EGFr mRNA was also found by Grandis et al. (1993), who analysed tumours from 24 patients with SCC of the head and neck, and found EGFr mRNA to be elevated in 92% of tumours. Another study examined expression of EGF receptors in fresh tumour biopsies taken from 60 patients with head and neck squamous cell carcinoma and found that EGFr levels were higher in the tumour than in the corresponding nontumoural controls from the same patient (Santini et al., 1991). A large study carried out by Itakura et al. (1994) immunohistochemically analysed 217 cases of human oesophageal squamous cell carcinoma for expression of EGFr. Over-expression of EGFr was detected in 71% of cases.

Prognostic Value of EGFr

The clinical significance of EGFr gene amplification and over-expression in squamous cell carcinomas of the head and neck, and oesophagus is under continual investigation, with varying results from different groups.

In the case of head and neck cancer, Santini et al. (1991) found a significant correlation between EGFr levels measured in adjacent nontumoural tissues, of 70 patients, and tumour size and stage. A simplified competition technique with a radiolabeled ligand allowed evaluation of functional EGFr. Of the patients treated with chemotherapy there was no relationship found between initial response to treatment and EGFr levels, although EGFr positive tumours exhibited a higher proportion of complete responses to chemotherapy than negative tumours. This correlation, of expression with size and

stage, was also observed by Kawamoto et al. (1991) who used a quantitative assay based on enzyme-labelled avidin-biotin interaction with anti-human EGFr to analyse the expression of EGFr in tumour tissue from nude mice implanted with human tumour cell lines. A large study carried out by Dassonville et al. (1993) evaluated head and neck tumours from 109 patients for EGFr levels. using the human recombinant ^{125}I-epidermal growth factor-binding assay. EGFr levels were detectable in all tumours, with the highest levels in stage 3 and 4 tumours. Patients whose tumours expressed the highest levels of EGFr had a significantly shorter relapse-free interval and poorer overall survival rates.

In contrast several studies have failed to show this correlation. In a study by Ishitoya et al. (1989) 21 squamous cell carcinoma tumours were examined for gene amplification and expression of the EGFr by Southern blot and Western blot analysis. The EGFr gene was found to be amplified in 19% of tumours, while 53% of tumours examined overexpressed the receptor. Although a relationship was identified with histological differentiation, no correlation was evident between amplification and/or overexpression and the clinical stage or tumour site. Furuta et al. (1991) retrospectively analysed EGFr gene amplification in 49 cases of SCC arising from the nasal cavities and paranasal sinuses by using slot blot analysis. No significant difference was observed between EGFr gene amplification and the presence of lymph node metastasis, local recurrence, or prognosis.

Ozawa et al. (1989) conducted a study to determine the prognostic value of EGFr in oesophageal SCC. On measurement of EGF receptor levels of 32 tumours by an ^{125}I-EGF binding assay, a significantly lower survival rate was observed for patients with a high EGF binding affinity than those with low EGF binding. Amplification of the EGFr

gene was observed in only 2 patients with the highest EGF receptor levels. In contrast an earlier study by Ozawa et al. (1987) of 31 patients, in which receptor levels were measured by the same binding assay, found no correlation between EGF receptor levels and the pathological characteristics of the tumours. However of this group of patients the two with the highest receptor levels developed secondary tumours 4-7 months after operation, thus, the authors conclude the EGFr levels may possibly be a useful prognostic factor - as they showed in their later study.

Yano et al. (1991) studied EGFr expression immunohistologically in 38 patients with oesophageal squamous cell carcinoma, and found a significant relationship between EGFr expression, lymph node metastases and prognosis. A relationship between the pattern of EGFr expression, mosaic and diffuse patterns, and survival was also found. Patients whose tumours stained a mosaic pattern, had a significantly lower survival rate than that of patients with a diffuse pattern. Results of a study in which EGFr levels were measured by ^{125}I-EGF binding assay, showed that survival rates of patients with high EGFr levels was significantly lower than that of patients with low EGFr levels (Mukaida et al., 1991). In a recent study, immunocytochemical analysis was performed on 217 cases of human oesophageal squamous cell carcinoma (Itakura et al., 1994). Results showed a statistically significant correlation between EGFr overexpression and sex, age, histological type, and the presence of invasion. No significant correlation was found between EGFr expression and tumour size, nodal status, or histological staining. Although patients with overexpression of EGFr had worse prognoses than those without, the difference was not statistically significant. Gene amplification was evaluated in 42 cases, and observed in 9 (21%) in which EGFr expression was also

identified. However EGFr gene amplification was not detected in 26 of 42 (79%) patients in whom EGFr overexpression was observed by immunohistochemistry.

From the studies discussed it is evident that overexpression of EGFr is not always associated with amplification of the EGFr gene. Thus EGFr gene amplification is not considered an important factor in determining the biologic behaviour of human oesophageal squamous cell carcinoma (Itakura et al., 1994). The same can be said for head and neck squamous cell carcinoma, where overexpression of EGFr is not always accompanied by gene amplification (Ozawa et al., 1989), and few studies have shown EGFr gene amplification to be of clinical significance (Ishitoya et al., 1989; Furuta et al., 1992).

Most studies discussed above have indicated that expression of EGFr, measured by means of a binding assay or immunohistochemical analysis, may prove to be a useful prognostic indicator for squamous cell carcinomas of the oesophagus, and head and neck.

Evaluation

To examine the expression of EGF receptors in squamous cell carcinoma tissues, a ^{125}I binding assay method or immunohistologic staining techniques with anti-EFGr monoclonal antibodies can be used. The binding assay involves incubating the tissue or cells with ^{125}I-EGF at 4°C for a desired time, the level of EGF receptors are determined by counting the radioactivity remaining after unbound ^{125}I has been washed off. The

binding assay is useful to measure the quantity and affinity of EGFr in tissues or cells, but it is not adequate to examine the location of EGFr in tissues (Yano et al., 1991).

Immunohistochemical staining with anti-EGFr monoclonal antibodies is used to determine the expression of EGFr and locate the binding site. In some studies EGFr immunoreactivity was observed predominantly on the cell membrane (Itakuru et al., 1994).

In this study immunocytochemical analysis was carried out on cells in a growth area cultured from explants of oesophageal and head and neck tumours. A monoclonal antibody, 2E9 (DAKO) specific to the EGF receptor, was used and the expression measured semiquantitatively. Expression was graded according to three ranges : 2 = 0-25% positivity; 4 = 25-50%; 6 = >50% positivity.

1.8.3 c-Myc Protein

The c-myc gene is the cellular homolog of the viral oncogene v-myc, which is found in a number of avian and feline retroviruses that induce leukaemias and carcinomas (Evan et al. 1992). The c-myc proto-oncogene has long been implicated in the control of normal cell growth and it's deregulation in the development of neoplasia (Spencer et al., 1991).

The c-myc gene encodes a 62 000 dalton nuclear protein, $p62^{c\text{-}myc}$ (Bishop, 1987), with a very short half life (20-30 minutes) and is tightly regulated by mitogens (Cole, 1986). The c-Myc protein is involved in growth regulation (Kelly et al., 1983), cell differentiation (Cole, 1986), DNA synthesis (Studzinski et al., 1986) transcriptional regulation and apoptosis (Evan et al., 1992).

The c-Myc protein can induce both proliferation and apoptosis in cells under differing conditions. A study in primary and immortalised fibroblasts - cells not normally observed to undergo programmed cell death - showed that c-myc expression induces apoptosis in fibroblasts but only if the cells are deprived of serum or blocked in proliferation by drugs or anti-proliferative cytokines (Evans et al., 1992).

Evan and Littlewood (1993) suggested that the c-Myc protein is a transcription factor that modulates two overlapping sets of genes, one involved in cell proliferation, the other in cell death, and that c-Myc regulates the overall decision of a cell as to whether to proliferate or not. Figure 1.4. The growth rate of neoplasms depends on the proliferation and death rates of cancer cells, which in part may represent apoptosis (Sachs and Lotem, 1993). The induction of apoptosis can be enhanced by deregulated expression of the c-myc gene and suppressed by the gene bcl2, thus allowing induction

of cell proliferation and inhibition of differentiation which are other functions of deregulated c-myc (Sachs and Lotem, 1993). Deregulation of the c-myc gene is due to chromosomal translocation and gene amplification (Munzel et al., 1991; Koskinen et al., 1993).

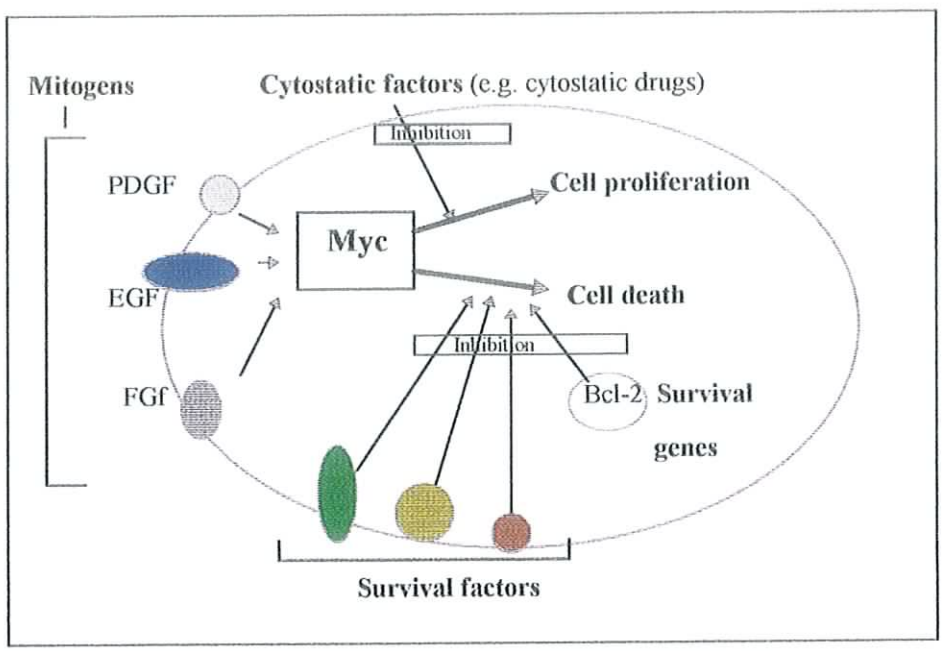


Figure 1.4. The c-Myc protein can induce both proliferation and apoptosis in cells under differing conditions. To accommodate these two apparently contradictory attributes of c-Myc, Evans and Littlewood (1993) suggested that the cell proliferation and cell death pathways are tightly coupled, or overlapping , processes that are both driven by c-Myc. Once established , cell growth and cell death are independently modulated by other genes, cytokines and other external factors.

Prognostic Value of c-Myc

C-myc gene amplification and overexpression of c-Myc protein has been reported in many human tumours, including transitional cell bladder cancer (Lipponen, 1995), renal adenocarcinoma (Lipponen et al., 1995), bladder cancer (Sauter et al., 1995), breast

cancer (Borg et al., 1992), head and neck cancer (Field et al., 1989; Gapany et al., 1994) and oesophageal cancer (He et al., 1995).

The prognostic significance of c-myc is variable. In a study of 311 cases of breast cancer, gene amplification was found in 8% of cases and correlated weakly with the presence of lymph node metastasis, advanced stage and DNA non-diploidy (Borg et al., 1992). However a strong correlation between c-myc amplification and poor clinical outcome in postmenopausal women was identified. Results of a recent study on bladder cancer, showed that c-Myc overexpression was associated with low grade and early stage tumours, while a strong association was found between c-myc copy number increase and tumour stage and grade (Sauter et al., 1995). Another study on bladder cancer in which c-Myc overexpression was evaluated immunocytochemically found a relationship between overexpression, histological grade, mitotic index and overexpression of epidermal growth factor receptor (Lipponen, 1995). But no relation was found to progression of tumours and overexpression had no prognostic value in survival analysis. Lipponen et al. (1995) also did a study on renal adenocarcinoma and found cytoplasmic expression of c-Myc, measured immunocytochemically, was related to favourable outcome, but in multivariate analysis expression of c-Myc had no independent prognostic value.

In head and neck cancer several studies have been carried out to determine the clinical significance of c-myc amplification and c-Myc overexpression. Amplification of the c-myc gene has not been shown to be an important feature of head and neck cancer in the western World (Leonard et al., 1991). On the other hand, overexpression of the c-Myc protein has been demonstrated in a number of tumours without amplification of the gene

(Sauter et al., 1995) and this also appears to be the case in head and neck cancer (Field, 1992).

In a study carried out on 44 SCC of the head and neck, c-Myc oncoprotein expression was evaluated using an enzyme-linked immunosorbance assay (ELISA) (Field et al., 1989). Although no statistical correlation was found between different clinicopathological parameters (patient age , sex, TNM staging, number of lymph nodes invaded, extracapsular rupture of the tumour, it's histopathological differentiation, or it's site), the survival rate of patients with tumours possessing elevated levels of c-Myc protein were found to be statistically shorter than those with lower levels of c-myc expression.

Results of a study by Gapany et al. (1994), in which 29 patients with SCC of the head and neck were immunocytochemically evaluated for c-Myc expression, showed a significant correlation between low levels of c-Myc protein in the tumour cells and metastatic lymph node involvement and advanced stage. These results are contrary to those obtained by Field et al. (1986) who showed a significant correlation between increasing levels of c-Myc expression and advancing stage measured in 14 patients with SCC of the head and neck.

The incidence of oesophageal carcinoma in China is more than twice that seen in the United States, and in some regions the incidence exceeds 100 per 100,000 (Coia and Sauter, 1994). Although there are many studies investigating the cause, treatment and pathogenesis of oesophageal carcinoma, from the literature there does not appear to be many studies investigating the role of the c-myc gene and it's prognostic significance in

this disease. Of two studies investigating the significance of c-myc amplification in oesophageal cancer, both were conducted by Chinese groups.

In a study by Lu et al. (1988), amplification of c-myc gene was found in 14% of oesophageal tumours analysed. These findings were supported by the results of a recent study, in which 41 samples of oesophageal SCC were analysed, and c-myc gene amplification was found in 39% (He et al., 1995). Amplification of the c-myc gene was found to relate to lymph node metastasis, TNM staging and also prognosis.

As for EGFr, amplification of a gene does not always result in overexpression of the gene product and therefore it's role in such neoplastic cells is unclear (Field, 1995). From the studies discussed above evaluation of c-Myc protein appears to have some clinical significance for head and neck cancer.

Evaluation of c-Myc

An enzyme-linked immunosorbence assay (ELISA) was developed by Moore et al. (1987) to measure the levels of c-Myc protein in normal and transformed cell lines. Field et al. (1989) used this assay to determine differential amounts of c-Myc in tissue lysates of head and neck tumours. However this assay does not allow the specific site of the protein to be identified within the cell and some studies have shown the location of the protein, whether in the cytoplasm or the nucleus, to be significant. Lipponen et al. (1995), in a study of 104 cases of renal adenocarcinoma, found cytoplasmic expression

of c-Myc to be related to high tumour grade, while nuclear expression was related to small tumour diameter.

Immunocytochemical analysis is the more common technique of evaluating c-Myc expression in human tumours, as it allows the binding site to be located. A standard indirect immunoperoxidase technique using monoclonal antibodies (see section 3.5) has been employed by many groups (Gapany et al., 1994; Lipponen, 1995; Sauter et al., 1995) and has also been used in this study.

1.8.4 The Bcl-2 Protein

Whereas c-Myc can promote apoptosis, the bcl-2 proto-oncogene encodes a protein that is a potent repressor of the process (Korsymeyer, 1992). The bcl-2 proto-ocogene is involved in the 14;18 translocation, a chromosomal abnormality present in follicular lymphomas (Tsujimoto et al., 1986). In this translocation the bcl-2 gene is juxtaposed with the immunoglobulin heavy-chain gene on chromosome 14 and is brought under the control of the promoter of this gene. In consequence, abnormally high levels of Bcl-2 protein are produced (Cleary et al., 1986). The Bcl-2 protein is a novel 239-amino acid, 25-kDa integral membrane protein that localises primarily to the mitochondrial membrane and nuclear membrane as well as other cellular membranes (Hockenbery et al., 1990). The Bcl-2 protein has a relatively long life of approximately 10 hours (Merino et al., 1994), and appears to constitute a member of a new category of oncogenes: regulators of programmed cell death (Korsmeyer, 1992).

The role of Bcl-2 in apoptosis has been studied by many groups. Hockenbery et al. (1990) noted that deregulated Bcl-2 expression prolonged the survival of the interleukin-3 (IL-3)-dependent pro-B lymphocyte cell line, FI5.12, when deprived of growth factor. On examination of the mechanism of cell death, it was revealed that death was by apoptosis, in which plasma membrane blebbing and volume loss was followed by nuclear condensation and an endonucleolytic cleavage of DNA into fragments of oligo-nucleosomal length. Evidence to support a normal physiologic role for Bcl-2 during B cell development, was provided by Merino et al. (1994), who found that susceptibility to apoptosis mediated by the glucocorticoid hormone dexamethasone is stage dependent in developing B cells and correlates with the levels of Bcl-2 protein.

One model for bcl-2 action in oncogenesis relies on the dual effects of proliferation and cell death in homeostatic regulation of cell populations, suggesting that abnormalities in both pathways may lead to clonal selection and neoplasia (Korsmeyer, 1992). The generation of bcl-2-transgenic mice with the translocation t(14;18), demonstrated the oncogenic consequences of deregulated Bcl-2 expression (McDonnell and Korsmeyer, 1991). An indolent follicular hyperplasia in these transgenic mice progressed to a malignant diffuse large cell lymphoma and showed that prolonged B-cell life increased tumour incidence. A high proportion of these tumours had rearrangements of c-myc as a second genetic alteration.

Another model would propose a more targeted role for bcl-2 in neoplasia in which bcl-2 is highly synergistic with specific cellular oncogenes. This concept follows the recognition that several oncogenes that function in cell activation and proliferation pathways have a paradoxical effect of promoting cell death by apoptosis (Hockenberry, 1994). One of these oncogenes, c-myc, induces apoptosis in adverse growth conditions

or as a result of a blockade of cell proliferation. This induction of apoptosis has been shown to be bypassed by the overexpression of Bcl-2 (Bissonnette et al., 1992). Bcl-2 mitigates the apoptotic affects of deregulated c-Myc expression without affecting it's ability to promote continuous cell growth (Fanidi et al., 1992).

Prognostic Value of Bcl-2

Deregulated expression of Bcl-2 has been found in non-small cell lung carcinoma (Pezzella et al., 1993), breast cancer (Silvestrini, et al., 1994) and renal adenocarcinoma (Lipponen et al., 1995). Pezzella et al. (1993) immunocytochemically investigated the prognostic importance of Bcl-2 in non-small cell lung carcinoma and found no association between expression and stage, but patients with Bcl-2 positive tumours had a higher rate of survival at 5 years; this rate was not significantly different from those patients with Bcl-2 negative tumours. Bcl-2 expression appeared to be associated with less aggressive tumour behaviour (Pezzella et al., 1993). Lipponen et al. (1995) immunocytochemically examined the expression of Bcl-2 in 104 cases of renal adenocarcinoma. Bcl-2 expression was significantly related to small tumour size, low T category and absence of metastasis. In a study on Bcl-2 expression in node-negative breast cancer patients, immunocytochemical analysis was carried out on 283 paraffin embedded sections (Silvestrini et al., 1994). Results showed that expression of Bcl-2 was related to small, slowly proliferating tumours, and that patients whose tumours expressed more than 30% positive cells showed a better prognosis. The results of these studies indicate that Bcl-2 expression is related to less aggressive tumours with a better prognosis.

Although, from the literature there appears to be no studies on the prognostic significance of Bcl-2 in head and neck cancer, from the studies discussed above, there appears to be a potential role for this protein in identifying patients with a better prognosis. Evaluation of the expression of this protein prior to treatment, could contribute to more accurate staging and assist in treatment planning.

Evaluation of Bcl-2 Protein

Evaluation of the expression of Bcl-2 protein can be carried out by immunocytochemical analysis using an antibody specific for the protein and an indirect immuno-peroxidase technique. Immunostaining with Bcl-2 has been shown to be localised in the cytoplasm alone of tumour cells (Silvestrini et al., 1994), and in a combination of the nucleus and cytoplasm (Lipponen et al., 1995).

1.8.5 The p53 Protein

The p53 tumour supressor gene is located on chromosome 17 and behaves as a negative regulator of cellular proliferation. The p53 gene encodes a nuclear phosphoprotein that appears to play an important role in control of the cell cycle, DNA repair and synthesis, cell differentiation and apoptosis.

p53 is thought to be involved in cell cycle checkpoints following damage of DNA. These cell cycle checkpoints presumably exist to prevent both replication of a damaged DNA template (the G_1 arrest) and segregation of damaged chromosomes (the G_2 arrest) (Kastan et al., 1992). Kastan et al. (1991) provided evidence for the participation of p53 protein in the cellular response to DNA damage. ML-1 myeloblastic cells were exposed to non-lethal doses of the DNA damaging agents, γ-irradiation or antinomycin D, which caused a transient inhibition of replicative DNA synthesis via both G_1 and G_2 arrest. The level of p53 protein in the ML-1 cells was observed to increase and decrease in temporal association with the G_1 arrest. Kastan et al. also showed in this study that cells that either lacked p53 gene expression or overexpressed a mutant form of the p53 gene did not exhibit G_1 arrest after γ-irradiation; however G_2 arrest was unaffected by the status of p53. These results suggested a mechanism for how loss of wild-type p53 might contribute to tumourigenesis by failure to induce G_1 arrest the cells continue to go through S-phase after DNA damage. Thus use of a damaged template for replicative synthesis might lead to significant mutation and possibly genomic instability, which in turn might lead to cancer. This hypothesis was supported by work of Kastan et al. (1992) who observed that patients with the radiosensitive, cancer-prone disease ataxia-telangiectasia (AT) lacked the ionising radiation induced increase in p53 protein levels seen in normal cells, and the pathway to G_1 arrest was defective.

p53 is not only an inhibitor of cell division, it can also cause apoptosis. Yonish-Rouach et al. (1991) originally observed that ectopic expression of p53 in the myeloid leukaemic cell line M1, which is devoid of endogenous p53, induced apoptosis. Lowe et al. (1993) illustrated that p53 is required for radiation induced apoptosis in mouse thymocytes. In the study thymocytes were isolated from p53 homozygous mutant, heterozygous and wild-type animals and subjected *in vitro* to treatments that induce apoptosis. Most of the apoptosis inducing treatments induced cell death with similar kinetics in all three genotypes, however p53 deficient cells displayed a dramatic resistance to the effects of ionising radiation. These findings on the requirement of p53 for radiation induced apoptosis, were further supported by Clarke et al. (1993), who observed that thymocytes with wild-type p53 readily undergo apoptosis after treatment with ionising radiation, while thymocytes lacking wild-type p53 were resistant to induction of apoptosis by radiation.

The protein product of the p53 tumour supressor gene appears to have two mechanism by which it suppresses tumour growth: by inducing a G_1 arrest after DNA damage, it allows for repair of damage and thus limits the propagation of heritable genetic errors; if the damage cannot be repaired then death by apoptosis is induced by p53.

Prognostic Value of p53

Inactivation of p53 by somatic allelic deletions and point mutations is the most frequent genetic alteration in human neoplasms (Hollstein et al., 1991). These aberrations, together with alterations of oncogenes and other tumour supressor genes , make up the

mutational network leading to malignancy (Hollstein et al., 1991). Aberrant expression of the p53 oncoprotein, measured immunocytochemically, has been found to be a common feature of a wide spectrum of human malignancies, including breast, colon, stomach, bladder, and testis carcinomas (Bártek et al., 1991).

In a study on bladder cancer, the expression of p53 immunocytochemically evaluated in 54 patients who had undergone surgery, was significantly associated with histological tumour grade and stage (Esrig et al., 1993). Patients with tumours positive for p53 mutation had a higher 2 year recurrence rate, but this was not significantly different from patients with p53 negative tumours. The results of a study on adenocarcinoma of the oesophagus, in which p53 was evaluated immunocytochemically in 42 patients who received neoadjuvant chemotherapy and radiation followed by resection, showed that p53 positivity correlated with residual disease in the resected specimen but not with disease free survival (Duhaylongsod et al., 1995). p53 overexpression did not correlate with stage, histological grade or survival rates in 107 epithelial ovarian cancers (Marks et al., 1991).

Mutations of p53 have also been frequently found in head and neck cancer, and have been shown to correlate with p53 expression measured immunocytochemically (Somers et al., 1992). In none of the studies into p53 expression in head and neck cancers has a correlation been found with any of the clinico-pathological data (Field, 1995). This includes a recent study of 103 patients, in which no correlation was found between p53 expression and survival, and a study by Nadal et al. (1995), who immunocytochemically examined 89 SCC of the larynx.

Radiotherapy is an important component of head and neck cancer treatment and some studies have suggested that cell lines expressing wild-type p53 are sensitive to ionising radiation while those with deregulated expression of the protein are resistant to radiation (Xia et al., 1995; McIlwrath et al., 1994; Lee et al., 1993).

McIlwrath et al. (1994) observed a significant correlation between the level of ionising radiation induced G_1 arrest, mediated by wild-type p53 and radiosensitivity. Cell lines having G_1 arrest were more radiosensitive. Xia et al. (1995) reported that a lymphoblast cell line resistant to the toxic effects of x-rays had p53 protein levels 4 times higher than the protein levels in a sensitive cell line. The resistant cell line was found to have a homozygous mutation, while the sensitive cell line contained a wild-type p53 sequence. Brachman et al. (1993) in a study on the radiosensitivity of 24 head and neck cancer cell lines, measured by surviving fraction at 2 Gy, found no correlation between radiosensitivity and p53 mutations. However the authors concluded that p53 alterations may predispose to increased radio-resistance.

Evaluation of p53 Protein

Since mutant p53 proteins typically have a much longer half-life than wild-type protein, evaluation of the expression of the mutant form is feasible by immunohistochemical accumulation in the cell. The relatively low levels of p53 protein in normal cells are generally undetectable when examined by immunohistochemical techniques, whereas in neoplastic cells carrying a missense mutation, the presence of p53 protein is easily demonstrated because of its prolonged half-life (Harris et al., 1993).

Some studies have shown that immunohistochemical detection of p53 nuclear accumulation is highly associated with mutations in the p53 gene (Esrig et al., 1993; Somers et al., 1992).

Although the concordance between a p53 gene mutation and the accumulation of p53 protein cannot be perfect, immunoreactivity is an approximate indicator of the tumours with altered p53 function (Harris et al., 1993).

1.9 Aim and Objectives

Aim

The aim of this thesis was to investigate the applicability of the Mothersill outgrowth assay in a) predicting the individual response of patients to treatment and b) in measuring parameters of potential prognostic use in oesophageal cancer and head and neck squamous cell carcinoma.

Objectives

- Optimisation of the Mothersill outgrowth assay in terms of the explant size and the culture vessel used. By standardising the area of tissue cut from a specimen it was hoped that a more standard outgrowth area and cell number cultured from the explant, would be achieved. A predictive assay that could be used routinely in a laboratory, would have to be compact, cost effective and easy to use. The culture vessels originally used were the Nunclon 25 cm^2 flasks, which are bulky and not that easy to work with. Investigation of other culture vessels as a suitable alternative to the present culture vessel was one of objectives of this thesis.
- Application of the outgrowth assay to the culture of biopsy material, which would make this system more applicable as a predictive assay, in that response to treatment could be determined *in vitro* for each patient, prior to commencement of treatment *in vivo*.
- Application of the outgrowth assay to the investigation of potential indicators of response to treatment in oesophageal carcinoma. Parameters examined were %

Growth Inhibition. % proliferating cells measured by Ki67, and level of expression of epidermal growth factor receptors (EGFr).

- Prediction of the individual response of patients with head and neck squamous cell carcinoma (SCC) to treatment with radiation and chemotherapy, which included cisplatin and 5-Flurouracil.
- Application of the outgrowth assay to the investigation of potential indicators of radiosensitivity in head and neck squamous cell carcinoma. Parameters to be examined *in vitro* included: population of proliferating cells measured by Ki67 and the level of expression of EGFr (these were to be measured in cultures receiving no treatment and in cultures treated with a single dose of 2 Gy); expression of c-Myc; expression of Bcl-2; co-expression of c-Myc and Bcl-2.
- Investigation of the potential of p53 protein as an indicator of radiosensitivity in head and neck SCC tumours and in 3 cell lines with varying radiosensitivities, originating from 2 colorectal tumours and 1 bladder tumour.

CHAPTER 2

MATERIALS

2.1 GENERAL APPARATUS

The following apparatus was used;

Nuaire Laminar flow cabinet.

Forma Scientific Incubator CO_2 - 5%

Temp - 37^0C

Humidity - 99%

Coulter Counter, Model D_N.

Standard laboratory equipment and glassware was used.

2.2 CULTURE FLASKS

Tissue was cultured in the following culture vessels:

a) Nunclon $25cm^2$ / 40 ml flasks

b) Nunclon 4 well tissue culture plate

c) Falcon 6 well tissue culture plate

d) Falcon 24 well tissue culture plate

2.3 CUTTING TEMPLATE

A cutting template was designed that cut tissue into standard size explants of 2 mm^2. The design and use of this instrument is described fully in section 3.

2.4 BUFFER

Phosphate buffered saline

Sodium Chloride (BDH)	8g
Potassium Chloride (BDH)	0.2g
Disodium hydrogen phosphate (BDH)	1.44g
Potassium dihydrogen phosphate (BDH)	0.24g

Adjust volume to one litre with distilled water.

Adjust the pH to 7.2.

2.5 FIXATIVES

10% Neutral Buffered Formalin

40% Formaldehyde Solution (BDH)	100ml
Distilled Water	900 ml
Sodium Dihydrogen Phosphate Monohydrate(BDH)	4g
Disodium Hydrogen Phosphate Anhydrous (BDH)	6.5g

Methanol:Acetic Acid (3:1)

Methanol (BDH)	90 ml
Acetic acid (BDH)	30 ml

2.6 DISAGGREGATION AGENTS

ENZYMATIC AGENTS

Weak Trypsin Solution

Earle's balanced salt solution (without calcium and magnesium) (Gibco)	500 ml
2.5% stock Trypsin (Gibco)	50 ml

Collagenase Type IV (Sigma)

Enzymatic digestive used in solution with weak trypsin to digest explants prior to plating in culture flasks.

CHELATING AGENT

Versene (Gibco); **Ethylenediaminetetraacetic Acid** (EDTA)

This agent acts as a calcium and magnesium chelator and is used in combination with weak trypsin (section 2.6) solution to remove cells from the substratum in preparation for subculture.

2.7 SURGICAL SPECIMENS

The surgical specimens used and their sources are shown in Table 2.1. Ethical approval was obtained were required.

Table 2.1. Specimens used for experimentation and their source.

Specimen	Surgical Procedure	Source
Ureter	Kidney transplant	Beaumont Hospital
Oesophagus	Oesophagectomy	St. James's Hospital
Oesophageal Biopsies	Endoscopy	St. James's Hospital
Head & Neck Tumour	Neck Dissection	Mater Misericordiae Hospital

2.8 CELL LINES

HT-29 Radio-resistant human tumour cell line, derived from a human colorectal tumour, with an SF2 value of 0.74.

RT-112 Human tumour cell line derived from a bladder tumour, with an SF2 value of 0.59.

SW-48 Radiosensitive human tumour cell line, derived from a colorectal tumour with an SF2 value of 0.16.

2.9 CULTURE MEDIA

Roswell Park Memorial Institute (RPMI) 1640 (GIBCO)

Supplemented to give final concentration of:

Foetal Calf Serum (Gibco)	10 %
Horse Serum (Gibco)	6%
Penicillin - Streptomycin (Gibco) [5000 IU/ml Penicillin,	50IU/ml
5000 µg/ml Streptomycin]	50µg/ml
L - Glutamine 200 mM (Gibco)	1 mM
Hydrocortisone (Sigma)	1 µg / ml
Insulin (Sigma)	10 IU/ ml
Fungizone Amphotericin B (Gibco)	1 µg / ml
Hepes Buffer Solution 1 M (Gibco)	12 ml

Dulbecco's Modified Eagle Medium (GIBCO)

Supplemented by the following to give final concentration of ;

Foetal Calf Serum (Gibco)	7.5%
Penicillin - Streptomycin (Gibco) [5000 IU/ml Penicillin,	50 IU/ml

5000 µg/ml Streptomycin]	50 µg /ml
L - Glutamine 200 mM (Gibco)	1 mM
Hydrocortisone (Sigma)	1 µg / ml
Hepes Buffer Solution 1M (GIBCO)	12 ml

Nut Mix F12 (GIBCO)

Supplemented as for Dulbecco's Modified Eagle Medium.

2.10 IMMUNOCYTOCHEMICAL REAGENTS

Monoclonal Antibodies

Cytokeratin	M821	(Dako)
Epidermal Growth Factor Receptor (EGFr)	2E9	(Monosan)
Nuclear Membrane Antigen	Ki-67	(Dako)
Mutant confirmation of the p53 protein	p53-240	(Novacastra)
c-myc proto-oncogene product	9E10	(Dr. G. Evans. St. Bartholomew's Hospital. London)
Bcl_2 oncoprotein	124	(Dako)

Labelling by all antibodies was developed using peroxidase-conjugated anti-mouse IgG (Vectastain, ABC Kit).

Chromogen for Peroxidase Enzyme

Diaminobenzaldehyde (DAB) Isopac 100 mg in sealed bottle, add 16 ml phosphate buffered saline (PBS) to bottle using needle and syringe. Mix well and aliquot into 500 ml or 1 ml amounts. Store in glass tubes at -20^{0}C.

3% Hydrogen Peroxide Solution (BDH)

This solution is used to block endogenous peroxidase activity.

One in ten dilution of a 30% solution of hydrogen peroxide (stored at 4^0C) in distilled water.

Glycergel Mounting Medium (DAKO C563).

An aqueous histological mounting medium for use when a permanent water-soluble mounting medium is required. In plastic culture flasks an organic mounting medium cannot be used due to corrosion of the plastic.

2.11 CHEMOTHERAPEUTIC AGENTS

Cisplatin Injection (1.0 mg/ml) Solution (David Bull Laboratories)

Flurouracil Injection B.P. 250 mg/ml (David Bull Laboratories)

2.12 OTHER REAGENTS

Harris's Haematoxylin (BDH)Used as nuclear counterstain.

5% Carbol Fuchsin (Clin-Tech) Used as an overall stain.

CHAPTER 3

METHODS

3.1 PRIMARY EXPLANT CULTURE (Mothersill et al. 1988)

A sample for culture e.g. endoscopic biopsy or tumour, on arrival in the laboratory was cleaned of any connective tissue etc., using a sterile scissors. The tissue was rapidly chopped using scissors and forceps to give pieces ~ 2-3 mm^3 . The pieces of tissue were placed in a solution of collagenase Type IV (Sigma) and weak trypsin (10 mg collagenase / 1 ml weak trypsin) and digested for 30 minutes at 37°C. The digested explants were placed in a 90 mm sterile petri dish and the trypsin neutralised using serum-containing media (RPMI 1640 containing Foetal Calf serum, hydrocortisone, insulin and antibiotics, see Section 2.9). Suitable explants, that were estimated 'by the eye' to be approximately 2-3 mm^3 , were plated singly in tissue culture vessels, containing an appropriate volume of growth medium (Section 4.1.3). Explants were manoeuvred to the middle of the flask and were carried gently to the incubator to prevent dislodgement. They were cultured at 37°C in a humidified atmosphere containing 5% CO_2 in air and left undisturbed for at least 72 hours, to allow for attachment of the tissue to the base of the flask. Cultures were incubated for a total of 14 days , then fixed and analysed.

3.2 CUTTING TEMPLATE

In experiments that involved the culture of standard size explants, as opposed to explants cut from a specimen as described above, a cutting template was used. The design and use of this template are described in Section 4.1.1.

3.3 IN VITRO TREATMENT OF TUMOURS

After approximately 72 hours in culture, or when the explants had attached to the base of the flask, the cultures were grouped according to the treatment to be administered as shown in Table 3.1.

Table 3.1. Treatment groups.

Group	Treatment
Control	none
Radiation	single dose of 2 Gy
Chemotherapy	Cisplatin + 5-Flurouracil
Combination	2 Gy + Cisplatin + 5-Flurouracil

3.4 Exposure of Tumour to Agent

The chemotherapeutic agents were diluted from available commercial preparations, in growth media, to give clinical equivalent doses when the drug solution was added to 2 ml of growth media. The chemotherapeutic solution was added directly to the media in the culture flask where it remained for the duration of the culture period. The concentration of the agents used are as shown.

Table 3.2. Dose of chemotherapeutic agents added to culture media. The clinical doses given are those used by St. James's Hospital, Dublin (Hickey et al., 1994).

Agent	Clinical Dose	Equivalent Dose (µg/2 ml)
Cisplatin	75 mg / m^2	0.1 - 1.0 µg / 2ml
5-Flurouracil	15 mg / kg	2-30 µg / 2 ml

3.5 Safe Handling of Agents

The transfer of the agents to syringes was carried out in a designated area only. The personnel carrying out these procedures were adequately protected with clothing, gloves and eye shield. In the event of contact with the skin or eyes, the affected area was washed with copious amounts of water or normal saline. Medical advice was sought if the eyes were affected. After use syringes, containers, absorbent materials, solutions and any other contaminated material was placed in a thick plastic bag or impervious container and incinerated.

3.6 Irradiation of Cultures

Using a Cobalt-60 teletherapy unit that delivered 0.8 Gy / minute at a Subject Source Distance (SSD) of 80, a single dose of 2 Gy was administered to the cultures. The cultures were irradiated within an hour of the chemotherapeutic agents being added to the culture media.

3.7 FIXATION

Media was aspirated from culture vessels and cultures washed with PBS.

Approximately 2 ml of fixative (or enough to cover culture) was pippetted into culture vessels and left at 4°C for 5 minutes. Fixative was removed and culture washed with PBS. Cultures were left in PBS until ready for examination.

3.8 IMMUNOCYTOCHEMICAL METHOD

Indirect Immuno-Peroxidase

The immunostaining was carried out directly on the explant tissue culture on the base of the flask. as for specimens mounted on glass slides. This method meant that the spatial organisation and distribution of the staining in the cultures were preserved. Immunostaining was performed using the Vectastain ABC kit, which employs a standard indirect immunoperoxidase method. The protocol for the indirect immunoperoxidase method was as follows:

1. Wash cultures with phosphate buffered saline.

2. Fix in a) 10% buffered formalin or b) methanol:acetic acid (3:1). at $4^{o}C$ for 4 min

3. Rehydrate in phosphate buffered saline (PBS).

4. Block endogenous peroxidase activity with fresh 3% H_20_2 in distilled water, incubate cultures for 30 minutes.

5. Wash in PBS for 20 minutes.

6. Incubate cultures for 20 minutes with diluted normal serum (large yellow labelled bottle)

7. Blot excess serum from cultures. (Do not rinse).

8. Incubate cultures for 30 minutes with primary antiserum diluted in buffer.

9. Wash cultures for 10 minutes in buffer.

10. Incubate cultures for 30 minutes with diluted biotinylated antibody solution (large blue labelled bottle).

11. Wash cultures for 10 minutes in buffer.

12. Incubate cultures for 30-60 minutes with Vectastain ABC Reagent.

13. Wash cultures for 10 minutes in buffer.

14. Incubate cultures for 2-7 minutes in peroxidase substrate solution.

15. Wash cultures for 5 minutes in tap water.

16. Counterstain nuclei with Haematoxylin for 1 minute, blue in warm water.

17. Mount in Glycergel.

Results: Positive - Brown reaction product.

Negative - Clear cytoplasm and blue nuclei.

3.9 TRANSMISSION ELECTRON MICROSCOPY

Cultures were fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer for 1 hour at room temperature. The culture was then washed in phosphate buffer, postfixed in 1% Osmium tetroxide for an additional hour and dehydrated through ascending grades of ethanol (30%, 50%, 70%). They were then embedded in epoxy resin. Thin sections (40 nm) were cut *en face* with a diamond knife, stained with uranyl acetate and lead citrate, and viewed in a Jeol 2000 electron microscope.

3.10 QUANTIFICATION OF RESULTS

Quantification of Attachment and Growth of Explant

During the growth period of 14 days the attachment of, and outgrowth from the explant was monitored at intervals. Initially the cultures were examined regularly (up to every day) and the extent of attachment and outgrowth estimated by the naked eye. However this daily examination proved to be unsatisfactory, since the explants were not allowed a

sufficient undisturbed period for attachment to occur. In later experiments the explants remained undisturbed in the incubator for at least 72 hours and as a result improved attachment was noted.

The extent of attachment of the explant to the base of the culture vessel was ranked according to three levels of attachment: $A_0 : A_1 : A_2$ as shown in Table 3.3.

Table 3.3. Ranking of attachment of the explant to the culture vessel.

A_0 - no attachment has occurred, explant moves freely through medium.
A_1 - some attachment is detectable, however other areas of explant are free to move and thus there is a large risk of detachment with disturbance due to examination.
A_2 - complete attachment has occurred; explant is held firmly in place, thus risk of detachment due to disturbance is decreased greatly.

The extent of the outgrowth (OG) is described according to six stages, from OG_0 to OG_5. Since the approximate area of the outgrowth is estimated by the eye, each area is described within a large area range, as shown in Table 3.4.

Table 3.4. Quantification of outgrowth area

Stage	Description	Range(mm^2)
OG_0	No Growth	Area = 0
OG_1	Minimal	0<Area<25
OG_2	Fair	25<Area<150
OG_3	Substantial	150<Area<400
OG_4	Very Good	400<Area<600
OG_5	Excellent	600<Area

Quantification of Growth Area and Cell Number

The outgrowth area of each explant was measured against a grid of $1mm^2$ areas. This was performed after immunocytochemical staining had been carried out on the outgrowth, so that it was macroscopically visible. The number of $1mm^2$ areas on the grid covered by the explant outgrowth gave the growth area for that explant.

The total cell number in each outgrowth area was estimated by counting the number of cells present in one millimetre square sections of the growth in three random areas and then multiplying the mean of these by the growth area determined above. This was done under x10 magnification, with a $1mm^2$ graticule in one eyepiece.

Quantification of Response to Treatment

The response of a culture to treatment, i.e. radiation or chemotherapy, was measured as the reduction of the cell number in the treated culture relative to the untreated control, and was expressed as % growth inhibition.

A patient from a group was said to be sensitive to a treatment, if the % growth inhibition produced by that treatment for that particular patient, was greater than the mean % growth inhibition of the group, for that treatment.

Immunocytochemical Assessment

The number of immunocytochemically positive cells in a growth area was estimated along a cross sectional area, which was drawn on the underside of the culture vessel (Figure 3.1). The total number of cells and the number of positive cells were counted along the lines of the cross section and the percentage positive cells calculated.

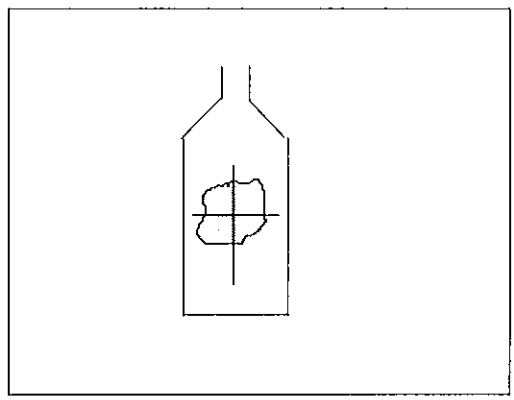


Figure 3.1. Determination of % positive cells: a cross section is drawn on the underside of the flask through the outgrowth area and the number of positive cells along that cross section counted.

CHAPTER 4

Optimisation of Culture Technique

Introduction

The primary explant culture technique, as described in Chapter 3, is a simple and efficient way of culturing epithelial and tumour cells. It does not require any great expertise and, it takes approximately an hour to set up 50 cultures. The main advantage of this technique is that a piece of the tissue is cultured, thus maintaining cell-cell communication, as opposed to those techniques which require single cell suspensions, for example the Courtenay Assay (Courtenay and Mills, 1978). Thus this outgrowth assay probably mimics a more *in vivo* like situation. Unlike other assays, large amounts of tissue are not required; from a 1 cm^2 piece of tissue it is possible to establish 50 cultures. Where a single cell suspension is required, large amounts of tissue are needed to produce enough cells to run an assay. Some areas of the primary explant culture technique were considered for optimisation, these included :

1. Standardisation of the size of the explant being cultured.

To achieve this a template had to be designed and produced that was capable of cutting human tissue specimens into explants of a standard size. To determine whether the culturing of standard size explants was advantageous over the culturing of those explants that were sized by the original method (i.e. subjectively chosen if thought to be ~ 2-3mm^3).

2. Culture Vessel.

Alternative culture vessels to the one presently used, the Nunclon 25 cm^2 flask, were assessed for their suitability to this culture system. The culture of explants in various vessels, using volumes of media adapted for the size of the vessel, was investigated and the growth areas produced compared to those produced from the Nunclon 25 cm^2 flask.

4.1 Standardisation of Explant Size

4.1.1 CUTTING TEMPLATE

The main criticism of this outgrowth assay was that the initial cell number plated in a culture vessel was unknown. It was not possible to measure the initial cell number using a Coulter Counter or Haemocytometer, since the tumour was not digested into a single-cell suspension, but rather fragments of the tumour were semi-digested in a solution of trypsin / collagenase for a short period of time (~ 20 minutes), and then plated in culture vessels. This allowed the substratum to be broken down and the cells to migrate from the piece of tumour and establish in a culture vessel. This meant that, while the final cell number could be calculated, as described in Section 3.7, the initial cell number was unknown. It was not essential to know the initial cell number as growth inhibition or surviving fraction was measured relative to an untreated control group.

The unknown initial cell number meant that for each culture a different number of proliferating cells was being 'plated' and thus a large standard error in the resultant growth areas could be expected. In an attempt to reduce this standard error and increase the reproducibility of the assay a template was designed to cut a piece of tissue into standard size explants.

The template had to fulfil a certain criteria;

a)it must be capable of cutting a sample into standard size explants.

b)it must be sterile and preferably disposable.

c)it must be manageable and suitable for inclusion in a kit.

d)it must be cost effective.

Three prototypes were designed and made (Figures 4.1.4.2 & 4.3). A feasibility test was carried out for each design to determine, if the prototype actually worked - was it capable of cutting a sample into standard sizes, and what the faults of the design were.

4.1.1.1 DESIGN 1

DESCRIPTION

This first design was based on the idea of a potato chipper. As can be seen in Figure 4.1.a and b, it was comprised of a grid of blades separated by 2 mm to form square areas of $2mm^2$. It was expected that a blade, of approximate thickness 0.5 mm and width 3 mm, approximately would be used. There were two possibilities for encasing the grid: 1) a surround of plastic or, 2) a surround of stainless steel. In theory this design would appear to fulfil the criteria listed above: by using the grid to cut the sample 2 mm^2 explants would be obtained; sterility could be achieved by autoclaving (or if the plastic was not autoclaveable, by gamma irradiation); it would appear to be cost effective, in that, if made of stainless steel it could be used repeatedly without fear of an inaccurate cut.

With these drawings and specifications a company (Comet Precision Ltd., Dublin) was approached and a prototype made up. The finished prototype was somewhat different from the original design, although the basic idea was maintained. Rather than a series of blades being used to form a grid, the grid was cut from a solid piece of metal.

USE

By pressing downward with the instrument upon the sample the cut would be achieved in one movement.

FEASIBILITY TEST

A piece of pig liver was cut into slices of various thickness, ranging from approximately 2mm - 5mm. A slice was placed upon the base of an upturned plastic petri dish and by pressing downwards with the instrument a cut was attempted. This was repeated for several slices of liver.

RESULT: All attempts failed.

In all cases the instrument failed to make any impression on the liver whatsoever.

DISCUSSION

The most apparent fault of the instrument was that the cutting edges were not sharp enough, however modifying this would entail a lot of man-hours and money, and even at that there was no guarantee of an improvement. For these reasons it was decided that an other design should be considered.

Figure 4.1,a. Plan and side elevation of design 1. This design comprised of a grid of blades, seperated by 2 mm, encased in a surround of stainless stell.

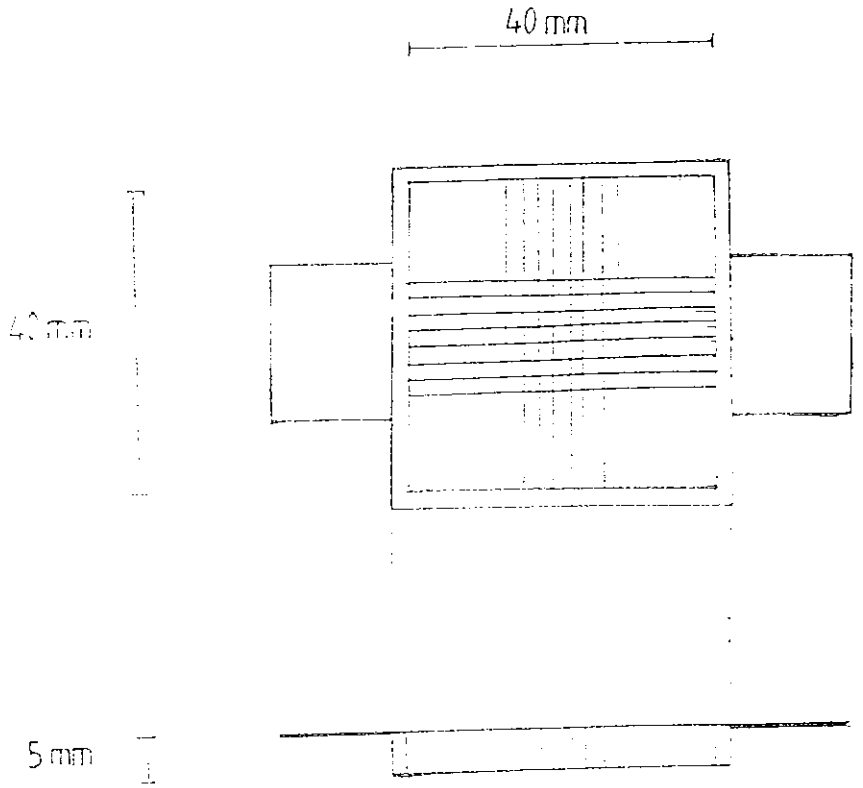
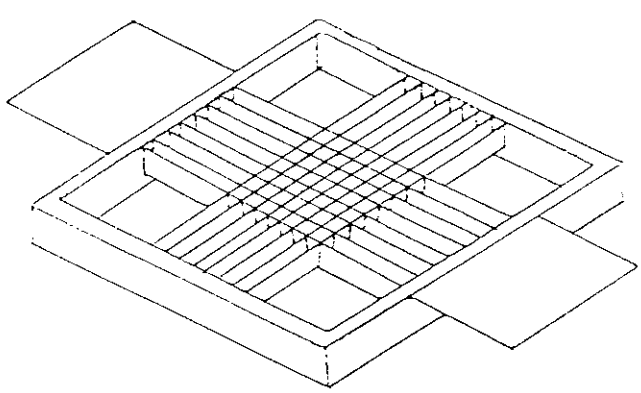


Figure 4.1,.b. Oblique view of design 1. By pressing downward with the instrument on a piece of tissue explants of 2 mm^2 would be cut, in one movement.



4.1.1.2 DESIGN 2

DESCRIPTION

A different approach was taken with this design, rather than the instrument itself cutting it acts as a guide to a cutting tool, for example, a scalpel blade.

As can be seen in Figures 4.2a, b and c, this design involved a series of slits cut into each arm of an L shaped piece of plastic. Each slit was separated from the other by 2mm. These acted as a guide to the cutting tool, a scalpel. On the base (the bend in the L) a heavily etched piece of plastic was affixed, the sample can be placed on this and held quite firmly in position while the cut is being executed. The plastic was bent into shape as shown in Figure 4.2c, to allow for the free forward movement of each arm a second hinge was made along a horizontal line 2mm above the original bend. These drawings and specifications were given to a company, (Industrial Print Ltd., Bray) and the required prototype made up.

USE

The sample is placed on the etched base of the instrument and the first flap bent into position over it. By using a scalpel the sample is cut through each successive slit into strips of 2 mm width. The flap is lifted, the second flap is positioned in the same manner but, perpendicular to the first flap and the cut repeated as above. The explants are then removed from the base using sterile tweezers.

FEASIBILITY TEST

A piece of pig liver was cut into slices of various thickness ranging from approximately 2 mm - 5 mm and placed singly on the etched surface of the instrument. The first flap was brought into position and using a disposable scalpel, the liver was cut along each successive slit. This flap was then lifted, the other positioned and the cut repeated as above to give square pieces of tissue.

The above was repeated for all slices of liver and the cut examined.

RESULT: Standard size pieces were successfully cut for all slices of liver.

DISCUSSION

Although the aseptic technique was not required for the above test, it was apparent that when in practice the use of this instrument would create some problems in terms of contamination. As a result of the design the users hands are in close proximity to the sample itself, and thus the risk of contamination is increased.

The instrument's size and light weight also make it difficult to manage for the operator. To overcome the above problems the size of the instrument could be modified, i.e. by increasing the size of the base and extending each arm out.

Being disposable (initial gamma irradiation) one is ensured of sterility at time of use and the simple design and use of plastic would prove cost effective.

Figure 4.2,a&b. Plan and side elevation of design 2. This design acted as a guide to a cutting instrument such as a scalpel blade. Each arm of an L-shaped piece of plastic had slits, seperated by 2 mm, cut out. The base comprised of a roughened surface, to which the tissue could be affixed.

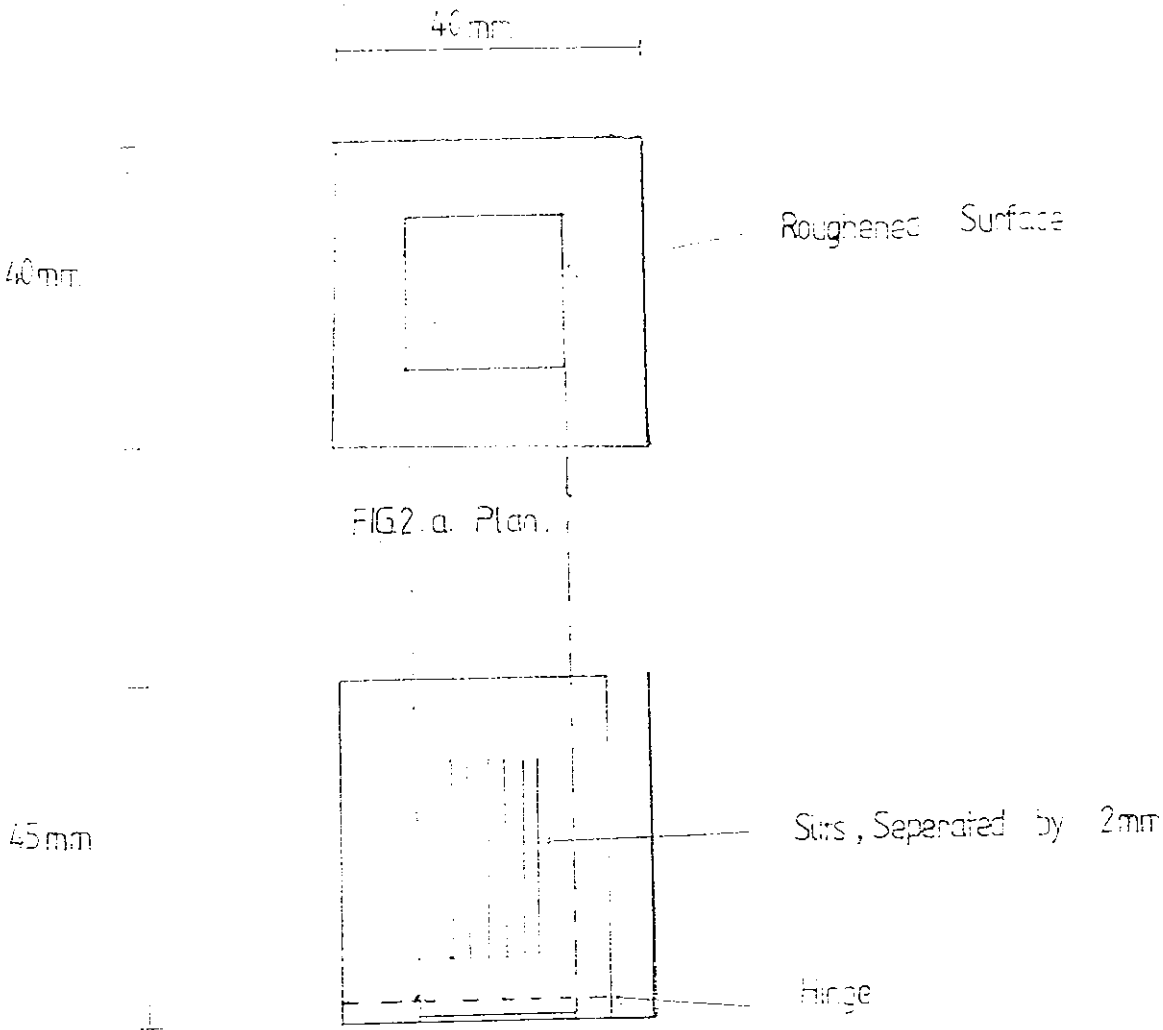
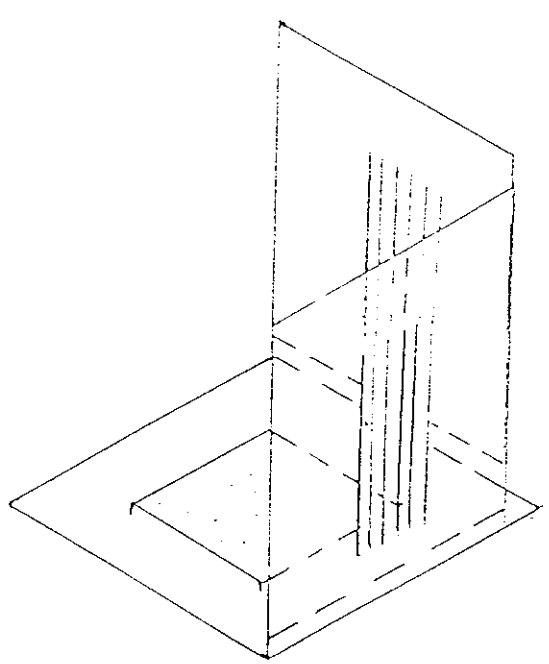


Figure 4.2,c. Oblique view of design 2. A piece of tissue placed on the roughened surface is cut, with a scalpel blade, through each successive slit, first through one arm and then, through the second perpendicular arm.



4.1.1.3 DESIGN 3

DESCRIPTION

This design was based on the same principle as that for Design 2, but is presented in a different manner. It is comprised of a plastic petri dish (Nunclon) - which are presently used in the laboratory - the base of which having being inverted acts as the insert, Figure 4.3a , and the lid as the base. Figure 4.3b.

Seven slits, separated by 2mm, were cut out of the insert, these were to act as the guide to the cutting instrument e.g. scalpel. Four rivets positioned at 90° to the next on the outer wall of the insert, fit into grooves cut at 90° to the next on the inner wall of the base. These allowed the insert to be positioned easily and accurately in one position and then in another perpendicular to the original. In the centre of the base a heavily etched piece of plastic was affixed. The sample can be placed on this and held firmly in position as the cut is being executed.

These drawings and specifications were given to a company (Industrial Print Ltd., Bray) and the prototype made up.

USE

The sample is placed on the etched base of the instrument and the insert placed in the base so that the rivets sit in the grooves and the slits rest upon the sample. While holding the base firmly the sample is cut along the slits using the cutting instrument e.g. scalpel. Having completed the cut, the insert is lifted, turned 90° either way and positioned in the

grooves. The sample is cut again in this position. The explants are removed using a sterile tweezers.

FEASIBILITY TEST

A piece of pig liver was cut into thin slices suitable for using in the cutting template. A slice was placed on the etched base of the instrument and the insert put in position. Using a scalpel blade the liver was cut along each successive slit. The insert was repositioned at 90^{o} to the original position and the liver cut along the slits. The cut fragments, or explants were removed from the base using a sterile tweezers.

RESULT: Standard size explants were successfully cut from the piece of liver.

DISCUSSION

This design appeared to satisfy all the requirements. It was capable of cutting the tissue into standard size explants. The instrument was disposable and since made of plastic, gamma irradiation after packaging would ensure sterility at time of use. The size and shape of the instrument mean that it was large enough to be manageable, yet compact and thus suitable for inclusion in a kit. Production costs would be greatly reduced due to the fact that the petri dishes are readily available and all that is required is modification.

The simplicity of the design and the use of plastic should prove cost effective.

In conclusion, this design appeared to match the requirements and, thus it was possible to proceed in verifying the need for an instrument which would exclude one unknown parameter and render this outgrowth assay more reproducible.

Figure 4.3,a. Plan and front elevation of insert of design 3. The insert was made from the inverted base of a petri dish. A series of slits, seperated by 2 mm, were cut into the insert and acted as a giude to a scalpel blade in cutting a piece of tissue. Rivets on the insert, positioned at 90° to one another, were positioned in line with grooves cut into the base. This allowed the insert to be positioned accurately and firmly, while the tissue was cut.

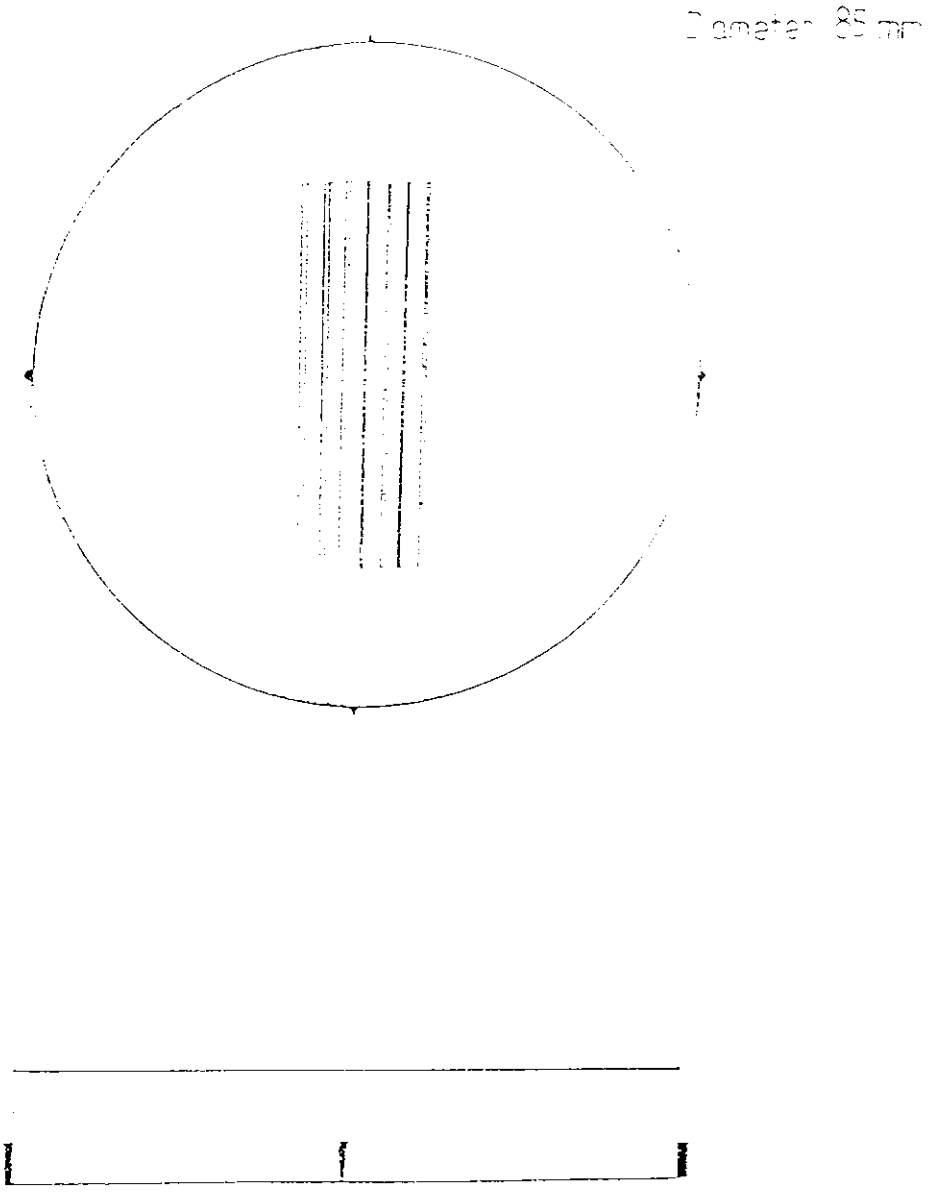
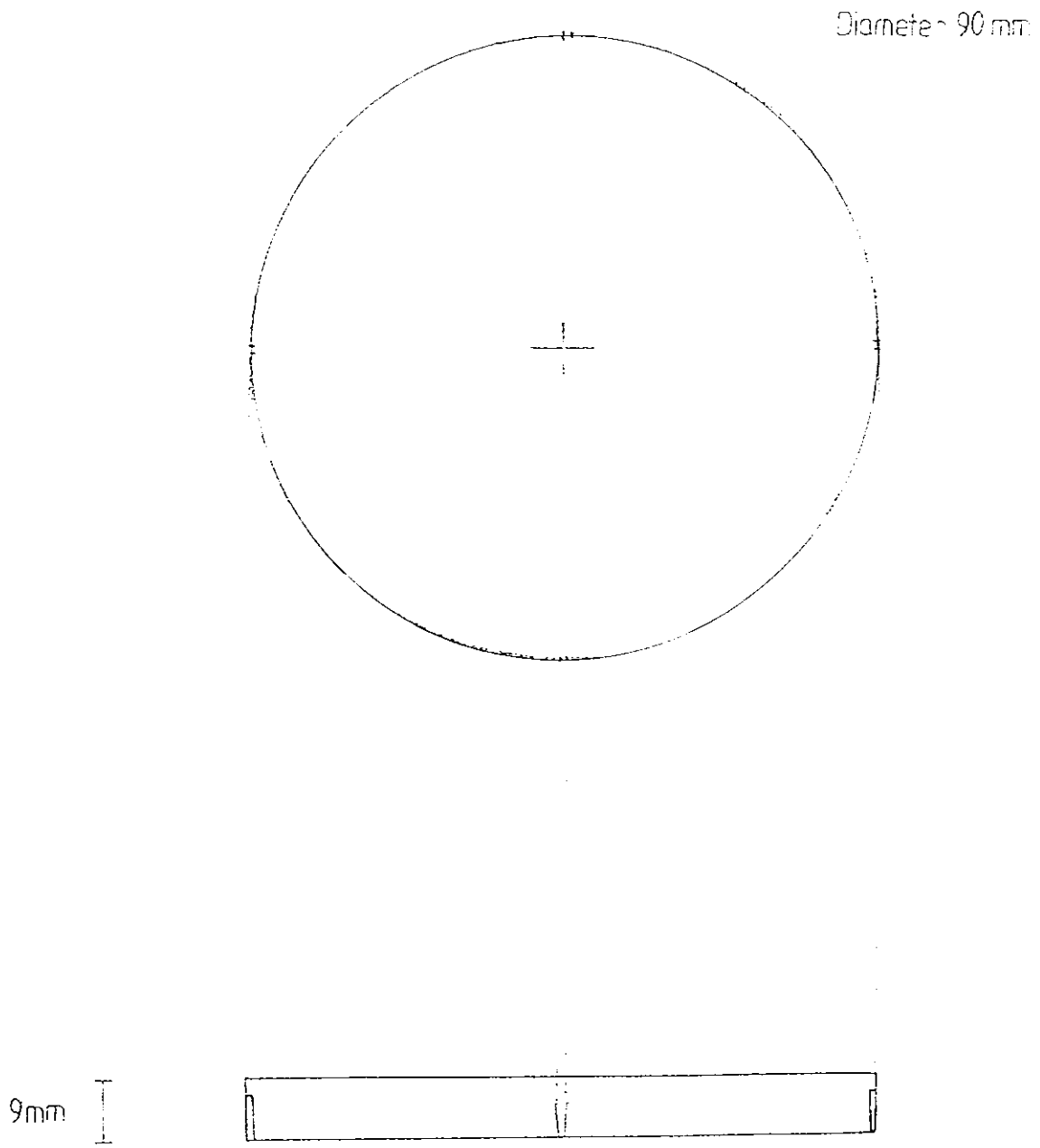


Figure 4.3,b. Plan and front elevation of base of design 3. The base of this design was made from the inverted lid of a petri dish. The centre of the base had a roughened surface, on which a piece of tissue to be cut was placed. Grooves in the wall of the base, positioned at 90^0 from one another, allowed the insert to be positioned accurately and firmly



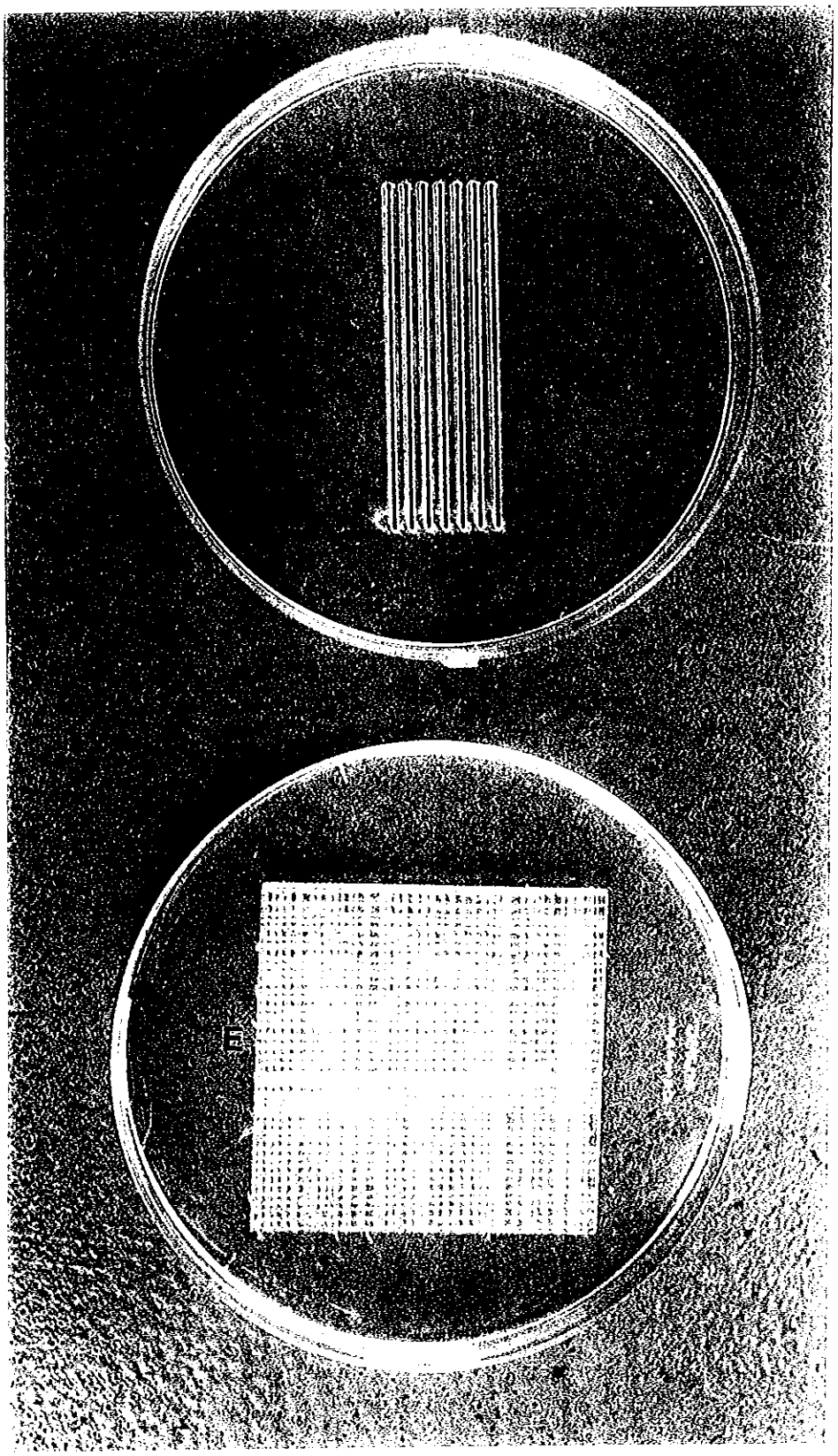


Plate 4.1. Prototype of Design 3. Use: A piece of tissue to be cut is placed on the etched surface, E, and the insert, I, positioned on top of the tissue. The tissue is cut with a scalpel blade through each successive slits. The insert is lifted and repositioned at 90° to the first position. The cut is repeated.

4.1.2 STANDARD SIZE EXPLANT

4.1.2.1 INTRODUCTION

A reduction in the standard error of the mean total cell number for a group of cultures would result in a more reproducible and reliable assay. To ascertain whether the culture of standard-size explants would reduce the standard error, standard-size explants and explants that were cut from the tissue according to the original method (Mothersill et al., 1988) were cultured in parallel. The resultant standard errors of the mean total cell number for each group of cultures was compared statistically using a two sample *t* test .

Standard-sized explants were cut from the tissue using the cutting template described in Section 4.1.3. In the control group explants were cut from the tissue by the normal technique of mincing it with a pair of scissors and choosing the explants that appeared to be approximately 2-3 mm^2 for culturing.

4.1.2.2 MATERIALS AND METHODS

Oesophageal specimens were obtained from 3 patients undergoing oesophagectomy. On removal the specimens were placed in sterile saline at ambient temperature and brought immediately to the laboratory for culture. Each specimen was cut in two, one half was fragmented by the normal procedure of mincing it with scissors, the other was cut into standard size pieces using the cutting template. The tissue samples were digested

separately in solutions of 0.25% trypsin / collagenase for 20 minutes at 37°C. The digested explants were then plated singly in Nunclon 25 cm^2 tissue culture flasks with 2 ml of growth media, and placed in the incubator at 37°C and 5% CO_2 . The plated explants were grouped according to the method of fragmentation. The growth media used was RPMI 1640 supplemented as described in Section 2.9. After 14 days of incubation the cultures are removed, fixed in 10% buffered formalin and stained with Harris Haemaotoxylin. The total cell number for each culture was determined, as described in Section 3.7 and the standard error of the mean total cell number for each group calculated and compared.

4.1.2.3 RESULTS

Tables 4.1.2.1-4.1.2.6 show the growth area and mean total cell number produced from each explant plated, from three different oesophageal specimens A, B and C. Tables 4.1.2.1, 4.1.2.3 and 4.1.2.5 show the mean total cell number for explants cut by the original technique and the standard error on the mean. The standard error is also expressed as a percentage of the mean; see brackets. Tables 4.1.2.2, 4.1.2.4 and 4.1.2.6 show the mean total cell number for standard size explants, cut using the template designed, and the standard error on the mean. Again the standard error is also expressed as a percentage of the mean; see brackets. Table 4.1.2.7 is a summary table of the three specimens, showing the % standard error on the mean total cell number of each group of cultures, that is the standard size explant group and the control explant group, for specimens A, B and C.

Table 4.1.2.1. Growth area and cell number of cultures produced from control size explants of Oesophagus A, which were cultured for 14 days.

Culture	Growth Area (mm^2)	Cell Number per mm^2			Mean Cell Number per mm^2	Total Cell Number ($\times 10^3$)
1	76	316	254	210	260 ± 31	19.8 ± 23
2	771	366	332	396	365 ± 18	281.1 ± 14
3	97	286	180	262	243 ± 32	23.5 ± 3
4	543	510	324	242	359 ± 79	194.7 ± 43
5	420	400	262	478	380 ± 63	159.6 ± 26
Mean	***381 ± 133 (35%)**					***135.7 ± 51 (37%)**

Table 4.1.2.2. Growth area and total cell number of cultures produced from standard size explants of Oesophagus A, which were cultured for 14 days.

Culture	Growth Area (mm^2)	Cell Number per mm^2			*Mean Cell Number per mm^2	Total Cell Number ($\times 10^3$)
1	455	594	276	434	435 ±92	197.8 ± 42
2	287	450	304	288	347 ±51	99.7 ± 15
3	255	498	406	358	421 ± 41	107.3 ± 10
4	446	396	398	522	439 ± 42	195.6 ± 19
5	232	348	358	378	361 ± 9	83.8 ± 2
6	436	490	404	300	398 ± 55	173.5 ± 24
Mean	***352 ± 43 (12%)**					***142.9 ± 21 (15%)**

* Mean values ± SEM

Table 4.1.2.3. Growth area and total cell number of cultures produced from control size explants of Oesophagus B, which were cultured for 14 day.

Culture	Growth Area (mm^2)	Cell Number per mm^2			*Mean Cell Number per mm^2	Total Cell Number ($\times 10^3$)
1	168	284	328	160	257 ± 50	43.2 ± 8.4
2	2.5	182	234		208 ± 26	0.5 ± .06
3	278	330	274	222	275 ± 31	76.5 ± 9
4	86	398	298	250	315 ± 43	27.1 ± 4
5	80	274	388	322	328 ± 33	26.2 ± 3
6	4	128	130	110	123 ± 6	0.5 ± 0.02
7	45	256	130	114	167 ± 45	7.5 ± 2
8	144	250	274	356	293 ± 32	42.2 ± 4.6
9	148	292	328	316	312 ± 11	46.2 ± 1.6
Mean	***106.2 ± 29 (27%)**					***29.9 ±8.3 (28%)**

* Mean value ± SEM

Table 4.1.2.4. Growth area and total cell number of cultures produced from standard sized explants of Oesophagus B, cultured for 14 days.

Culture	Growth Area (mm^2)	Cell Number per mm^2			*Mean Cell Number per mm^2	Total Cell Number ($\times 10^3$)
1	28	192	144	96	144 ± 28	4.0 ± 0.8
2	375	202	218	116	179 ± 32	67 ± 1.2
3	418	226	104	198	176 ± 37	73.6 ± 15.5
4	255	178	236	214	209 ± 17	53.4 ± 4.3
5	98	168	272	194	211 ± 31	20.7 ± 3.0
6	303	226	248	130	201 ± 36	61.0 ± 11
7	263	204	232	244	227 ± 12	59.6 ± 3.1
8	231	336	276	200	261 ± 48	60.4 ± 11
9	391	316	292	172	260 ± 44	101.7 ± 17
Mean	***262.4 ± 44 (17%)**					***55.7 ± 9.5 (17%)**

* Standard error of mean

Table 4.1.2.5. Growth area and cell number produced from cultures of control size explants of Oesophagus C, cultured for 14 days.

Culture	Growth Area (mm^2)	Cell Number per mm^2	*Mean Cell Number per mm^2	Total Cell Number ($\times 10^3$)
1	397	638 626 460	575 ± 57	228.1 ± 22.6
2	305	926 636 783	783 ± 84	238.7 ± 25.5
3	432	700 474 546	573 ± 67	247.7 ± 29
4	450	388 668 614	557 ± 86	250.5 ± 38.7
Mean	***396 ± 32 (8.2%)**			***241.25 ± 5 (2%)**

Table 4.1.2.6. Growth and cell number of cultures produced from standard sized explants of Oesophagus C, cultured for 14 days.

Culture	Growth Area (mm^2)	Cell Number per mm^2	*Mean Cell Number per mm^2	Total Cell Number ($\times 10^3$)
1	424	452 430 544	475 ± 35	201.5 ± 15
2	498	454 550 508	504 ±16	250.9 ± 7.9
3	191	544 520 428	497 ± 35	94.9 ± 6.7
4	383	518 432 464	471 ± 25	180.5 ± 9.6
5	548	314 540 468	441 ± 67	241.5 ± 36.7
6	315	512 302 634	483 ± 97	152.0 ± 30.5
7	481	492 546 438	492 ± 31	236.6 ± 14.9
8	253	282 620 680	527 ± 124	133.4 ± 31.4
9	516	394 452 464	437 ± 22	225.3 ± 11.3
Mean	***401 ± 42 (10.4%)**			***190.7 ± 18 (9%)**

* Mean value ± SEM

Table 4.1.2.7. % standard error of mean total cell number of each group, control and standard size, for specimens, A, B and C. Although the % standard error was reduced when standard size explants were cultured, this reduction was not significant, P = 0.2.

Specimen	Control Explants	Standard Explants
A	37	15
B	28	17
C	2	9
Mean % SEM	**22**	**14**
P-value	**0.2**	

A.

B.

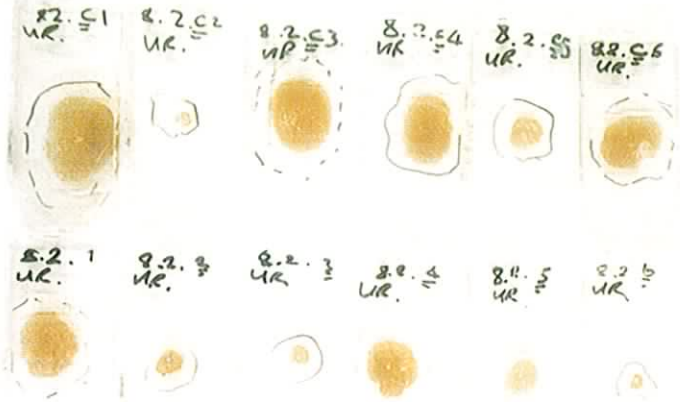


Plate 4.2. Explants of urothelium cut by a) using the cutting template designed and b) by the origional method of mincing the tissue with a sterile scissors and selecting explants of approximately 2-3 mm^3 for culturing. All explants were plated singly in Falcon 25 cm^2 flasks with 2 ml of growth media and incubated for 14 days. Row A are the growth areas produced from explants cut using the template. Row B are the growth areas produced from explants cut by the origional method. The growth areas in row A appear to be more uniform in size than those in row B.

4.1.2.4 DISCUSSION

In the case of specimen A and C the regularity of size of the explants did not appear to have any effect on the mean total cell number, while in specimen B the mean total cell number for the standard size explants was nearly double that of the random size explants. The standard error on the mean total cell number for specimens A and B was greatly reduced when standard size explants were cultured, however this was not the case for specimen C.

Overall the mean total cell number did not vary much between standard and control size explants, but the % standard error on the mean total cell number was almost halved for standard size group, in the case of specimen A and C, Table 4.1.2.7. The % standard error of the mean total cell number for standard size explants cultured was not significantly different from the % standard produced when explants cut by the original method were cultured, Plate 4.2.

From these results we can conclude that the culture of standard size explants reduces the standard error of the mean total cell number, but this reduction was not significant.

Although the problem of the unknown initial cell number has not been overcome, this set of experiments has shown that it is possible to reduce one of the variables involved. This reduction of the standard error may render the assay more reproducible and reliable as a predictive assay.

4.2 The Culture Vessel

4.2.1 INTRODUCTION

The culture vessel used in the laboratory for primary explant culture was the Nunclon 25 cm^2 flask and, although this flask provided very good results in terms of the total cell number produced from explants cultured, the use of other vessels was investigated, Plate 4.3. These culture vessels, Table 4.2.1, were more compact and would perhaps be suitable for inclusion in a kit form of the assay. The suitability of the culture vessels to this outgrowth assay was examined in terms of attachment of, and outgrowth from explants plated in these culture vessels with varying volumes of media. Attachment and outgrowth were monitored over a 14 day period, while the cell number produced from each culture was measured on day 14. The mean cell numbers achieved from cultures grown in each type of vessel were compared and the optimum vessel compared to the 'gold standard' of the laboratory - the Falcon 25 cm^2 flask.

Table 4.2.1. Culture vessels investigated for their suitability to the outgrowth assay and as a possible alternatives to the Nunclon 25 cm^2 flask.

Culture Vessel	Surface Area Per Well
Falcon 6-well Plate	960 mm^2
Falcon 24-well Plate	200 mm^2
Nunclon 4-well Multidish	190 mm^2

4.2.2 MATERIALS AND METHODS

Ureter taken at kidney transplantation were obtained from 8 different patients. On removal the samples were placed in sterile saline and brought immediately to the laboratory for culture. The samples were minced, using sterile scissors into explants of approximately 2-3 mm^3 and digested in a solution of trypsin / collagenase for 20 minutes. The digested explants were plated singly in each well of the above-mentioned culture vessels and varying volumes of growth medium added.

The minimum volume of growth media to be added to each well of the three vessels was calculated from the ratio of surface area to volume of media used in the Falcon 25 cm^2 flask, as shown in Table 4.2.2. This ratio of volume to surface area allows the explant to attach to the base of the well, this is known from experience with the Falcon 25 cm^2 flask. The explants were allowed 14 days in culture, after which time the resultant growth (if any) was fixed and stained. The attachment and growth development was examined and noted (as described in Section 3.7) at stated intervals during the 14 day growth period.

Table 4.2.2. Minimum volume of growth media added to each well, for each vessel. Calculated from the ratio; surface area : volume of growth media, of the Falcon 25 cm^2 flask. Using this ratio of media to surface area allows the explant to attach to the base of the flask.

Vessel	Surface Area (cm^2)	Volume Growth Media (ml)
Falcon culture flask	25	2
Falcon 6-well well plate	9.6	0.8
Falcon 24-well plate	2.0	0.2
Nunclon 4-well multidish	1.9	0.15

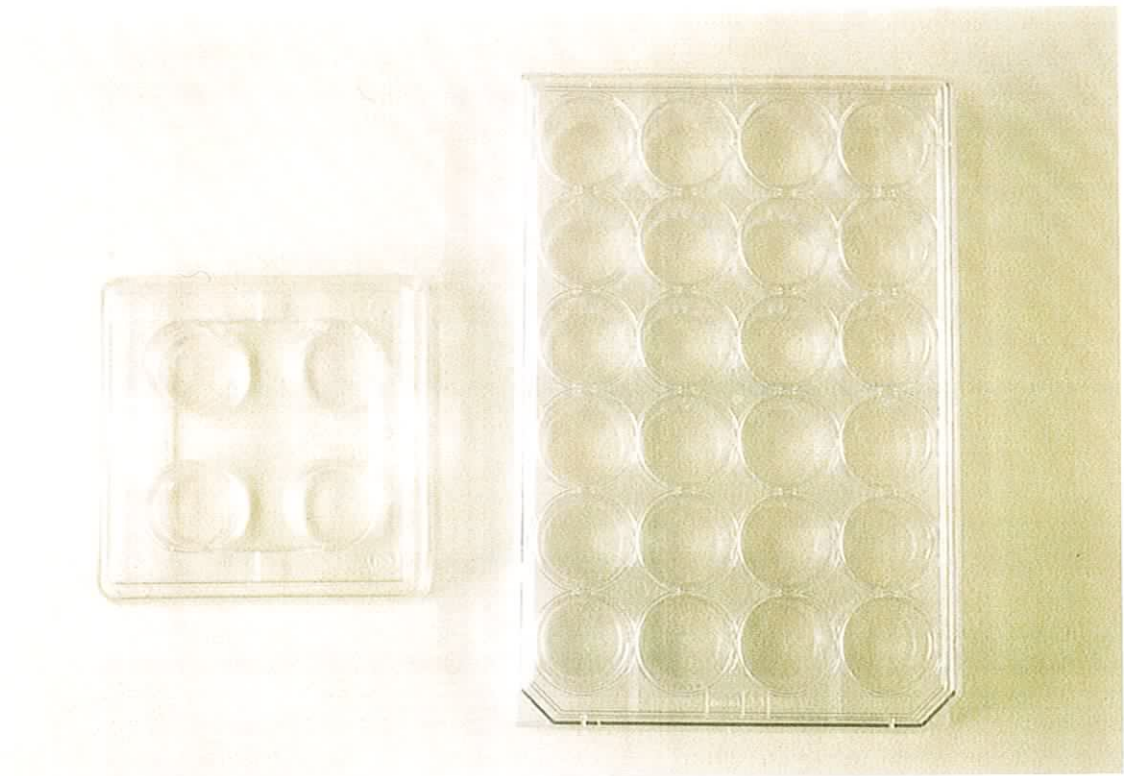


Plate 4.3. Culture vessels investigated as alternatives to the Nunclon 25 cm^2 culture flask. These culture vessels were more compact and easy to use. The suitability of the Falcon 24-well plate (A) and the Nunclon 4-well dish (B) to the explant culture system was measured in terms of attachment of and outgrowth from explants plated in each well of these dishes.

4.2.3 RESULTS

Tables 4.2.3-10 show the attachment, outgrowth and total cell number of cultured urothelium explants plated in Falcon 6-well plates with varying volumes of growth media. The same results are shown for urothelium explants plated in Falcon 24-well plates, Tables 4.2.11-16, and Nunclon 4-well Multidishes, Tables 4.2.17-19.

The optimum volume of media added to each well of the Falcon 6-well plate was 0.8 ml. This volume produced 100% attachment after 4/5 days in culture and a mean total cell number of 63,400 ± 27,200, Table 4.2.20. A volume of 1.5 ml added to each well resulted in attachment of 60% of explants after 4/5 days in culture and a mean total cell number of 126,700 ± 98,000. Although this mean total cell number was higher than that produced for 0.8 ml, 1.5 ml was not considered the optimum volume due to the reduced attachment and high standard error of the mean total cell number, 77% of the mean.

The optimum volume of media added to the Falcon 24-well plate was 0.3 ml. This volume resulted in attachment of 83% of explants after 4/5 days in culture and a mean total cell number of 19,090 ± 12,810. This standard error was very high at 67%, Table 4.2.21.

The optimum volume of media added to each well of the Nunclon 4-well plate was 0.3 ml. This volume resulted in 33% attachment of explants after 4/5 days in culture and a mean total cell number of 1,010 ± 80, Table 4.2.22.

Of all the vessels investigated for their suitability to this culture system, the Falcon 6-well plate, produced the highest total cell number and 100% attachment after 4/5 days, Table 4.2.23. In Table 4.2.24, this total mean number is compared to that achieved from a sample of urothelium explants cultured in the 'gold standard' of the laboratory, the Nunclon 25 cm^2 flasks.

Table 4.2.3. Assessment of suitability of the Falcon 6-well plate to explant culture, in terms of attachment (A) and outgrowth (OG) development of urothelium explants, cultured for 14 days with varying volumes of media, RPMI 1640. Attachment and outgrowth was graded according to the key below.

Culture	Volume Media	4 th Day		6th Day		7th Day		8th Day		11th Day		13th Day	
No.	(ml)	A	OG	A	OG	A	OG	A	OG	A	OG	A	OG
1	1	1	1	2	1	2	1	1	1	2	1	2	1
2	1	0	0	2	0	2	1	1	1	2	2	2	1
3	1.5	1	1	2	1	2	1	1	1	2	1	2	1
4	1.5	2	1	2	1	2	2	2	2	2	3	2	2
5	2	1	0	1	0	2	1	1	1	2	2	2	2
6	2	0	0	0	0	0	0	0	0	2	1	1	0

KEY:

	Attachment (A)	**Growth (G)**
0	no attachment	no growth
1	minimal attachment	0>25 mm^2
2	complete attachment	25>150 mm^2
3		150>400 mm^2

Table 4.2.4. Assessment of suitability of the Falcon 6-well plate to explant culture, in terms of outgrowth area and total cell number of urothelium cultures, grown from explants plated with varying volumes of media and cultured for 14 days.

Well No.	Volume Media (ml)/well	Outgrowth Area (mm^2)	Cell Number./mm^2			Mean Cell Number / mm^2	Total Cell Number ($\times 10^3$)
1	1	11.5	83	87	104	91.7± 6.2	1.0 ± 0.07
2	1	23	297	229	370	298.7 ± 40.7	6.9 ± 0.9
3	1.5	5	284	68	91	147.7 ± 68.5	0.7 ± 0.3
4	1.5	74	208	249	353	270 ± 43.1	20.0 ± 3.2
5	2	97	513	205	193	303.7 ± 104.7	29.4 ± 10.1
6	2	0					0

Table 4.2.5. Assessment of suitability of the Falcon 6-well plate to explant culture, in terms of attachment (A) and outgrowth (OG) from urothelium explants cultured in varying volumes of media and monitored at intervals over a 14 day growth period. Attachment of and outgrowth from the explants was graded according to the key below.

Well No.	Volume Media /Well (ml)	4th Day		10th Day	
		A	OG	A	OG
1	1	2	0	2	1
2	1	2	1	2	2
3	1	2	0	2	1
4	1.5	2	1	2	3
5	1.5	0	0	2	1
6	1.5	0	0	0	0

KEY

	Attachment (A)	**Growth (G)**
0	no attachment	no growth
1	minimal attachment	0>25 mm^2
2	complete attachment	25>150 mm^2
3		150>400 mm^2

Table 4.2.6. Assessment of suitability of the Falcon 6-well plate to explant culture, in terms of outgrowth area and total cell number of urothelium cultures, grown from explants plated with varying volumes of growth medium and cultured for 14 days.

Well No.	Volume Media (ml)/well	Outgrowth Area (mm^2)	Cell Number./mm^2			Mean Cell Number / mm^2	Total Cell Number ($\times 10^3$)
1	1	10	86	187	117	130 ± 29.9	1.3 ± 0.3
2	1	164	1225	1500	1333	1353 ± 80	221.8 ± 13
3	1	62	566	1133	560	753 ± 190	46.7 ± 11.8
4	1.5	418	633	1533	833	999.7 ± 273	417.8 ± 114
5	1.5	65	1200	1033	933	1055 ± 78	68.6 ± 5
6	1.5	0					

Table 4.2.7. Assessment of suitability of the Falcon 6-well plate to explant culture,, in terms of attachment (A) and outgrowth (OG) from urothelium explants plated with 0.8 ml of media and monitored at intervals over a 14 day growth period. Attachment of and outgrowth from the explants was graded according to the key below.

Well No.	Volume Media	4th Day		8th Day	
	(ml)/Well	A	OG	A	OG
1	0.8	1	1	1	1
2	0.8	1	1	2	2
3	0.8	1	0	2	1
4	0.8	1	0	1	1
5	0.8	1	0	2	1
6	0.8	1	1	2	1

KEY:

	Attachment (A)	**Growth (G**
0	no attachment	no growth
1	minimal attachment	0>25 mm^2
2	complete attachment	25>150 mm^2
3		150>400 mm^2

Table 4.2.8. Assessment of suitability of the Falcon 6-well to explant culture, in terms of outgrowth area and total cell number of urothelium cultures, grown from explants plated with 0.8 ml of media and cultured for 14 days.

Well No.	Volume Media (ml)/well	Outgrowth Area (mm^2)	Cell Number./mm^2			Mean Cell Number / mm^2	Total Cell Number ($\times 10^3$)
1	0.8	24	100	49	69	73 ± 15	1.7 ± 0.3
2	0.8	614	760	303	206	423 ± 17	259.7 ± 10
3	0.8	156	431	146	211	263 ± 86	41.0 ± 13
4	0.8	21	198	142	152	164 ± 17	3.4 ± 0.3
5	0.8	178	327	398	773	499 ± 138	88.9 ± 24
6	0.8	73	196	580	232	336 ± 122	24.5 ± 8.9

Table 4.2.9. Assessment of suitability of the Falcon 6-well plate to explant culture, in terms of attachment and outgrowth from urothelium explants plated with 0.8 ml of media and monitored at intervals over a 14 day growth period. Attachment of and outgrowth from the explants was graded according to the key below.

Well Number	Volume Media (ml)/Well	5th Day	
		A	OG
1	0.8	2	1
2	0.8	2	1
3	0.8	1	0
4	0.8	1	1
5	0.8	1	0
6	0.8	2	2

KEY:

	Attachment (A)	**Growth (G)**
0	no attachment	no growth
1	minimal attachment	0>25 mm^2
2	complete attachment	25>150 mm^2
3		150>400 mm^2

Table 4.2.10. Assessment of suitability of the Falcon 6-well plate to explant culture, in terms of outgrowth area and total cell number of urothelium cultures, grown from explants plated with 0.8 ml of media and cultured for 14 days.

Well No.	Volume Media (ml)/well	Outgrowth Area (mm^2)	Cell Number./mm^2			Mean Cell Number / mm^2	Total Cell Number ($\times 10^3$)
1	0.8	48	650	238	230	373 ± 139	17.9 ± 6.7
2	0.8	96	239	292	379	303 ± 41	29.1 ± 3.9
3	0.8	0					
4	0.8	27	375	456	827	583 ± 132	15.7 ± 3.5
5	0.8	5	70	80	69	73 ± 3	0.4 ± 0.02
6	0.8	280	569	231	593	464 ± 117	215.6 ± 54.4

Table 4.2.11. An initial volume of media was added to each well of a Falcon 24-well plate, this initial volume was the same in rows A and C, and rows B and D. The initial volume was added to allow the explant to attach to the base of the well. After 72 hours in culture a second volume was added to each well, see the last column, in order to sustain the growth for a further 11 days.

Well No.	Row A Initial Vol. (ml)	Row B Initial Vol. (ml)	Row C Initial Vol. (ml)	Row D Initial Vol. (ml)	Vol. Added to all Wells After 72hrs (ml)
1	0.15	0.1	0.15	0.1	0.4
2	0.15	0.1	0.15	0.1	0.4
3	0.15	0.1	0.15	0.1	0.2
4	0.15	0.1	0.15	0.1	0.2
5	0.15	0.1	0.15	0.1	0.1
6	0.15	0.1	0.15	0.1	0.1

Table 4.2.12. Assessment of suitability of the Falcon 24-well plate to explant culture, in terms of attachment (A) and outgrowth (OG) of urothelium explants plated with varying volumes of growth media and monitored at intervals during a 14 day growth period. Attachment of and outgrowth from the explants was graded according to the key below.

Well	4th Day								6th Day							
No.	Row	A	Row	B	Row	C	Row	D	Row	A	Row	B	Row	C	Row	D
	A	OG	A	OG	A	OG	A	OG	A	OG	A	O	A	OG	A	OG
1	2	1	2	0	2	0	2	f	2	f	1	0	2	0	2	f
2	2	0	2	0	2	f	2	0	2	f	2	f	2	f	2	f
3	2	f	2	f	2	0	2	0	2	f	2	1	0	0	2	1
4	2	f	2	1	0	0	2	f	2	1	1	f	2	1	2	0
5	2	f	2	f	0	0	2	1	2	f	2	f	2	f	2	1
6	2	f	2	0	2	1	0	0	2	f	1	f	2	1	2	0

f = Fibroblastic cells

KEY:

	Attachment (A)	Growth (G
0	no attachment	no growth
1	minimal attachment	0>25 mm^2
2	complete attachment	25>150 mm^2
3		150>400 mm^2

Table 4.2.13a: Assessment of suitability of the Falcon 24-well plate to explant culture, in terms of outgrowth area and total cell number of urothelium cultures, grown from explants plated with varying volumes of media.

Well No.	Total Volume Media/well(ml)	Outgrowth Area (mm^2)	Cell Number /mm^2			Mean Cell Number/mm^2	Total Cell Number ($\times 10^3$)
A 1	0.55	f					
2	0.55	f					
3	0.35	0					
4	0.35	13	655	523	466	548± 56	7.1 ± 0.7
5	0.25	f					
6	0.25	8	158	88	123	123± 20	1.0 ± 0.2
B 1	0.5	f					
2	0.5	f					
3	0.3	60	780	700	760	747 ± 24	44.7 ± 1.4
4	0.3	f					
5	0.2	f					
6	0.2	f					

f – Fibroblastic cells

Table 4.2.13b. Assessment of suitability of the Falcon 24-well plate to explant culture, in terms of outgrowth area and total cell number of urothelium cultures, grown from explants plated with varying volumes of media.

Well No.	Vol.Med. (ml)/well	Outgrowth Area (mm^2)	Cell Number /mm^2			Mean	Total Cell Number ($\times 10^3$)
C 1	0.55	f					
2	0.55	f					
3	0.35	f					
4	0.35	20	340	449	404	398 ± 32	7.9 ± 0.6
5	0.25	f					
6	0.25	36	234	218	307	253 ± 27	9.1 ± 1.0
D 1	0.5	f					
2	0.5	f					
3	0.3	22	598	366	578	514 ± 74	11.3 ± 1.6
4	0.3	f					
5	0.2	89	358	581	563	501 ± 71	44.5 ± 6.3
6	0.2	6	177	330	339	282 ± 52	1.7 ± 0.2

f = Fibroblastic cells.

Table 4.2.14. . Initial volume of medium (ml) added to each well of a Falcon 24-Well plate, to allow the explant to attach to the base of the flask. A subsequent volume was added after 72 hours in culture, see last column. The initial volumes were the same for rows A and B, and C and D.

Well No.	Row A-Initial (ml)	Row B- Initial (ml)	Row C- Initial (ml)	Row D- Initial (ml)	After 72hrs (ml)
1	0.1	0.1	0.15	0.15	0.2
2	0.1	0.1	0.15	0.15	0.1
3	0.1	0.1	0.15	0.15	0.1

Table 4.2.15. Assessment of the suitability of the Falcon 24-well plate to explant culture, in terms of attachment (A) and outgrowth (OG) of urothelium explants plated with varying volumes of media (table) and monitored over a 14 day period. The attachment of and outgrowth from the explants was graded according to the key below.

Well No.	**5th Day**								**11th Day**							
	Row A		**Row B**		**Row C**		**Row D**		**Row A**		**Row B**		**Row C**		**Row D**	
	A	OG	A	OG	A	OG	A	OG	A	OG	A	OG	A	OG	A	OG
1	0	1	2	1	2	1	2	1	0	0	2	1	0	0	2	1
2	1	0	0	0	2	1	0	0	2	1	0	0	2	1	0	0
3	2	0	2	0	2	0	2	0	2	1	1	1	2	1	0	0

KEY:

	Attachment (A)	Growth (G
0	no attachment	no growth
1	minimal attachment	0>25 mm^2
2	complete attachment	25>150 mm^2
3		150>400 mm^2

Table 4.2.16. Assessment of suitability of the Falcon 24-well plate to explant culture, in terms of outgrowth area and total cell number of cultures produced from urothelium explants plated with varying volumes of growth media and incubated for 14 days.

Well No.	Total Volume Med.(ml)	Growth Area (mm^2)	Cell Number /mm^2			Mean Cell Number/ mm^2	Total Cell Number ($\times 10^3$)
A 1	0.3	0					
2	0.2	0					
3	0.2	0					
B 1	0.3	36	206	119	130	151.7 ± 27	5.46 ± 0.97
2	0.2	0					
3	0.2	0					
C 1	0.35	9	60	32	50	47.3 ± 8	0.43 ± 0.07
2	0.25	28	98	113	94	101.7 ± 6	2.85 ± 0.17
3	0.25	3	56	88	85	76.3 ± 10	0.23 ± 0.03
D 1	0.35	26	80	102	122	101.3 ± 12	2.63 ± 0.31
2	0.25	0					
3	0.25	0					

Table 4.2.17. Assessment of the suitability of the Nunclon 4-well dish to explant culture, in terms of attachment (A) and outgrowth (OG) development of urothelium explants plated with an initial volume of growth media, to allow the explants to attach and a second volume added at various times. The cultures were monitored over a 14 day growth period. The attachment of and outgrowth from the explants was graded according to the key shown for table 4.2.14.

Well No.		Volume Media (ml)/well		2nd Day		4th Day		6th Day		8th Day		11th Day		12th Day	
		Init.	18hr	A	OG	A	OG	A	OG	A	OG	A	OG	A	OG
A	1	0.1	0.1	1	0	1	1	1	1	1	1	1	0	1	1
	2	0.1	0.2	1	0	0	0	0	0	0	0	0	0	0	0
	3	0.1	0.3	1	1	0	0	1	0	0	0	0	0	0	0
	4	0.1	0.4	1	0	0	0	1	1	1	1	1	1	1	1
		Init	113hrs												
B	1	0.1	0.1	0	0	0	0	1	0	1	0	0	0	0	0
	2	0.1	0.2	0	0	1	1	1	0	1	1	1	1	1	1
	3	0.1	0.3	1	1	1	1	1	1	1	2	1	2	2	2
	4	0.1	0.4	1	0	0	0	1	0	0	0	0	0	0	0
		Init	89hrs												
C	1	0.1	0.1	1	0	1	1	1	1	1	1	1	1	1	1
	2	0.1	0.2	0	0	0	0	0	0	0	0	0	0	0	1
	3	0.1	0.3	1	0	1	1	0	0	1	1	2	0	2	0
	4	0.1	0.4	1	0	1	1	1	1	1	2	1	3	2	3

Table 4.2.18 . Assessment of suitability of the Nunclon 4-well dish to explant culture, in terms of outgrowth area and total cell number of cultured urothelium explants, plated with varying volumes of growth media added initially, to allow the explant to attach, and a second volume at varying times. The total growth period was 14 days.

Well No.		Volume Media (ml)/well		Growth Area (mm^2)	Cell No./mm^2			Mean	Total Cell No.
		Init	18hr						
A	1	0.1	0.1	0					
	2	0.1	0.2	f					
	3	0.1	0.3	0					
	4	0.1	0.4	6	900	900	875	891.6 ± 8	5.35 ± 0.05
		Init	113hr						
B	1	0.1	0.1	0					
	2	0.1	0.2	8	142	94	173	136 ± 23	1.09 ± 0.18
	3	0.1	0.3	45	256	175	400	277 ± 66	12.46 ± 2.96
	4	0.1	0.4	f					
		Init	89hrs						
C	1	0.1	0.1	5	232	246	120	199 ± 40	0.99 ± 0.20
	2	0.1	0.2	1	1,166			1,166	0.93
	3	0.1	0.3	f					
	4	0.1	0.4	265	1,325	1,140	1,600	1,355± 134	359.07 ± 35.5

f = Fibroblastic cells

Table 4.2.19. Assessment of the suitability of the Nunclon 4-well dish to explant culture, in terms of outgrowth area and total cell number of cultures of urothelium plated with varying volumes of media. No growth was produced from the explants cultured in these plates.

Well No.	Vol. Med. (ml)			Outgrowth Area (mm^2)	Total Cell No.
	Plate 1	Plate 2	Plate 3		
1	0.08	0.08	0.1	0	
2	0.08	0.08	0.1	0	
3	0.1	0.1	0.1	0	
4	0.1	0.1	0.1	0	

Table 4.2.20. Assessment of the suitability of the Falcon 6-well plate to explant culture, in terms of: % explants attached after 4/5 days in culture; % explant that grew; mean outgrowth area and mean total cell number, for all cultures of urothelium plated with varying volumes of media.

Vol. Media (ml)/ Well	% Explants Attached after 4/5 Days	% Explants Grew	Mean Outgrowth Area ($mm2^{}$)	Mean Total Cell Number ($\times 10^3$)
0.8 (n=11)	100	100	138.36 ± 54	63.4 ± 27.2
1.0 (n=5)	80	100	54.1 ± 29	55.5 ± 42.4
1.5 (n=5)	60	80	140.5 ± 93.7	126.7 ± 98
2.0 (n=2)	50	50	97	29.4

Table 4.2.21. Assessment of suitability of the Falcon 24-well plate to explant culture, in terms of, % explants attached after 4/5 days in culture; % explants that grew; mean growth area and mean total cell number for all cultures of urothelium explants plated with varying volumes of media and grown for 14 days.

Volume Media (ml)/Well	% Attached after 4/5 Days	% Explants Grew	Mean Growth Area (mm^2)	Mean Total Cell No. ($\times 10^3$)
0.2 (n=8)	62.5	75	47.5 ± 41.5	23.1 ± 21.40
0.25 (n=8)	75	37.5	18.75 ± 7.9	11.69 ± 8.36
0.3 (n=6)	50	50	(n=3) 39.33 ± 11.09	19.09 ± 12.81
0.35 (n=6)	83	83	(n=4) 19.25 ± 3..6	5.58 ± 2.48
0.5 (n=4)	50	0	0 (fibroblastic cells only)	
0.55 (n=4)	100	0	0 (fibroblastic cells only)	

Table 4.2.22. Assessment of suitability of the Nunclon 4-well dish to explant culture, in terms of: % explants attached after 4/5 days in culture; % explants that grew; mean growth area and mean total cell number for all cultures of urothelium explants plated with varying volumes of media and grown for 14 days.

Volume Media (ml)/Well	% Attached after 4/5 Days	% Explants Grew	Mean Growth Area (mm^2)	Mean Total Cell No. ($\times 10^3$)
0.08 (n=4)	0	0	0	
0.1 (n=8)	0	0	0	
0.2 (n=3)	66	33	5	0.99
0.3 (n=3)	33	66	4.5 ± 3.5	1.01 ± 0.08
0.4 (n=3)	66	33	45	12.46
0.5 (n=3)	33	66	135.5 ±129.5	182.21 ± 176.86

Table 4.2.23. Optimum volume of growth media for each culture vessels and the mean growth area and mean total cell number of cultures grown using this volume.

Culture Vessel	Optimum Volume Media (ml)/Well	% Explants Grew	Mean Growth Area (mm^2)	Mean Total Cell No. ($\times 10^3$)
Falcon 6-well	0.77	100	138.36 ± 54	63.4 ± 27.2
Falcon 24-well	0.3	50	36.33 ± 13.6	19.09 ± 12.81
Nunclon 4-well	0.3	66	4.5 ± 3.5	1.01 ± 0.08

Table 4.2.24. Mean growth area and mean total cell number of cultures of urothelium explants plated in the 'gold standard', Nunclon 25 cm^2 , flask and the Falcon 6 Well plate.

Culture Vessel	Volume Media (ml)	Mean Growth Area (mm^2)	Mean Total Cell No. ($\times 10^3$)
Nunclon 25 cm^2	2	294.4	135.6
Falcon 6 Well Plate	0.77	138.4	63.4

Table 4.2.25. Mean outgrowth area and mean total cell number for six cultures of urothelium explants, plated in Nunclon 25 cm^2 flasks with 2 ml of growth media.

Culture No.	Outgrowth Area (mm^2)	Cell Number./mm^2			Mean Cell Number / mm^2	Total Cell Number ($\times 10^3$)
1	594	314	304	304	307 ± 3	182.3 ± 1.8
2	292	566	460	582	536 ± 38	156.5 ± 11.1
3	505	534	402	362	433 ± 52	218.7 ± 26.3
4	676	304	316	286	302 ± 9	204.1 ± 6.1
5	28	410	202	240	284 ± 64	7.9 ± 1.8
6	424	280	306	194	260 ± 34	110.2 ± 14.4
Mean	**420 ± 95**				**353.7 ± 44**	**146.6 ± 32***

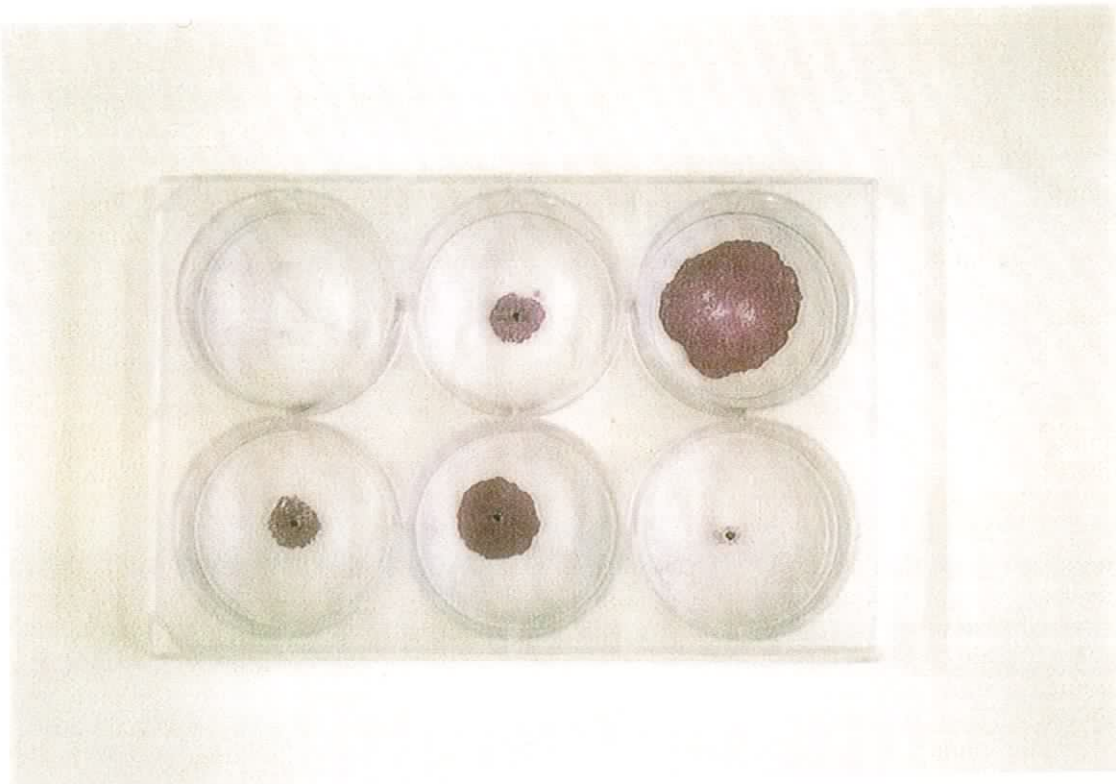


Plate 4.4. Explants of normal urothelium cut according to the original method of mincing the tissue with sterile scissors and then plated singly in each well of a Falcon 6-well plate with 0.8 ml of growth media. The resultant growth areas produced vary greatly in size producing a high standard error on the mean total cell number. This variation and poor growth could be attributed to evaporation of the small volume of media, however this small volume was necessary to allow the explant to attach to the base of the well.

4.2.4 DISCUSSION

Overall, the Falcon 6-well plate produced the best results in terms of attachment, growth and mean total cell number. 100% of the explants plated in the 6-well plate grew, Table 4.2.23. The mean total cell number of the cultures grown from explants plated in this culture plate was greater than for all other culture vessels investigated, however the standard error of the mean was unacceptably high, 43%, Plate 4.4. The high standard error might be due to evaporation of the media in the well. It was necessary to use these small volumes to enable the explant to attach to the base of the well. When the volume was increased to overcome the problem of evaporation, the percentage of explants attached after 4/5 days was reduced. The mean growth area and mean total cell numbers achieved using this vessel are compared to those achieved when explants were cultured in the 'gold standard' culture vessel, the Nunclon 25 cm^2 flask, it can be seen that the Nunclon 25 cm^2 flask produced the optimum mean growth area and mean total cell number with a much lower standard error, 22%, Table 4.2.24.

In conclusion none of the alternative vessels investigated were ideally suited to this culture system. The main problem appeared to be the small volumes of growth media used, which were necessary for attachment of the explant to the base of the well. Evaporation of the media resulted in poor growth and high standard errors. Increasing the volume resulted in poor attachment and therefore unsuccessful culture of the explants. The Falcon 25 cm^2 culture flask remains the best culture vessel for this outgrowth assay.

CHAPTER 5

Culture of Human Oesophageal Endoscopic Biopsies

5.1 INTRODUCTION

Many groups have attempted to establish a system to determine human tumour response to cytotoxic therapy *in vitro* (Hoffman et al., 1984). This group has established a primary culture system (Mothersill et al., 1988) which has successfully been applied to the culture of surgical specimens of squamous cell carcinomas and adenocarcinomas of the oesophagus (Mothersill et al., 1991). However these surgical specimens are taken at oesophagectomy when a patient may have already received chemo- and radiotherapy, and the results of a predictive assay would be too late to assist in treatment planning.

As with Trifillis et al (1993), who attempted to culture normal epithelium from cystoscopic biopsies of human bladder, the goal was to culture human epithelium cells from small amounts of tissue available from, in this case, endoscopic biopsy material.

5.2 MATERIALS AND METHODS

PATIENTS

Fresh endoscopic biopsies were obtained from 10 patients with histologically confirmed oesophageal carcinoma. Three patients had squamous cell carcinomas and 7 had adenocarcinomas.

IN VITRO CULTURE

The biopsies were placed in growth medium, (RPMI 1640, Gibco) and brought immediately to the laboratory for culture. In the laboratory the biopsies were cut into explants of approximately $1mm^3$, placed in a solution of 1ml / 10mg

trypsin/collagenase (Sigma Type IV) and allowed to digest at 37°C for 10 minutes. The digested explants were then plated singly in Nunclon 25cm^2 flask, or each well of a Falcon 6-well plate with specific volume of the supplemented growth medium (RPMI 1640 containing serum, hydrocortisone, insulin and antibiotics), as shown in Table 5.1, and placed in a Forma Scientific incubator at 37°C and 5% CO_2 in air.

Table 5.1 . Volume of growth media added to culture vessel initially and at day 2. The initial small volume allowed the explant to attach to the base of the vessel.

Vessel	Vol. Media Added to Vessel(ml)	
	Day 1	Day 2
Nunclon 25cm^2 Flask	1.0	1.0
	0.8	1.2
Falcon 6 Well Plate	0.4	0.4
	0.6	0.2

The explants became attached to the surface of the culture flasks after approximately 24 hours, and after 2-3 days in culture epithelium like cells could be seen migrating from the explant. A total of 14 days in culture was allowed after which time the cultures were removed from the incubator, fixed in 10% buffered formalin and immunocytochemical examination carried out.

IMMUNOCYTOCHEMICAL ANALYSIS

The epithelial nature of the cells was characterised using a universal cytokeratin antibody. The population of proliferating cells was identified using an antibody to nuclear membrane antigen, Ki67. Labelling by all antibodies was developed using peroxidase-conjugated anti-mouse IgG

(Vectastain, ABC kit) and diaminobenzaldehyde (DAB) (see Section 3.8).

5.3 RESULTS

Nine of 10 biopsies grew successfully *in vitro*. Table 5.2[1] shows the mean growth area and mean total cell number achieved from explants cultured in varying conditions as described in Table 5.1. The optimum conditions for growth were achieved using the Nunclon 25 cm^2 flask, with an initial volume of growth media of 1ml and a subsequent volume of 1ml added on day 2.

The epithelial nature of the cells constituting the outgrowth was confirmed immunocytochemically, as shown in Plate 5.1. The population of proliferating cells was also identified, Plate 5.2. The malignant nature of the cultured cells from the biopsies can be characterised by the variation in the cell size and shape, and lack of orientation of the cells, as shown in Plate 5.3.

Table 5.2[1]. Mean growth areas and mean total cell number for explants plated in Falcon 6 Well plates and Nunclon 25 cm flasks with varying initial and additional volumes of media being added to each culture

Culture Vessel	Vol.of Growth Media		Mean Growth Area	Mean Total Cell
	Initial	Day 2	(mm^2)	Number ($\times 10^3$)
Falcon 6-well Plate	0.4	0.4	106 ± 106	51.1 ± 51.1
Falcon 6-well Plate	0.6	0.2	25 ± 20	4.6 ± 4
Nunclon 25 cm^2 Flask	1.0	1.0	618 ± 36	315.4 ± 15
Nunclon 25 cm^2 Flask	0.8	0.8	258 ± 108	116.4 ± 51.9

[1]The data shown in Table 5.2 is a summary of the data presented in appendix 1.

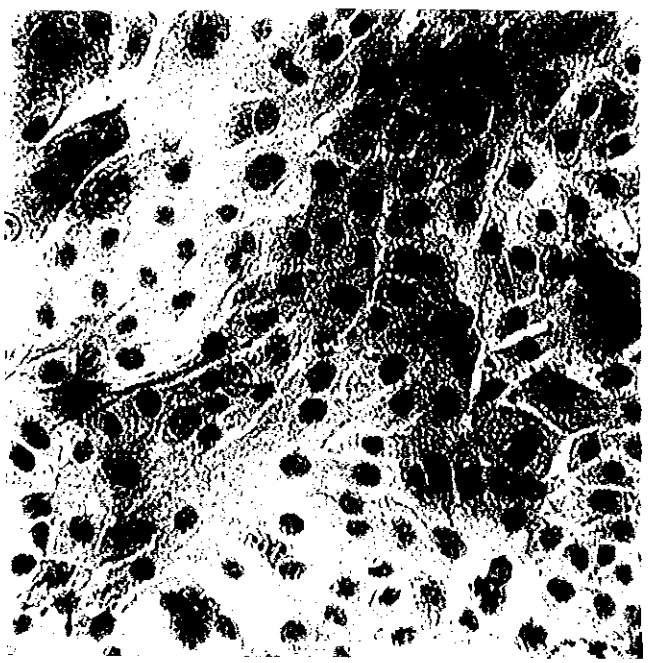


Plate 5.1. Cellular outgrowth from an oesophageal endoscopic biopsy, cultured for 14 days at 37^{o}C, stained for expression of cytokeratin. Note the dark reaction product in the cytoplasm of the cellular outgrowth.

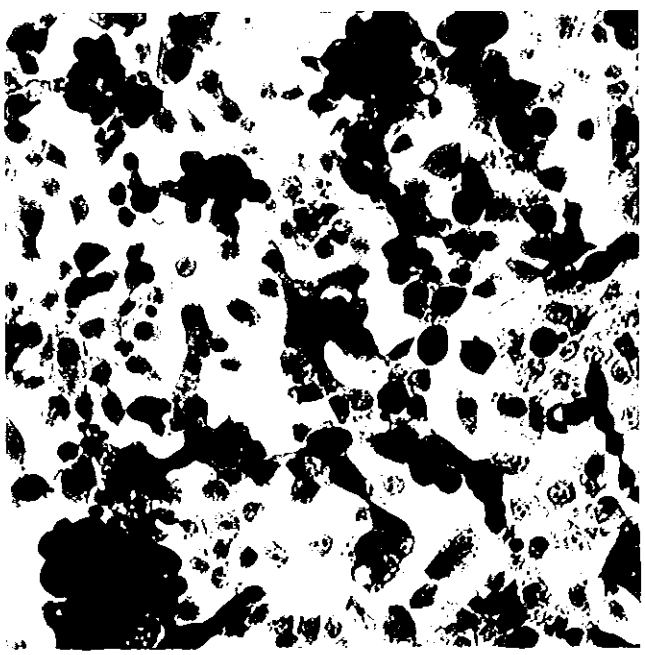


Plate 5.2. Dark staining (Ki67 positive) focus on a cellular outgrowth from an oesophageal endoscopic biopsy.

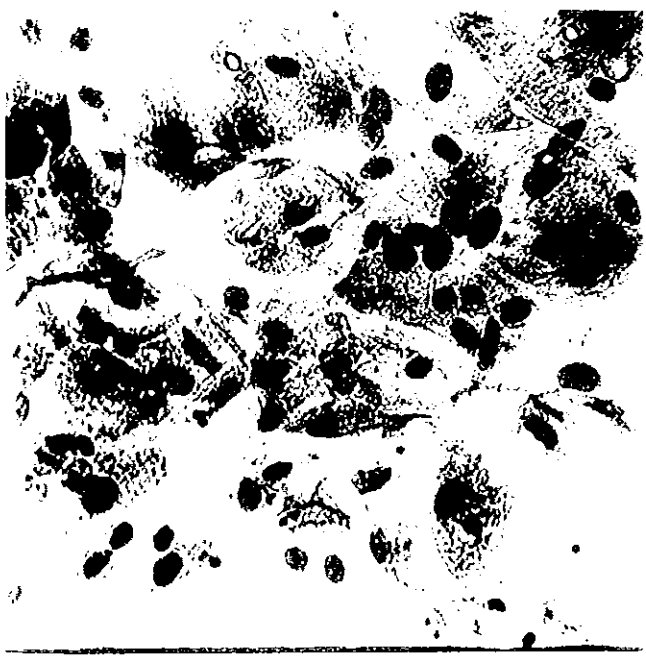


Plate 5.3. Characterization of malignant nature of cellular outgrowth from oesophageal endoscopic biopsy. Note the variation in the cell size and shape, and lack of orientation of the cells.

5.4 Discussion

Due to the small size of the explant of the biopsy attachment is enhanced with a smaller initial volume of growth media. this volume was increased on day 2 to the standard optimum volume for that culture vessel: 2 ml for the Nunclon 25 cm^2 flask and 0.8 ml for the Falcon 6-well plate (see Section 4.2). This increase in volume sustained the culture over the 14 day growth period.

From these results it is evident that cultures of human tumour oesophageal epithelium can be established from endoscopic biopsies.

This system has previously shown its potential as a tool to measure the response of tumours to cytotoxic agents (Mothersill et al. 1993), however the ability to culture these pre-treatment endoscopic biopsies enhances the clinical relevance of the system as an individual predictor of patient response to cytotoxic therapy.

CHAPTER 6

Potential Indicators of Response of Oesophageal Carcinoma Tumours to Treatment

6.1 Introduction

The prognosis of a patient with oesophageal carcinoma is worse than for other types of gastrointestinal tumours due to poor general condition in many patients and the rapidity of tumour progression (Masanori, 1992). The response of patients to combined chemo- and radiotherapy varies widely; in some it results in complete tumour regression, whereas others show little histological response. To improve the response rate to treatment and prognosis of these patients the selection of treatment is very important not only in terms of administration of active agents but to avoid the adverse effects of ineffective agents.

Cisplatin and 5-flurouracil (5-FU) have known activity in oesophageal carcinoma, and appear to act as radiosensitisers, but a combination of chemo- and radiotherapy is expensive and potentially toxic. Furthermore, it entails a delay of 6-8 weeks before surgery. The ability to predict the response of individual tumours to chemo- and radiotherapy prior to treatment would allow selection of patients likely to respond and avoid administration to those resistant.

The expression of epidermal growth factor receptor (EGFr) is thought to be of prognostic significance in oesophageal cancer (Mukaida et al., 1990; Yano et al., 1990) and may be indicative of response to chemo-radiotherapy (Hickey et al., 1994). Proliferation, as measured by Ki67, has been shown to correlate with tumour grade in breast tumours (Sullivan et al. 1993).

The *in vitro* predictive testing system proposed here entails culturing pre-treatment endoscopic biopsies and treating them with clinical equivalent doses of chemo- and radiotherapy. The individual tumour response is estimated by measuring the % growth

inhibition of the treated cultures relative to the untreated control. The expression of epidermal growth factor receptor (EGFr) and Ki67 was also estimated using this system. A correlation was sought between levels of expression of EGFr and Ki67, and tumour grade and response to treatment.

6.2 MATERIALS AND METHODS

BIOPSIES

Fresh endoscopic biopsies were obtained from 14 patients with suspected oesophageal carcinoma. These included 5 squamous cell carcinomas (SCC) and 9 adenocarcinomas. The malignant nature of the tissue was confirmed by histological examination.

IN VITRO CULTURE OF BIOPSIES

The primary explant culture assay has been described in full in Section 3.1. Briefly, endoscopic biopsies taken from the patient were placed in growth medium (RPMI 1640, Gibco) at ambient temperature and brought immediately to the laboratory for culture. In the laboratory the biopsies were cut, using scissors and forceps, into fragments of approximately 1 mm^3 and placed in a solution of weak trypsin/collagenase at a concentration of 1ml / 10 mg, for 10 minutes at 37°C. After digestion the explants were plated singly in Nunclon 25 cm^2 tissue culture flasks with 2 ml of supplemented growth medium (RPMI -1640 containing serum, hydrocortisone, insulin and antibiotics). Cultures were then placed in a CO_2 incubator at 37°C. After 72 hours incubation the cultures were treated with clinically equivalent doses of chemo- and radiotherapy, and

returned to the incubator immediately. After a further 11 days incubation the cultures were fixed and immunocytochemically stained.

IN VITRO CHEMOTHERAPY AND RADIATION TREATMENT

Depending on the size of the tissue and the amount of cultures set up, the cultures were divided into four groups according to the treatment to be given, priority being given to the radiation group. These groups were as follows:

GROUP	TREATMENT
Control	None
Chemotherapy	Cisplatin & 5FU
Radiation	A single dose of 2 Gy
Combination	Chemotherapy & Radiation

After 72 hours of undisturbed growth the cultures were treated according to the grouping above. The control group remained untreated.

Chemotherapeutic drugs were diluted from available commercial preparations, using growth medium, to give clinical equivalent doses when the drug solution was added to 2 ml of medium in the culture flask. The clinical dose of 5-Flurouracil was 15 mg/kg and the equivalent dose when added to 2 ml was 30 μg. The clinical dose of Cisplatin was 75 mg/m^2 and the equivalent dose varied according to the individual patient surface area, e.g. for a patient of surface area 1.74 m^2 a clinical dose of 130.5 mg of Cisplatin would be administered and the equivalent dose in 2 ml of growth media would be 4 μg. Table 6.1. Radiation doses were given using a Cobalt-60 teletherapy unit delivering 2 Gy/min. Where a combination of chemotherapy and radiation were investigated, the

radiation dose was administered within an hour of addition of the chemotherapeutic agents to the medium.

Table 6.1. Clinical equivalent dose calculated from weight of each patient, converted to litres and dose estimated for 2 ml of growth media.

Treatment		Dose
5-Flurouracil		15mg/ Kg
		15mg/ L
	»	30µg/ 2ml
Cisplatin		75mg/ m^2
e.g. Patient 1: weight = 63.5kg. height = 172.2 cm		
» Surface area of 1.74 m^2		130.5mg/ 1.74m^2
	or	130.5mg/63.5Kg
		130.5mg/ 63.5l.
	»	4µg/ 2ml
This calculation was repeated for each individual patient.		

IMMUNOCYTOCHEMISTRY

The population of proliferating cells was determined using an antibody to nuclear membrane antigen, Ki-67 (DAKO). The epithelial nature of the cells constituting the outgrowth was examined using a universal cytokeratin antibody (Amersham Bucks., UK.). Epidermal growth factor receptor (EGFr) was evaluated semi-quantitatively using the monoclonal antibody against EGFr, 2E9 (Monosan). Labelling by all the antibodies was developed using peroxidase-conjugated anti-mouse IgG (Vectastain, ABC kit) and the chromagen diaminobenzidine (DAB). Ki67 was measured as a percentage of positive cells in a population of cells along a cross section of the growth area (see Section 3.10). EGFr was measured semi-quantitatively and graded as follows: 0-25% positive cells = 2, 25-50% positive cells = 4, > 50% positive cells = 6.

EVALUATION OF PATIENT RESPONSE TO TREATMENT

The response of each patient to treatment was measured as a percentage growth inhibition (%GI) of the treated cultures relative to the untreated controls. Increasing %GI indicated increasing sensitivity. This percentage was estimated as described in Section 3.10.

6.3 RESULTS

Clinical Data

Table 6.2 shows the clinical data for all the patients from whom biopsies were taken. These data includes sex, age, histological type, TNM stage, status of margins, differentiation (as defined by the histopathologist), treatment received, follow up time and current status. Of the 14 biopsies cultured, 9 (64%) were histologicaly confirmed to be adenocarcinoma and 5 (36%), to be squamous cell carcinoma (SCC). Of the 9 patients with adenocarcinomas 55% were treated with surgery alone, 1 patient (11%) received a combination of chemo- and radiotherapy and 2 patients (22%) were treated with primary surgery followed by a combination of chemo- and radiotherapy (unknown treatment for 1 patient). The follow-up time ranges from 17 - 0.1 months and currently of the 9 patients with adenocarcinoma 5 (55%) are still alive, while 2 (40%) of the 5 patients with squamous cell carcinoma are alive.

Culture of Endoscopic Biopsies

Epithelial cultures were produced from explants of the biopsies cultured in Nunclon 25 cm^2 flasks with 2 ml of supplemented growth media. The epithelial nature of the cells was confirmed immunocytochemically using a cytokeratin antibody, see Plate 6.1. The cultures also exhibited a typical tumour morphology, with huge variations in the cell size and shape, and lack of orientation of the cells, Plate 6.2.

Growth Inhibition

Tables 6.3 and 6.4 show the *in vitro* response of each biopsy to treatment grouped according to histology. These tables illustrate the huge variation in patient response to treatment. In the case of the adenocarcinomas 55% of the tumours were sensitive to a single dose of 2 Gy, that is to say the % growth inhibition (%GI(2Gy)) in these cultures after irradiation was greater than the mean %GI for all the cultures treated with a single dose of 2 Gy (mean %GI(2Gy) = 47%). The %GI(2Gy) of the sensitive tumours was significantly different from that of the resistant tumours ($p< 0.001$). Of the 5 squamous cell carcinoma tumour biopsies cultured, only one appeared to be sensitive to irradiation, with a %GI(2Gy) greater than the mean %GI(2Gy) (mean %GI = 28).

Due to the small size of the biopsies it was not always possible in all cases to establish enough cultures to allow for the investigation of response to chemotherapy, however for 3 adenocarcinomas and 3 SCC this was possible. Two of the three adenocarcinomas appeared to be sensitive to chemotherapy alone and a combination of chemo- and radiotherapy, Table 6.3. One SCC appeared to be sensitive to chemotherapy alone *in vitro*, but when treated with a combination of chemo- and radiotherapy another tumour exhibited marked sensitivity, Table 6.4.

Table 6.2. Clinical data of the patients from which the biopsies were taken. This information includes sex, age, histological type, TNM, stage, differentiation, status of margins, treatment received, follow up time and current status. Nine of the biopsies were adenocarcinomas and 5 were squamous cell carcinomas (SCC).

Patient	Sex	Age	Tumour Type	T	N	M	Stage	Differentiation	Resection Margins	Treatment	Follow-up Time	Current Status
1	M	75	Adeno				4	NR	NR		2	Alive
2	F	76	Adeno				4	moderate	NR	chemoRt	0.1	Dead
3	M	70	Adeno	3	1	0	3	poor	free	surgery		Dead
4	M	63	Adeno	0	0	0	0	moderate	free	surgery + chemoRt	17	alive
5	M	72	Adeno					moderate				alive
6	M	72	Adeno	3	1	0	3	moderate	free	surgery	5	dead
7	F	67	Adeno	3	0	0	2a	moderate	free	surgery + chemoRt	1	alive
8	M	64	Adeno	3	1	0	3		free	surgery	10	alive
9	M	69	Adeno	3	1	1	4	moderate	free	surgery	0.25	dead
10	F	78	SCC	3	1	0	3	moderate	free	surgery	5	dead
11	F	63	SCC	3	0	0	3	moderate	free	surgery		dead
12	M	78	SCC	1	0	0	1	moderate	free	surgery	0	dead
13	F	74	SCC			1	4	moderate		chemoRt	14	alive
14	F	34	SCC	3	0	0	2a	moderate	free	surgery	15	alive

NR = no record
chemoRt = chemo- and radiotherapy

Table 6.3[1]. Response of adenocarcinoma tumour biopsies to treatment. Biopsies taken from 9 patients with adenocarcinoma of the oesophagus, were cultured *in vitro* and treated with clinical equivalent doses of radiation, chemotherapy or a combination of both. These patients went on to receive treatment. The follow up time and current status of each patient is presented.

Patient	2 Gy	Response to Cis. + 5FU	Treatment (%GI.) 2Gy+Cis. +5FU	Treatment	Follow up time(months)	Status
1	0	NT	NT		2	alive
2	0	NT	NT	chemoRt	0.1	dead
3	60	NT	NT	surgery		dead
4	56	NT	NT	surgery + chemoRt	17	Alive
5	85	NT	NT	surgery		alive
6	44	NT	NT	surgery	5	dead
7	100	100	100	surgery + chemoRt	1	alive
8	0	0	0	surgery	10	alive
9	77	78	97	surgery	0.25	dead
Mean	47 ± 13	59 ± 30	66 ± 33		4	

Table 6.4. Response of SCC tumour biopsies to treatment. Biopsies taken from patients with Squamous cell carcinoma of the oesophagus were cultured *in vitro* and treated with clinical equivalent doses of radiation, chemotherapy or a combination of both. These patients went on to receive treatment, their follow up time and current status is presented.

Patient	2 Gy	Response to Cis. + 5FU	Treatment (%GI.) 2Gy+Cis+5FU	Treatment	Follow-up time (months)	Current Status
1	0	NT	NT	surgery	5	dead
2	100	NT	NT	surgery		dead
3	18	83	99	surgery	0	dead
4	0	0	0	chemoRt	12	alive
5	23	8	100	surgery	12	alive
Mean	28 ± 18	30 ± 26	66 ± 33		5	

5FU = 5-Flurouracil; Cis = cisplatin; NT = no treatment administered

[1]The mean growth areas cultured from explants of the tumour biopsies is shown for each patient in appendix 2.

Growth Inhibition and Current Status

No patient received pre- or post-operative radiotherapy but since only 6 biopsy specimens were large enough to investigate the use of chemotherapy alone or in combination with radiotherapy, and of these 6 only 2 patients went on to receive chemo-radiotherapy, it was not possible to statistically determine whether the *in vitro* results were predictive. Of the 2 patients who received chemo-radiotherapy both are alive. The *in vitro* predictive results for these 2 patients indicated that one tumour was sensitive to a combination of chemo- and radiotherapy while, the other was resistant.

In Tables 6.5 and 6.6 the current status of the patients is compared to the mean % growth inhibition after a single dose of 2 Gy, to determine whether this parameter could predict for patient outcome irrespective of treatment received. There was no significant difference found between the % growth inhibition of those patients alive and those dead, for either histological type.

Table 6.5. Current status and mean %GI(2Gy) of tumour biopsies taken from patients with adenocarcinoma of the oesophagus. No significant difference was found between the mean % GI after 2 Gy of those patients alive or dead.

Current Status	n	Mean %GI(2Gy)
Alive	5	48
Dead	4	45
P-value		**0.46**

Table 6.6. Current status and %GI(2Gy) of tumour biopsies taken from patients with SCC of the oesophagus. No significant difference was found between the mean % GI after 2 Gy of those patientalive or dead.

Current Status	n	Mean %GI(2Gy)
Alive	2	11
Dead	3	39
P-value		**0.27**

Ki67, EGFr and Stage

Positive staining of Ki-67 was localised in the nucleus. Plate 6.3, while EGFr immunoreactivity was observed predominantly on the cell membrane. Plate 6.4. Figure 6.1 shows the expression of Ki67 and EGFr, measured in control cultures of each adenocarcinoma, plotted against stage. There is a positive correlation between expression of both these proteins and the stage of the tumour, this correlation is stronger for EGFr, correlation coefficient (corr. coeff.) = 0.87, than for Ki67, corr. coeff. = 0.64. No correlation was found between expression of Ki67 and EGFr measured in cultures of squamous cell carcinoma tumours, and tumour stage. This result will obviously be affected by the very small patient number.

EGFr, Ki67 - Indicators of Response

ADENOCARCINOMA

Table 6.7 shows the expression of Ki67 and EGFr for each adenocarcinoma patient and the *in vitro* response of each patient to a single dose of 2 Gy, measured in terms of %GI. In Figures 6.2 and 6.3 the expression of Ki67 and EGFr, measured in control cultures of adenocarcinoma tumours, was plotted against the %GI (2Gy) in cultures treated with a single dose of 2 Gy. A strong negative correlation was found between the expression of both proteins and radiosensitivity after 2 Gy, this correlation was -0.78 for Ki67 and -1 for EGFr. The expression of both Ki67 and EGFr for each patient is plotted against %GI(2Gy) in Figure 6.4.

Cultures with expression of Ki67 < 12% (the mean) had an average %GI(2Gy) of 69% (radiosensitive) which was significantly higher than the value from cultures with

expression of Ki67 >12%(the mean) (%GI(2Gy) = 19; P= 0.02). Table 6.8. Cultures expressing EGFr at a level less than or equal to the mean, 4 (4 = 25-50%) had an average %GI(2Gy) of 72% (radiosensitive) which was significantly higher than the value from cultures expressing EGFr greater than the mean (%GI(2 Gy) = 0 (radio-resistant) : P=0.02). Table 6.7.

Table 6.7. Response of adenocarcinoma tumours to treatment *in vitro*. Biopsies taken from patients were cultured as described, treated after 3 days in culture and the response to treatment estimated after a total of 14 days in culture. Expression of Ki67 and EGFr was measured immunocytochemically in the control cultures. Expression of EGFr was measured semi-quantitatively: % Positive Cells: 2 = <25% , 4 = 25 - 50%, 6 = >50%

PATIENTS	%Ki67	EGFr	% Growth Inhibition after 2 Gy
1	4	0	100
2	3	0	90
3	6	4	60
4	6	4	44
5	12	NR	56
6	16	NR	77
7	23	6	0
8	23	6	0
9	15	6	0
Mean	**12 ± 3**	**4 ±1**	**47 ± 13**

Figure 6.4. Expression of Ki-67 and EGFr measured in the control cultures of each adenocarcinoma biopsies (n=7) and the corresponding inhibition of growth in the cultured biopsies treated with 2 Gy.

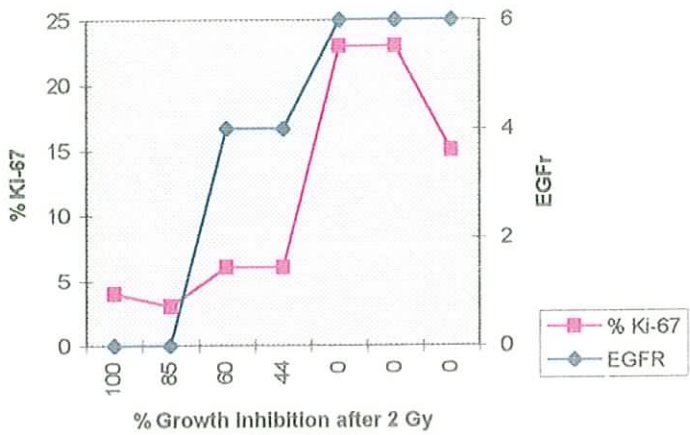


Table 6.8. % Ki67 positive cells and expression of EGFr as indicators of radiosensitivity. The % GI(2Gy) of tumours expressing levels of Ki67 and EGFr less than the mean is significantly higher than % GI at 2 Gy of those tumours expressing levels of Ki67 and EGFr greater than the mean.

	% GI(2 Gy)	P-value
% Ki67< mean	69	
% Ki67> mean	19	0.02
EGFr < mean	72	
EGFr > mean	0	0.02

SQUAMOUS CELL CARCINOMA

Table 6.9 shows the expression of Ki67 and EGFr measured immunocytochemically in control cultures of SCC biopsies and the *in vitro* response of those biopsies to a single dose of 2 Gy, measured in terms of growth inhibition (%GI). No correlation was found between expression of Ki67 and *in vitro* radiosensitivity (correlation coefficient = -0.1). A very weak correlation was found between expression of EGFr and *in vitro* radiosensitivity (correlation coefficient = -0.4).

Table 6.9. Response of SCC tumours to treatment *in vitro*. Biopsies taken from patients were cultured as described, treated after 3 days in culture and the response to treatment estimated after a total of 14 days in culture. Expression of Ki67 and EGFr was measured immunocytochemically in the control cultures. Expression of EGFr was measured semi-quantitatively: % Positive Cells: 2 = <25% , 4 = 25 - 50%, 6 = >50%

PATIENTS	%Ki67	EGFr	% Growth Inhibition after 2 Gy
1	NR	6	0
2	9	NR	100
3	36	4	18
4	5	6	0
5	1	6	23
Mean	**13**	**5.5**	**28 ± 18**

NR = no record

Ki67, EGFr and Patient Outcome

No significant difference was found between the level of expression of Ki67 or EGFr in cultures of biopsies of those patients presently alive, and the level of expression in cultures of biopsies of those patients dead. This was true for both adenocarcinoma and squamous cell carcinoma, Tables 6.10-6.13. Expression of Ki67 and EGFr was not predictive of patient outcome.

Table 6.10. % Ki67 positive cells as an indicator of patient outcome. **Adenocarcinoma**: Mean %Ki67 = 12%

Current Status	n	Mean % Ki67
Alive	5	13
Dead	4	11
P-value		**0.35**

Table 6.11. Expression of EGFr as indicator of patient outcome. **Adenocarcinoma**: Mean positive EGFr = 4

Current Status	n	Mean EGFr
Alive	4	3
Dead	3	5
P-value		**0.23**

Table 6.12. % Ki67 positive cells as an indicator of patient outcome. **SCC**: Mean %Ki67=13%

Current Status	n	Mean % Ki67
Alive	2	3
Dead	2	22
P-value		**0.21**

Table 6.13. Expression of EGFr as an indicator of patient outcome. **SCC**; Mean positive EGFr=5

Current Status	n	Mean EGFr
Alive	2	6
Dead	2	5
P-value		**0.25**

Figure 6.1. Correlation between in vitro expression of Ki67and EGFR, and stage of adenocarcinoma tumours.

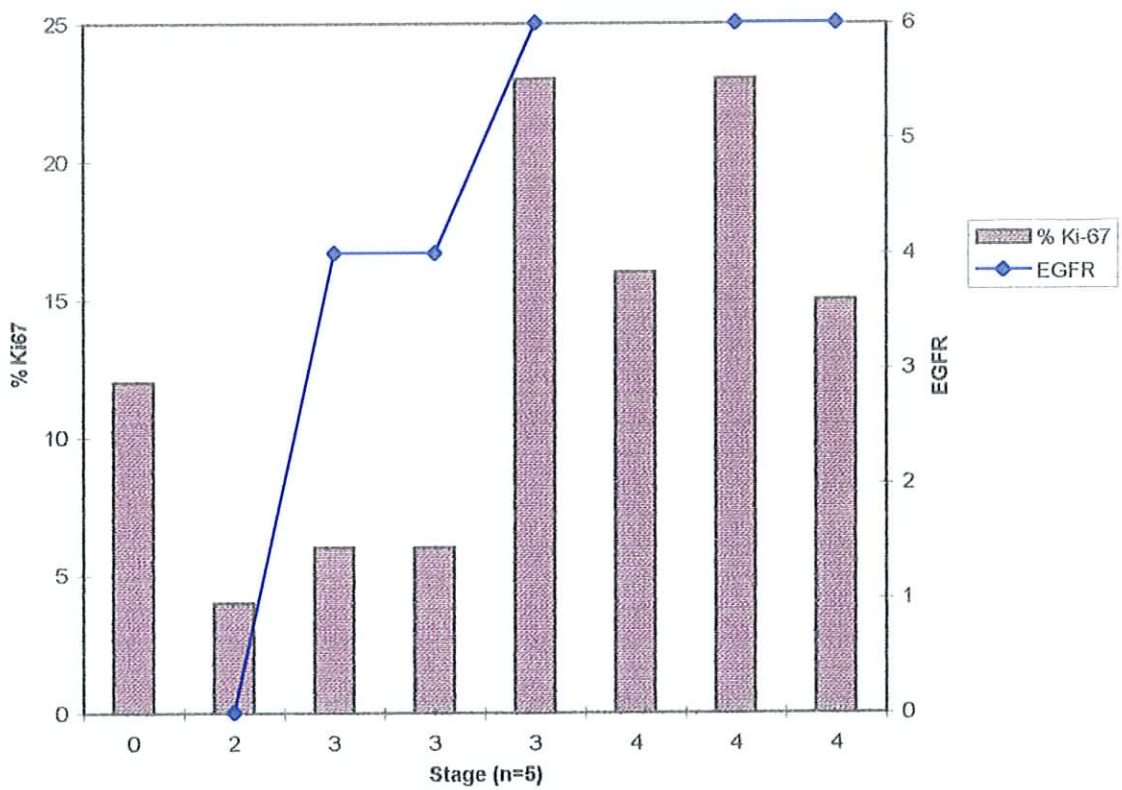


Figure 6.2. Expression of % Ki-67 measured immunocytochemically correlates with in vitro radioresistance (correl. coeff. = -0.78).

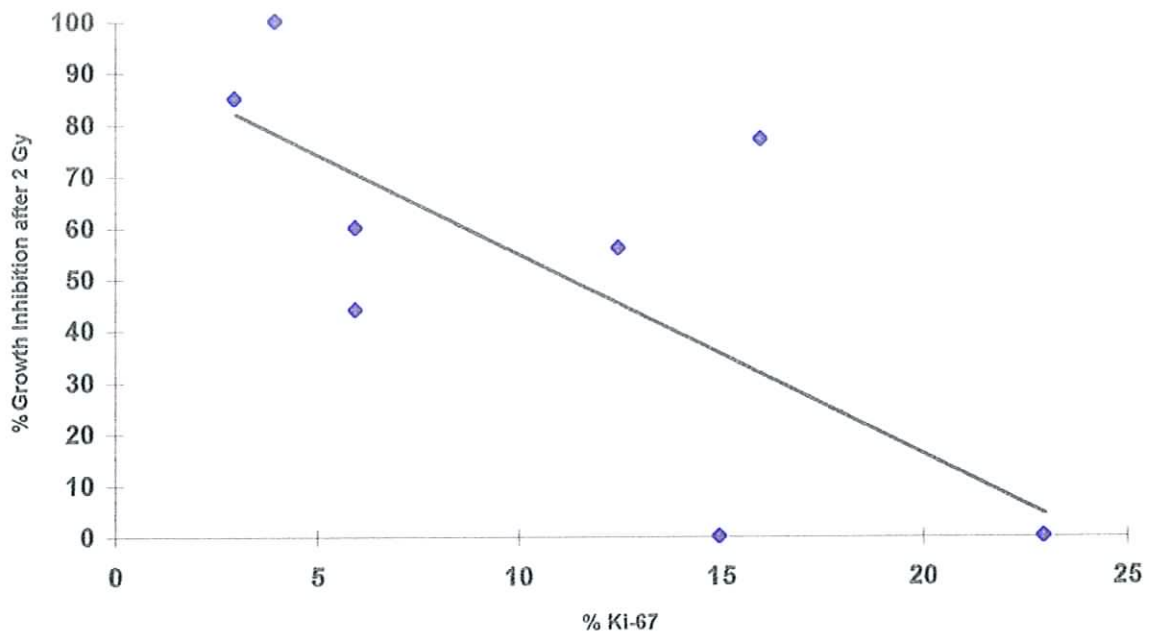
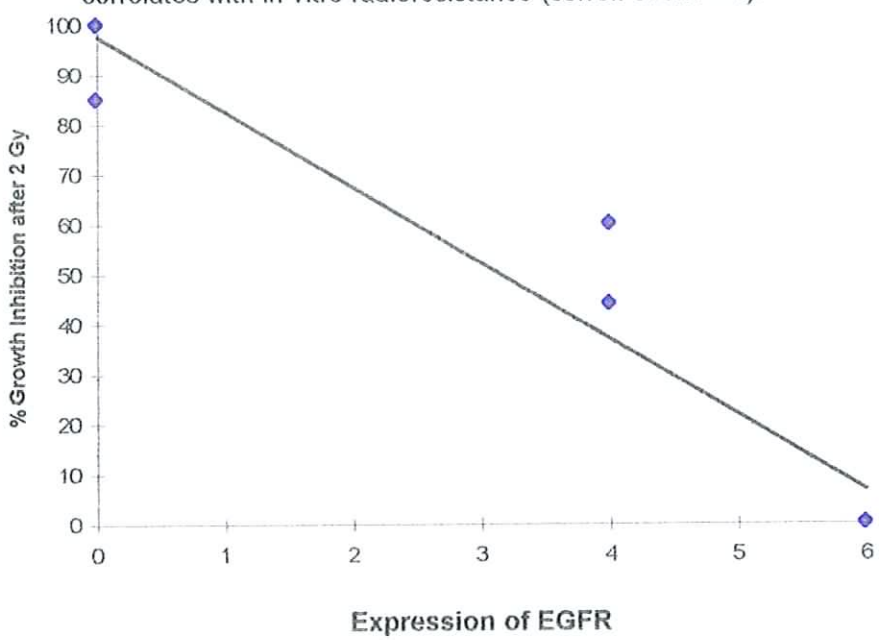


Figure 6.3. Expression of EGFR measured immunocytochemically correlates with in vitro radioresistance (correl. coeff. =-1).



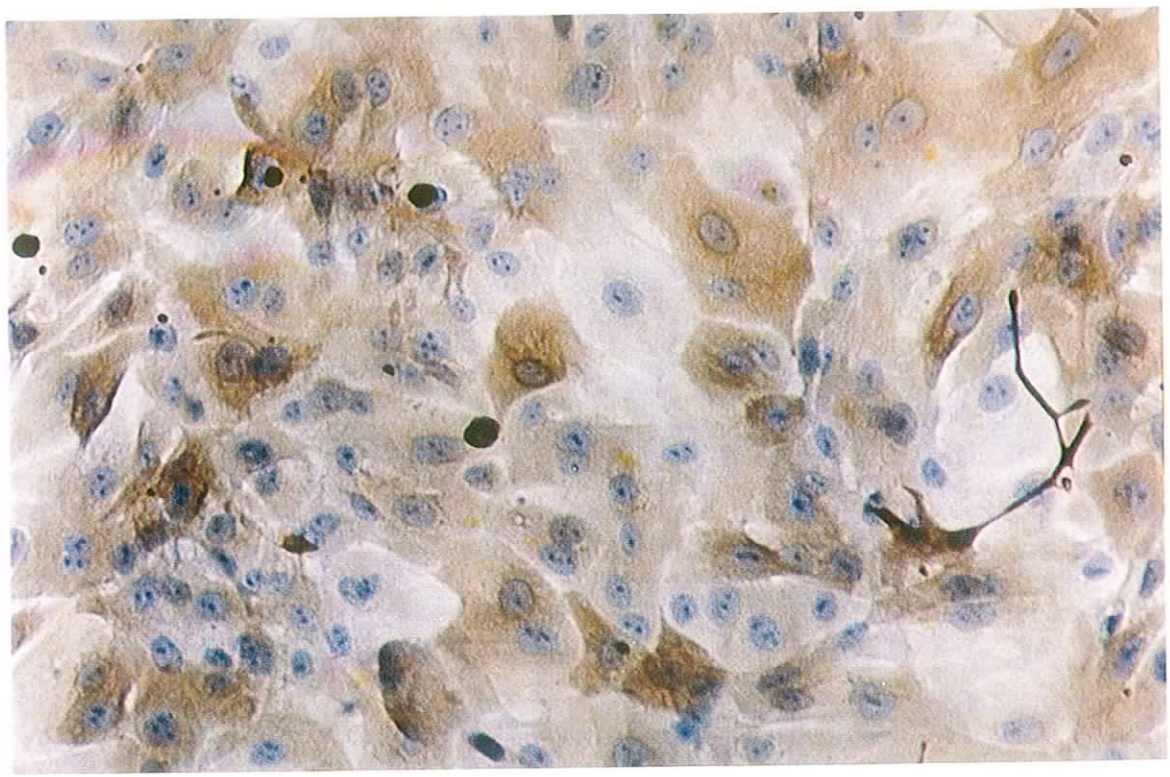


Plate 6.1. Epithelial nature of the cells was confirmed immunocytochemically; control culture of oesophageal endoscopic biopsy was stained using a cytokeratin antibody. Positive brown staining is localised in the cytoplasm of cells.

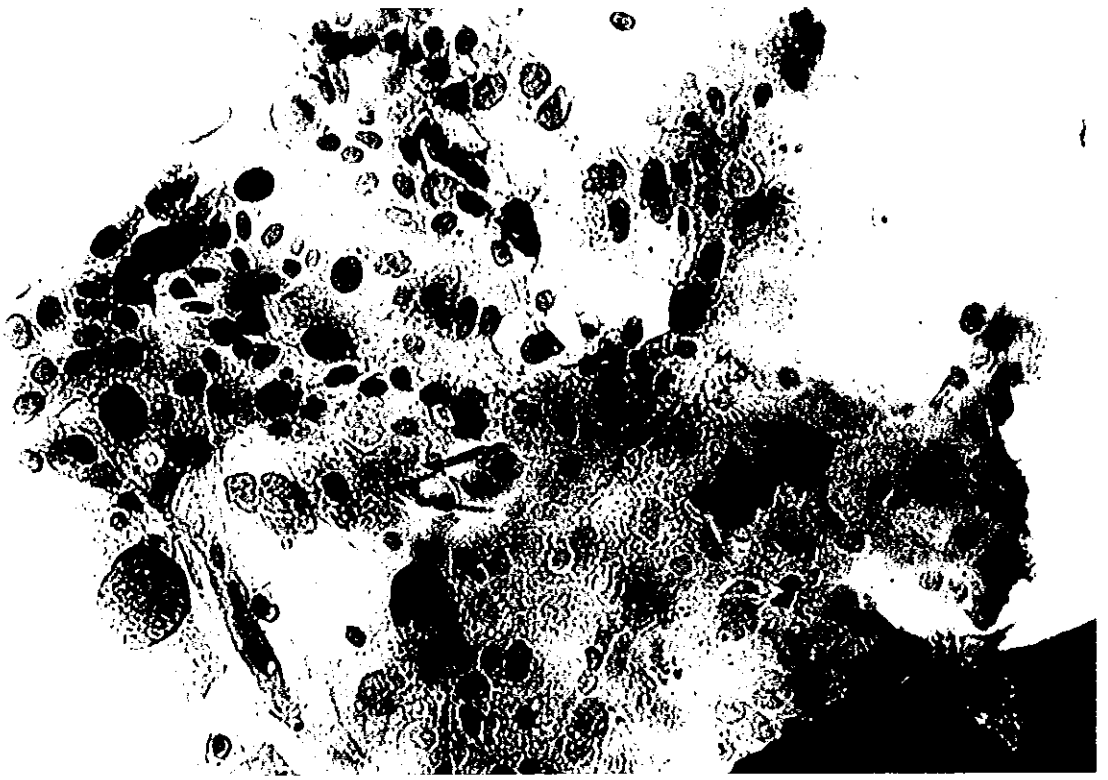


Plate 6.2. Control culture of oesophageal endoscopic biopsy specimen displaying a morphology characteristic of malignant cells; variation in cell size and shape and lack of orientation.

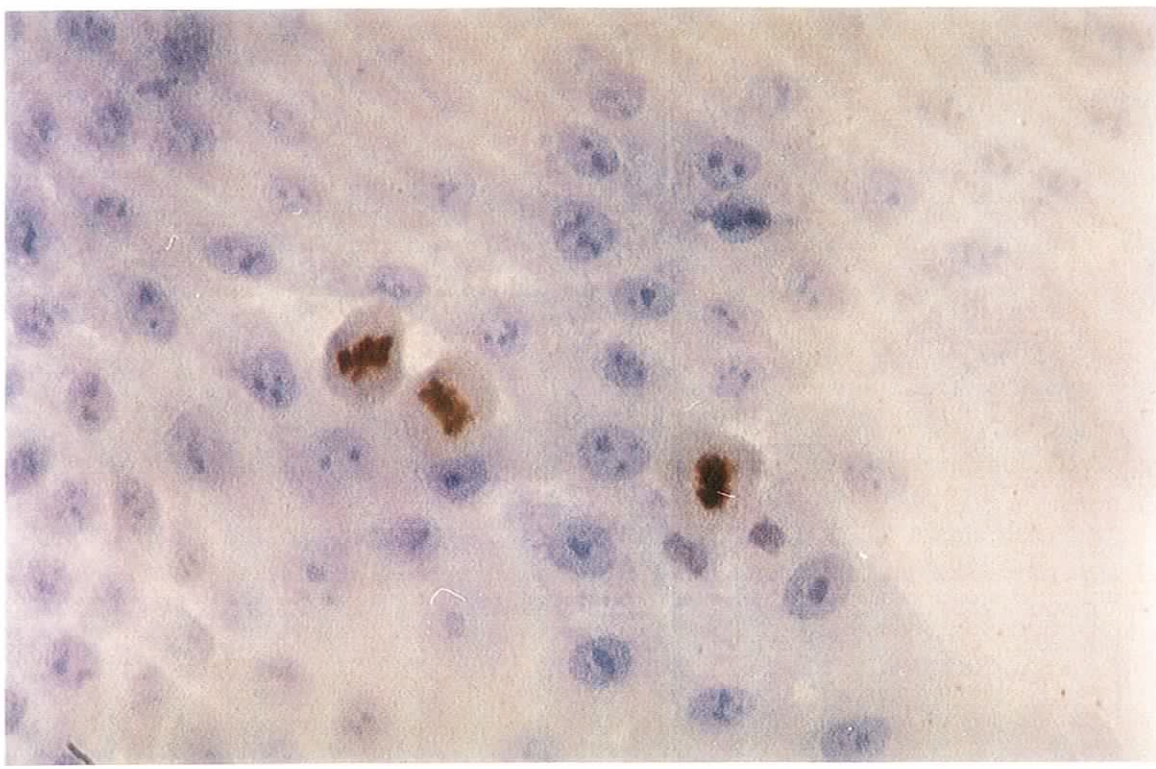


Plate 6.3. Outgrowth from a cultured oesophageal endoscopic biopsies was examined immunocytochemically for expression of Ki67. Brown positive staining is localised in the nucleus of the cells.

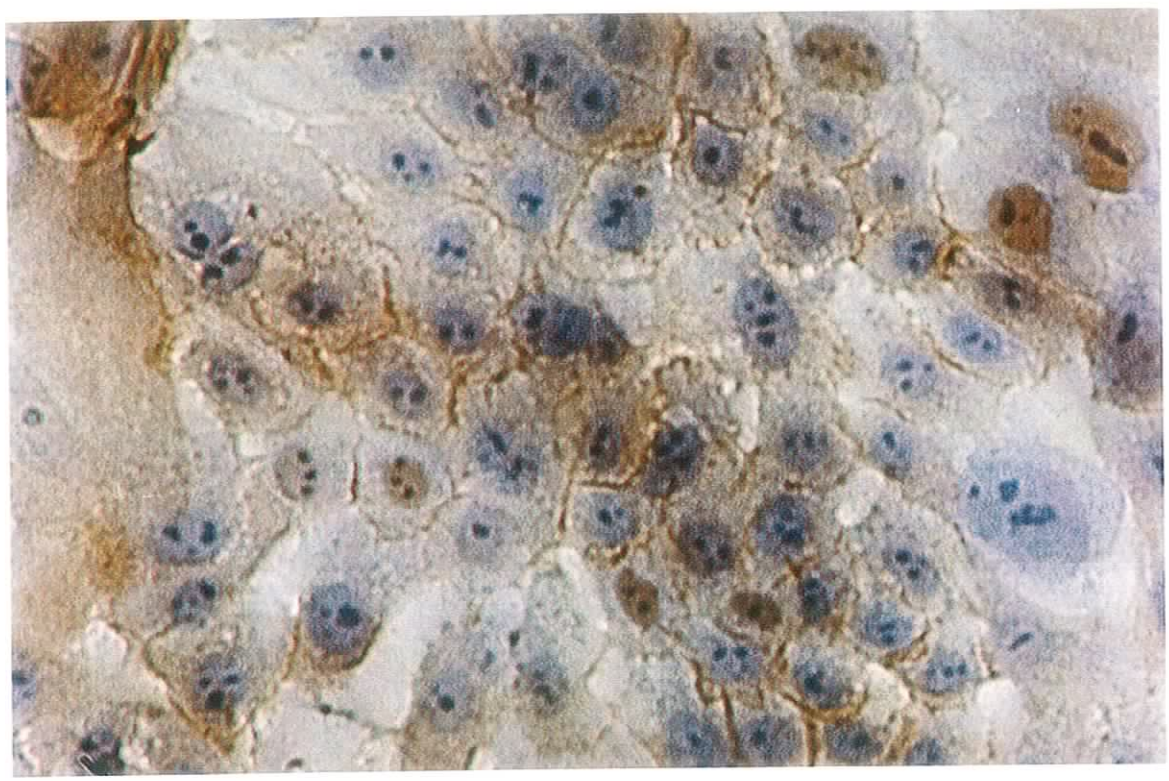


Plate 6.4. Outgrowth from a cultured oesophageal endoscopic biopsies was examined immunocytochemically for expression of EGFr (2E9 antibody). Brown positive staining is localised on the cell membrane and in the cytoplasm of the cells.

6.4 DISCUSSION

Using the Mothersill outgrowth assay it was possible to establish epithelial cultures from oesophageal endoscopic biopsies and obtain *in vitro* values for growth inhibition after treatment with irradiation and clinical equivalent doses of chemotherapy. However, due to the small size of the biopsies it was only possible to establish enough cultures for treatment with chemotherapeutic drugs in a total of 6 cases. The very small patient number illustrates the difficulty in obtaining fresh human specimens for a study such as this.

The variation in the *in vitro* response of patients to treatment, reflects the *in vivo* situation where a patient may have no response, a partial response or a complete response to radiotherapy. In the case of the adenocarcinoma tumours cultured 45% were resistant (%GI(2Gy) < mean) to a single dose of 2 Gy, while 55% were sensitive (%GI(2Gy)>mean). Some studies have shown a relationship between the response of a tumour to radiotherapy *in vivo* and the 5-year survival. Wang et al. (1989) found that those patients who had a complete response to radiotherapy had an improved 5-year survival (50% of patients) than those patients with a partial response (31% of patients with 5-year survival). *In vitro* studies using the Courtenay Mills clonogenic assay have also found a correlation with the response of a tumour to radiotherapy and the survival rates of patients from whom the tumours were taken. West et al. (1993) in a study of carcinoma of the cervix, found that patients with radio-resistant tumours *in vitro*, measured by surviving fraction at 2 Gy, had a significantly lower 3 year survival than those with radiosensitive tumours *in vitro*. In this study *in vitro* radiosensitivity,

measured by %GI(2Gy) did not correlate with current status of patients, but this is not surprising since none of the patients received radiotherapy alone and for those patients that received chemo-radiotherapy, *in vitro* results were obtained for only 2 patients.

For patients treated with chemotherapy and radiation combined with surgery, some studies have shown an improved survival for a subset of patients who had a complete response to chemo-radiotherapy, that is those patients with no cancer in the resected oesophagus. In a study at the University of Michigan patients were treated with cisplatin, 5-FU and Velban, along with concurrent radiotherapy and followed by oesophagectomy (Forastiere et al., 1990). Survival at 5 years was 60% for patients with no cancer in the resected specimen. These results have been supported by a more recent study, where the preoperative regime consisted of cisplatin, 5-FU and concurrent radiotherapy. The 2-year survival was 52% for those patients with a pathologic complete response (Forastiere et al., 1994). If these patients could be identified prior to commencement of treatment, those resistant patients could be considered for alternative treatment and the administration of ineffective agents could be avoided. Using this outgrowth assay it was possible to determine the *in vitro* response of individual patients to treatment, however due to small specimen size it was not always possible to determine response to a whole range of treatment modalities. If larger specimens could be obtained it would be possible to measure the response to these different treatment modalities that were actually administered to the patients, and thus determine the predictive potential of this assay.

The expression of EGFr and Ki67 were measured immunocytochemically, directly on the fixed cultures. Immunocytochemical expression of EGFr was localised on the cell membrane, Plate 6.4, which corresponds to the findings of Ozawa et al. (1987).

Expression of Ki67 was localised in the nucleus. Within this system the Ki67 monoclonal antibody appeared to be specific only for mitotic or near mitotic cells. Plate 6.3.

Expression of both antigens measured in this way was found to correlate with tumour stage for adenocarcinoma tumours. Other studies have found that expression of EGFr, measured by ^{125}I binding assay or immunohistochemical analysis, does not correlate with stage. Itakura et al. (1994) found no significant correlation between EGFr expression, measured immunohistochemically, and histological staging for SSC. This lack of correlation was also found by Ozawa et al.(1989) who measured the level of EGFr in 32 patients with oesophageal SCC by means of the ^{125}I binding assay. Another study by Mukaida et al. (1990) also found no correlation between stage and EGFr levels in 56 oesophageal cancer tissues, but in this study there was no distinction between histological types. Since most studies have concentrated on SCC it is difficult to comment on the role of EGFr expression in adenocarcinoma of the oesophagus.

Some studies have examined the role of EGFr expression and proliferating index as indicators of response to treatment. In particular, a study by Hickey et al. (1994) immunohistochemically evaluated EGFr and PCNA on pre-treatment biopsies of oesophageal SCC and found a relationship between expression of these antigens and response to chemo-radiotherapy. A minimal response to treatment was associated with expression of both markers, while a complete response was achieved in patients who were negative for both markers. In the results presented here, a similar correlation was seen between expression of EGFr and the proliferating index, measured by the monoclonal antibody Ki67, and response to radiotherapy *in vitro*, for adenocarcinomas.

High levels of EGFr and Ki67 were associated with radio-resistance, while low levels of both were associated with radiosensitivity. This strong correlation suggests that expression of EGFr and Ki67 may indicate the aggressiveness of the tumours and their curability.

In conclusion, EGFr and Ki67 expression measured immunocytochemically in pre-treatment biopsies of oesophageal adenocarcinoma correlates with *in vitro* radiosensitivity. No correlation was found between these parameters and *in vitro* sensitivity of squamous cell carcinomas.

The culture system described here allows pre-treatment endoscopic biopsies to be cultured and the response to treatment to be evaluated *in vitro*. Using this system it was also possible to evaluate the expression of EGFr and Ki67, which is not possible with other predictive assay, such as the Courtney Mills clonogenic assay.

The ability to predict the response of an individual patient to treatment from endoscopic biopsies prior to commencement of treatment, would allow treatment planning to be tailored to the needs of that patient. In the case of oesophageal carcinoma, where palliative treatment is so important, a predictive assay would identify effective agents and avoid administration of ineffective treatment modalities, thus improving the patients quality of life. Where there is a possibility of cure, prognostic indicators such as EGFr and Ki67 and a predictive assay could assist in staging the cancer more accurately, identify patients resistant to a treatment modality and ensure an effective treatment regime is administered.

CHAPTER 7

Prediction of Individual Response of Head and Neck Squamous Cell Carcinoma Tumours to Treatment *In Vitro*

7.1 Introduction

Approximately one third of patients, with head and neck cancer, present with highly confined T1(a lesion confined to organ of origin) and T2 (localised lesion characterised by deep extension in adjacent structures or tissues, see Section 1.2) lesions. Most others present with local or regionally advanced disease (T3 or T4, N1-3, M0; see Section 1.2) (Vokes et al., 1993). Three active modalities of therapy - surgery, radiotherapy and chemotherapy - are being used in all phases of treatment of all stages of head and neck cancer. Radiotherapy is used primarily in the treatment of early stages (T1 and T2) with excellent results (Al-Sarraf et al., 1995). For patients with local or regionally advanced disease (stages T3 and T4), the prognosis is much worse. Frequently, extensive surgery and radiation therapy are used in sequence unless the patient is unable to undergo surgery or has unresectable disease. Fewer than 30 percent of patients are cured. The majority of patients will die of local or regionally persistent disease (Vokes et al., 1993).

Chemotherapy and radiotherapy are being investigated to improve local control and survival rates, and as an alternative to radical surgery for organ preservation in laryngeal cancer. While most trials comparing chemo- and radiotherapy to radiotherapy alone have failed to show any improvement in survival for chemo-radiotherapy (Laramore et al., 1992, Jaulerry et al., 1992), some have shown an improvement in local control rates (Jaulerry et al., 1992, Merlano et al., 1992). Merlano et al. (1992) showed a significant difference in the overall survival of patients with advanced, unresectable squamous cell carcinoma, treated with alternating chemotherapy (cisplatin and flurouracil) and radiotherapy compared to those patients treated with radiotherapy alone.

Parameters of intrinsic radiosensitivity have been investigated as potential predictors of individual patient response to treatment, in the hope that treatment planning can be

tailored to the individual. The surviving fraction at 2 Gy (SF_2) has been investigated by many groups (West et al., 1993; Grinsky et al., 1994; Brock et al., 1990), with varying degrees of success. West et al. have had the most promising results for carcinoma of the cervix (1994). In this study patients with radio-resistant tumours (SF_2 > median) had a significantly lower 3-year survival than patients with radiosensitive tumours (SF_2 < median). The SF_2 value also correlated with locoregional control. Several studies on head and neck cancer have been carried out to determine whether *in vitro* parameters were predictive of treatment outcome. Brock et al. (1990) determined the SF_2 value for 72 patients with squamous cell carcinoma of the head and neck, but failed to show any correlation with local tumour control. In a more recent study by Grinsky et al. (1994), *in vitro* parameters (SF_2, alpha values(the initial slope of the survival curve), and calculated cell growth fraction) were determined for 113 patients with squamous cell carcinoma of the head and neck, using the cell adhesion matrix (CAM) plate assay. The results of this study showed that in terms of long-term local control, alpha values but not SF_2 values were predictive of treatment outcome.

The aim of this study was to investigate the potential of the Mothersill outgrowth assay (Mothersill et al., 1988) in identifying patients with squamous cell carcinoma of the head and neck, likely to respond to treatment. The *in vitro* parameter used was the % growth inhibition (%GI) of cultures exposed to treatment relative to the untreated controls.

7.2 Materials and Methods

PATIENTS

Tumour specimens from 47 patients with histologicaly confirmed squamous cell carcinoma of the head and neck were taken at surgery.

IN VITRO CULTURE OF TUMOUR SPECIMENS

The primary explant culture method has been described in detail elsewhere (Section 3.1). Briefly, tumour specimens taken from the patient were placed in sterile saline at ambient temperature and brought immediately to the laboratory for culture. In the laboratory the specimens were cut into explants of approximately 2-3 mm^3 and digested in a solution of weak trypsin/collagenase at a concentration 1 ml / 10 mg, at 37°C for 20 minutes. After digestion the explants were plated singly in Nunclon 25 cm^2 flasks with 2 ml of growth media (RPMI -1640 containing 20% serum, hydrocortisone, insulin and antibiotics). Cultures were then placed in a CO_2 incubator at 37°C. After 72 hours incubation the cultures were treated with clinically equivalent doses of chemo- and radiotherapy, and returned to the incubator immediately. After a further 11 days incubation the cultures were fixed and immunocytochemically stained.

IN VITRO CHEMOTHERAPY AND RADIATION TREATMENT

Depending on the size of the specimen and the number of cultures that could be set up from it, the cultures were grouped according to treatment to be given, Table 7.1, priority being given to the radiation group.

Table 7.1. Treatment groups. Cultures were grouped according to the treatment to be administered.

GROUP	TREATMENT
Control	None
Radiation	A single dose of 2 Gy
Chemotherapy	Cisplatin – 5 Flurouracil
Combination	Chemotherapy – Radiation

After 72 hours of undisturbed growth the cultures were treated, the control group remaining untreated. Chemotherapeutic agents were diluted from available commercial preparations, using growth medium, to give varying concentrations of cisplatin and 5-flurouracil, as shown in Table 7.2.

Table 7.2. Concentration of cisplatin and 5 flurouracil added to cultures in the chemotherapy and combination groups.

Cisplatin (µg/ 2ml)	5 Flurouracil (µg/ 2ml)
0.1	2
0.1	30
1	2
1	30

The diluted agents were added directly to the medium in each culture and a single dose of 2 Gy was given to each culture in the radiation and combination group.

IMMUNOCYTOCHEMICAL ANALYSIS

The epithelial nature of the outgrowth was determined using a cytokeratin antibody. The population of proliferating cells, was identified using the antibody Ki67(DAKO) to the proliferating cell nuclear antigen. Immunostaining was performed using the Vectastin

ABC kit, which employs a standard indirect immunoperoxidase method. The protocol for the indirect immunoperoxidase is described in Section 3.8.

The percentage positive cells was measured along a cross section through each growth area, as described in Section 3.10.

RESPONSE TO TREATMENT

The response of the cultures to treatment was expressed as the percentage growth inhibition in the treated cultures relative to the untreated control. The growth area and total cell number was estimated for each culture and the mean total cell number for each treatment group calculated. The reduction in the mean total cell number of the treated cultures relative to the control cultures is expressed as % growth inhibition (% GI). The higher the % GI is for a tumour the more sensitive it is deemed to be.

ELECTRON MICROSCOPY

The epithelial origin and the malignant nature of the cells was also determined using electron microscopy. Cultures were fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer for 1 hour at room temperature. The culture was then washed in phosphate buffer, postfixed in 1% osmium tetroxide for an additional hour and dehydrated through ascending grades of ethanol. They were then embedded in epoxy resin. Thin sections (40 nm) were cut *en face* with a diamond knife, stained with uranyl acetate and lead citrate, and viewed in a Jeol 2000 electron microscope.

7.3 Results

Over a 15 month period 47 specimens were received, and cultured. Of these, 28 (60%) grew successfully, producing epithelial-like cells, while predictive results were obtained for 24 specimens, Table 7.3. The failure to obtain predictive results in other cases was due to the small size of the specimen received. The epithelial origin and the malignant nature of the cells was confirmed by immunocytochemical analysis, Plate 7.1, and electron microscopy, Plate 7.2. Unsuccessful culture was due to fungal and fibroblastic contamination, and no growth, Table 7.4.

Table 7.3. Tumour specimens cultured successfully.

Specimens cultured	**47**
Unsuccessful culture	19
Successful culture	28
Predictive results	**24**

Table 7.4. Reasons for unsuccessful culture. Unsuccessful culture of 19 specimens caused by contamination and no growth.

Unsuccessful Culture	**No. of Specimens**
Fungal contamination	5
Fibroblastic contamination	5
No growth	9

Clinical Data

Table 7.5 shows clinical information regarding each patient including, sex, age, stage, treatment received, follow-up time and current status. Of the 24 patients 62% had Stage 4 disease, 8% had Stage 3, another 8% had Stage 2 and 21% had Stage 1(for definition of stages see Section 1.2). 75% of the patients are alive and well (A&W), 8% are alive with disease (AWD) and 17% are dead of disease (DOD). The follow-up time ranged from 2-15 months.

Due to the variation in the treatment modality administered to each patient, the patients have been grouped according to treatment received. Table 7.6 shows the percentage of patients that received each modality.

Table 7.6. Treatment modalities administered to patients.

No. of Patients	% of Total	Treatment Modality
8	33	surgery
1	4	radiotherapy
7	29	surgery + radiotherapy
4	17	surgery + chemo-radiotherapy
2	8	radiotherapy - surgery - chemo-radiotherapy
2	8	radiotherapy - salvage surgery

% Growth Inhibition and Clinical Data

There was no correlation between the *in vitro* parameter of radiosensitvity, measured by %GI (2 Gy) and the clinical prognostic factor, stage. Figure 7.1. Mean values for %GI (2 Gy) for stages 1, 2, 3 and 4, were 51, 35, 52 and 44% respectively. No significant differences were found between the %GI (2 Gy) for the various stages.

Table 7.5. Clinical data of 24 patients for who in vitro predictive results were obtained. Six different treatment regimens were administered to the patients, patients are ranked in order of increasing radiosensitivity.

Patient	Sex	Age	Stage	Treatment	Follow Up Time(months)	Current Status	% GI (2 Gy)
1	M	71	2	Rt + salvage surg	9	A&W	0
2	M	77	1	Surg	9	A&W	0
3	M	73	4	Surg			0
4	M		4	Surg + chemoRt	11	A&W	0
5	M	56	4	Rt - Surg + chemoRt	11	A&W	0
6	F		4	Surg + Rt	15	A&W	0
7	M		4	Surg + Rt	14	AWD	0
8	M	53	4	Surg + Rt	12	A&W	6
9	F	74	3	Rt		DOD	20
10	M	72	1	Rt		A&W	38
11	M	61	4	Rt - Surg + chemoRt		DOD	39
12	M	73	4	Rt + salvage surg.		DOD	45
13	F	23	1	Surg	recurred - surg + chemoRt	AWD	48
14	M	62	4	Surg + Rt	9	A&W	57
15		70	4	Surg + Rt	2	A&W	61
16	M	59	2	Surg	3	A&W	70
17		52	1	Surg	11	A&W	78
18	M	57	4	Surg + chemoRt	11	A&W	79
19	M	68	3	Surg	2	A&W	83
20	M		4	surg + chemoRt	9	DOD	85
21	F	68	1	Surg	3	A&W	91
22	M		4	Surg + chemoRt	11	A&W	91
23	M		4	Surg + Rt	7	A&W	100
24	M		4	Surg + Rt	3	A&W	100

The average age of patients for whom radiosensitivty was assessed was 63. with a range of 23-77. Figure 7.2 illustrates the lack of correlation between patient age and tumour radiosensitivity.

% Growth Inhibition as Indicator of Outcome

The individual response of each patient to treatment *in vitro* is shown in Table 7.7 . This table shows the huge variation in patient response to treatment which reflects the *in vivo* situation of patient response. The radiosensitivities of the cultures derived from these patients ranged from %GI of 0% to 100%. with a median of 46% and a mean of 45%. Considering radiotherapy alone. 54% of tumours treated *in vitro* were sensitive (%GI > mean) to a single dose of 2 Gy.

Tables 7.8-13 show the current status and mean response to treatment *in vitro* for patients grouped according to the treatment modality received. The patient groups are too small and the follow up too short to establish whether there is a significant difference between the % growth inhibition of patients with different current status. i.e. % growth inhibition of patients that are alive and well versus % growth inhibition of patients dead of disease after treatment with primary surgery and postoperative radiotherapy.

Table 7.7. *In vitro* response of tumour specimens from each patient, to a single dose of 2 Gy, clinical equivalent doses of chemotherapy and a combination of chemo- and radiotherapy. Response to treatment is measured in terms of % growth inhibition (%G.I.) in treated cultures relative to untreated controls. The higher the %G.I. the more sensitive the tumour is to treatment, in vitro.

Patient	2 Gy	0.1ug Cis+2ug 5FU	1ug Cis+2ug 5FU	0.1ug Cis+30ug 5FU	1ug Cis+30ug 5FU	0.1ugCis +2ug 5Fu+2Gy	1ugCis+2ug 5FU +2Gy
1	0	100	100			80	87
2	0					96	73
3	0	0	0			0	0
4	0	92	100	100	100	83	85
5	0	0	100	100	100	64	94
6	0	43	77			73	66
7	0	73	76	99	96	89	99
8	6	93	94	96	97	98	96
9	20						
10	38	28	0			41	41
11	39	100	100	100	100	100	100
12	45	88	92			98	
13	48	95	96	93	100	99	99
14	57					0	
15	61						
16	70	f	70			87	100
17	72	85	100	100	100	81	93
18	79	99	90	99	100	93	97
19	83						
20	85	93	95	f	97	89	94
21	91					98	95
22	91	100	100	100	100	100	100
23	100	100	100	100	100	100	100
24	100	100	100	100	100	100	100
Mean	45	76	83	99	99	79	85

Table 7.8. Current status of patient that received radiotherapy alone. One patient received radiotherapy alone, and failed to respond (DOD). The in vitro response of the specimen to radiation indicated resistance, 20% growth inhibition.

Current Status	N	Mean % G.I. after treatment						
		2 Gy	0.1ug Cis+2ug 5FU	1ug Cis+2ug 5FU	0.1ug Cis+30ug 5FU	1ug Cis+30ug 5FU	0.1ugCis +2ug 5Fu+2Gy	1ugCis+2ug 5FU +2Gy
A&W								
AWD								
DOD	1	20						

Table 7.9. Current status of 8 patients that were treated with surgery alone. Of 8 patients who received surgery alone, 6 are alive and well (A&W), while one is alive with disease (AWD). The mean % growth inhibition of 5 patients A&W is 64% and the % growth inhibition of the patient who is AWD is 48%.

Current Status	N	Mean % G.I. after treatment						
		2 Gy	0.1ug Cis+2ug 5FU	1ug Cis+2ug 5FU	0.1ug Cis+30ug 5FU	1ug Cis+30ug 5FU	0.1ugCis +2ug 5Fu+2Gy	1ugCis+2ug 5FU +2Gy
A&W	6	60± 16	56 ± 28 (n=2)	57 ± 30 (n=3)	100 (n=1)	100 (n=1)	81 ± 10 (n=5)	80 ± 11 (n=5)
AWD	1	48	95	96	96	100	99	99
DOD								

Table 7.10. Current status of 7 patients that were treated with surgery and postoperative radiotherapy. Six patients received surgery followed by postoperative radiotherapy, 5 of these patients are alive and well and one is alive with disease. The mean growth inhibition after 2 Gy in vitro is 45% for the A&W group and 0% for the patient AWD.

Current Status	N	Mean % G.I. after treatment						
		2 Gy	0.1ug Cis+2ug 5FU	1ug Cis+2ug 5FU	0.1ug Cis+30ug 5FU	1ug Cis+30ug 5FU	0.1ugCis +2ug 5Fu+2Gy	1ugCis+2ug 5FU +2Gy
A&W	6	54 ± 8	83 ± 14 (n=4)	92 ± 6 (n=4)	99 ± 1 (n=3)	99 ± 1 (n=3)	74 ± 19 (n=5)	90 ± 8 (n=4)
AWD	1	0	73	76	99	96	89	99
DOD								

Table 7.11. Current status of 4 patients that were treated with surgery followed by chemo- and radiotherapy. The in vitro results indicated that all the patients were sensitive, however one patient died at 9 months. The other 3 are alive and well at 11 months.

Current Status	N	Mean % G.I. after treatment						
		2 Gy	0.1ug Cis+2ug 5FU	1ug Cis+2ug 5FU	0.1ug Cis+30ug 5FU	1ug Cis+30ug 5FU	0.1ugCis +2ug 5Fu+2Gy	1ugCis+2ug 5FU +2Gy
A&W	3	57 ± 28	98 ± 2	97 ± 3	99 ± 0.3	100 ± 0	93 ± 4	95 ± 3
AWD								
DOD	1	85	93	95		97	89	94

Table 7.12. Current status of 2 patients treated with primary radiotherapy and salvage surgery. One is dead of disease and the other is alive and well. The in vitro results indicated none and a partial response to radiotherapy.

Current Status	N	Mean % G.I. after treatment						
		2 Gy	0.1ug Cis+2ug 5FU	1ug Cis+2ug 5FU	0.1ug Cis+30ug 5FU	1ug Cis+30ug 5FU	0.1ugCis +2ug 5Fu+2Gy	1ugCis+2ug 5FU +2Gy
A&W	1	0	100	100			82	88
AWD								
DOD	1	45	88	92	100	100	98	94

Table 7.13. Current status of 2 patients that received primary radiotherapy followed by surgery and chemoradiotherapy. One patient is dead of disease and the other is alive and well at 11 months. In vitro results for both patients showed a partial and no response to radiotherapy, and a complete response of both to chemoradiotherapy.

Current Status	N	Mean % G.I. after treatment						
		2 Gy	0.1ug Cis+2ug 5FU	1ug Cis+2ug 5FU	0.1ug Cis+30ug 5FU	1ug Cis+30ug 5FU	0.1ugCis +2ug 5Fu+2Gy	1ugCis+2ug 5FU +2Gy
A&W	1	0	0	100	100	100	64	100
AWD								
DOD	1	39	100	100	100	100	100	100

Figure 7.1. Lack of correlation between in vitro radiosensitivity and stage (corr.coeff. =-0.1).

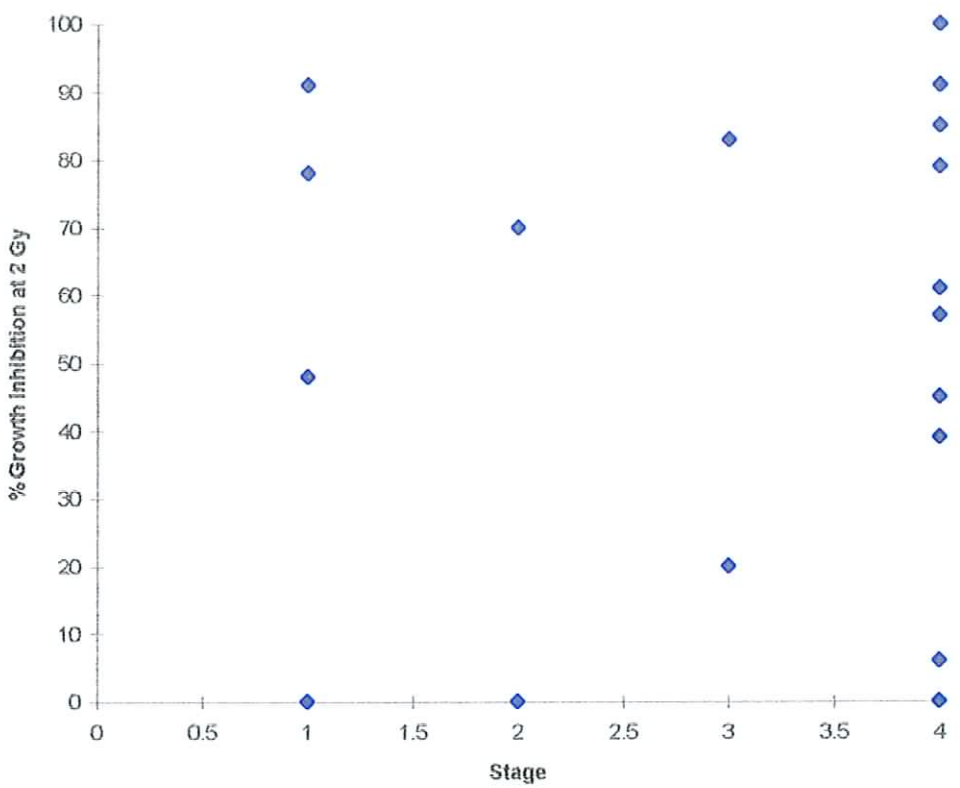
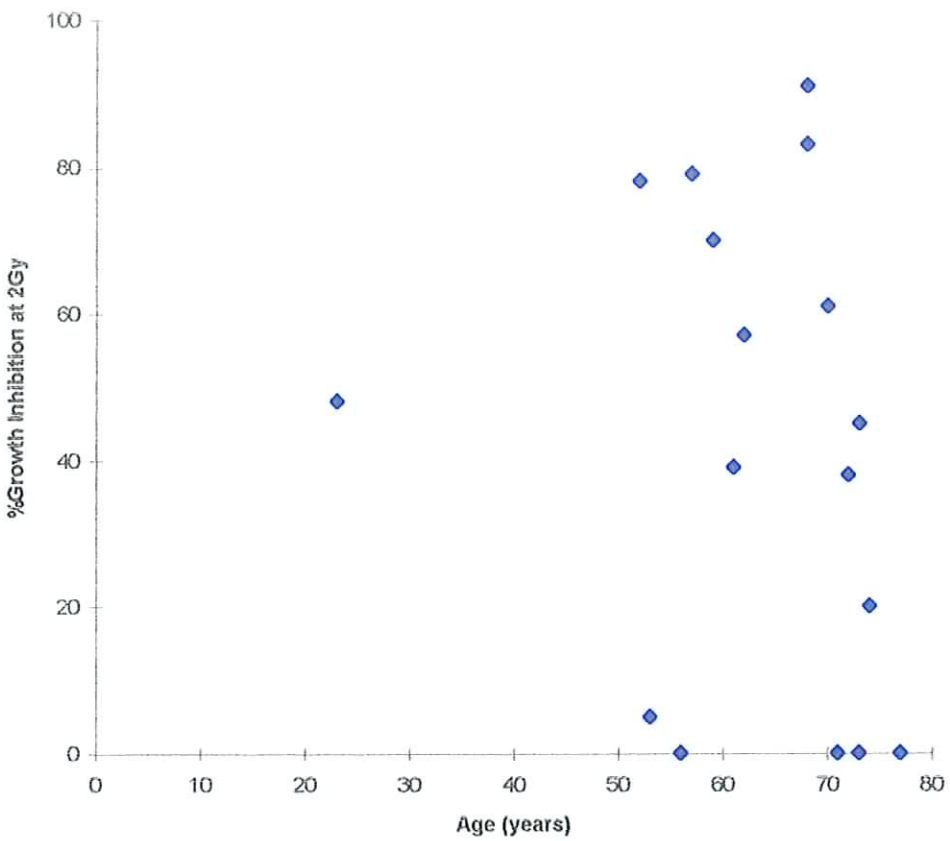


Figure 7.2. Lack of correlation between tumour radiosensitivity (%GI at 2Gy) and patient age.



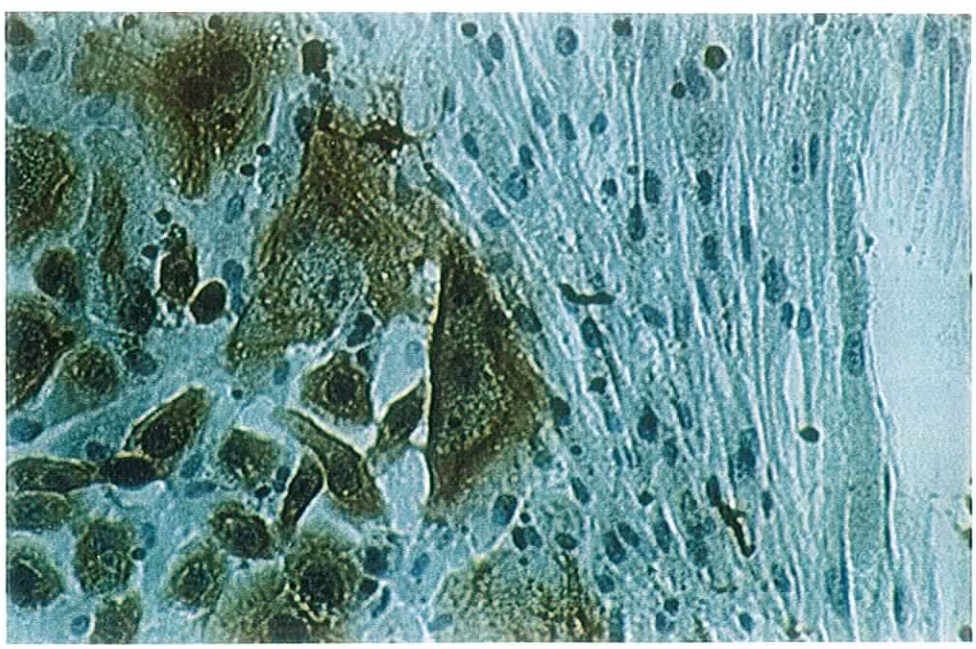


Plate 7.1. The epithelial nature of the cells was confirmed immunocytochemically using a cytokeratin antibody. A culture established from a head and neck tumour was fixed after 2 weeks incubation and immunocytochemically stained. Brown positive stain was localised in the cytoplasm and differentiated epithelial from fibroblast cells.

Plate 7.2. Epithelial origin of the cells in the outgrowth from an explant of tumour was confirmed using electron microscopy. The presence of desmosones and tonofilaments are characteristic of epithelial cells.

7.4 Discussion

Using the Mothersill outgrowth assay (Mothersill et al., 1988) epithelial growth was obtained from 28 of 46 (60%) specimens received and of these predictive results were obtained from 24 (52%). This success rate is lower than the success rate of 60% achieved in two studies which evaluated SF_2 values for head and neck tumours using the CAM plate assay (Grinsky et al., 1994; Brock et al., 1990). This low success rate was mainly due to unsuccessful culture as a result of contamination in the earlier stages of the study and occurred in biopsy specimens that were taken from out-patients (where conditions were not as sterile as in theatre), as the study progressed the success rate improved. On average 3 tumour specimens were received per month, which is equivalent to the number of fresh tumour specimens received by West et al. (1993) in a study on carcinoma of the cervix.

The lack of correlation between intrinsic radiosensitivity and stage is consistent with results reported elsewhere for primary head and neck cancers (Grinsky et al., 1992, 1994), and cervical cancers(West et al., 1995) using a clonogenic assay. The lack of correlation between tumour radiosensitivity and patient age has also been reported by West et al. (1995) for cervical cancer tumours.

Due to the small patient number, short follow up time and variable treatment modalities, it is impossible to derive meaningful conclusions. Taking individual patients it is possible to comment on the predictive ability of this parameter, but it is not possible to make any statistical analysis due to the small patient numbers in each group and the short follow up time.

One patient received radiotherapy alone. Table 7.8. the *in vitro* response of cultures of this patient's tumour to 2 Gy showed a % growth inhibition of 20%. indicating that this patient was resistant to radiotherapy. This patient is now 'dead of disease'.

There are 7 patients in the surgery alone group. Table 7.9. whose status is known. 6 of these are alive and well and one is alive with disease. Of the 6 alive and well. 4 had a % GI at 2 Gy of greater than 70% (sensitive). while two had % GI at 2 Gy of < 38%(resistant). The patient alive with disease had a % GI value of 48%. Perhaps in this case the % growth inhibition indicated the curability of the tumours by any treatment. or the aggressiveness of the tumour.

Surgery and post-operative radiotherapy was administered to 6 patients. Table 7.10. 5 are alive and well. and one is alive with disease. Three of the 5 patients alive and well had a % GI (2 Gy) of > 57% (sensitive) and 2 had a % GI (2 Gy) of <5% (resistant). The patient alive with disease had a % GI (2 Gy) of 0%. In this case the % GI (2 Gy) was predictive of outcome for 4 patients, the 3 alive and well and the one patient alive with disease.

Four patients were treated with surgery followed by chemo-radiotherapy. 3 are alive and well at 11 months and one is dead of disease. Table 7.11. The *in vitro* results showed 3 of these patients to be radiosensitive and all 4 to be sensitive to a combination of chemo- and radiotherapy. The *in vitro* results were predictive of patient response to treatment in three cases, those 3 patients alive and well.

Also worth noting are the *in vitro* results of those 4 patients whose primary treatment was radiotherapy. Tables 7.12 & 7.13. the % growth inhibition at 2 Gy for all these patients was < 45%. with a mean of 21%. indicating that they were resistant to

radiotherapy. Two of these specimens were taken at salvage surgery, when the tumour had recurred or did not respond to radiotherapy. The *in vivo* radioresistance of these tumours was reflected in the *in vitro* system by low values for the % growth inhibition after 2 Gy.

Other studies have shown that *in vitro* intrinsic radiosensitivity measured at 2 Gy, using clonogenic assays, may predict an individual patients response to radiotherapy (Grinsky et al., 1992; West et al., 1993). The preliminary results presented here using the outgrowth assay suggest a trend, within each treatment group, between *in vitro* response to treatment and patient outcome. An increase in patient number and longer follow up time would allow one to better define this trend.

The Mothersill outgrowth assay has some technical advantages over the clonogenic assays whether using the Courtenay-Mills soft agar system (Courtenay and Mills, 1978) or the CAM plate assay (Baker et al., 1986). Firstly, a single cell suspension is not required, this is a difficult task which requires a large amount of tissue. Often cell clumps which have not been completely disagregated are seeded and counted as colonies, thus giving false results. The methodology of the clonogenic assays is laborious and time-consuming, the outgrowth assay is quick and does not require a great deal of expertise.

In conclusion, the preliminary results presented suggest a possible role for the Mothersill outgrowth assay in measuring the response of cultures of tumour from individual patients to treatment *in vitro*. An increase in patient number, a longer follow-up time and a more homologous group of patients, in terms of stage and treatment modalities administered would produce more conclusive results on the ability of this

parameter, measured in terms of growth inhibition, to predict the individual patient response to treatment.

CHAPTER 8

Potential Indicators of Radiosensitivity in Squamous Cell Carcinoma of the Head and Neck

8.1 Introduction

The clinical observation that patients with head and neck squamous cell carcinomas in comparable stages may have diverse clinical courses and responses to similar treatments is, as yet, unexplained (Kearsley et al., 1990). The TNM classification (see Section 1.2), although widely utilised in clinical practice, is associated with a well recognised difficulty in predicting clinical outcome. Not infrequently, tumours staged on the basis of their origin, size, and metastatic node involvement will differ dramatically in their clinical course and response to treatment (Gapany et al., 1994).

Recently, attention has been focused on the potential of oncogenes, tumour suppressor genes and assessment of cell proliferation as potential biomarkers in human cancer (Hall et al., 1995). Studies have investigated the role of Ki67, a marker of cellular proliferation, the protein products of the oncogenes EGFr, c-myc, bcl2 which are regulators of cell growth and, the tumour suppressor gene, p53 as prognostic indicators in squamous cell carcinoma of the head and neck (SCCHN), and as potential indicators of radiosensitivity. Immunocytochemical evaluation of the protein products of these oncogenes considered alone or in terms of co-expression has been shown to correlate with clinical pathologic parameters and overall survival in breast cancer (Bland et al., 1995).

In the case of head and neck cancer, recent studies have shown that immunocytochemical evaluation of the percentage of Ki67 positive cells (%Ki67) after radiotherapy provided an independent variable of responsiveness to radiotherapy (Ogawa, 1992; Valente et al., 1994).

The evaluation of EFGr is considered more valuable as a potential prognostic indicator, than EGFr gene amplification, as overexpression of EGFr is not always as a result of

amplification of the gene (Ishitoya et al., 1989; Itakura et al., 1994). Some studies have shown that overexpression of EGFr correlates with poorer response to treatment (Hickey et al., 1994; Santini et al., 1991) and poorer prognosis (Santini et al., 1991; Yano et al., 1995).

The role of c-Myc and Bcl2 in the regulation of apoptosis, the development of cancer and as a potential target in treatment (Carson and Ribeiro, 1993), is an area receiving particular attention. Less attention has focused on the role of these oncoproteins as potential prognostic indicators, in particular for head and neck cancer. A study by Gapany et al. (1994) immunohistochemically evaluated c-Myc levels in head and neck tumours and found a definite correlation between low levels of c-Myc and metastatic lymph node involvement, and advanced stages. The expression of c-Myc has been shown to be related to clinical parameters, such as histological grade , mitotic index and stage, in renal adenocarcinoma (Lipponen et al., 1995) and bladder cancer (Lipponen, 1995). Expression of Bcl-2 has been immunocytochemically evaluated in breast cancer (Silvestrini et al., 1994), lung cancer (Pezzella et al., 1993) and renal cancer (Lipponen et al., 1995). In all these studies Bcl-2 expression was found to be related to a better prognosis.

A model for bcl-2 action in oncogenesis proposes that bcl-2 works in synergy with specific cellular oncogenes (Hockenberry, 1994), one of these oncogenes is c-myc (Bissonnette et al., 1993; Fanidi et al., 1993). Bcl-2 mitigates the apoptotic effects of deregulated c-Myc expression without effecting it's ability to promote continuos cell growth (Fanidi et al., 1993).

In this study immunocytochemical analysis was used to evaluate the expression of Ki67, EGFr, c-Myc and Bcl-2 in cultures of tumours taken from patients with head and neck SCC. The ability of these proteins to predict *in vitro* radiosensitivity was examined.

8.2 Materials and Methods

PATIENTS

Immunocytochemical analysis of potential indicators of radiosensitivity was carried out on tumour specimens from 24 patients with histologically confirmed squamous cell carcinoma of the head and neck, taken at surgery.

IN VITRO CULTURE OF TUMOUR SPECIMENS

The primary explant culture method has been described in detail in Section 3.1. Briefly, tumour specimens taken from the patient were placed in sterile saline, at ambient temperature, and brought immediately to the laboratory for culture. In the laboratory the specimens were cut into explants of approximately 2-3 mm^3 and digested in a solution of weak trypsin/ collagenase, at a concentration of 1 ml/ 10 mg, for 20 minutes at 37°C. After digestion the explants were plated singly in Nunclon 25 cm^2 flasks with 2 ml of growth media (RPMI -1640 containing 20% serum, hydrocortisone, insulin and antibiotics). Cultures were then placed in a CO_2 incubator at 37°C. After 72 hours incubation the cultures were treated with radiotherapy, and returned to the incubator immediately. After a further 11 days incubation the cultures were fixed and immunocytochemically analysed.

IN VITRO RADIATION TREATMENT

After 72 hours of undisturbed growth the cultures were split into two groups, the control group which was to remain untreated and the irradiated group which was to receive a single dose of 2 Gy. The single dose of 2 Gy was administered using a cobalt 60 teletherapy unit.

IMMUNOCYTOCHEMICAL ANALYSIS

The epithelial nature of the outgrowth was determined using a cytokeratin antibody. The parameters examined immunocytochemically and the respective antibodies used are shown in Table 8.1.

Table 8.1. Antibodies used to measure the parameters being investigated as potential predictors of radiosensitivity.

Parameter	Monoclonal Antibody
Proliferation Index	Ki67 (DAKO)
Expression of Epidermal Growth Factor Receptors (EGFr)	2E9 (Monosan)
Expression of oncoprotein, c-Myc	9E10*
Expression of oncoprotein, bcl2	124 (Dako)

Immunostaining was performed using the Vectastain ABC kit, which employs a standard indirect immunoperoxidase method. The protocol for the indirect immunoperoxidase method is described fully in Section 3.5.

Evaluation of immunostaining was determined by measuring the percentage positive cells along a cross section through each growth area (see Section 3.7), for all the

* 9E10 antibody provided by kind permission from Dr G Evans, St Bartholomew's Hospital, London.

antibodies except EGFr. The expression of EGFr was measured semi-quantitatively (see Section 3.7), as shown in Table 8.2.

Table 8.2. Expression of EGFr was measured semi-quantitatively. Depending on the expression of positive cells, the above grades were given.

EGFr positive cells	Grade
0 -25%	2
25-50%	4
>50%	6

In the immunocytochemical analysis of the expression of c-Myc and Bcl2 each culture for each patient was ranked either positive, 1, or negative, 0. The culture was considered positive if the % positive cells measured was greater than the mean % positive cells for all the 15 patients, and considered negative if less than, or equal to the mean % positive cells. Where the effect of co-expression on radiosensitivity was investigated, the patients were given a score of 1, 0 or -1 depending on whether the patients cultures were positive for one protein, positive for both proteins measured or negative for both. The scores were allocated as shown in Table 8.3.

Table 8.3. Co-expression of proteins were scored 1, 0 or -1. Where either protein was positive a score of 1 or -1 was given. Where both were either positive or negative a score of 0 was given. A positive score, 1, was always given when only protein A was positive, while a negative score was given when only protein B was positive.

Expression of Protein A	Expression of Protein B	Score
1	0	1
1	1	0
0	0	0
0	1	-1

RESPONSE TO TREATMENT

The response of the cultures to treatment was expressed as the percentage growth inhibition in the treated cultures relative to the untreated control. The growth area and total cell number was estimated for each culture and mean total cell number for each treatment group calculated. The reduction in the mean total cell number of the treated cultures relative to the control cultures is expressed as % growth inhibition (% GI). The higher the % GI was for a tumour the more sensitive it was deemed to be.

8.3 Results

Ki67

Positive staining with the Ki67 antibody was localised in the nucleus, Plate 8.1. Table 8.4 shows the expression of Ki67 measured in control cultures (Ki67(C)) of head and neck tumours and in cultures that received a single dose of 2 Gy (Ki67(2Gy)), for each patient. Also shown is the stage and *in vitro* response to irradiation for each patient. Patients are ranked in order of increasing radio-resistance. Those patients with a %GI(2Gy) value less than the mean are considered resistant.

Expression of Ki67 measured in cultures of head and neck tumours that received no treatment, %Ki67(C), positively correlated with the stage of each patient (correl. coeff. = 0.6). The expression of Ki67 measured in cultures after irradiation, Ki67(2Gy), very weakly correlated with patient stage (correl. coeff. = 0.4), Figure 8.1.

There was no significant difference between the % Ki67 expressed in the control cultures in the resistant and sensitive groups ($p = 0.47$), Table 8.5. There was, however,

a significant difference between the level of expression of Ki67 measured in cultures after a single dose of 2 Gy, in the resistant and sensitive groups ($p = 0.045$), Table 8.6.

The percent change in the expression of Ki67 from the control cultures to the irradiated cultures was calculated. Table 8.4. No significant difference was found between the percent change in the resistant and sensitive groups ($p = 0.3$). Table 8.7.

In summary, the expression of Ki67 measured before irradiation, i.e. in the control cultures, had no influence on radiosensitivity. The expression of Ki67 after irradiation did predict for *in vitro* radiosensitivty: expression of Ki67 in the sensitive group was significantly lower than the resistant group. Table 8.8.

Table 8.4. Expression of Ki67 measured immunocytochemically in cultures of head and neck tumours before treatment (%Ki67(C)) and after a single dose of 2Gy. The percent change in the expression from the control cultures to the irradiated was calculated. Patients are ranked according to increasing radio-resistance and the stage of each patient is given.

Patient	Stage	%GI(2Gy)	%Ki67(C)	%Ki67 (2 Gy)	%change
1	4	91	68		0
2	4	85	2	4	100
3	4	79	18	4	-78
4	1	78	1	1	0
5	2	70	0	1	1
6	1	48	3	6	100
7	4	39	30	50	67
8	3	20	13	17	31
9	4	0	12	50	316
10	4	0	29	9	-69
11	4	0	11	6	-45
12	1	0	1	1	0
Mean		**43**	**16**	**12**	**27**

Table 8.5. Expression of Ki67 measured in cultures before treatment was not significantly different between radio-sensitive and resistant groups.

Ki67(C)	Resistant	Sensitive
	12	1
	30	18
	13	68
	29	2
	11	1
	1	13
	3	0
Mean	14	15
P-value	0.47	

Table 8.6. Expression of Ki67 measured in cultures after irradiation was significantly different between radio-sensitive and resistant groups.

Ki67(2Gy)	Resistant	Sensitive
	50	1
	50	4
	17	0
	9	4
	6	6
	1	
Mean	22	3
P-value	0.045	

Table 8.7. Percent change in expression of Ki67 from cultures before treatment to cultures after irradiation was not significantly different between radio- sensitive and resistant groups.

%change	Resistant	Sensitive
	67	-100
	31	100
	316	-78
	-69	0
	-45	1
	0	100
Mean	50	4
P-value	0.3	

Table 8.8. Expression of Ki67 measured in cultures after irradiation was significantly lower in the radiosensitive group than in the radio-resistant group. No significant difference was found between expression of Ki67 measured in cultures before treatment. and percent change in expression in resistant and sensitive groups.

	Resistant	Sensitive	P-value
Mean %Ki67(C)	14	15	0.47
Mean %Ki67(2Gy)	22	3	0.045
Mean % change	50	4	0.3

EGFr

The expression of EGFr was localised in the cell membrane. Plate 8.2. Table 8.9 shows the expression of EGFr measured in cultures of head and neck tumours that received no treatment (EGFr(C)), and that were irradiated with a single dose of 2 Gy (EGFr(2Gy)). Also shown is the stage and *in vitro* response to irradiation for each patient. Patients are ranked in order of increasing radio-resistance. Those patients with a %GI(2Gy) value less than the mean are considered resistant.

There was no correlation found between the expression of EGFr measured in control cultures of head and neck tumours or after irradiation with 2 Gy, and the stage of each patient. The correlation coefficients were 0.2 and -0.1 respectively, Figure 8.2.

The was a highly significant difference between the expression of EGFr(C), of the resistant and sensitive group ($p = 0.002$). Low expression of EGFr correlated with *in vitro* sensitivity to 2 Gy, while high expression correlated with resistance, Table 8.10. This correlation was also found for expression of EGFr(2Gy). There was a significant difference between the expression of EGFr(2Gy) in the radio- resistant and sensitive groups ($p = 0.03$), Table 8.11.

In summary, the expression of EGFr measured in the control cultures and the cultures that were treated with a single dose of 2 Gy, differed significantly in the resistant and sensitive groups, Table 8.12.

Table 8.9. Expression of EGFr measured in control cultures, EGFr(C), of head and neck tumours and in cultures that were irradiated with 2 Gy, EGFr(2Gy). The stage and *in vitro* response to 2 Gy, %GI(2Gy), is shown for each patient. Patients are ranked according to increasing radio-resistance.

Patient	Stage	%GI (2Gy)	EGFr (C)	EGFr(2Gy)
1	1	100	4	
2	4	100	2	2
3	4	91	0	
4	4	85	2	2
5	4	79	0	0
6	1	78	0	0
7	2	70	2	
8	4	39	6	6
9	1	38	2	6
10	3	20	6	6
11	4	5	6	6
12	4	0	2	2
13	4	0	2	2
14	4	0	6	0
15	4	0	6	
16	4	0	6	
Mean		**44**	**3**	**3**

Table 8.10. Expression of EGFr measured in control cultures, EGFr(C), of head and neck tumours was significantly different in the radio-resistant and sensitive groups (p = 0.002).

EGFr(C)	Resistant	Sensitive
	2	0
	2	2
	6	2
	6	0
	6	0
	2	4
	6	2
	6	
	6	
Mean	**5**	**1**
P-value	**0.002**	

Table 8.11. Expression of EGFr measured in cultures treated with a single dose of 2 Gy, is significantly different in the radio- resistant and sensitive groups (p = 0.03)..

EGFr(2Gy	Resistant	Sensitive
	2	0
	2	2
	0	0
	6	2
	6	
	6	
	6	
Mean	**4**	**1**
P-value	**0.03**	

Table 8.12. Expression of both EGFr(C) and EGFr(2Gy) differed significantly between radio- resistant and sensitive groups.

	Resistant	Sensitive	P-value
Mean EGFr(C)	5	1	0.002
Mean EGFr(2Gy)	4	1	0.03

c-Myc & Bcl2

Immunocytochemical staining of c-Myc and Bcl-2 was localised in the cytoplasm. Plate 8.3 and 8.4. Table 8.13 shows the expression of c-Myc and Bcl2 for each patient. The corresponding *in vitro* radiosensitivity for each patient is also shown and is marked as either sensitive, s, if the %GI(2Gy) was greater than the mean, or resistant, r, if the %GI(2Gy) is less than the mean. The mean value for %GI(2Gy) was 52 % and the mean expression of c-Myc and Bcl2 was 44% and 12% respectively. The patients are ranked in order of increasing radio-resistance.

Table 8.13. Expression of c-Myc and Bcl2 for each patient. Each patient is marked either resistant, r, or sensitive, s, depending on whether the %GI(2Gy) was less than or greater than the mean. Patients are ranked in order of increasing radio-resistance.

Patient	Radiosensitivity	%GI (2Gy)	% c-Myc	% Bcl2
1	s	100	100	0
2	s	91	100	0
3	s	91	100	1
4	s	85	35	2
5	s	83	100	100
6	s	79	10	0
7	s	78	1	10
8	s	61	100	10
9	s	57	100	0
10	r	48	50	0
11	r	5	18	12
12	r	0	4	17
13	r	0	30	3
14	r	0	100	0
Mean		**52**	**44**	**12**

The expression of c-Myc was ranked as either 1, if the % positive cells was greater than the mean (44%), or 0, if the %positive cells was less than or equal to the mean. There was no significant difference between the level of expression of c-Myc between the radio- resistant and sensitive groups ($p = 0.1$). Table 8.14.

The expression of Bcl2 for each patient was ranked as 1, if the % positive cells was greater than 12%, and 0, if % positive cells was less than or equal to 12%. There was no significant difference between the level of expression of Bcl2 in the radio- resistant or sensitive groups ($p = 0.32$). Table 8.15.

Table 8.14. Expression of c-Myc was ranked as 1, if > mean, 44%, or 0 if <= 44%. No significant difference between the level of expression of c-Myc in the radio- resistant or sensitive groups.

c-Myc	Resistant	Sensitive
	1	1
	0	1
	0	1
	0	0
	1	1
		0
		0
		1
		1
Mean	0.4	0.7
P-value	0.19	

Table 8.15. Expression of Bcl2 was ranked as 1, if > mean, 12%, or 0 if <= 12%. No significant difference found between the level of expression of Bcl2 in the radio- resistant or sensitive groups.

bcl2	Resistant	Sensitive
	0	0
	1	0
	1	0
	0	0
	0	1
		0
		0
		0
		0
Mean	0.4	0.1
P-value	0.12	

The co-expression of c-Myc and Bcl2 is shown in Table 8.16. A score of 0 was given if the level of expression was negative for both proteins in the cultures of a patient, or positive for both. A score of 1 was given if c-Myc was positive and Bcl2 was negative. A score of -1 was given if Bcl2 was positive (i.e. > mean) and c-Myc was negative

(i.e.< mean). This scoring allowed the influence of the proteins on radiosensitivity to be determined. There was a significant difference found between the co-expression of the proteins between the radio- resistant and sensitive groups (p = 0.096). The mean score in the resistant group was 0. indicating that neither protein had an independent influence on radio-resistance. The mean score in the sensitive group was 0.6 indicated that c-Myc had a influence on radiosensitivity. over Bcl2.

Table 8.16. Co-expression of c-Myc and Bcl2 for a patient was scored as 0 if the level of expression was negative for both proteins or positive for both. A score of 1 was given if c-Myc was positive and Bcl2 was negative. A score of -1 was given if Bcl2 was positive while c-Myc was negative. A significant difference was found between the co-expression of these proteins in the radio- resistant and sensitive groups.

c-Myc & bcl2	Resistant	Sensitive
	1	1
	-1	1
	-1	1
	0	0
	1	0
		0
		0
		1
		1
Mean	**0**	**0.6**
P-value	**0.096**	

Figure 8.1. Expression of Ki67 measured in cultures of head and neck tumours before treatment (C) and after a single dose of 2 Gy vs. stage. (Correl. coeff.; Ki67(C) = 0.6: Ki67(2Gy) = 0.4).

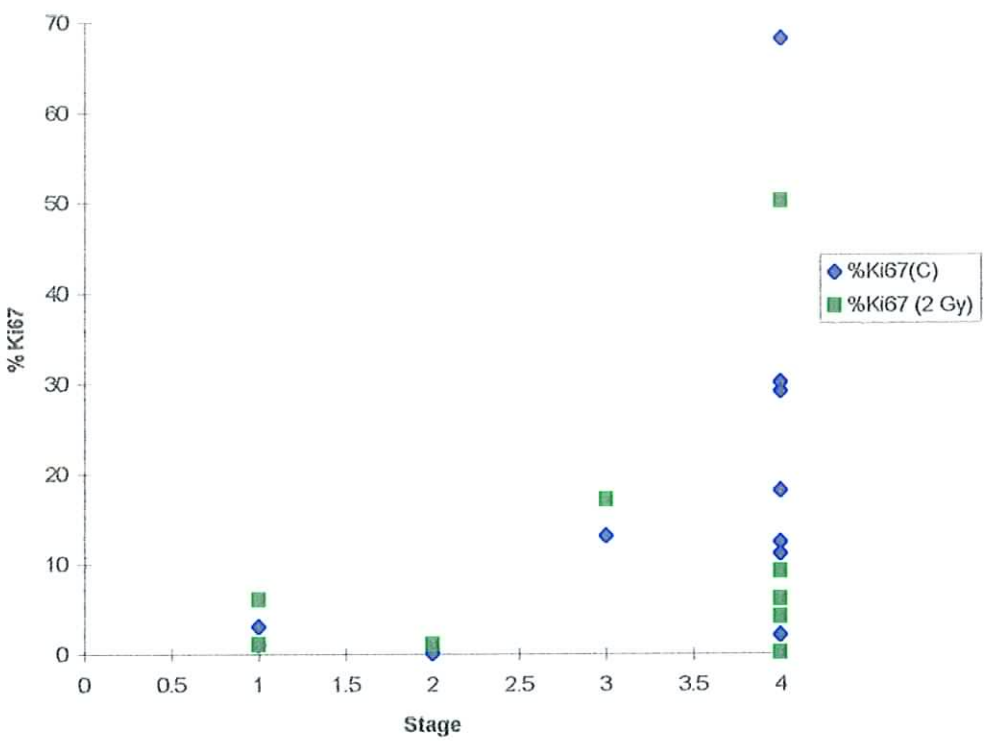
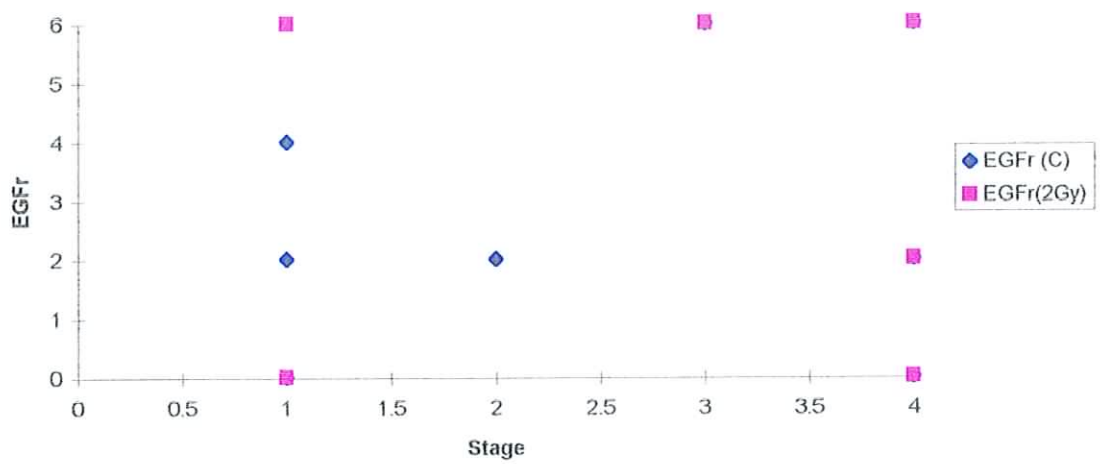


Figure 8.2. Expression of EGFr measured in cultures before treatment (C) and after a single dose of 2 Gy (2Gy), does not correlate with stage (correl. coeff.: C = 0.2; 2Gy = -0.1)



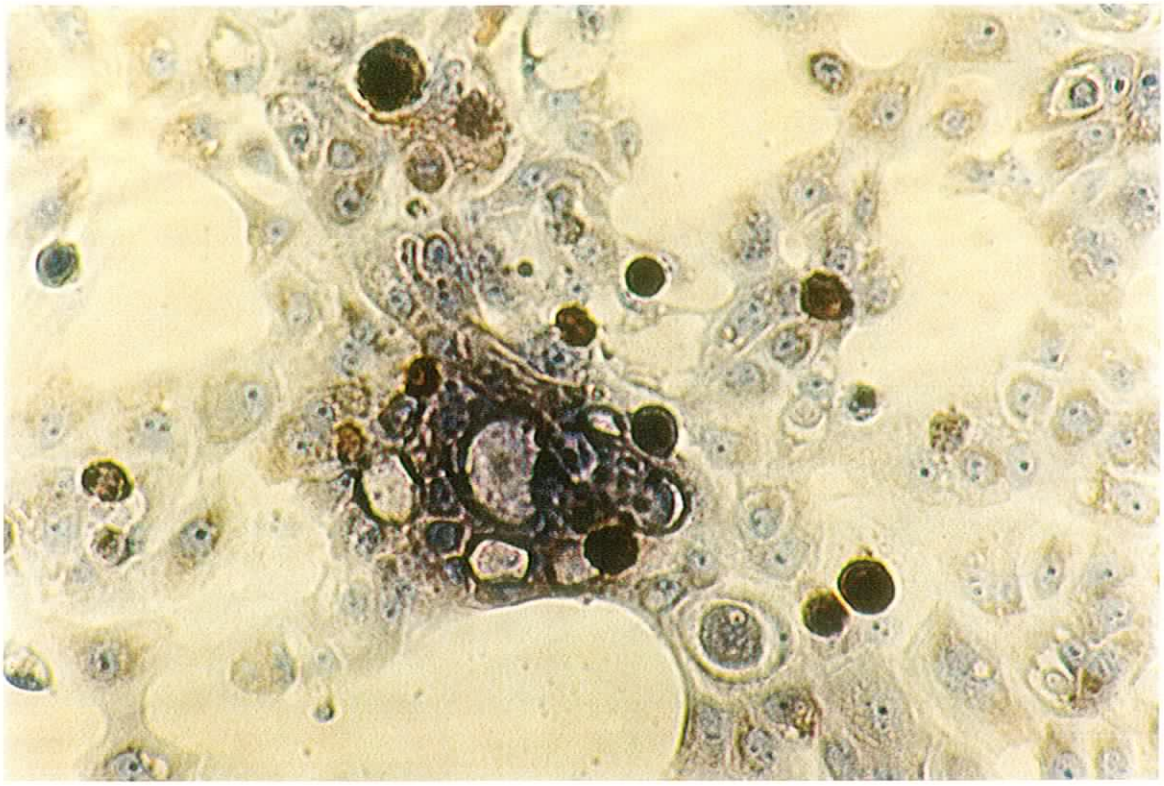


Plate 8.1. Cells cultured from an explant of head and neck SCC tumour, immunocytochemically stained for Ki67. Positive brown staining was localiised in the nucleus.

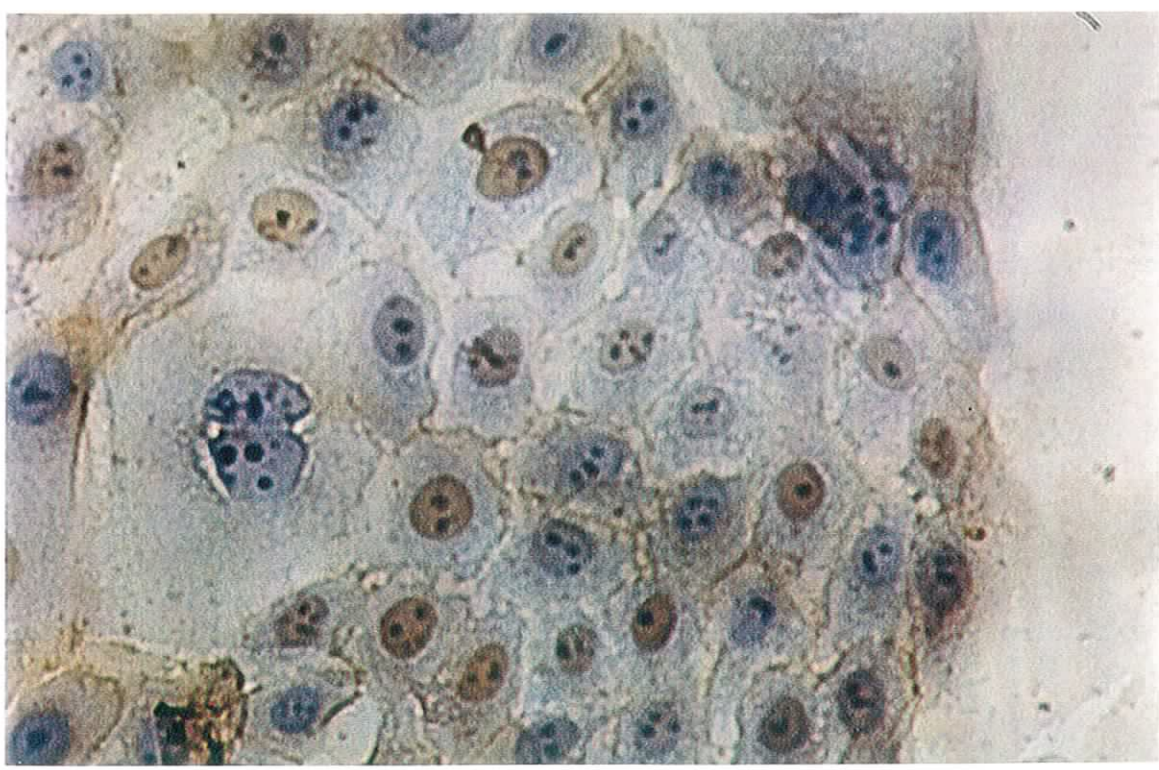


Plate 8.2. Cells cultured from an explant of head and neck SCC tumour, immunocytochemically stained for EGFr. Positive staining was localised in the cell membrane and the cytoplasm.

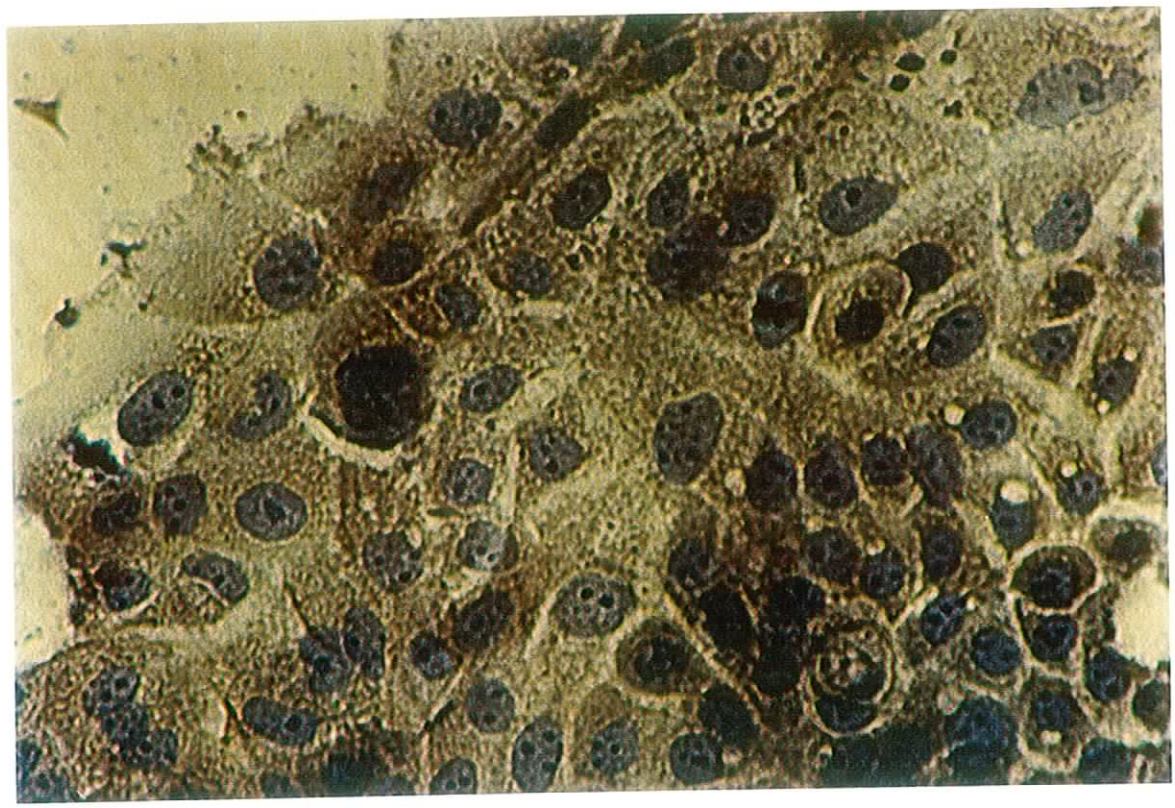


Plate 8.3. Cells cultured from an explant of head and neck SCC tumour, immunocytochemically stained for c-Myc. Positive brown staining was localised in the cytoplasm.

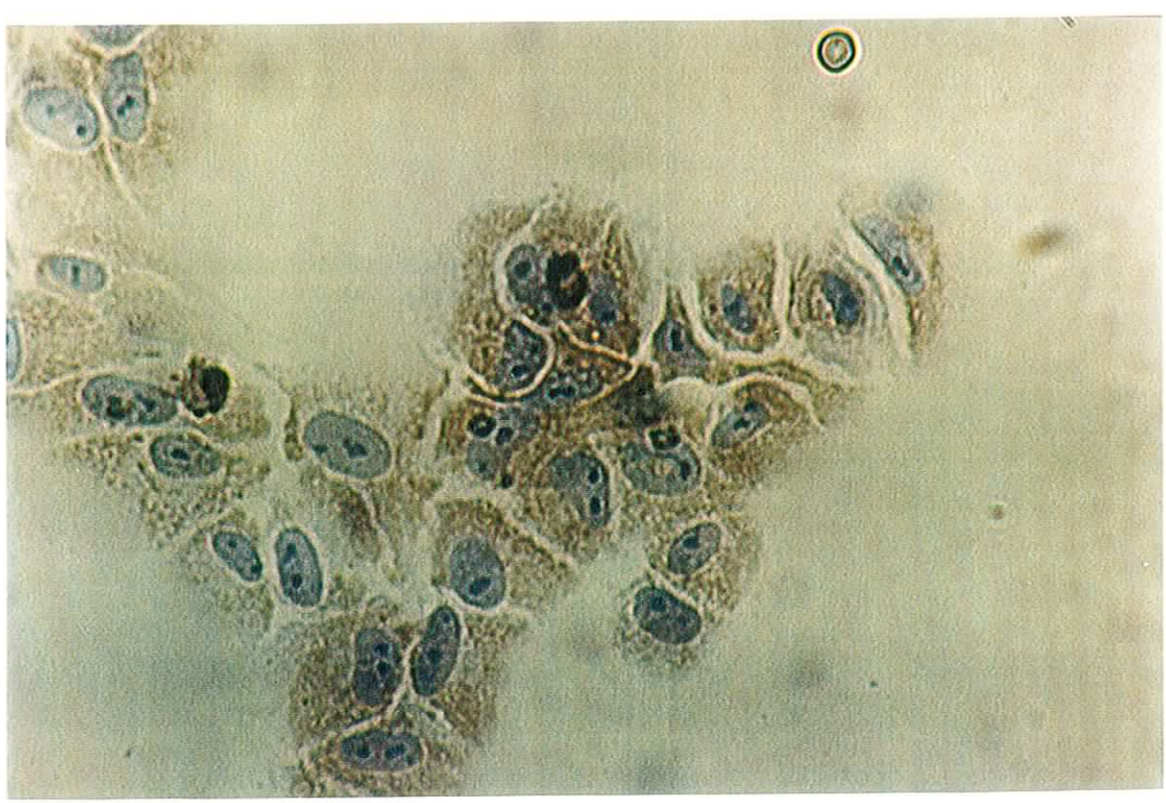


Plate 8.4. Cells cultured from an explant of head and neck SCC tumour, immunocytochemically stained for Bcl-2. Positive brown staining was localised in the cytoplasm.

8.4 Discussion

The positive correlation between the initial expression of Ki67 (%Ki67(C)) and stage, disagrees with the findings of two studies on HNSCC. Both Valente et al. (1994) and Ogawa et al. (1992) observed no correlation between the initial value of Ki67 and clinical stage.

In this study no correlation was found between the % Ki67 positive cells in the control cultures (i.e. before irradiation) and radiosensitivity, but the %Ki67 positive cells measured after irradiation with 2 Gy (%Ki67(2Gy)) significantly differentiated the responders from the non-responders. The findings of the two studies mentioned above support this.

Ogawa et al. (1992) immunocytochemically evaluated the Ki67 labelling index in 21 SCCHN biopsies taken before and during preoperative radiation therapy, at doses of 10 and 20 Gy. The results showed no correlation between Ki67 labelling indexes before radiation therapy and the antitumour effect of radiation. A marked decrease in Ki67 labelling was observed during radiotherapy (rates below 3%) and was related to poor clinical outcome. Valente et al. (1994) also evaluated the % Ki67 positive cells from biopsy specimens taken before treatment and after 10 Gy of radiotherapy, from 31 patients with oral SCC. This group also observed no correlation between the initial % Ki67 positive cells and response to radiotherapy, but found that the reduction in % Ki67 positive cells after 10 Gy was significantly correlated with a complete response.

Valente et al. (1994) also found that the % change in Ki67 positive cells after 10 Gy of radiotherapy significantly differentiated the responders from the non-responders, unlike the findings in this study which showed no correlation between the % change in Ki67 positive cells after 2 Gy and radiosensitivity.

The results of the present study can be likened to those of Valente et al.(1994), in that those patients with a marked decrease in % Ki67 positive cells after 2 Gy were sensitive to radiotherapy *in vitro*. Although the doses administered are different, the 2 Gy administered *in vitro* is equivalent to the clinical single fraction of radiotherapy.

The radiosensitivity of cells *in vitro* varies considerably as they pass through the cell cycle. There seems to be a general tendency for cells in the S-phase to be the most resistant and for cells in G_2 and mitosis to be the most sensitive (Steel, 1993). This is one of the basic findings in radiobiology and would indicate that a tumour with a large population of cycling cells would be more sensitive to radiotherapy than a tumour with a low population of cycling cells. Therefore, one would expect that evaluation of the population of cycling cells would be a useful indicator of radiosensitivity. However conflicting results have been reported.

Response to radiotherapy may not be restricted to the actively proliferating tumour cells. If the S phase represents the major part of the cell cycle in a tumour, that tumour should proliferate at a faster rate but also have a higher resistance to radiotherapy. This could explain the occurrence of higher numbers of Ki67 positive cells after treatment - resistant clones with higher proliferative activity may supervene, or quiescent cells may be recruited after irradiation (Valente, 1994).

It seems that overexpression of the EGF receptor is a frequent, if not constant, step in the malignant transformation of squamous cells (Ozanne et al., 1986). However overexpression of EGFr is not always accompanied by amplification of the EGFr gene, and in studies on head and neck SCC gene amplification has been found to have no prognostic value (Furuta et al., 1992). Therefore evaluation of EGFr overexpression is considered more valuable as a prognostic indicator in some cancers.

In this study the expression of EGFr was measured immunocytochemically in cultures established from 16 tumours from patients with head and neck SCC. EGFr was evaluated in cultures that received no treatment, EGFr(C), and in cultures that were irradiated with a single dose of 2 Gy. No correlation was found between expression of EGFr and stage, these results are in accord to those of Ishitoya et al. (1989), who found that overexpression of EGFr in 8 head and neck tumours was not related to stage. Itakura et al. (1994) also observed this lack of correlation between EGFr levels, immunocytochemically analysed in 217 cases of oesophageal SCC, and stage. Santini et al. (1991) evaluated EGFr by an EGF-binding assay in a much larger group of patients, 59 head and neck SCC tumour biopsies and found a significant correlation between EGFr levels and stage: EGFr levels increased with stage.

In this study there was a significant correlation found between the level of EGFr(C) and sensitivity to radiation treatment, those patients with high levels of EGFr were resistant to radiation while those with low levels were sensitive. Santini et al., (1991) found that head and neck SCC tumours positive for EGFr had a greater probability of complete response to chemotherapy than EGFr negative patients. In radiobiological terms a highly proliferative population of cells is considered to be more sensitive to the effects of DNA damaging agents such as radiation and some chemotherapeutic drugs. As EGFr is involved in signal transduction which results in the growth and proliferation of various cells (Carpenter and Cohen, 1979), one would expect that the level of EGFr would reflect the proliferative activity of the population and thus it's sensitivity to DNA damaging agents. However in the system presented here the expression of EGFr appears to indicate the aggressiveness of the tumour and thus its resistance to treatment. A study by Hickey et al. (1994) on the ability of the expression of EGFr and PCNA to predict the response of oesophageal SCC to chemo-radiotherapy, found that expression

of both these identified a group of patients who showed little response to treatment and had a universally poor outcome. These results also conflict with radiobiological findings.

No significant difference was found between the expression of c-Myc or Bcl-2 in the radio- resistant and sensitive groups. although c-Myc appeared to influence radiosensitivity. Table 8.14. The co-expression of c-Myc and Bcl-2 was examined to determine if one protein had more influence on radiosensitivity. than the other. A score of 0 was given when the expression of both proteins were positive. or both were negative. this allowed for the evaluation of the influential factor in the radio-sensitivity of that tumour. There was a significant difference between the co-expression of c-Myc and Bcl-2 in the radio- resistant and sensitive groups. In the sensitive group a mean value of 0.6 indicated that high expression of c-Myc and low expression of Bcl-2 influenced the radiosensitivity of the tumours. In the resistant group a mean value of 0, indicates that neither protein appeared to play an independent role.

Deregulated c-Myc expression induces the rapid onset of apoptosis in cells arrested by a variety of cytostatic and cytotoxic agents (Evan et al., 1992). Radiation also induces apoptosis. perhaps in this case radiation treatment induced apoptosis mediated by deregulated c-Myc in the radiosensitive tumours. In the resistant tumours where Bcl-2 appears to be the influencing factor. the expression of Bcl-2 could be preventing the apoptotic death induced by c-Myc, as shown by Bissonnette et al., (1992) and Wagner et al. (1993). This would allow the radiation damaged cells to survive and result in a resistant tumour.

In most studies evaluating the prognostic significance of Bcl-2 and c-Myc the patients have received surgery alone, or were some have received radiotherapy or chemotherapy. these have not been considered separately. Lipponen et al. (1995) in

study on renal adenocarcinoma found the expression of c-Myc to be related to a favourable outcome for patients treated by surgery alone and Bcl-2 to be related to smaller tumour size and the absence of metastasis. In a study on lung cancer all patients underwent surgery. 4 of these received postoperative radiotherapy and one received postoperative chemotherapy. In evaluating the prognostic significance those patients who received radio- and chemo-therapy were not evaluated separately. The results of the study suggested that Bcl-2 positive tumours had a better clinical outcome, but was not significantly different from Bcl-2 negative tumours.

In conclusion, of the parameters evaluated immunocytochemically in this study, some appeared to have the potential to indicate the radio-responsiveness of head and neck SCC tumours. The % Ki67 positive cells measured in cultures treated with a single dose of 2 Gy, predicted the *in vitro* response of tumours to irradiation: high levels correlated with radio-resistance. The level of expression of EGFr measured in control cultures and in irradiated cultures correlated significantly with radiosenstivity; high levels of EGFr correlated with radio-resistance. Radiosensitive tumours appeared to be influenced by expression of c-Myc, while radio-resistant tumours were positive for Bcl-2 and negative for c-Myc.

CHAPTER 9

p53 as an Indicator of Radiosensitivity

9.1 Introduction

Tumour supressor genes express a product that can suppress the expression or function of other genes involved with cell growth and proliferation. However, if the tumour suppressor gene is inactivated by a genetic lesion, then the supressor function is lost and the constraints imposed on the growth and proliferation genes is lifted, thereby leading to the growth of the cancer cell (Field et al., 1993).

Mutation of the p53 gene is the most common genetic alteration detected in squamous cell carcinoma of the head and neck (SCCHN) (Somers et al., 1992). The normal gene product is required for G_1 arrest following ionising radiation: cells having mutant or no p53 genes fail to demonstrate this response (Kastan et al., 1993). This suggests that p53 acts as a checkpoint control protein that halts the cell cycle in G_1 to allow for repair of DNA damage, or if the damage is deemed to be too severe, p53 can induce apoptosis (Lowe et al., 1993; Clarke et al., 1993).

Some studies have shown that radiosensitivity depends on the ability of p53 to induce a G_1 arrest following radiation. O'Connor et al. (1993) studied the role of p53 in the radiosensitivity of Burkitt's lymphoma and lymphoblastoid cell lines and found that cell lines with mutant p53 showed minimal G_1 arrest and were more radio-resistant than those cell lines that contained normal p53 genes. These findings were further supported by McIlwrath et al. (1994) who found that mutant p53 transfects of the radiosensitive human ovarian cell line A2780 were more resistant to ionising radiation than the parental line.

Studies on head and neck cancer cell lines have so far failed to show any correlation between p53 status and radiosensitivity (Jung et al., 1992; Brachman et al., 1993).

Preliminary results on the significance of p53 as an indicator of tumour radiosensitivity, measured immunocytochemically in primary cultures of head and neck tumours, suggested an association between low expression of p53 and radiosensitivity (Sheridan et al., 1995).

The aim of this study was to further establish this association with a larger patient number, and to investigate the significance of p53 expression on radiosensitivity of cell lines with varying radiosensitivities, measured as surviving fraction at 2 Gy (SF_2).

9.2 Materials and Methods

PATIENTS

Tumour specimens from 15 patients with head and neck squamous cell carcinoma were immunocytochemically analysed for expression of the non-functional conformation of the protein product of the p53 tumour supressor gene.

CELL LINES

Three cell lines, derived from a bladder tumour and 2 colorectal tumours, of varying radiosensitivity, Table 9.1, were immunocytochemically analysed for expression of the protein product of p53. Radiosensitivity was expressed in terms of surviving fraction at 2 Gy (SF_2).

Table 9.1. Cell lines of varying radiosensitivity and source, were examined immunocytochemically for expression of the non-functional protein product of p53.

Cell Line	Source	SF_2 Value
RT-112	Bladder tumour	0.59
HT-29	Colorectal tumour	0.74
SW-48	Colorectal tumour	0.16

IN VITRO CULTURE OF TUMOUR SPECIMENS

The primary explant culture method has been described in Section 3.1. Briefly, tumour specimens taken from the patient were placed in sterile saline, at ambient temperature and brought immediately to the laboratory for culture. In the laboratory the specimens were cut into explants of approximately 2-3 mm^3 and digested in a solution of weak trypsin/ collagenase, at a concentration of 10 mg/ ml, for 20 minutes at 37°C. After digestion the explants were plated singly in Nunclon 25 cm^2 flasks with 2 ml of growth media (RPMI -1640 containing 20% serum, hydrocortisone, insulin and antibiotics). Cultures were then placed in a CO_2 incubator at 37°C. After 72 hours incubation the cultures were treated with radiotherapy, and returned to the incubator immediately. After a further 11 days incubation the cultures were fixed and immunocytochemically stained.

IN VITRO RADIATION TREATMENT

After 72 hours of undisturbed growth the cultures were split into two groups, the control group which was to remain untreated, and the irradiated group which was to receive a

single dose of 2 Gy. The single dose of 2 Gy was administered using a cobalt 60 teletherapy unit.

IMMUNOCYTOCHEMICAL ANALYSIS

Protein expression of p53 was evaluated using a monoclonal antibody, p53-240 (DAKO) specific for wild and mutant forms of the p53 protein. Immunostaining was performed using the Vectastain ABC kit, which employs a standard indirect immunoperoxidase method. The protocol for the indirect immunoperoxidase method is described fully in Section 3.5.

For the cultured tumour specimens, immunocytochemical staining was carried out directly on the outgrowth produced from the explant, and expression of p53 was evaluated by counting the percentage positive cells along a cross section through the outgrowth area (see Section 3.6).

Cytospin preparations of each cell line were made in triplicate, and immunostaining carried out on the slide. The expression of p53 protein was determined for each cell line by counting the number of positive cells in a population of at least 200 cells, for each of the three slides and a mean calculated. Measurement of p53 expression was carried out without knowledge of the SF_2 values for each cell line.

9.3 Results

Head and Neck Tumours

The expression of p53 was localised in the nucleus and cytoplasm. Plate 9.1. Table 9.2 shows the expression of p53 measured in the original set of 10 patients, grouped according to their radiosensitivity. Although there was a trend towards low expression of p53 and sensitivity to a single dose of 2 Gy, the difference between the expression of p53 in the sensitive and resistant groups was not significant ($p = 0.12$).

Table 9.3 shows the expression of p53 for 15 patients, including the original 10 shown in Table 9.2. The expression of p53 was measured in control cultures, %p53(C), that received no treatment and in cultures that received a single dose of 2 Gy, %p53(2Gy).

Table 9.2. Expression of p53 measured in cultures of head and neck tumours, grouped according to the *in vitro* radiosensitivity of that patient to a single dose of 2 Gy. The difference between the expression of p53 in the sensitive and resistant groups was not significant.

%p53	Sensitive	Resistant
	1	25
	1	100
	3	14
	8	2.5
		17
		5
Mean	**3**	**27**
P-value	**0.12**	

The stage and *in vitro* response to irradiation, is shown for each patient. Patients are ranked in order of increasing radio-resistance. There were very weak correlations

between the %p53(C) and %p53(2Gy), and stage of tumour, Figure 9.1. The correlation coefficients were 0.3 and 0.2 respectively.

Table 9.3. Expression of p53 measured in cultures of head and neck tumours that received no treatment, %p53(C), and that were treated with a single dose of 2 Gy, %p53(2Gy). The stage and *in vitro* radiosensitivity is shown for each patient. Patients are ranked according to increasing radio-resistance.

Patient	Stage	%GI (2Gy)	% p53(C)	%p53(2Gy)
1	1	91	8	39
2	4	91	3	0
3	4	85	1	6
4	3	83	100	100
5	4	61	100	100
6	4	57	17	
7	1	48	1	1
8	1	38	9	3
9	4	5	14	
10	2	0	5	31
11	1	0	0	
12	4	0	2.5	
13	4	0	17	
14	4	0	100	
15	4	0	25	
Mean	**3**	**37**	**27**	**26**

The expression of p53 measured in the control cultures, %p53(C), did not differ significantly between radio- resistant and sensitive groups ($p = 0.38$). Table 9.4. The same was found for the expression of p53 measured in the irradiated cultures, p53(2Gy) ($p = 0.47$). Table 9.5.

In summary, no correlation was found between expression of p53, measured in control cultures or irradiated cultures, and the *in vitro* radiosensitivity of each patient to a single dose of 2 Gy, Table 9.6.

Table 9.4. Expression of p53 measured in control cultures does not differ significantly between radio-resistant and sensitive groups.

%p53(C)	Resistant	Sensitive
	14	8
	5	3
	0	1
	2	100
	17	100
	100	17
	25	1
		9
Mean	23	30
P-value	0.38	

Table 9.5. Expression of p53 in cultures treated with a single dose of 2 Gy does not differ significantly in radio- resistant and sensitive groups.

%p53(2Gy)	Resistant	Sensitive
	31	39
	1	6
	3	0
	100	100
Mean	34	36
P-value	0.47	

Table 9.6. Expression of p53 measured in control cultures or after irradiation did not correlate with *in vitro* radiosensitivity.

	Resistant	Sensitive	P-value
Mean %p53(C)	23	30	0.38
Mean %p53(2Gy)	34	36	0.47

Cell Lines

Table 9.7 shows the expression of p53 measured in each cytospin preparation for each cell line. The mean % p53 and the SF_2 value is shown for each cell line. The cell line SW-48 was radiosensitive, with an SF_2 value of 0.16. The cell line HT- 29 was radio-resistant, SF_2 value of 0.74 and RT -112 was intermediate, SF_2 value of 0.59. Immunocytochemical staining for p53 was localised in the nucleus. The level of expression of p53 did not differ significantly between the radiosensitive cell line, SW-48, Plate 9.2, and the intermediate cell line, RT-112 ($p = 0.17$), Plate 9.3. However there was a significant difference between the level of expression of p53 measured in the radiosensitive SW-48 and the radio-resistant HT-29 ($p = 0.0005$), Plate 9.4. There was also a significant difference between the level of expression of p53 measured in the radio-resistant HT-29 and the intermediate RT-112 ($p = 0.004$).

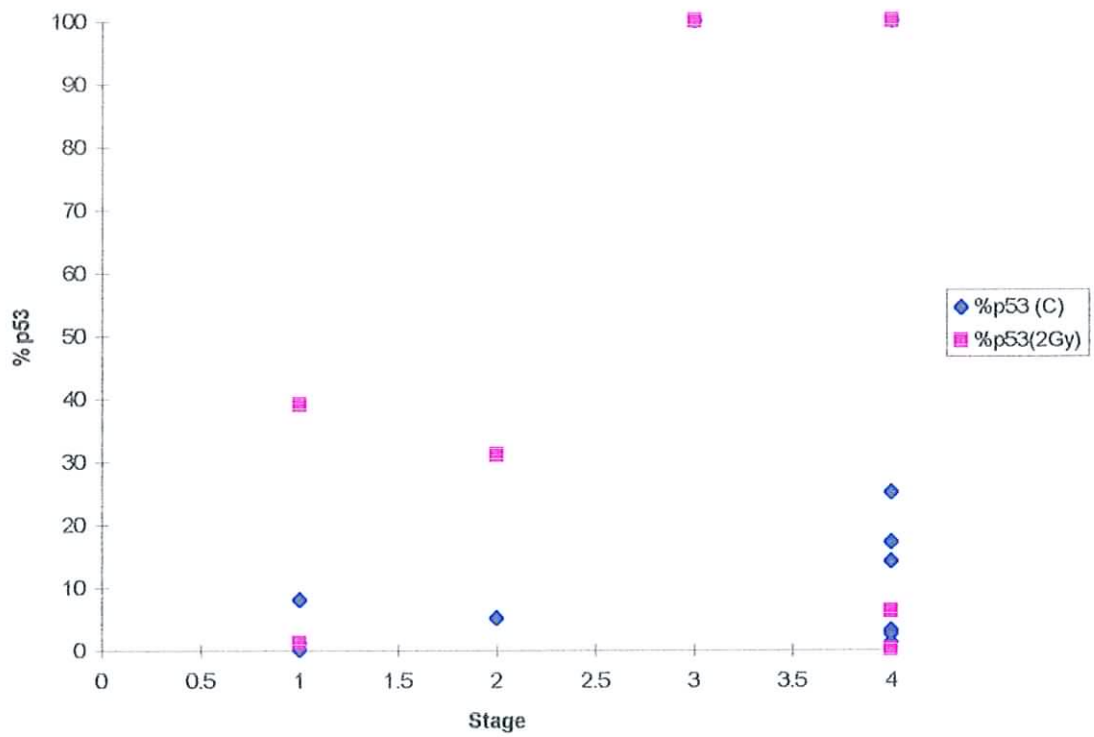
Table 9.7. % positive cells in a population of at least 200 cells, for three cytospin preparations from each cell line. The mean %p53 positive cells is shown for each cell line ± standard error of the mean (SEM).

	RT-112	SW-48	HT-29
% p53 - slide 1	2	1	74
% p53 - slide 2	7	0	86
% p53 - slide 3	26	9	
Mean % p53 ± SEM	**12 ± 7.3**	**3 ±3**	**80 ± 6**
SF_2 value	**0.59**	**0.16**	**0.74**

Table 9.8. P-values, indicating whether the expression of p53 measured in a cell lines differed significantly between cell lines of varying radiosensitivity. The radiosensitivity status of each cell lines is given with the corresponding p-value, calculated from a student t-test comparing the level of expression of p53 in both cell lines.

Compared % p53 of Cell Lines	P-value
Sensitive & Resistant	0.0005
Intermediate & Resistant	0.004
Intermediate & Sensitive	0.17

Figure 9.1. Expression of p53 in cultures of head and neck tumours before treatment (C) and after a single dose of 2 Gy, plotted against stage (Corr. coeff. = 0.3, 0.2).



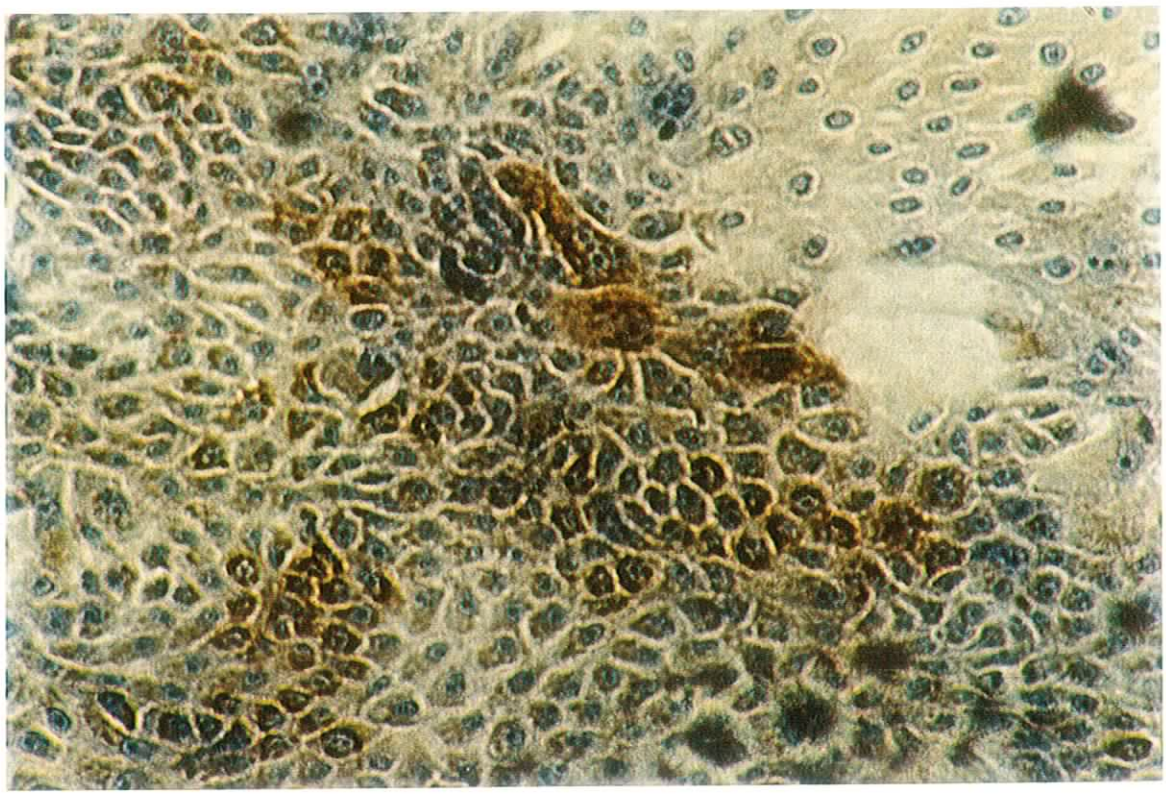


Plate 9.1. Expression of p53 protein evaluated immunocytochemically in cultures derived from explants of head and neck tumours, was localised in the nucleus and the cytoplasm of the cells.

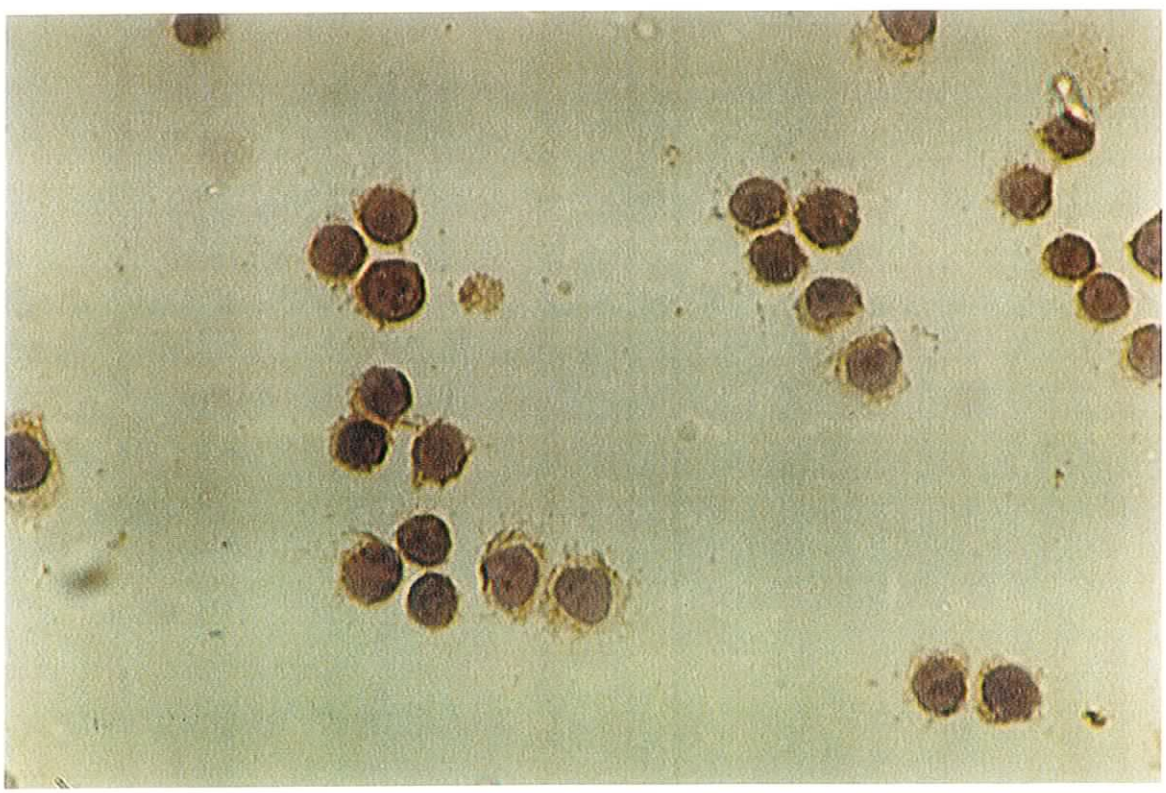


Plate 9.2. Expression of p53 protein in cytospin preparations of the radiosensitive SW48 cell line, derived from a human colorectal tumour. Positive staining was localised in the nucleus.

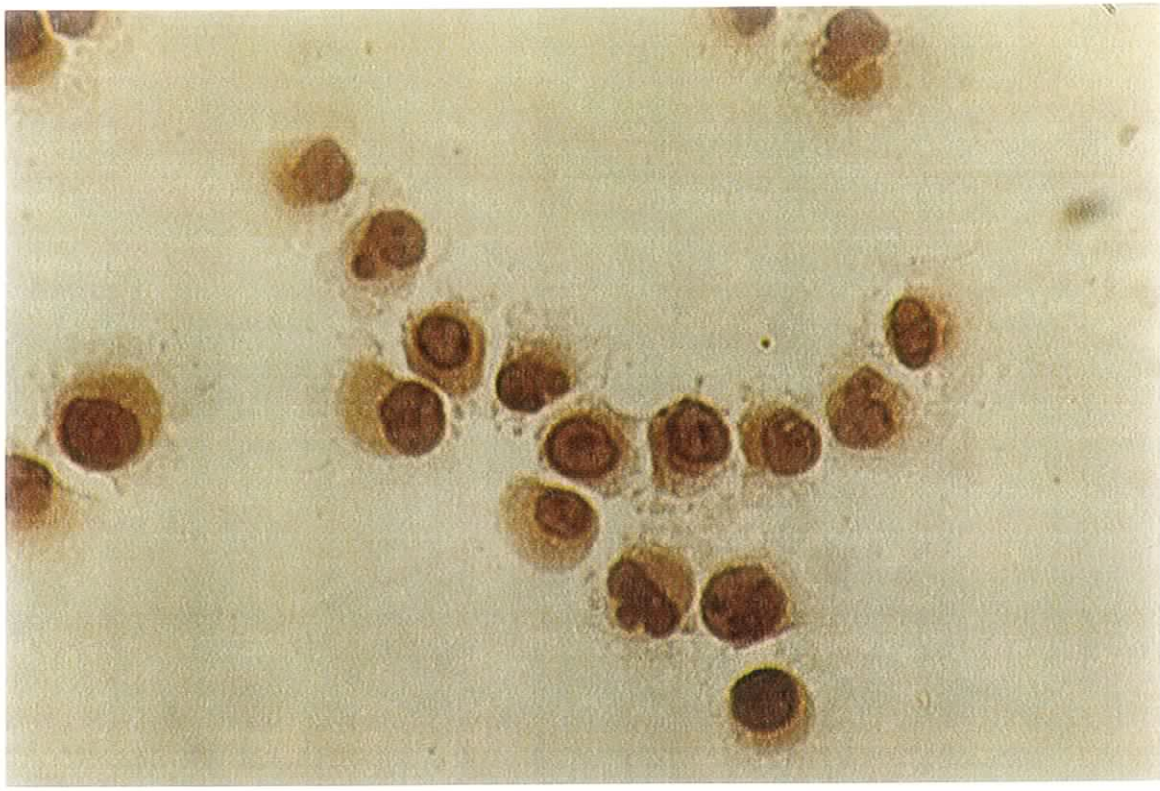


Plate 9.3. Expression of p53 in a cytospin preparation of the RT112 cell line (intermediate sensitivity),, derived from a bladder tumour, was localised in the nucleus.

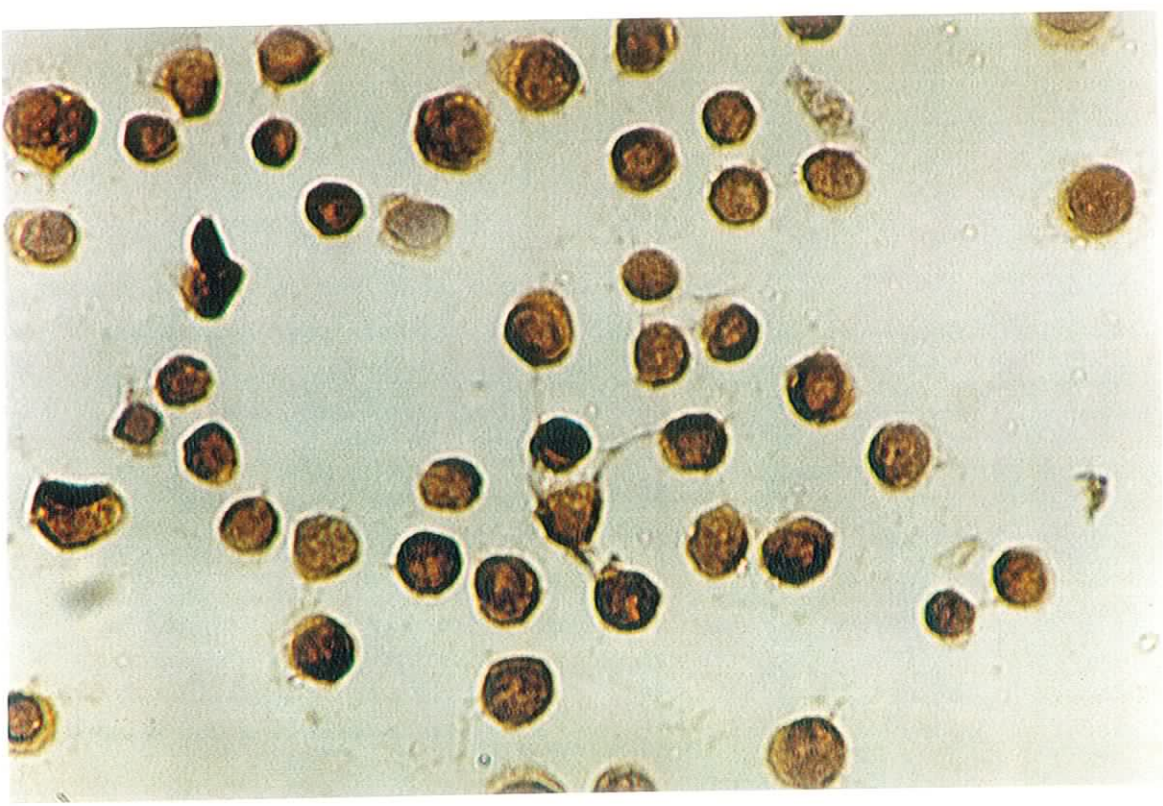


Plate 9.4. Expression of p53 protein evaluated immunocytochemically in a cytospin preparation of the radioresistant Ht29 cell line, derived from a human colorectal tumour. Positive staining was localised in the nucleus.

9.4 Discussion

The results for the original 10 primary head and neck tumours, indicated a trend towards expression of p53 and radio-resistance, however in the larger patient group the trend was lost. These results agree with the findings of most previous studies on head and neck cancer cell lines which concluded that mutations in the tumour supressor gene do not correlate with radio-resistance.

Jung et al. (1992) examined p53 mutation status by single-stranded conformational polymorphism in 6 head and neck SCC cell lines, and p53 overexpression by immunocytochemical analysis in one cell line. Three of the radiation sensitive and two of the radiation resistant cell lines contained mutations in the p53 gene, indicating that mutations to the gene did not correlate with radiosensitivity. Two of the 3 radio-resistant cell lines were immunocytochemically positive for the p53 protein, while all 3 of the sensitive cell lines were negative for the protein. The authors commented that there was no correlation between p53 expression and clinicopathological parameters, but no comment was made concerning the possible trend between p53 protein expression and radio-resistance. A trend which was also seen in preliminary results of this study.

Brachman et al. (1993) also examined the p53 mutational status of 24 head and neck SCC cell lines and correlated it with the radiosensitivity of the cell lines, measured by surviving fraction at 2 Gy. The results suggested that p53 mutation may predispose to increased radio-resistance, p53 protein expression was not evaluated.

While most studies in head and neck cancer have evaluated the p53 gene mutation status, few have evaluated the expression of the p53 protein. A recent study which did examine p53 protein levels in 33 primary head and neck SCC tumours treated with

surgery and radiotherapy, found no indication of a clinical or prognostic significance for p53 expression (Nylander et al., 1995).

The results for the 3 cell lines, none of which originated from a head and neck tumour, showed a highly significant difference between expression of p53 in the radio-resistant and sensitive cell lines. This is consistent with studies showing that in many human cancers, overexpression of p53 protein is not due to increased transcription, but results from increased protein stability caused by mutations Xia et al., 1995).

A study on 12 human tumour cell lines originating from a variety of human tumours including bladder, neuroblastoma and ovarian, evaluated p53 protein levels using the ELISA technique (McIlwrath et al., 1994). The results showed that the radio-resistant cell lines expressed high levels of p53, which may have been indicative of non-functional p53 protein (McIlwrath et al., 1994), and thus supported the results of this study. Another study which examined p53 protein levels in 17 Burkitt's lymphoma and lymphoblastoid cell lines which exhibited three types of response in G_1 following irradiation (O'Connor et al., 1993). The results showed that the radiosensitivity of most of the cell lines appeared to depend on the ability of p53 to induce a G_1 arrest, and in cell lines that exhibited minimal G_1 arrest elevated levels of mutant p53 protein were found. The results also showed that the mutant p53 lines did not show any further accumulation of the p53 protein following radiation. These findings are comparable to the results presented here, in that, the p53 protein levels either remained the same or were reduced after irradiation, in 62% of the tumours examined. Xia et al. (1995) in a study on the p53 status of two closely related human lymphoblast cell lines that vary significantly in their response to ionising radiation, found that the p53 protein levels of

the radio-resistant line were four times higher than that in the sensitive line. Irradiation also lead to higher levels of p53 in both lines but to a greater extent in the sensitive cell line, indicating that the p53 was functional in the sensitive cell line.

Conflicting results by Biard et al. (1994) showed that epithelial cells derived from an embryonic rat lung bearing mutation of the p53 gene displayed enhanced sensitivity to ionising radiation with doses of 2-12 Gy, compared with their counterparts carrying wild-type p53 alleles.

From other studies and from this study there appears to be evidence to support a role for non-functional p53 induced radio-resistance in cell lines of varying radiosensitivity and origins. This does not however extend to head and neck tumours or cell lines derived from head and neck tumours, although Brachman et al. (1993) suggested that p53 alterations may predispose to increased radio-resistance. The earlier results of this study also suggested a trend towards high levels of p53 protein and radio-resistance. Taken together, these results suggest that p53 protein plays a complex role in modulating radio-resistance and in the case of head and neck cancer, p53 does not appear to directly influence radiosensitivity.

CHAPTER 10

General Conclusions

Optimisation of the Outgrowth Assay

Standardising the explant size by using the cutting template did achieve the reduction in the standard error of the mean total cell number of cultures from a specimen, however this difference did not reach statistical significance.

The three culture vessels investigated, although more compact and easier to use, did not prove to be suitable for culturing explants in this system. The attachment was poor in these vessels resulting in fewer cultures, and cell numbers were less than those achieved when explants were cultured in the Nunclon 25 cm^2 flasks. The original culture vessel therefore remains the most suitable vessel for culturing explants in this system.

Culture of Biopsy Material

Using this assay epithelial cultures were successfully established from biopsy material taken from patients with suspected oesophageal cancer. The epithelial nature of the cultures was confirmed immunocytochemically and by electron microscopy. The ability to culture pre-treatment biopsy material makes this system more applicable to the clinical situation: determination of the *in vitro* response of a patient to treatment, prior to commencement of treatment could aid treatment planning.

Indicators of Response - Oesophageal Carcinoma

Using the Mothersill outgrowth assay it was possible to measure the *in vitro* response of individual patients to treatment, from pre-treatment biopsy material. It was also possible to carry out immunocytochemically analysis on the same cultures that were assessed for response to treatment. In the clonogenic assays used by West et al. (1995)

and Grinsky et al. (1994) it is not possible to measure multiple immunocytochemical parameters and obtain predictive results.

The parameters measured, as described in Chapter 6, were % growth inhibition after irradiation with a single dose of 2 Gy (%GI(2Gy)), % Ki67 positive cells and the level of expression of EGFr. As none of the patients received radiotherapy alone, it was not possible to correlate %GI(2Gy) with patient response to treatment, or patient outcome. It was possible to correlate the %Ki67 positive cells and expression of EGFr, measured in cultures that received no treatment, with the *in vitro* response to treatment. A significant difference was found between the %Ki67 positive cells, expression of EGFr and the %GI(2Gy) measured in adenocarcinoma biopsies. High expression of both these parameters correlated with radio-resistance. A study by Hickey et al. (1994) on the ability of PCNA (another measurement of population of proliferating cells) and EGFr to predict the outcome of oesophageal cancer patients to treatment, found that tumours positive for both markers showed little response to chemo-radiotherapy and had a universally poor outcome.

Taken together these results suggest a role for Ki67 and EGFr as indicators of the aggressiveness of a tumour.

Indicators of Response- Head and Neck Squamous Cell Carcinoma

As stated above it was possible to measure a range of parameters as possible indicators of response to treatment, from the same tumour, using this assay. In the case of head and neck cancer the parameters measured where: % growth inhibition (%GI) after treatment with radiotherapy, chemotherapy or a combination of both; % Ki67 positive cells; level of expression of EGFr; expression of c-Myc; expression of Bcl-2; co-expression of c-Myc and Bcl-2; expression of p53.

It was not possible to make a conclusion on the ability of the *in vitro* %GI after treatment to predict the patient outcome to that treatment. The main reasons for this were, the number of patients within one treatment group were too small (e.g. 7 patients treated with surgery followed by radiotherapy, 4 patients treated with surgery followed by chemo-radiotherapy):, and the follow up time was too short. However within these groups the results look promising. There appears to be a trend between patient status and response to *in vitro* irradiation. %GI(2Gy), for example; 6 patients were treated with surgery and post-operative radiotherapy, 5 are alive and well (A&W), and one is alive with disease (AWD). Of the 5 patients A&W, three had a %GI(2Gy) of >57% (sensitive) and 2 had a %GI(2Gy) of < 5%(resistant). The %GI(2Gy) measured *in vitro* appears to have been predictive of the outcome of 4 patients: the 3 A&W and 1 AWD. A larger patient group and longer follow up time will allow for conclusive results.

Of the other parameters measured, %Ki67 measured in cultures irradiated with a dose of 2 Gy, significantly differentiated the *in vitro* radiosensitive and radio-resistant tumours. The level of expression of EGFr, measured in cultures that received no treatment and that were irradiated with a dose of 2 Gy, was significantly different in the radiosensitive and radio-resistant tumours (section 8.3). The independent expression of c-Myc and Bcl-2 did not differentiated the radio- resistant and sensitive groups. However, the *in vitro* radiosensitivity of a tumour appeared to be significantly influenced by the positive expression of c-Myc, where Bcl-2 was negative, Table 8.16.

p53 as an Indicator of Radiosensitivity

The role of p53 in influencing radio-resistance is a controversial one, as reflected in the contradictory reports in the literature. Earlier results in this study suggested a possible

trend between expression of non-functional p53 protein and radio-resistance, however this trend was lost on increasing the patient number (Section 9.3). These findings are in accord with those of the literature, however most studies in the literature, particularly in the case of head and neck cancer, have examined the p53 gene mutation status and not the level of expression of the non-functional protein.

The expression of non-functional p53 protein measured immunocytochemically in three cell lines, originating from human colorectal and bladder tumours, significantly differentiated the radio- resistant cell line from the radiosensitive (Section 9.3). A number of studies on the role of p53 in influencing radiosensitivity, measured p53 protein levels in cell lines derived from a variety of tumours (other than head and neck) and found that non-functional p53 protein expression appeared to correlate with radio-resistance.

These results suggest a role for expression of non-functional p53 in influencing radio-resistance, this role appears to be more complex in head and neck squamous cell carcinoma.

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APPENDIX 1

The data presented in this appendix refers to Chapter 5. and is summarised in Section 5.3.

Table 5.1. Growth area and cell number achieved from explants of an oesophageal biopsy cultured in Nunclon 25 cm^2 flasks. An initial volume of 1 ml of growth media was added to each flask, with an additional 1 ml of media added at day 2. A total of 14 days in culture was allowed.

Area of Outgrowth	Cell No/mm^2	Mean Cell No./mm^2	Total Cell No. ($\times 10^3$)
511	754 484 382	541 ± 110	276.3 ± 56.2
659	372 590 448	470 ± 64	309.7 ± 42.2
693	602 510 456	523 ± 43	362.2 ± 29.8
556	810 354 432	532 ± 141	295.8 ± 78.4
672	536 524 426	495 ± 35	332.9 ± 23.5
Mean 618 ± 36			**315.4 ± 15**

Table 5.2. Growth area and cell number achieved from explants of an oesophageal biopsy plated in Nunclon 25 cm^2 flasks. An initial volume of 0.8 ml of growth media was added to each flask, with an additional volume of 1.2 ml added at day 2. A total of 14 days in culture were allowed.

Area of Outgrowth	Cell No./ mm^2	Mean Cell No./ mm^2	Total Cell No. ($\times 10^3$)
632	470 528 356	451 ± 50	285.2 ± 31.6
343	526 596 492	538 ± 31	184.5 ± 10.6
152	318 238 542	366 ± 91	55.6 ± 13.8
162	422 368 260	350 ± 48	56.7 ± 7.8
2	16 174	95 ± 79	0.2 ± 0.17
Mean 258 ± 108			**116.4 ± 51.9**

Table 5.3. Growth area and cell number achieved from explants of an oesophageal biopsy cultured in a Falcon 6-well plate. An initial volume of 0.4 ml was added to each well, with an additional volume of 0.4 ml added at day 2. A total of 14 days in culture were allowed.

Area of Outgrowth	Cell No./ mm^2	Mean Cell No./ mm^2	Total Cell No. ($\times 10^3$)
0			
319	400 532 520	484 ± 42	154.4 ± 13.4
0			
Mean 106 ± 106			**51.1 ± 51.5**

Table 5.4. Growth area and cell number produced from explants of an oesophageal biopsy cultured in a Falcon 6-well plate. An initial volume of 0.6 ml of growth media was added to each well, with an additional volume of 0.2 added at day 2. A total of 14 days in culture were allowed.

Area of Outgrowth	Cell No./ mm^2	Mean Cell No./ mm^2	Total Cell No. ($\times 10^3$)
64	268 112 210	197 ± 45	12.6 ± 2.9
0			
11	104 94 106	101 ± 4	1.1 ± 0.04
Mean 25 ± 20			**4.6 ± 4**

APPENDIX II

The data in this appendix refers to Chapter 6. Tables 1-14 represent patients 1-14. Each table shows the mean growth areas achieved from endoscopic biopsies taken from a patient, cultured *in vitro* and treated with clinical equivalent doses of chemo- and radiotherapy. The type of tumour is given for each table. The sensitivity of the tumour to treatment was measured in terms of the % growth inhibition (%GI) in treated cultures relative to the untreated controls. Increasing %GI correlates with increasing *in vitro* sensitivity.

The % Ki67 positive cells and expression of EGFr measured immunocytochemically in control cultures, is shown for each patient. The level of expression of EGFr was evaluated semi-quantitatively (see Section 3.10) as follows:

0 - 25% positive cells - 2; 25-50% positive cells - 4; > 50% positive cells - 6.

PATIENT 1

Table 1 . Adenocarcinoma

	Treatment			
	Control	**2 Gy**	**Cisplatin + 5FU**	**2 Gy + Cisplatin + 5FU**
Mean Growth Area (mm^2)	18 ± 1	19 ± 2	NT[1]	NT
% Growth Inhibition		0%		
% Ki67	23			
%Expression of EGFr	6			

PATIENT 2

Table 2. . Adenocarcinoma

	Treatment			
	Control	**2 Gy**	**Cisplatin + 5FU**	**2 Gy + Cisplatin + 5FU**
Mean Growth Area (mm^2)	4 ± 1	6 ± 1	NT	NT
% Growth Inhibition		0		
% Ki67	15			
%Expression of EGFr	6			

PATIENT 3

Table 3. Adenocarcinoma

	Treatment			
	Control	**2 Gy**	**Cisplatin + 5FU**	**2 Gy + Cisplatin + 5FU**
Mean Growth Area (mm^2)	17 ± 3	7 ± 1.5	NT	NT
% Growth Inhibition		60		
% Ki67	6			
Expression of EGFr	4			

PATIENT 4

Table 4. Adenocarcinoma

	Treatment			
	Control	**2 Gy**	**Cisplatin + 5FU**	**2 Gy + Cisplatin + 5FU**
Mean Growth Area (mm^2)	16 ± 7	7	NT	NT
% Growth Inhibition		56		
% Ki67	12.5			
%Expression of EGFr				

[1]NT = no treatment administered

PATIENT 5

Table 5. Adenocarcinoma

	Treatment			
	Control	**2 Gy**	**Cisplatin + 5FU**	**2 Gy + Cisplatin + 5FU**
Mean Growth Area (mm^2)	27 ± 19	4 ± 1	NT	NT
% Growth Inhibition		85		
% Ki67	3			
Expression of EGFr	0			

PATIENT 6

Table 6. Adenocarcinoma

	Treatment			
	Control	**2 Gy**	**Cisplatin + 5FU**	**2 Gy + Cisplatin + 5FU**
Mean Growth Area (mm^2)	9 ± 1	5 ± 2	NT	NT
% Growth Inhibition		44		
% Ki67	6			
Expression of EGFr	4			

PATIENT 7

Table 7. Adenocarcinoma

	Treatment			
	Control	**2 Gy**	**Cisplatin + 5FU**	**2 Gy + Cisplatin + 5FU**
Mean Growth Area (mm^2)	2 ± 0.3	0	0	0
% Growth Inhibition		100	100	100
% Ki67	4			
Expression of EGFr	0			

PATIENT 8

Table 8. Adenocarcinoma

	Treatment			
	Control	**2 Gy**	**Cisplatin + 5FU**	**2 Gy + Cisplatin + 5FU**
Mean Growth Area (mm^2)	2.5 ± 0.5	18.5 ± 13	3.5	2 ± 0.5
% Growth Inhibition		0	0	20
% Ki67	23	19		
Expression of EGFr	6	4		

PATIENT 9

Table 9. Adenocarcinoma

	Treatment			
	Control	**2 Gy**	**Cisplatin + 5FU**	**2 Gy + Cisplatin + 5FU**
Mean Growth Area (mm^2)	30	7 ± 5	6.5 ± 4	1 ± 0.25
% Growth Inhibition		77	78	97
% Ki67	16		2	
Expression of EGFr				

PATIENT 10

Table 9. Squamous Cell Carcinoma

	Treatment			
	Control	**2 Gy**	**Cisplatin + 5FU**	**2 Gy + Cisplatin + 5FU**
Mean Growth Area (mm^2)	12 ± 8	55 ± 30	NT	NT
% Growth Inhibition		0		
% Ki67				
Expression of EGFr	6			

PATIENT 11

Table 10. Squamous Cell Carcinoma

	Treatment			
	Control	**2 Gy**	**Cisplatin + 5FU**	**2 Gy + Cisplatin + 5FU**
Mean Growth Area (mm^2)	5 ± 1	0	NT	NT
% Growth Inhibition		100		
% Ki67	9			
Expression of EGFr				

PATIENT 12

Table 11. Squamous Cell Carcinoma

	Treatment			
	Control	**2 Gy**	**Cisplatin + 5FU**	**2 Gy + Cisplatin + 5FU**
Mean Growth Area (mm^2)	260	212	45	1
% Growth Inhibition		18	83	99
% Ki67	36			
Expression of EGFr	4			

PATIENT 13

Table 12. Squamous Cell Carcinoma

	Treatment			
	Control	**2 Gy**	**Cisplatin + 5FU**	**2 Gy + Cisplatin + 5FU**
Mean Growth Area (mm^2)	4	46	35	8
% Growth Inhibition		0	0	0
% Ki67	5			
Expression of EGFr			6	

PATIENT 14

Table 13. Squamous cell carcinoma

	Treatment			
	Control	**2 Gy**	**Cisplatin + 5FU**	**2 Gy + Cisplatin + 5FU**
Mean Growth Area (mm^2)	6.5 ± 2	5 ± 1	6	0
% Growth Inhibition		23	8	100
% Ki67	1.4			
Expression of EGFr	6			

APPENDIX III

The data in this appendix refers to Chapter7. Tables 1-24 represent patients 1-24. Each table shows the treatment added to each culture established from a squamous cell carcinoma of the head and neck (SCCHN) tumour (concentration of chemotherapeutic agents, μg/2ml and dose of radiotherapy, Gy) and the resultant growth area in that culture after treatment. The growth area was measured against a grid of 1 mm^2 boxes, the area on the grid covered by the growth area was equal to the area of the growth from the explant. The cell number of each growth area was counted in three randomly chosen areas within the growth, the mean cell number calculated and from this the total cell number calculated for that growth area;

mean cell number × growth area = total cell number

The % growth inhibition in cultures after treatment was determined relative to the untreated control, which was considered to have 0% growth inhibition (%GI). Increasing growth inhibition correlates with increasing *in vitro* sensitivity to treatment.

SEM = standard error of the mean

S.E. = standard error

f = fibroblastic cell growth

Patient 1

Cisplatin(ug/2ml)	5-Flurouracil(ug/2ml)	Rad. (Gy)	Growth Area (mm2)	Cell No./mm2	Mean	S.E.	Total Cell No(x103)	S.E.	Inhibition(%)
0	0	0	f						
0	0	0	f						
0	0	0	10	192 128 202	174	23	1.74	0.23	
						Mean	**1.74**		
0	0	2	f						
0	0	2	f						
0	0	2	12	120 186 184	163	22	1.96	0.26	
						Mean	**1.96**		**0**
0.1	2	0	0			0			
0.1	2	0	f						
						Mean	**0**		**100**
1	2	0	2	54 44	49	5	0.1	0.01	
1	2	0	0				0		
						Mean	**0.05**		**100**
0.1	2	2	f						
0.1	2	2	f						
0.1	2	2	1.5	226			0.34		
						Mean	**0.34**		**80**
1	2	2	f						
1	2	2	f						
1	2	2	3	78 90 52	73	11	0.22	0.03	
						Mean	**0.22**		**87**

Patient 2

Cisplatin(ug/2ml)	5-Flurouracil(ug/2ml)	Rad. (Gy)	Growth Area (mm2)	Cell No./mm2	Mean	S.E.	Total Cell No(x103)	S.E.	Inhibition(%)
0	0	0	77	676 718 316	570	127	43.89	9.78	
0	0	0	16	426 412 400	413	7	6.61	0.11	
0	0	0	f						
						Mean	**25.25**	**SEM=18.45**	
0	0	2	74	444 486 354	428	39	31.67	2.88	
0	0	2	271	710 572 546	609	51	165.04	13.82	
0	0	2	f						
						Mean	**98.35**	**SEM=66.01**	**0**
0.1	2	2	6	164 172 158	165	4	0.99	0.02	
0.1	2	2	f						
							0.99		**96**
1	2	2	19	514 684 486	561	62	10.66	1.19	
1	2	2	8	362 334 308	335	15	2.68	0.12	
						Mean	**6.67**	**SEM=3.95**	**73**

Patient 3

Cisplatin(ug/2ml)	5-Flurouracil(ug/2ml)	Rad. (Gy)	Growth Area (mm2)	Cell No./mm2	Mean	S.E.	Total Cell No(x103)	S.E.	Inhibition(%)
0	0	0	6	356 304 408	356	30	2.14	0.18	
0	0	0	14	236 220 270	242	15	3.39	0.21	
0	0	0	2	166 178	172	6	0.34	0.01	
						Mean	**1.957**	**SEM =0.88**	
0	0	2	21	288 200 248	245	25	5.15	0.52	
0	0	2	71	284 292 290	288	2	20.49	0.14	
						Mean	**12.82**	**SEM = 7.59**	**0**
0.1	2	0	1	86			0.09		
0.1	2	0	31	167 192 154	171	11	5.3	0.34	
						Mean	**2.69**	**SEM = 2.58**	
1	2	0	15	126 124 97	116	9	1.73	0.13	
1	2	0	68	260 274 354	296	29	20.13	1.97	
						Mean	10.93	SEM = 9.11	**0**
0.1	2	2	37	178 290 350	273	50	10.09	1.85	
0.1	2	2	16	210 198 236	215	11	3.43	0.17	
						Mean	**6.76**	**SEM = 3.3**	**0**
1	2	2	33	168 160 208	179	15	5.9	0.49	**0**

Patient 4

Cisplatin(ug/2ml)	5-Flurouracil(ug/2ml)	Rad. (Gy)	Growth Area (mm2)	Cell No./mm2	Mean	S.E.	Total Cell No(x103)	S.E.	Inhibition(%)
0	0	0	6	226 209 306	260	28	1.56	0.17	
0	0	0	11	320 324 220	288	34	3.17	0.12	
0	0	0	13	264 314 330	303	20	3.93	0.07	
0	0	0	9	242 192 280	238	25	2.14	0.22	
0	0	0	42	296 408 364	356	35	14.95	1.47	
						Mean	**5.15**	**SEM = 2.48**	
0	0	2	29	278 234 256	256	13	7.42	0.38	
0	0	2	22	222 186 190	199	11	4.38	0.24	
0	0	2	0						
						Mean	**5.9**	**SEM = 1.5**	**0**
0.1	2	0	0						
0.1	2	0	4	142 152 106	133	14	0.53	0.05	
0.1	2	0	5	81 39 28	49	16	0.25	0.08	
						Mean	**0.39**	**SEM = 0.14**	**92**
1	2	0	0				0		
1	2	0	0				0		
						Mean	**0**		**100**
0.1	30	0	0				0		
							0		**100**
1	30	0	0				0		
							0		**100**
0.1	2	2	2.5	546 608	577	31	1.44	0.08	
0.1	2	2	1	292			0.29		
						Mean	**0.86**	**SEM = 0.57**	**83**
1	2	2	1.5				0.79		
1	2	2	2.5	314 264	289	25	0.72	0.06	
						Mean	**0.75**	**SEM = 0.03**	**85**

Patient 5

Cisplatin(ug/2ml)	5-Flurouracil(ug/2ml)	Rad. (Gy)	Growth Area (mm2)	Cell No./mm2	Mean	S.E.	Total Cell No(x103)	S.E.	Inhibition(%)
0	0	0	13	302 344 384	343	24	4.46	0.07	
0	0	0	10	491 373 434	433	34	4.33	0.34	
0	0	0	15	407 345 290	347	34	5.21	0.51	
						Mean	**4.66**	**SEM = 0.27**	
0	0	2	21	574 412 416	467	53	9.81	1.11	
0	0	2	27	710 572 588	623	43	16.83	1.16	
0	0	2	32	454 556 504	505	29	16.15	0.93	
						Mean	**14.26**	**SEM = 2.24**	**0**
0.1	2	0	1				0.4		
0.1	2	0	22	483 478 352	423	37	9.3	0.81	
						Mean	**4.85**	**SEM = 4.40**	**0**
1	2	0	0				0		
1	2	0	0				0		
						Mean	**0**		**100**
0.1	30	0	0				0		
							0		**100**
1	30	0	0				0		
							0		**100**
0.1	2	2	7	354 428 372	385	22	2.69	0.15	
0.1	2	2	3	182 242 204	209	17	0.63	0.05	
						Mean	**1.66**	**1.02**	**64**
1	2	2	2.5	152 148 140	147	4	0.37	0.01	
1	2	2	1				0.16		
						Mean	**0.26**	**SEM = 0.10**	**94**

Patient 6

Cisplatin(ug/2ml)	5-Flurouracil(ug/2ml)	Rad. (Gy)	Growth Area (mm2)	Cell No./mm2	Mean	S.E.	Total Cell No(x103)	S.E.	Inhibition(%)
0	0	0	17	184 162 206	184	13	3.13	0.2	
0	0	0	22	186 174 110	157	23	3.45	0.5	
0	0	0	19	248 202 189	213	18	4.05	0.3	
0	0	0	19	180 116 132	143	19	2.71	0.4	
0	0	0	18	280 200 228	236	23	4.25	0.4	
0	0	0	8	156 116 140	137	12	1.1	0.1	
0	0	0	9	128 172 288	196	48	1.76	0.4	
						Mean	**2.92**	**SEM=0.44**	
0	0	2	11	382 256 216	285	50	3.13	0.5	
0	0	2	24	334 338 332	335	2	8.06	0.04	
0	0	2	f						
0	0	2	f						
						Mean	**5.60**	**SEM=4.19**	**0**
0.1	2	0	3	92 66 49	69	12	0.21	0.04	
0.1	2	0	5	76 50 52	59	8	0.3	0.04	
0.1	2	0	17	220 402 174	265	70	4.5	1.19	
						Mean	**1.67**	**SEM=1.42**	**43**
1	2	0	12	71 67 75	71	2	0.85	0.02	
1	2	0	4	116 139 126	127	7	0.51	0.03	
1	2	0	f						
						Mean	**0.68**	**SEM=0.17**	**77**
0.1	2	2	14	93 108 96	99	4	1.39	0.06	
0.1	2	2	6	52 83 44	60	12	0.36	0.01	
0.1	2	2	7	90 100 84	91	5	0.64	0.03	
						Mean	**0.80**	**SEM=0.31**	**73**
1	2	2	7	104 94 79	98	3	0.69	0.02	
1	2	2	13	97 105 102	101	2	1.32	0.03	
						Mean	**1.01**	**SEM=0.31**	**66**

Patient 7

Cisplatin(ug/2ml)	5Flurouracil(ug/2ml)	Rad. (Gy)	Growth Area (mm2)	Cell No./mm2	Mean	S.E.	Total Cell No(x103)	S.E.	Inhibition(%)
0	0	0	229	414 356 400	390	17	89.3	4	
0	0	0	12	224 248 248	240	8	2.9	0.1	
0	0	0	182	524 280 480	428	75	77.9	14	
0	0	0	106	296 404 328	340	33	36	3.5	
						Mean	**51.53**	**SEM=19.85**	**0**
0	0	2	52	388 444 468	433	24	22.5	1	
0	0	2	302	400 360 382	381	9	115	3	
0	0	2	105	378 326 324	343	18	36		
0	0	2	208	378 290 344	337	26	70.2	5	
						Mean	**60.93**	**SEM=20.63**	**0**
0.1	2	0	5	136 178 64	126	33	0.6	0.1	
0.1	2	0	217	184 140 168	164	13	35.6	3	
0.1	2	0	25	206 212 128	182	27	4.5	1	
						Mean	**13.57**	**SEM=11.07**	**73**
0.1	30	0	2.5	216 202	209	7	0.5	0.02	
0.1	30	0	8	107 74 100	94	10	0.7	0.07	
0.1	30	0	4	178 38 212	143	53	0.6	0.2	
						Mean	**0.6**	**0.06**	**99**
1	2	0	106	174 144 144	154	10	16.3	1	
1	2	0	86	190 152 144	162	14	13.9	1	
1	2	0	45	150 152 180	161	10	7.2	0.4	
						Mean	**12.47**	**SEM=2.72**	**76**
1	30	0	16	152 212 210	191	20	3	0.3	
1	30	0	2	172 102	137	35	0.3	0.1	
						Mean	**1.65**	**SEM=1.34**	**97**
0.1	2	2	44	188 238 156	194	24	8.5	1	
0.1	2	2	24	106 90 92	96	5	2.3	0.1	
						Mean	**5.4**	**SEM=3.07**	**89**
1	2	2	9	88 70 54	71	10	0.6	0.1	
1	2	2	2	24 32	28	4	0.05	0.01	
						Mean	**0.33**	**SEM=0.27**	**99**

Patient 8

Cisplatin(ug/2ml)	5-Flurouracil(ug/2ml)	Rad. (Gy)	Growth Area (mm2)	Cell No./mm2	Mean	S.E.	Total Cell No(x103)	S.E.	Inhibition(%)
0	0	0	27	198 316 170	228	45	6.16	1.2	
0	0	0	74	242 222 256	240	10	17.76	0.74	
0	0	0	17	644 626 556	609	27	10.35	0.46	
0	0	0	56	462 444 404	437	17	24.45	0.95	
0	0	0	37	398 260 416	358	49	13.25	1.81	
0	0	0	18	358 308 290	319	20	5.74	0.36	
						Mean	**12.95**	**SEM=3.23**	
0	0	2	51	284 330 296	303	14	15.47	0.71	
0	0	2	23	572 520 640	577	35	13.28	0.81	
0	0	2	26	390 242 276	303	45	7.87	1.17	
						Mean	**12.21**	**SEM=2.25**	6
0.1	2	0	8	146 142 158	149	5	1.19	0.04	
0.1	2	0	14	112 93 75	93	11	1.31	0.15	
0.1	2	0	4	59 51 30	47	9	0.19	0.04	
						Mean	**0.9**	**SEM=0.35**	93
1	2	0	12	89 104 71	88	9	1.06	0.11	
1	2	0	6	87 120 146	118	17	0.71	0.1	
1	2	0	4	87 141 91	106	17	0.42	0.07	
						Mean	**0.73**	**SEM=0.18**	94
0.1	30	0	4	140 129 138	136	3	0.54	0.01	
							0.54		96
1	30	0	4	115 72 106	98	13	0.4	0.05	
							0.4		97
0.1	2	2	0				0		
0.1	2	2	0				0		
0.1	2	2	3	122 77 101	100	13	0.3	0.04	
							0.3		98
1	2	2	1.5				0.37		
1	2	2	1				0.14		
1	2	2	9	117 77 96	97	11	0.87	0.1	
						Mean	**0.46**	**SEM=0.2**	96

Patient 9

Cisplatin(ug/2ml)	5-Flurouracil(ug/2ml)	Rad. (Gy)	Growth Area (mm2)	Cell No./mm2	Mean	S.E.	Total Cell No(x103)	S.E.	Inhibition(%)
0	0	0	16	514 428 418	453	30	7.26	0.5	
0	0	0	30	198 248 242	229	16	6.88	0.5	
						Mean	**7.07**	**SEM=0.2**	
0	0	2	22	232 240 226	233	4	5.12	0.02	
0	0	2	24	280 239 270	263	12	6.31	0.29	
						Mean	**5.71**	**SEM=0.59**	**20**

Patient 10

Cisplatin(ug/2ml)	5-Flurouracil(ug/2ml)	Rad. (Gy)	Growth Area (mm2)	Cell No./mm2	Mean	S.E.	Total Cell No(x103)	S.E.	Inhibition(%)
0	0	0	16	450 370 420	413	23	6.6	0.37	
0	0	0	15	280 172 294	249	38	3.7	0.57	
						Mean	**5.1**	**SEM=1.42**	
0	0	2	10	422 168 210	267	79	2.7	0.79	
0	0	2	16	248 190 234	224	17	3.6	0.27	
						Mean	**3.15**	**SEM=0.45**	**38**
0.1	2	0	7	302 302 254	286	16	2	0.1	
0.1	2	0	20	244 350 196	263	45	5.3	0.9	
						Mean	**3.65**	**SEM=1.63**	**28**
1	2	0	11	198 342 344	295	48	3.2	0.5	
1	2	0	32	324 344 318	329	88	10.5	0.2	
						Mean	**6.85**	**SEM=3.61**	**0**
0.1	2	2	12	172 366 250	263	56	3.1	0.7	
0.1	2	2	10	290 292 296	293	2	2.9	0.02	
						Mean	**3**	**SEM=0.1**	**41**
1	2	2	10	299 295 297	297	1	3	0.01	
1	2	2	f						
						Mean	**3**		**41**

Patient 11

Cisplatin(ug/2ml)	5-Flurouracil(ug/2ml)	Rad. (Gy)	Growth Area (mm2)	Cell No./mm2	Mean	S.E.	Total Cell No(x103)	S.E.	Inhibition(%)
0	0	0	308	628 636 556	607	25	186.85	8	
0	0	0	294	628 682 550	620	38	182.28	11	
						Mean	**184.56**	**SEM=2**	
0	0	2	244	542 570 574	562	10	137.13	2	
0	0	2	200	382 480 430	431	28	86.13	6	
						Mean	**111.63**	**SEM=25**	**39**
0.1	2	0	2	138 69	103	34	0.21	0.1	
0.1	2	0	1	124			0.12		
						Mean	**0.16**	**SEM= .04**	**100**
1	2	0	2	78 38	58	20	0.12	0.04	
1	2	0	0				0		
							0.12		**100**
0.1	30	0	0				0		
0.1	30	0	0				0		
						Mean	**0**		**100**
1	30	0	0				0		
1	30	0	0				0		
						Mean	**0**		**100**
0.1	2	2	2	100 98	99	1	0.2	0.002	
0.1	2	2	2	222 282	252	30	0.5	0.06	
						Mean	**0.35**	**SEM=0.15**	**100**
1	2	2	2	170 126	148	22	0.3	0.04	
1	2	2	3	120 87 94	100	10	0.3	0.03	
						Mean	**0.3**	**SEM=0**	**100**

Patient 12

Cisplatin (ug/2ml)	5-Flurouracil (ug/2ml)	Radiation (Gy)	Growth Area (mm2)	SEM	Growth Inhibition (%)
0	0	0	24		
0	0	0	29		
0	0	0	73		
		Mean	**42**	**SEM=15.6**	**0**
0	0	2	41		
0	0	2	5		
		Mean	**23**	**SEM=17.8**	**45**
0.1	2	0	4		
0.1	2	0	6		
		Mean	**5**	**SEM=1**	**88**
1	2	0	4		
1	2	0	3		
		Mean	**3.5**	**SEM=0.5**	**92**
1	2	2	0		
1	2	2	1.5		
		Mean	**0.7**	**SEM=0.7**	**98**

Patient 13

Cisplatin(ug/2ml)	5-Flurouracil(ug/2ml)	Rad. (Gy)	Growth Area (mm2)	Cell No./mm2	Mean	S.E.	Total Cell No(x103)	S.E.	Inhibition(%)
0	0	0	52	626 604 504	578	37	30.06	1.92	
0	0	0	51	554 624 618	599	22	30.53	1.12	
0	0	0	35	640 614 540	598	30	20.93	1.05	
0	0	0	97	540 438 458	479	31	46.43	3	
0	0	0	44	562 432 546	513	41	22.59	1.8	
						Mean	**30.11**	**SEM=4.51**	
0	0	2	36	494 422 442	453	21	16.3	0.75	
0	0	2	32	528 426 604	519	51	16.62	1.63	
0	0	2	23	602 548 662	604	33	13.89	0.76	
						Mean	**15.6**	**SEM=0.86**	**48**
0.1	2	0	14	99 93 79	90	6	1.26	0.08	
0.1	2	0	13	171 122 145	146	14	1.9	0.18	
						Mean	**1.58**	**SEM=0.32**	**95**
1	2	0	1						
1	2	0	4	268 306 322	299	16	1.19	0.06	
						Mean	**1.19**		**96**
0.1	30	0	10	207 194 185	195	6	1.95	0.06	**93**
1	30	0	0				0		**100**
0.1	2	2	3	53 70 62	62	5	0.18	0.01	
0.1	2	2	3	45 62 54	54	5	0.16	0.01	
						Mean	**0.17**	**SEM=0.01**	**99**
1	2	2	3	48 51 50	50	1	0.15	0.003	
1	2	2	4	70 60 75	68	4	0.27	0.01	
						Mean	**0.21**	**SEM=.06**	**99**

Patient 14

Cisplatin(ug/2ml)	5-Flurouracil(ug/2ml)	Rad. (Gy)	Growth Area (mm2)	Cell No./mm2	Mean	S.E.	Total Cell No(x103)	S.E.	Inhibition(%)
0	0	0	12	224 182 158	188	19	2.26	0.23	
0	0	0	3	142 146 188	159	15	0.48	0.04	
0	0	0	f						
						Mean	1.37	SEM=0.88	
0	0	2	2	138 156	147	9	0.29	0.02	
0	0	2	5	184 190 152	175	12	0.88	0.06	
0	0	2	f						
						Mean	0.58	SEM=0.29	57
0.1	2	2	8	240 242 186	223	18	1.78	0.14	0
1	2	2	f						

Patient 15

Cisplatin(ug/2ml)	5-Flurouracil(ug/2ml)	Rad. (Gy)	Growth Area (mm2)	Cell No./mm2	Mean	S.E.	Total Cell No(x103)	S.E.	Inhibition(%)
0	0	0	5	626 718 642	662	28	3.31	0.14	
0	0	0	11	406 524 428	453	36	4.98	0.63	
0	0	0	5	474 622 508	535	45	2.67	0.22	
						Mean	**3.65**	**SEM=0.69**	
0	0	2	4	350 338 268	319	25	1.27	0.09	
0	0	2	6	278 248 256	261	9	1.56	0.05	
						Mean	**1.41**	**SEM=.14**	**61**

Patient 16

Cisplatin(ug/2ml)	5-Flurouracil(ug/2ml)	Rad. (Gy)	Growth Area (mm2)	Cell No./mm2	Mean	S.E.	Total Cell No(x103)	S.E.	Inhibition(%)
0	0	0	11	508 494 450	484	17	5.3	0.2	
0	0	0	9	748 260 390	466	146	4.2	1.3	
0	0	0	f						
						Mean	**4.75**	**SEM= 0.5**	
0	0	2	1	392			0.4		
0	0	2	4	798 617 964	793	100	3.2	0.4	
0	0	2	f						
						Mean	**1.8**	**SEM=1.4**	**62**
0.1	2	0	f						
0.1	2	0	f						
1	2	0	7	204 188 210	201	6	1.4	0.04	
1	2	0	f						
						Mean	**1.4**		**70**
0.1	2	2	6	108 121 68	99	16	0.6	0.1	
0.1	2	2	f						
						Mean	**0.6**		**87**
1	2	2	0				0		
1	2	2	0				0		
						Mean	**0**		**100**

Patient 17

Cisplatin(ug/2ml)	5-Flurouracil(ug/2ml)	Rad. (Gy)	Growth Area (mm2)	Cell No./mm2	Mean	S.E.	Total Cell No(x103)	S.E.	Inhibition(%)
0	0	0	9	472 466 444	461	8	4.15	0.07	
0	0	0	12	328 446 382	406	39	4.87	0.47	
0	0	0	29	596 600 600	599	1	17.36	0.02	
						Mean	**8.79**	**SEM - 4.29**	**0**
0	0	2	6	252 214 240	235	11	1.41	0.07	
0	0	2	9	330 432 422	395	32	3.55	0.29	
						Mean	**2.48**	**SEM = 1.06**	72
0.1	2	0	7	186 338 246	257	44	1.8	0.3	
0.1	2	0	7	132 108 120	120	7	0.84	0.05	
						Mean	**1.32**	**SEM - 0.47**	**85**
1	2	0	0				0		
1	2	0	0				0		
						Mean	**0**		**100**
0.1	30	0	0				0		
						Mean	**0**		**100**
1	30	0	0				0		
						Mean	**0**		**100**
0.1	2	2	2	246 118	182	64	0.36	0.13	
0.1	2	2	12	163 296 282	247	42	2.96	0.5	
						Mean	**1.66**	**SEM = 1.29**	**81**
1	2	2	2	137 92	114	22	0.23	0.04	
1	2	2	3	241 206 165	204	22	0.61	0.06	
1	2	2	9	86 107 84	92	7	0.83	0.06	
						Mean	**0.56**	**SEM = 0.17**	**93**

Patient 18

Cisplatin(ug/2ml)	5-Flurouracil(ug/2ml)	Rad. (Gy)	Growth Area (mm2)	Cell No./mm2	Mean	S.E.	Total Cell No(x103)	S.E.	Inhibition(%)
0	0	0	64	616 558 414	529	60	33.88	3.8	
0	0	0	23	810 678 738	742	38	174.37	8.93	
0	0	0	26	316 380 384	360	22	9.36	0.57	
						Mean	**72.51**	**SEM=51.4**	
0	0	2	41	288 410 346	348	35	14.27	1.43	
0	0	2	60	236 270 302	269	19	16.14	1.14	
						Mean	**15.2**	**SEM=0.92**	**79**
0.1	2	0	12	100 114 87	100	8	1.2	0.1	
0.1	2	0	4	74 88 89	84	5	0.33	0.02	
						Mean	**0.76**	**SEM=0.43**	**99**
1	2	0	30	294 468 290	351	59	10.52	1.77	
1	2	0	12	284 326 344	318	18	3.82	0.22	
						Mean	**7.17**	**SEM=3.32**	**90**
0.1	30	0	4	144 130 115	130	8	0.52	0.03	
							0.52		**99**
1	30	0	0				0		
							0		**100**
0.1	2	2	20	232 258 200	230	17	4.6	0.34	
0.1	2	2	29	234 180 192	202	16	5.86	0.46	
						Mean	**5.23**	**SEM=0.62**	**93**
1	2	2	11	242 182 292	239	32	2.62	0.35	
1	2	2	9	276 210 172	219	30	1.97	0.27	
						Mean	**2.29**	**SEM=0.32**	**97**

Patient 19

Cisplatin(ug/2ml)	5-Flurouracil(ug/2ml)	Rad. (Gy)	Growth Area (mm2)	Cell No./mm2	Mean	S.E.	Total Cell No(x103)	S.E.	Inhibition(%)
0	0	0	7	560 592 548	567	13	3.97	0.09	
0	0	0	34	580 454 596	543	45	18.47	1.53	
						Mean	**11.22**	**SEM=7.18**	
0	0	2	7	334 286 396	339	32	2.37	0.22	
0	0	2	5	282 292 326	300	13	1.5	0.06	
						Mean	**1.93**	**SEM=0.43**	**83**

Patient 20

Cisplatin(ug/2ml)	5-Flurouracil(ug/2ml)	Rad. (Gy)	Growth Area (mm2)	Cell No./mm2	Mean	S.E.	Total Cell No(x103)	S.E.	Inhibition(%)
0	0	0	85	664 596 770	677	51	57.52	4.33	
0	0	0	77	432 612 590	545	57	41.94	4.39	
0	0	0	111	708 628 1044	793	127	88.06	14.1	
0	0	0	128	788 604 736	709	55	90.79	7.04	
0	0	0	103	762 724 658	715	30	73.61	3.09	
						Mean	**70.38**	**SEM=9.26**	
0	0	2	18	546 512 424	494	36	8.89	0.65	
0	0	2	15	568 570 458	532	37	7.98	0.55	
0	0	2	29	408 514 428	450	32	13.05	0.93	
0	0	2	20	486 576 596	553	34	11.05	0.68	
						Mean	**10.24**	**SEM=1.13**	**85**
0.1	2	0	48	216 166 158	180	18	8.64	0.86	
0.1	2	0	15	248 248 334	277	29	4.15	0.43	
0.1	2	0	16	182 184 170	179	4	2.86	0.06	
						Mean	**5.22**	**SEM=1.75**	**93**
1	2	0	11	208 184 178	190	9	2.09	0.05	
1	2	0	15	216 206 168	197	15	2.95	0.22	
1	2	0	18	240 356 390	329	45	5.92	0.14	
						Mean	**3.65**	**SEM=1.16**	**95**
0.1	30	0	f						
1	30	0	5	458 282 284	341	58	1.71	0.29	
							1.71		**97**
0.1	2	2	44	256 236 234	242	7	10.65	0.31	
0.1	2	2	34	146 150 168	155	7	5.26	0.24	
						Mean	**7.95**	**SEM=2.67**	**89**
1	2	2	17	234 218 200	217	10	3.69	0.17	
1	2	2	12	214 344 274	279	53	3.35	0.64	
1	2	2	32	222 210 176	203	14	6.48	0.45	
						Mean	**4.51**	**SEM=1.0**	**94**

Patient 21

Cisplatin(ug/2ml)	5-Flurouracil(ug/2ml)	Rad. (Gy)	Growth Area (mm2)	Cell No./mm2	Mean	S.E.	Total Cell No(x103)	S.E.	Inhibition(%)
0	0	0	42	634 714 816	721	53	30.29	2.2	
0	0	0	10	422 656 424	501	78	5	0.8	
0	0	0	342	776 740 792	769	15	263.1	5.1	
						Mean	**99.5**	**SEM=82.14**	
0	0	2	13	742 522 672	645	65	8.4	0.8	
0	0	2	42	250 166 221	212	25	8.9	1	
						Mean	**8.6**	**SEM=0.25**	**91**
0.1	2	2	5	216 186 220	207	11	1	0.05	
0.1	2	2	23	128 129 140	132	4	3	0.09	
						Mean	**2**	**SEM=1**	**98**
1	2	2	14	246 352 254	284	34	3.9	0.5	
1	2	2	12	510 656 410	526	71	6.3	0.8	
						Mean	**5.1**	**SEM=1.19**	**95**

Patient 22

Cisplatin(ug/2ml)	5-Flurouracil(ug/2ml)	Rad. (Gy)	Growth Area (mm2)	Cell No./mm2	Mean	S.E.	Total Cell No(x103)	S.E.	Inhibition(%)
0	0	0	20	226 240 196	221	13	4.41	0.26	
0	0	0	29	410 306 378	365	31	10.58	0.9	
0	0	0	28	414 362 192	323	67	9.04	1.88	
						Mean	**8.01**	**SEM=1.85**	
0	0	2	3	324 218 308	283	33	0.85	0.1	
0	0	2	5	236 224 194	218	12	1.09	0.06	
0	0	2	1.5	198			0.3		
						Mean	**0.75**	**SEM=0.23**	**91**
0.1	2	0	0				0		
0.1	2	0	0				0		
						Mean	**0**		**100**
1	2	0	0				0		
1	2	0	0				0		
						Mean	**0**		**100**
0.1	30	0	0				0		
							0		**100**
1	30	0	0				0		
							0		**100**
0.1	2	2	0				0		
0.1	2	2	0				0		
0.1	2	2	0				0		
						Mean	**0**		**100**
1	2	2	0				0		
1	2	2	0				0		
1	2	2	0				0		
						Mean	**0**		**100**

Patient 23

Cisplatin(ug/2ml)	5-Flurouracil(ug/2ml)	Rad. (Gy)	Growth Area (mm2)	Cell No./mm2	Mean	S.E.	Total Cell No(x103)	S.E.	Inhibition(%)
0	0	0	f						
0	0	0	52	844 840 656	780	62	40.56	3.22	
0	0	0	57	240 194 282	239	25	13.6	1.42	
						Mean	**27.08**	**13.34**	
0	0	2	f						
0	0	2	0				0		
0	0	2	0				0		
						Mean	**0**		**100**
0.1	2	0	0				0		
0.1	2	0	0				0		
						Mean	**0**		**100**
1	2	0	0				0		
1	2	0	0				0		
						Mean	**0**		**100**
0.1	30	0	0				0		**100**
1	30	0	0				0		**100**
0.1	2	2	0				0		
0.1	2	2	0				0		
0.1	2	2	0				0		
						Mean	**0**		**100**
1	2	2	0				0		
1	2	2	0				0		
1	2	2	0				0		
						Mean	**0**		**100**

Patient 24

Cisplatin(ug/2ml)	5-Flurouracil(ug/2ml)	Rad. (Gy)	Growth Area (mm2)	Cell No./mm2	Mean	S.E.	Total Cell No(x103)	S.E.	Inhibition(%)
0	0	0	8	288 242 282	271	14	2.2	0.1	
0	0	0	14	250 208 204	221	15	3.1	0.2	
0	0	0	6	272 92 72	145	63	0.9	0.4	
0	0	0	2	240 230	235	5	0.6	0.01	
						Mean	**1.7**	**SEM=0.58**	
0	0	2	0				0		
0	0	2	0				0		
0	0	2	0				0		
						Mean	**0**		**100**
0.1	2	0	0				0		
0.1	2	0	0				0		
						Mean	**0**		**100**
1	2	0	0				0		
1	2	0	0				0		
						Mean	**0**		**100**
0.1	30	0	0				0		
0.1	30	0	0				0		
						Mean	**0**		**100**
1	30	0	0				0		
1	30	0	0				0		
						Mean	**0**		**100**
0.1	2	2	0				0		
0.1	2	2	0				0		
0.1	2	2	0				0		
						Mean	**0**		**100**
1	2	2	0				0		
1	2	2	0				0		
1	2	2	0				0		
						Mean	**0**		**100**

Publications arising from this work

Sheridan, M., Reid, I., Mothersill, C., Seymour, C.B. (1995). A Predictive Assay for Individual Patient Response of Oesophageal Carcinoma to Chemoradiotherapy. Journal of Experimental & Clinical Cancer Research: 14 : 74-75.

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Sheridan, M., Mothersill, C., Seymour, C.B. and Harney, J. (1994)
Development of an Optimised *in vitro* System for Measurement of Tumour Response to Cytotoxic Therapy. Int. J. Radiation Biol. 65: 139.

Sheridan, M., Reid, I., Mothersill, C., Seymour, C.B., Walsh, T.N., Hennessy, T.P.J. (1994). A Predictive Assay for Response of Oesophageal Carcinoma to Chemoradiotherapy. Gut 2: 35.

Sheridan, M., Reid, I., Mothersill, C., Seymour, C.B., Walsh, T.N., Hennessy, T.P.J. (1994). Development of Optimised *in vitro* System for Measurement of Individual Patient Response to Cytotoxic Therapy prior to Surgery. Ir. J. Med. Sci. (abstract).

Mothersill, C., Sheridan, M., Harney, J., Bonnar, J., Hennessy, .P. J. and Seymour, C.B. (1993). Development of an Optimised *in vitro* System for Measurement of Human Tumour Response to Cytotoxic Agents. In Animal Cell Culture Technology, eds. Spier and Griffiths. Butterworth: PP723-28.

Sheridan, M., Mothersill, C., Seymour, C.B. and Harney, J. (1993). Development of an Optimised *in vitro* System for the Measurement of Tumour Response to Cytotoxic Therapy. Ir. J. Med. Sci. (abstract).

Glossary

Apoptosis: a mode of cell death commonly observed when death is a "desirable" or programmed event. Characteristic features include, nuclear and cytoplasmic condensation, blebbing of the plasma membrane followed by fragmentation of the cell into individual membrane-bound "apoptotic bodies". These apoptotic bodies are phagocytosed by neighbouring epithelial cells and resident macrophages so that, typically, apoptosis is not accompanied by an inflammatory response.

Barrett's Oesophagus: replacement of the stratified squamous epithelium of the distal oesophagus, by metaplastic columnar epithelium, caused by gastric reflux.

Capsula: (pl. capsulae) a membranous structure, usually dense collagenous connective tissue, that envelops an organ, a joint, or any other part.

Clonogenic cells: cells that have the capacity to produce an expanding family of descendants (usually at least 50). Also called 'colony-forming cells' or 'clongens'.

Colony: aggregate or small family of cells derived from a single cell.

Cytokine: hormone-like low molecular weight proteins, secreted by many different cell types, which regulate the intensity and duration of immune responses and are involved in cell-to-cell communication.

Differentiation: the complex of changes involved in the progressive diversification of the structure and functioning of the cells of an organism. For a given line of cells, differentiation results in a continual restriction of the types of transcription that each cell can undertake.

Dysphagia: difficulty in swallowing.

Dyspnea: shortness of breath, a subjective difficulty or distress in breathing, usually aasociated with disease of the heart or lungs.

Exophytic: denoting a neoplasm or lesion that grows outward from an epithelial surface.

Fistula: an abnormal passage from one epithelialised surface to another epithelialised surface. [L. a pipe, a tube]

Growth fraction: the proportion of cells in cycle in a population.

Microinvasion: invasion of tissue immediately adjacent to a carcinoma in situ, the earliest stage of malignant neoplastic invasion.

Missense mutant: a mutant in which a codon is mutated to one directing the incorporation of a different amino acid. This substitution may result in an inactive or unstable product.

Mitogen: a compound that stimulates cells to undergo mitosis.

Nonsense mutation: a mutation that converts a sense codon to a chain-terminating codon or vice-versa. The results following translation are abnormally short or long polypeptides, generally with altered functional properties.

Oncogenes: genes that initiate uncontrollable growth of mammalian cells. Oncogenes are typically mutated variants of normal cell genes (proto-oncogenes or c-*onc* genes) that have lost their regulatory function for normal nonmutated proteins.

Otalgia: earache

Proto-oncogene: a cellular gene that functions in controlling the normal proliferation of cells and either (1) shares nucleotide sequences with any of the known viral *onc* genes, or (2) is thought to represent a potential cancer gene that may become carcinogenic by mutation, or by overactivity when coupled to a highly efficient promoter.

Retroviruses: RNA viruses that utilise reverse transcriptase during their lifecycle. This enzyme allows the viral genome to be transcribed into DNA. The name retrovirus alludes to this "backwards" transcription. The transcribed viral DNA is integrated into the genome of the host cell where it replicates in unison with the genes of the host chromosone. The cell suffers no damage from this relationship unless the virus carries an oncogene. If it does, the cell will be transformed into a cancer cell.

Sense codon: any of the 61 triplet codons in mRNA that specify an amino acid.

Stem cells: cells capable of both self-renewal and supply of daughter cells that differentiate to produce all the various types of cells in a lineage.

Stridor: a high-pitched, noisy respiration; a sign of respiratory obstruction, especially in the trachea or larynx.

Transcription: formation of an RNA molecule upon a DNA template by complementary base pairing; mediated by RNA polymerase.

Translocation: a chromosome abberration which results in a change in position of a chromosomal segment within the genome, but does not change the total number of genes present.