Whey growth factor protection against chemotherapy drug-induced toxicity in vitro.

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Errata

page 6, line 10 - sentence beginning line 10 should be deleted.

page 9, line 20 -'periwinkle' replaces 'myrtle'.

page 11, line 18 - 'extensively' replaces 'primarily'

page 20, line 5 - sentence replaced with 'Apoptosis induced by serum-deprivation or confluence of in vitro cultures appears to be a general phenomenon in cells which do not produce autocrine survival factors, however relevant exogenous survival factors vary with cell type.'

page 31, line 20 - 'a' replaces 'an'

page 37, Figure 2.1 legend, line 2 - sentence replaced with 'Cultures would then be pre-treated with growth inhibitory agents, followed by the addition of chemotherapeutic agents, whilst constantly maintained in the presence of medium containing 5% serum.'

page 43, lines 21,22 - sentence replaced with 'Each culture monolayer was trypsinised and re-combined with it's culture supernatant to ensure both adherent and non-adherent cells were recovered.'

page 53, line 13 - sentence replaced with 'When cell counts were performed again after the 30h re-culture period there was only a slight increase in the total number of detached cells when compared to untreated cultures (Table 2.3).'

page 69, sentence 2 -The quality control data is shown in Figure 3.4. Treatment of Mv1Lu cell cultures with 3.0 ng/ml TGFβ for 30 h resulted in a final A_{630} of approximately 0.28 units. As the A_{630} for the TGFβ-treated drug-free cultures in Figure 2.11 have increased to approximately 0.42 units, I conclude that some cell growth has occurred.

page 126, line 6 - The last sentence in the main text is unsubstantiated by statistical analysis.

page 129, Figure 4.3 legend - '154.0 ng/ml' replaces '154.0 ug/ml'.

page 153, line 6 - sentence replace with 'In this regard, the studies described in the next chapter investigate the growth modulatory actions of orally administered WGFE to ascertain the requirement for growth inhibition in an *in vivo* model.'

page 153, line 21- include following sentence 'This does not, however, discount the possibility that IGF, EGF and PDGF may have synergies between themselves or with other peptides, producing an entirely different response to that observed in these studies.'

page 163, line 11 - '40 x' replaces '4 x'.

page 177, line 6 - 'may be' replaces 'are'

Abstract

Cheese whey contains a mixture of growth factors, some of which are known to minimise the toxicity associated with exposure to chemotherapeutic drugs, both *in vitro* and *in vivo*. This thesis describes the development and application of an *in vitro* model used to investigate the cytoprotective effects of a whey-derived growth factor extract (WGFE) in reducing epithelial cell death caused by chemotherapy agents. A mink lung (Mv1Lu) epithelial cell line was selected for model development by virtue of its sensitivity to growth inhibitory factors such as transforming growth factor ß (TGFß) and its susceptibility to two chemotherapy agents, etoposide and vinblastine, which are commonly associated with the clinical development of dose-limiting side-effects during chemotherapy treatment. As WGFE contains biologically active concentrations of $TGF\beta_2$, and induces an overall inhibitory response in epithelial cells, studies confirmed that the addition of $rTGF\beta_2$ prior to drug exposure reduced subsequent cell loss. Four WGFE preparations with different growth factor compositions mediated varying degrees of cytoprotection when added to cultures prior to and during drug exposure. However, none of these preparations enhanced cell regeneration when added after drug treatment. Comparison of the WGFE preparations with equivalent growth inhibitory doses of $rTGF\beta_2$ indicated that the survival activity of three preparations could be solely attributed to TGFß-like activity. However, the fourth fraction (control WGFE) possessed additional cytoprotective activity which was not neutralised by an antibody directed against $TGF\beta_{1,2,3}$. Individual preparations of other recombinant growth factors known to be present in control WGFE failed to account for this residual activity. Size exclusion chromatographic separation of control WGFE at neutral pH resulted in the co-elution of growth inhibitory and survival activity. Transient acidification of the eluted pools increased growth inhibitory activity, due to the release of latent TGFß complexes, but did not concomitantly increase cell survival. Oral administration of control WGFE to rats reduces the incidence of drug-induced apoptosis in the small intestinal crypts. However, such a feeding strategy did not alter the proportion or distribution of crypt cell

proliferation in normal animals. These studies confirm that WGFE preparations are able to ameliorate drug toxicity, and suggest that the growth inhibitory components of WGFE are not entirely responsible for mediating the cytoprotection observed.

Abbreviations

This thesis adopts the use of standard abbreviations. All non-standard abbreviations included in this thesis are listed below.

$A_{570-600}$	absorbance at (570 nm minus 600 nm)
A_{630}	absorbance at 630 nm
BrdU	5-bromo-2′-deoxyuridine
CHO	Chinese hamster ovary cell line
DAB	diaminobenzidine
DMEM	Dulbecco's modified Eagle's medium
EGF	epidermal growth factor
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
FBS	fetal bovine serum
G_1	gap 1 phase of the cell cycle
G_2	gap 2 phase of the cell cycle
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]

HGF	hepatocyte growth factor
HL-60	human promyelocytic leukemia cell line
HPLC	high pressure liquid chromatography
ID_{50}	dose producing 50% maximal inhibition
IEC-6	intestinal epithelial cell line
IGF	insulin-like growth factor (type I or II)
IL-3	interleukin 3
IL-11	interleukin 11
Kav	$\frac{\text{elution volume (test protein) - (blue dextran 2000)}}{\text{void volume - elution volume (blue dextran 2000)}}$
LAP	latency associated peptide
LD_{50}	dose producing 50% maximum lethality
M	mitotic phase of the cell cycle
Mv1Lu	mink lung epithelial cell line
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PI	propidium iodide
pRb	retinoblastoma gene product
RIA	radio-immunoassay
RIE	rat intestinal epithelial cell line

RT	room temperature
S	DNA synthesis phase of the cell cycle
TGFß	transforming growth factor ß (isotypes 1, 2 or 3)
THF	tetrahydrofolate
WGFE	whey-derived growth factor extract

Statement of originality

This work contains no material which has been accepted for the award of any other degree or diploma in any other University of other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying.

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Vicki Taylor

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Chapter One

Introduction

Chapter1

Chemotherapy agents typically function by preferentially targeting a feature of the cancerous cell. Such drug strategies often focus on the hyper-proliferative state of neoplastic tissues, producing deleterious effects in those normal tissues with rapid turnover rates. Certain regions of the gastrointestinal tract are often damaged in this manner due to the high rate of proliferation of the epithelial cells lining the gut. Drug-induced damage in the gastrointestinal tract can be reduced by the administration of peptides such as transforming growth factor ß and interleukin 11, which may serve to reduce epithelial cell proliferation prior to drug exposure. Previous studies in our laboratory have indicated that a growth factor preparation extracted from cheese whey was able to reduce the intestinal damage caused by chemotherapeutic drug administration in an animal model. Thus, the main objective of this thesis was to develop an *in vitro* model of drug toxicity, using various chemotherapy agents, in which to examine the contributions of the composite growth factors to identify probable sources of such survival activity.

The first section of this literature review examines (1) the use of chemotherapy agents in clinical practice and the side-effects often associated with such treatments; (2) the biochemical and molecular pathways which are thought to lead to drug-induced cell death in non-cancerous tissues; (3) potential strategies for reducing such side-effects; and (4) a review of mediators which have been shown to improve cell survival following exposure to chemotherapeutic drugs. The discussion of chemotherapy agents and their mechanisms of action is a very broad topic, relevant only to the extent that they are able to induce detrimental side-effects in non-target tissues. Therefore, rather than provide exhaustive lists of drugs and their actions, examples have been supplied on the basis of (i) their ability to induce intestinal damage and (ii) their inclusion in my experimental research.

The second section of the literature review considers the application of a whey-derived growth factor extract in promoting protective responses in cells of epithelial origin. The review examines (1) the functions of whey, both *in vitro* and *in vivo*; (2) the production and

characterisation of the growth factor extract; (3) the actions of individual growth factors on epithelial cells; (4) the activity of the growth factor mixture on epithelial cells *in vitro*; and (5) the ability of this preparation to attenuate the toxicity associated with chemotherapy treatment in an *in vivo* model.

Due to the commercial nature of the research performed with the whey growth factor extract, no research findings with this mixture have been generated outside of the CRC for Tissue Growth and Repair. At the commencement of my PhD, very little work concerning this growth factor extract had in fact been published. However, the information directly relating to the production and characterisation of the extract was accessible within the CRC, and has therefore been included in the appropriate sections of this review. Apart from this exception, I have restricted the scope of this review to include only such knowledge as was available prior to September 1995, shortly after the start of this research. All other pertinent information published after this time is included in the relevant chapter discussions.

1. Introduction

1.1 CHEMOTHERAPEUTIC DRUGS

Chemotherapy is the treatment of choice against a broad range of cancers, including choriocarcinoma, acute lymphocytic leukemia, and tumours of the breast and testis (Anonymous, 1995). In addition to single drug treatments, many chemotherapy schedules involve the use of combination drug treatments, or drugs used in conjunction with radiotherapy.

It is often quite feasible to eliminate tumour cells by employing a recurrent low dose drug administration schedule, having minimal impact on healthy tissues. However, combination chemo/radiotherapy, high dose ablative chemotherapy, or indeed low concentrations of particular agents, will tend to exacerbate the toxic damage inflicted on non-cancerous cells. Such toxicity frequently results in the presentation of dose-limiting side effects, requiring a reduction or complete cessation of chemotherapy treatment. Aside from social implications, the economic cost of treatment escalates with the onset of drug-induced side effects, as these patients typically require extended hospitalisation. Thus, there is an urgent need to focus on the effective administration of chemotherapeutic agents whilst at the same time minimising the risk of damage to healthy cell populations.

1.1.1 Chemotherapy

Cancerous cells originate from normal cells which have lost the ability to regulate their own growth. As such, one of the features that most readily distinguishes the cancerous cell from its healthy counterpart is its increased rate of uncontrolled proliferation.

The majority of chemotherapy treatments in current use operate on the underlying assumption that a drug which preferentially affects proliferating cells will be substantially more toxic to cancerous cells than to healthy cells. In most instances this is the case, as cancerous cells

proliferate more rapidly than the bulk of normal cells in the body. However, some cells, such as those which comprise the bone marrow or the epithelial lining of the gastrointestinal tract, proliferate quickly under normal conditions and are therefore more susceptible to the damage caused by chemotherapy.

1.1.2 Side-effects of chemotherapy treatment

1.1.2.1 Non-intestinal toxicities

Whilst the side-effects of chemotherapy treatment are often associated with rapidly proliferating cells, this is not always so. Cytotoxic drug exposure can also result in irreversible neurological toxicity, and cardiac or renal complications. These conditions, however, are usually associated with a small number of chemotherapeutic agents, allowing specific monitoring and therapies to be employed to minimise such effects (Blijham, 1993).

In the bone marrow, drug exposure induces ablation of the stem cell population, resulting in a loss of haematopoietic cells. Bone marrow restitution has been accelerated with the advent of marrow cell transplantation and autologous replacement therapies. The collection of circulating haematopoietic stem cells for autologous reconstitution after chemotherapy has also improved survival outcomes (Juttner *et al.,* 1985). Meanwhile, research into the cytoprotective properties of granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) suggested that both of these cytokines were able to enhance the recovery of peripheral blood cell populations (Katano *et al.,* 1995; Morstyn *et al.,* 1988). The application of these techniques, often as a combined strategy, provided the opportunity to increase either the dose of cytotoxic drug, or the length of the treatment schedule. Clinical trials employing doxorubicin therapy for breast or ovarian cancers showed that post-infusion of G-CSF reduced haematological toxicities, enabling the safe administration of repeated high drug doses (Bronchud *et al.,* 1989). The use of large drug doses proved successful in reversing tumour progression, but was restricted by the onset of epithelial toxicity in 100% of the patients (Bronchud *et al.,* 1989). Thus, advances in cell survival technologies for the bone marrow

surpassed those for the gastrointestinal tract, leaving oral and intestinal mucositis as the primary obstacles to more intensive chemotherapy treatment.

1.1.2.2 Intestinal toxicity

1.1.2.2.1 Mucositis

In the gastrointestinal tract of the rat, drug toxicity reduces the rate of renewal of the epithelial cells, and depletes crypt populations due to the continual migration of cells toward the villus (Altmann, 1974). As the effects of chemotherapy intensify, villi become distorted and cell migration becomes erratic. Such disorganisation leads to gross tissue disturbances, entailing exposure of the underlying mucosa and ulcerative damage, which manifest clinically as mucositis. Current practice in the management of oral mucositis involves application of pain relief, antiseptic mouth washes and ice cubes to numb the afflicted area. The treatment of the symptoms of intestinal mucositis, such as diarrhoea, is fairly straightforward. However, intestinal ulceration can lead to a significant loss of nutrient uptake and the need for extended patient hospitalisation.

1.1.2.2.2 Cellular localisation of drug-induced damage

The gastrointestinal tract is lined with a layer of epithelial cells, forming a continuous barrier from the mouth to the anus. In the small intestine, the epithelium is functionally divided into two main compartments - the highly proliferative crypts and the terminally differentiated villi (Figure 1.1). It is within the crypts of the small intestine that chemotherapeutic drugs can cause damage. In part, this localisation of drug toxicity can be attributed to the high rate of cell division observed in the intestinal crypts of many animals. Typically, crypt cells divide once every 12 hours and 15 hours, in the human and rodent respectively (Lipkin *et al.,* 1963; Wright, 1978). Cell cycle time appears to be minimally influenced by position along the gastrointestinal tract (Lipkin *et al.,* 1963), however it is adversely affected by factors such as nutritional deprivation and toxic damage (Wright, 1978). Paradoxically, drug-induced damage varies along the length of the gastrointestinal tract, indicating that parameters other than cell cycle time are

responsible for drug sensitivity.

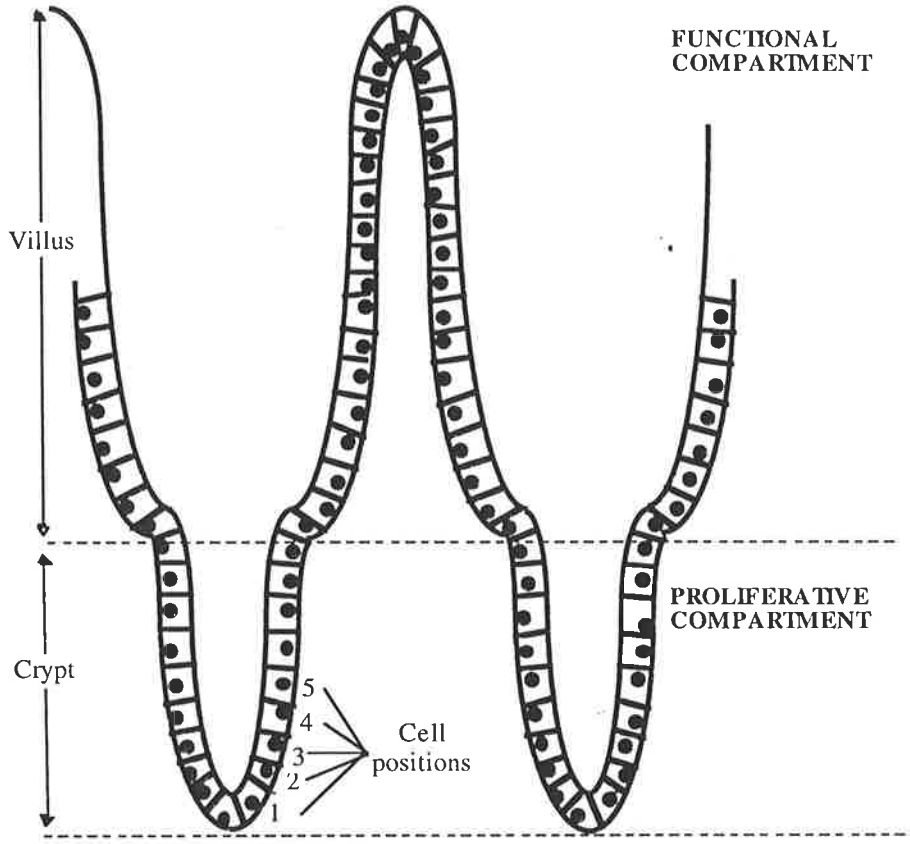


Figure 1.1 Schematic representation of intestinal structure

A number of studies have identified the cell positions along the crypt which are targeted by chemotherapeutic drugs in an *in vivo* model. Ijiri and Potten (1983) have shown that different toxic agents, including radiation and chemotherapeutic drugs, exhibit different target cell specificities when inducing cellular damage in the mouse small intestine. For example, methotrexate targeted crypt cells between positions 11-14, whilst busulphan was most toxic to cells at positions 3-6 along the crypt (Ijiri and Potten, 1987). This again suggests that, whilst drug toxicity is directed towards the proliferative zone of the intestinal crypt, there are likely to be other factors involved in defining the susceptibility of a cell to cytotoxic insult. One such factor may be the different biochemical actions of the drugs themselves.

1.1.3 Cytotoxic drugs have diverse mechanisms of action

Chemotherapy agents can be broadly classified into different groups, according to their primary mode of action. These include **(1)** anti-metabolites, which disrupt metabolism along a pathway associated with cell proliferation, eg. methotrexate, 5-fluorouracil; **(2)** alkylating agents, such as cisplatin and melphalan, which bind to the cellular DNA and prevent replication; **(3)** hormone drugs, which antagonise the effects of cancer-promoting hormones, eg. tamoxifen, stilboestrol; **(4)** cytotoxic antibiotics, such as doxorubicin, which act within the nucleus to damage the DNA; and **(5)** plant alkaloids, including vincristine and vinblastine, which target various cellular functions. Many of these drugs are able to cause damage at any temporal stage of the cell cycle, but induce a quantitatively greater degree of disruption in rapidly proliferating cells.

1.1.3.1 Methotrexate

Methotrexate is a structural analogue of dihydrofolate, and therefore is classified as an anti-metabolite. This drug binds in an irreversible manner to sites within the dihydrofolate reductase enzyme. Formation of methotrexate-enzyme complexes prevents the chemical reduction of dihydrofolate to tetrahydrofolate (THF), thus depleting intracellular pools of THF. Reduced folates are essential for the production of co-factors involved in both purine and pyrimidine biosynthesis. During pyrimidine biosynthesis, N^5, N^{10}-methylene THF is required by the enzyme thymidylate synthetase for successful conversion of deoxyuridine monophosphate to deoxythymidine monophosphate. Another folate derivative, N^{10}-formyl THF, is integral to the formation of inosine monophosphate, a precursor of both purine nucleotides. Thus, methotrexate has the capacity to indirectly deplete cells of three of the nucleotides necessary for DNA synthesis (Smith *et al.*, 1987).

In many cell lines cultured *in vitro*, methotrexate initially delays cells in the DNA synthesis (S) phase of the cell cycle. Such cell cycle arrest is thought to result from a delay whilst the cell scavenges for nucleotides required for DNA strand extension or for the repair of mismatched DNA bases. Subsequently, methotrexate impedes the entry of cells into S phase due to

inhibition of RNA synthesis required for the transition from G_1 to S phase of the cell cycle.

1.1.3.2 Etoposide

Etoposide is an epipodophyllotoxin, derived from an extract of the plant *Podophyllum peltatum*. Whilst the parent molecule inhibits proper microtubule formation, necessary for mitotic division, etoposide does not affect cells in mitotic (M) phase. Instead, etoposide exposure results in the cellular accumulation of DNA strand breaks by interfering with the function of the topoisomerase II enzyme. Topoisomerase II alters the topographical conformation of DNA by producing transient double-stranded breaks in the DNA helix in order to release torsional stresses produced during DNA replication (Henwood and Brogden, 1990). The enzyme binds to the DNA in dimeric form, producing a stable enzyme-DNA complex during the uncoiling process and then re-ligates the cleaved DNA. Etoposide acts by forming a stable aggregate with the DNA-enzyme complex, reversibly inhibiting the re-ligation process and shifting the normal reaction equilibrium towards the cleavable DNA complex (Anderson and Berger, 1994).

Etoposide exposure delays cell cycle progression at the interface between the S and second gap (G_2) phases, presumably whilst the cell endeavours to religate the cleaved DNA and release the topoisomerase II complex (Henwood and Brogden, 1990).

1.1.3.3 Vinblastine

Vinblastine, along with vincristine, is a clinically important alkaloid derivative of an extract from the myrtle, *Vinca rosea*. Vinblastine binds to tubulin, blocking or reversing its polymerisation into microtubule bodies. Microtubules are required for the maintenance of cell shape and contractility, movement of subcellular organelles and phagocytic functions, in addition to their role in chromosome organisation prior to cell division. The loss of microtubule activity results in the arrest of cell cycle progression at metaphase, the stage of mitosis during which the microtubular spindles align the chromosomal DNA.

1.1.4 Chemotherapeutic drug-induced toxicity

1.1.4.1 Chemotherapy agents induce a common form of cell death

Whilst the anti-proliferative effects of most cytotoxic drugs are understood, the factors that lead to drug-induced death remain unclear. A number of studies have established that exposure to radiation or chemotherapy induces a consistent pattern of cellular death in both *in vitro* (Barry *et al.,* 1990; Marks and Fox, 1991) and *in vivo* (Searle *et al.,* 1975; Pipan and Sterle, 1979; Potten *et al.,* 1994; Anilkumar *et al.,* 1992) models. Indeed, it has been suggested that the responses to a diverse range of toxic insults may ultimately converge upon a single genetic program of cell death, commonly termed apoptosis (Kerr *et al.,* 1972).

It is not yet clear whether chemotherapeutic drugs are able to directly induce this apoptotic state, or activate it indirectly as a by-product of accumulated DNA damage. In the case of etoposide, a discrepancy certainly exists between the temporal locations of maximal drug sensitivity and the maximal cytotoxic response. Whilst topoisomerase II enzyme levels and DNA cleavage are greatest during G_2 and M phases of the cell cycle, etoposide-mediated cytotoxicity is optimal during incubation with S phase cells (Chow and Ross, 1987). Rather, it would seem more probable that a cell might be programmed to undergo apoptosis in response to cellular perturbations, such as irreparable DNA damage, from which it was not able to recover.

The induction of apoptosis is dependent upon the intensity of the cytotoxic insult, as excessive drug doses are known to induce necrosis in addition to apoptosis (Marks and Fox, 1991). At low drug doses, a brief exposure may induce no immediate evidence of cell toxicity, instead producing a delayed response many hours after drug removal (Sorenson *et al.,* 1990). This delay may be due in part to the requirement for protein and RNA synthesis prior to DNA cleavage, as observed in thymocyte populations (Cohen and Duke, 1984; Wyllie *et al.,* 1984).

1.1.4.2 Characteristics of apoptosis

Apoptosis is a term originally suggested by Kerr *et al.* (1972) to describe a form of cell death,

observed in a number of normal and damaged tissues, which appeared to be distinct from necrosis. Apoptotic cells were originally characterised by morphological assessment, featuring a loss of volume and concomitant increase in density, aggregation of chromatin, distortion of the endoplasmic reticulum and the activation of phagocytosis *in vivo*. However, research findings have since expanded to encompass biochemical markers of apoptotic death, such as DNA fragmentation, and genetic markers associated with the induction of apoptosis.

1.1.4.2.1 Changes in cell morphology

The morphology of a cell deleted by apoptosis is notably distinct from that generated during a necrotic response. Apoptosis, both *in vivo* and *in vitro*, is differentiated from necrosis by the early preservation of cell membrane integrity and the absence of swelling of organelles. During the apoptotic response the nucleus contracts and the chromatin condenses, accumulating near the periphery of the nuclear membrane. Cells dying by apoptosis *in situ* become segregated from surrounding cells and extrude their contents through small packages of the cell membrane, giving rise to the cell surface "blebbing" associated with this form of death. These apoptotic bodies are then either phagocytosed by neighbouring cells or shed from the tissue surface (Kerr *et al.*, 1972).

Induction of the apoptotic program *in vitro* by chemotherapy agents has been investigated primarily in a human promyelocytic leukemia (HL-60) cell line, in which the addition of a cytotoxic stimulus results in a rapid and collective shift of the entire population through the apoptotic pathway (Tanizawa *et al.*, 1989; Del Bino *et al.*, 1991). However, the apoptotic progression typified by this cell line is not representative of the response observed by other cell lines in culture, particularly adherent cell lines. In Chinese hamster ovary (CHO) cells, exposure to cisplatin produced a transient increase in volume before cell contraction, attributed to the temporary arrest of the cell population in G_2 phase. Exposure of CHO cells to different chemotherapeutic agents consistently resulted in a loss of cell volume, but variable delays in the appearance of DNA fragmentation products, depending upon the particular toxin applied (Barry *et al.*, 1990). Desjardins and MacManus (1995) have further characterised the progressive

appearance of apoptotic markers in an adherent cell line, which occur over an extended timecourse when compared to more the traditionally studied cells. This model suggests that the damaged cells remain in a latent "pre-apoptotic" phase prior to the transient increase in cell volume and concurrent appearance of high molecular weight DNA fragments. Dying cells then progress to detach from neighbouring cells, with further degradation of the DNA to intermediate molecular weight fragments and condensation of the chromatin. Finally, the cells begin to shrink in size, exhibit 180bp DNA fragmentation patterns and detach from the culture monolayer. The continuing decay of the nuclear and plasma membranes leads to the loss of ability to exclude vital dyes, a stage termed "secondary necrosis".

1.1.4.2.2 DNA fragmentation

In some cell types, apoptosis is associated with the regular cleavage of DNA into fragments which are multiples of 180-200 base pairs in length (Wyllie, 1980). The DNA fragments are produced by the actions of an endogenous endonuclease which is able to digest the DNA strands as they remain wound around structural histones. This fragmentation produces a widely recognised pattern of DNA laddering on electrophoretic gels which has become almost exclusively associated with the induction of apoptosis.

The DNA fragments produced during apoptosis can be used to aid in the identification of apoptotic cells both *in situ* and *in vitro*, by employing a terminal deoxy-transferase enzyme to incorporate labelled nucleotides onto the ends of the cleaved DNA strands (Gavrieli *et al.*, 1992). However, not all cells appear to undergo such extensive DNA fragmentation. Reports by Oberhammer *et al.* (1993) and Cohen *et al.* (1992) have highlighted the lack of 180 base pair DNA fragments in a number of different cell types in response to various toxic stimuli. This distinction between DNA cleavage and other features of apoptosis, such as cellular morphology, emphasise the importance of examining a variety of apoptotic markers, which may emerge at different times following the induction of the cell death program.

1.1.4.3 Gene products associated with the induction of apoptosis

There are many genes and proteins that have been identified as contributing factors along the apoptotic pathway, depending in part on the cell type examined. In humans, apoptosis is controlled by elements such as the Bcl-2 family of proteins, c-Myc and p53.

1.1.4.3.1 Role of the Bcl-2 family in the apoptotic process

The *bcl-2* gene family encodes for a group of structurally conserved proteins, including both promoters and suppressors of apoptosis. The *bcl-2* gene product is a 26 kDa transmembrane protein that sustains cell viability by providing protection against oxidative damage (Hockenbery *et al.,* 1993). Indeed, expression of the Bcl-2 protein in a range of tissues is known to correspond to cell sub-populations with the greatest propensity for survival, such as stem cells or proliferating cells (Hockenbery *et al.,* 1991). Proteins with partial homology to Bcl-2, such as Mcl-1 and Bcl-xL, also modulate the suppression of apoptosis (Boise *et al.,* 1993), (Reynolds *et al.,* 1994). In contrast, Bax, Bad and Bcl-xs, which retain some of the conserved domains found in Bcl-2, facilitate the induction of apoptosis by down-regulating Bcl-2 activity via the formation heterodimers (Boise *et al.,* 1993), (Oltvai *et al.,* 1993), (Yang *et al.,* 1995). Over-expression of *bcl-2* in mammary epithelial cell lines restricts apoptosis in situations where the cells would normally die, such as over-confluence or serum-deprivation of the cultures (Lu *et al.,* 1995). Bcl-2 also influences other growth regulatory pathways, such as blocking c-*myc*-induced apoptosis, but not c-*myc*-induced mitogenesis (Fanidi *et al.,* 1992).

1.1.4.3.2 Role of c-Myc in the induction of apoptosis

C-Myc is a nuclear phosphoprotein associated with DNA binding, possibly functioning as a transcription factor for specific growth regulatory genes. The deregulation of c-*myc* gene expression leads to continual cell proliferation, but also produces concomitant increases in apoptosis (Evan *et al.,* 1992). The signals that direct cells towards either a proliferative or apoptotic response are unknown, however Evan *et al.* (1994) suggest that c-*myc* induced apoptosis may represent a constitutive pathway that is normally blocked by the presence of

survival factors.

Activation of the c-*myc* gene is central to the proliferative response in many cell types. Unrestricted expression of the c-*myc* gene results in excessive proliferative activity, evident even under conditions where cells would normally become quiescent (Evan *et al.,* 1992). In mouse keratinocytes, transforming growth factor ß (TGFß) specifically down-regulates c-*myc* expression, mediated by the actions of the *Rb* gene product (Coffey *et al.,* 1988; Pietenpol *et al.,* 1990b). Blocking the *c-myc* gene with anti-sense oligonucleotides produced an identical growth inhibitory response, suggesting that *c-myc* is a primary target of TGFß action in these cells (Pietenpol *et al.,* 1990a).

The induction of an apoptotic pathway by the c-*myc* gene requires that it bind to its heterodimeric partner, Max. This heterologous dimerisation mediates transcriptional activity, leading to the induction of apoptosis in a Myc concentration-dependent manner (Evan *et al.,* 1992). An important feature of Myc-induced cell death is that expression of the protein can induce apoptosis in growth arrested cells, irrespective of the temporal cell cycle location of the arrest, or the nature of the arresting factor (Evan *et al.,* 1992). Studies in a kidney epithelial cell line showed that c-Myc-induced death could occur through both p53-dependent and p53-independent pathways, both of which were effectively negated by the presence of Bcl-2 (Sakamuro *et al.,* 1995). Similar research in Chinese hamster ovary cells indicated that Mcl-1, a member of the Bcl-2 family, was able to delay the induction of apoptosis but could not totally override c-Myc-mediated apoptosis (Reynolds *et al.,* 1994).

1.1.4.3.3 Role of p53 in the apoptotic pathway

Mutation or deletion of the p53 gene is one of the most frequently observed genetic defects associated with cancer development in humans (Hollstein *et al.,* 1991; Levine *et al.,* 1991). The production of mice with homozygous deletions of the p53 gene has permitted a functional analysis of p53-dependence in the induction of apoptosis. Mice with p53 null alleles have a greater susceptibility to tumour formation (Hooper, 1994), and resist radiation-induced

apoptosis of the epithelial cells in the small intestine (Merritt *et al.,* 1994; Clarke *et al.,* 1994). Thus, p53 appears to play a dual role in cellular control, implicated in both the regulation of hyper-proliferative states and the induction of apoptosis.

The anti-proliferative activity of the p53 protein is associated with a hyper-phosphorylated state, and can be rapidly reversed *in vitro* by the addition of serum (Raynal *et al.,* 1994). Cells sensitive to the growth inhibitory effects of TGFß would be expected to possess mostly hyper-phosphorylated protein. However, serum-induced de-phosphorylation of p53 is enhanced by concurrent exposure to growth inhibitory doses of TGFß, an anomaly which the authors attribute to differential phosphorylation of specific amino acid residues (Raynal *et al.,* 1994).

The p53 protein has a putative function as a transcription factor, implied by the presence of a p53-binding site within the WAF1 gene (El-Deiry *et al.,* 1993). Functional p53 activates $p21^{WAF1/CIP1}$, an inhibitor of cyclin-dependent kinase (Cdk) complex activity (Xiong *et al.,* 1993). In turn, inhibition of Cdk complexes preserves the under-phosphorylated form of the retinoblastoma gene product (pRb), blocking the activation of early DNA synthesis and consequently prompting arrest of cell-cycle progression in the first gap (G_1) phase (Dulic *et al.,* 1994). This activity of the hypo-phosphorylated pRb is associated with its ability to obstruct the initiation of DNA synthesis, normally initiated by the E2F family of transcription factors.

The p53 protein has also been shown to bind to a consensus site located within the promoter region of the *bax* gene, suggesting a role in upregulating the expression of this Bcl-2 protein inhibitor (Miyashita and Reed, 1995). The validity of such a hypothesis is enhanced by the observation that Bax expression is elevated in epithelial cells undergoing apoptosis in a p53-dependent fashion, but not in a p53-independent manner (Sakamuro *et al.,* 1995). *In vitro* studies with *p53*-deleted mouse fibroblasts have shown that the p53 gene is necessary to elicit an apoptotic response against various cytotoxic drugs (Lowe *et al.,* 1993). Hence, the DNA damage induced in some normal cells by chemotherapy treatment is dependent upon increased p53 protein activity. The p53 protein is then able to either transiently stall cells in G_1 phase, whilst DNA damage is repaired, or induce the cells to undergo apoptosis. By following either

pathway, the p53 protein effectively ensures the reliable genetic heritability of any cell permitted to re-enter the replicative process.

The pathway triggered by the p53 protein appears to be dependent upon the presence of external factors, possibly mediated by the transduction of survival signals. In a murine haematopoietic cell line, the presence of interleukin 3 (IL-3) during cell irradiation causes a p53-dependent growth arrest in G_1 phase (Canman *et al.,* 1995). In contrast, cells irradiated in the absence of IL-3 bypass the G_1 restriction point, and proceed to S phase where they undergo apoptosis. Other proteins, such as the insulin-like growth factors, may play a similar role in cells of fibroblast origin (Sell *et al.,* 1995).

1.2 STRATEGIES FOR MEDIATING EPITHELIAL CELL SURVIVAL

Enhanced protection against drug-induced damage in normal tissues has become a primary focus in the search for more effective chemotherapeutic strategies. Improved cell survival, particularly in the stem cells of the gastrointestinal tract, would clearly reduce the unpleasant and sometimes critical nature of the damage resulting from chemotherapy treatment.

The ability to manipulate cell cycle progression offers an opportunity to restrain cells from particular phases within the cycle where they would be most susceptible to accumulating DNA damage or becoming targets for direct drug action. Alternatively, there may be a role for the use of growth-stimulatory agents to promote the rapid regeneration of a cell population from a smaller residual population of surviving stem cells. Cell survival may also ultimately rely upon the presence of "survival factors" - those agents whose mechanism of action is currently not understood, but nevertheless improve the survival outcome in various situations.

1.2.1 Induction of growth arrest

The hypothesis that temporary cessation of normal growth patterns may render cells less susceptible to cytotoxic damage has gained acceptance as a result of studies with both TGFß and interleukin 11 (IL-11). The mechanism(s) underlying the cytoprotection conferred by these agents has not been fully elucidated, but may be linked to factors such as the straightforward de-sensitisation of cells through the induction of G_1 arrest, or perhaps the down-regulation of c-*myc* gene expression and c-*myc* induced apoptosis.

1.2.1.1 Transforming growth factor ß

In many cell types, exposure to TGFß results in the retention of the hypo-phosphorylated conformation of the retinoblastoma gene product, pRb, effectively stalling cell cycle progression in late G_1 phase. However, the effects of TGFß on both growth and apoptotic responses can vary with cell type. Normal human prokeratinocytes are reversibly arrested in G_1 phase (Shipley *et al.*, 1986). Whilst a similar pattern of growth inhibition is observed in uterine

epithelial cells, exposure to TGFß also stimulates the emergence of apoptotic cells (Rotello *et al.,* 1991). In contrast, the growth of immortalised ectocervical epithelial cells is reversibly inhibited by low concentrations of TGFß, but is permanently blocked at high concentrations in conjunction with the induction of apoptosis (Rorke and Jacobberger, 1995).

Studies with a rat intestinal epithelial (IEC-6) cell line indicate that TGFß promotes growth arrest without inducing classical markers of terminal differentiation (Ko *et al.,* 1994; Barnard *et al.,* 1989). This correlates well with *in vivo* observations in the mouse small intestine, where both crypts and villi were stunted following TGFß administration (Migdalska *et al.,* 1991). These *in vivo* findings were thought to be caused by a slower rate of division of the crypt stem cells as well as partial inhibition of migrating crypt cells. Thus, the ability of TGFß to inhibit progenitor cell division in the small intestine, and a derivative cell line *in vitro*, suggested a potential application in protecting against cytotoxic damage.

Various *in vivo* models of drug-induced toxicity have been employed to study the potential for TGFß-mediated protective effects. Pierce and Coffey (1994) hypothesised that TGFß-induced growth arrest in G_1 phase would diminish the impact of S phase toxicity associated with cytarabine administration in mice. Their theory proved to be well founded, as pre-treatment of mice with recombinant $TGFß_1$ significantly reduced mortality following cytarabine exposure.

Further validation of this cytoprotective strategy was provided by a model of oral mucositis, employing Syrian golden hamsters exposed to 5-fluorouracil with irritation of the cheek epithelium (Sonis *et al.,* 1990). In this model, Sonis *et al.* found that pre-treatment of the cheek pouch with recombinant $TGFß_3$ reduced the incidence and severity of drug-induced ulceration, as well as improved animal survival rates (Sonis *et al.,* 1994). This work was consolidated by *in vitro* studies, in which prior treatment with $TGFß_3$ mediated improved cell survival in a mink lung epithelial (Mv1Lu) line exposed to vinblastine.

1.2.1.2 Interleukin 11

Interleukin 11 is a multi-functional cytokine originally applied to the reduction of haematological complications arising from chemotherapy treatment. Subsequent investigations by Du *et al.* (1994) indicated that this factor was also able to improve survival of the intestinal epithelium following high dose drug administration. These findings were later confirmed in the hamster model of oral mucositis previously employed for studies of TGFß efficacy (Sonis *et al.,* 1995). Furthermore, IL-11 was implicated as a cytoprotective agent in two models of inflammatory bowel disease (Keith *et al.,* 1994), suggesting a broad protective role for this cytokine within the gastrointestinal tract.

The effect of interleukin 11 on epithelial cells remained uncharacterised at the time that the afore-mentioned drug toxicity studies were performed. Indeed, it was only in light of the potential clinical benefit to be gained from this cytokine that studies into the effects of IL-11 on epithelial cells were initiated. A normal intestinal epithelial cell line (IEC-18), but not transformed cell lines (SW620 and HT29), was able to be growth inhibited in response to IL-11 exposure (Booth and Potten, 1995). This response was detected as a reduction in DNA synthesis as early as 12 hours after the addition of IL-11, and was reversed by removal of the cytokine from the growth medium. Thus, the transient growth inhibition induced by IL-11 may prove to be a factor in protecting epithelial cells from drug toxicity in much the same way as it appears to be implicated in TGFß-mediated survival.

1.2.2 Anti-apoptotic activity

Cell survival may be enhanced by the addition of factors which currently operate via an uncharacterised mechanism. Recently, Evan *et al.* (1994) have devised a hypothetical model to account for the actions of c-Myc in mediating proliferative and apoptotic responses, and to explain the impact of exogenously administered "survival factors". In brief, c-Myc controls the extent of proliferation and apoptosis in many cells by regulating the transcriptional activation of target genes. These authors suggest that c-Myc has constitutive pro-apoptotic activity,

remaining latent in the presence of requisite survival signals often supplied *in vitro* by the addition of serum. Thus, cell survival becomes dependent upon two distinct signals, one for mitogenic stimulation of cell proliferation and another to block the induction of c-Myc-induced apoptosis.

Apoptosis induced by serum-deprivation or confluence of *in vitro* cultures appears to be a general phenomenon, however relevant survival factors vary with cell type. Cell death is restricted by the addition of epidermal growth factor (EGF) or insulin to cultured mammary epithelial cells (Merlo *et al.,* 1995), and by the exposure of oligodendrocytes to platelet-derived growth factor (PDGF) or either insulin-like growth factor (IGF) -I or -II (Barres *et al.,* 1992). In haematopoietic cell lines reliant upon the presence of IL-3, the apoptotic program normally triggered by the absence of this cytokine can be averted by the addition of IGF-I. Additionally, studies in serum-deprived fibroblasts have shown that both IGF-I and PDGF are capable of protecting cells from apoptotic death, a function found to occur independently of their mitogenic potential (Harrington *et al.,* 1994). Similar inhibition of apoptosis was also observed in the presence of either IGF-II or insulin, whilst EGF, TGFß and fibroblast growth factor (FGF) had no such effect.

1.2.2.1 Epidermal growth factor-mediated protection against drug toxicity

Epidermal growth factor stimulates DNA synthesis in intestinal cells cultured *in vitro*, and partially abrogates the growth inhibition induced by TGFß (Kurokawa *et al.,* 1987). EGF also exerts trophic effects on explanted cultures of duodenal mucosa, increasing the rate of crypt cell proliferation by approximately five-fold (Challacombe and Wheeler, 1991). Yet, in addition to this potent growth stimulatory effect, EGF is also able to protect mammary epithelial cells against apoptosis associated with serum-deprivation (Merlo *et al.,* 1995).

This survival function, however, does not appear to translate to a cytoprotective effect against drug-induced apoptosis. Epidermal growth factor shows no propensity for protecting mice against melphalan-induced intestinal toxicity, or for promoting enhanced regeneration following

drug exposure (Robinson *et al.,* 1985). Indeed, the administration of EGF prior to melphalan treatment exacerbates crypt cell depletion and animal weight loss.

The potential for EGF-mediated protective properties have also been investigated in the Syrian hamster model of oral mucositis. In a similar manner to the model of melphalan-induced toxicity, EGF intensified the severity of oral mucositis induced by 5-fluorouracil (Sonis *et al.,* 1992). The degree of damage, and the progression of the disease, was affected by the timing of EGF administration, suggesting that toxicity was closely interrelated with the rate of cell proliferation. The fact that EGF did not display any survival activity in either model of drug-induced toxicity suggests that it operates solely as a mediator of mitogenic stimulation in intestinal epithelial cells.

1.2.2.2 Insulin-like growth factor-I-mediated protection against drug toxicity

In contrast to EGF, IGF-I is able to prevent apoptosis induced by chemotherapeutic agents in both cancerous and non-transformed cell lines (Guo *et al.,* 1995a; Sell *et al.,* 1995). Furthermore, IGF-mediated protection against apoptosis occurs independently of mitogenesis, as this factor is able to restrict apoptosis induced by drug exposure without alleviating the concomitant growth arrest (Harrington *et al.,* 1994). Paradoxically, co-administration of IGF-I and methotrexate increased the severity of small intestinal mucositis in rats, suggesting that IGF-I had no anti-apoptotic role in this model (Read *et al.,* 1994). It would appear that in this instance, IGF-I may promote mitogenesis selectively over cell survival, thereby committing a larger proportion of the cell population to proliferate in the presence of methotrexate, resulting in increased apoptosis and derangement of tissue structure.

1.2.3 Accelerated regeneration following drug exposure

There is little evidence in the literature to indicate that the stimulation of rapid stem cell proliferation has been embraced as a survival strategy following cytotoxic drug exposure. One such study involves the infusion of rats with IGF-I in the days following methotrexate administration. This IGF-I post-treatment produced significant improvements in intestinal

architecture, as determined by regeneration of both crypt and villus structures (Read *et al.,* 1994). However, such indications of improved survival may be deceptive, as any overt acceleration of the cell cycle machinery may actually reduce the accuracy of detection and deletion of cells bearing DNA damage, thus potentially increasing the likelihood of mutations being borne through to the daughter cells.

1.2.4 Dietary components as survival factors

Amongst the numerous studies undertaken on the basis of one of the above strategies, there is a growing amount of literature relating to the application of dietary components for cytoprotective purposes.

1.2.4.1 Vitamin A

Oral administration of vitamin A is able to maintain the integrity of the small intestine in rats administered concurrent doses of methotrexate (Tsurui *et al.,* 1990). This protection corresponds with the retention of protein and lipid content within the small intestine, and stabilisation of gut weight. Further studies by Kosakai *et al.* (1991) showed that concurrent administration of vitamin A led to the partial preservation of thymidine kinase activity in crypt cells following methotrexate exposure. Thymidine kinase is one of two enzymes responsible for the synthesis of thymidine via the salvage pathway, and highlights an important rescue capacity of this vitamin. Importantly, however, vitamin A treatment has no effect on tumour growth or methotrexate-induced elimination of tumour material (Nagai *et al.,* 1993).

1.2.4.2 Glutamine

Glutamine is an essential amino acid required for the maintenance of cell growth, both *in vitro* and *in vivo*. Dietary supplementation with this nutrient produces no benefits in normal animals, but markedly reduces intestinal damage following abdominal irradiation. Oral administration of glutamine improved mucosal integrity and structure, and stabilised body weight when incorporated into the diet of animals treated either prior to, or following, radiation therapy

(Klimberg *et al.,* 1990a; Klimberg *et al.,* 1990b). A similar cytoprotective role has been identified for glutamine following exposure of rats to the chemotherapy agent, 5-fluorouracil. In this model of drug-induced damage of the gastrointestinal tract, intravenous administration of glutamine either before or after drug therapy promoted significant restoration of gut structure and an increase in long-term animal survival (O'Dwyer *et al.,* 1987).

1.3 APPLICATION OF A GROWTH FACTOR PREPARATION, EXTRACTED FROM CHEESE WHEY, TO PROTECT AGAINST CHEMOTHERAPEUTIC TOXICITY

Research and clinical trials directed towards the alleviation of the symptoms of mucositis and underlying structural damage have traditionally focussed on the application of a single agent, such as those discussed in Section 1.2. We have hypothesised that the use of combination treatments may promote additive, or even synergistic, levels of cell survival. In such an instance, it may be feasible to restrict cell cycling prior to drug addition, coupled with the post-drug application of a "survival factor" to enhance long-term cell survival. Further supplementation with dietary factors may also contribute to a reduction in the onset and severity of mucositis. However, the preparation of such a mixture would certainly become a complex procedure, in part due to the uncharacterised nature of the interactions between individual components. Furthermore, the cost of the recombinant growth factors required for clinical use may prove to be too prohibitive for routine application.

As an alternative approach, we have adopted the use of a natural product which contains many such proteins, and which is likely to provide a source of factors which are suited to the protection and repair of the gastrointestinal tract. This preparation is a composite of growth factors and other proteins prepared from a by-product of the cheese-making process. These proteins, and other extraneous matter, accumulate into the residual whey run-off following coagulation of casein to produce cheese curd.

The constituents of cheese whey comprise approximately 20% of total bovine milk protein, and include relatively abundant proteins such as ß-lactoglobulin, α-lactalbumin, bovine serum albumin and immunoglobulins. In addition, whey contains a number of less abundant proteins (eg. lactoferrin, $ß_2$-microglobulin, folate binding protein), growth factors and enzymes (eg. lactoperoxidase, lysozyme) (Whitney, 1988). It must be noted, however, that the relative abundance and overall composition of whey is affected by seasonality, stage of lactation and many of the treatments applied to the milk prior to coagulation (Kinsella and Whitehead, 1988).

1.3.1 Cellular responses to whey proteins

Milk has long been recognised as a rich source of nutrients for the maintenance of cell growth. Whilst milk does not contain the necessary adhesion factors for the initiation of monolayer cultures, it can act as a suitable replacement for serum in promoting cell proliferation in a variety of cell lines (Sereni and Baserga, 1981; Klagsbrun, 1978). Importantly, both human and goats milk contain growth stimulatory factors which are potent mitogens in cultured intestinal epithelial cells (Corps and Brown, 1987). Similar growth stimulatory activity is present in whey, promoting the dose-dependent stimulation of DNA synthesis in fibroblast cells (Damerdji *et al.,* 1988; Belford *et al.,* 1995). Aside from proliferative effects, animals fed bovine whey show improved cell-mediated and humoral immune responsiveness (Wong and Watson, 1995), highlighting the broad-ranging effects of this protein mixture.

Whilst whey induces growth modulatory responses in cell culture, the effects cannot be solely attributed to the presence of growth factors. Ellis *et al.* (1993) have shown that certain whey proteins can partially inhibit the proliferation of mammary epithelial cells. Both purified α-lactalbumin and ß-lactoglobulin elicit a reduction in DNA synthesis, suggesting that the actions of whey-derived growth factors may be complicated by the presence of these proteins. Partial purification of the whey extract may therefore be necessary in order to facilitate the identification of other active constituents.

1.3.2 Preparation and characterisation of whey-derived growth factor extract

The initial purification of whey involves micro-filtration through a 1.4 μm membrane to remove fat and particulate matter, followed by additional steps to remove many of the major whey proteins. Francis *et al.* (1995) have recently developed a method which utilises the basic isoelectric points of many bovine milk growth factors to remove contaminating ß-lactoglobulin, α-lactalbumin and BSA peptides by cation exchange chromatography. The eluting fraction, termed whey-derived growth factor extract, or WGFE, contains only 0.5% of the original whey proteins, but retains most of the growth factor activity.

The WGFE preparation is composed of lactoperoxidase, lysozyme, immunoglobulins and a variety of minor components, all at greatly increased specific activities when compared with the initial whey material (Francis *et al.,* 1995). Table 1.1 lists the general composition of WGFE. The minor components include residual casein and ß-lactoglobulin, as well as a number of growth factors and other unidentified material. The typical growth factor composition of WGFE is summarised in Table 1.2, and to date includes the identification of IGF-I and -II, IGF binding proteins, acidic and basic FGF (Rogers *et al.,* 1995), PDGF, and TGFß (Rogers *et al.,* 1996; Belford *et al.,* 1997).

EGF has previously been measured in milk from both human (Carpenter, 1980) and bovine (Iacopetta *et al.,* 1992) sources. However, in contrast to the findings of Iacopetta *et al.*, there does not appear to be a detectable quantity of EGF in WGFE. Rather, WGFE contains an EGF-like molecule capable of potently stimulating Balb/c 3T3 fibroblast growth *in vitro* in the absence of serum-supplementation (Dunbar *et al.* 1997).

The majority of the TGFß present in WGFE is in a latent form, but is readily activated by transient acidification, thermal denaturation or enzymatic cleavage of the latent complex. Over 85% of the total TGFß activity in WGFE is present as the $TGF\beta_2$ isoform, with the remaining 15% likely to be attributable to the $TGF\beta_1$ isoform (Rogers *et al.,* 1996).

component	% protein (w/w)
lactoperoxidase	60
lysozyme	15 - 20
immunoglobulin	15 - 20
minor components	~10

Table 1.1 Compositional analysis of whey-derived growth factor extract
(derived from Francis *et al.*, 1995)

growth factor	concentration (ng/mg WGFE)
IGF-I[1]	22
IGF-II	24
acidic FGF[2]	0.2
basic FGF[2]	0.2
PDGF[1]	4
TGFß (active)[3]	3
TGFß (total)[3]	100

Table 1.2 Growth factor composition of whey-derived growth factor extract
(adapted in part from 1. Belford *et al.*, 1997; 2. Rogers *et al.*, 1995 and 3. Rogers *et al.*, 1996)

1.3.3 Actions of WGFE *in vitro*

The ability of WGFE to temper growth responses *in vitro* has been assessed in a range of cell lines, including fibroblast, myoblast and epithelial cultures. In cells of mesodermal origin, such as Balb/c 3T3 fibroblasts and L6 myoblasts, WGFE stimulates cell proliferation above that normally observed in the presence of 10% foetal calf serum (Belford *et al.,* 1995). Furthermore, the presence 1% or 5% FBS in WGFE-treated cell cultures produced additive growth responses, suggesting that WGFE may either supplement identical growth factors present in serum, or provide a source of additional factors not found in serum. Other cell lines, including Chinese hamster ovary cells, were less proliferative in response to WGFE than to 10% serum, whilst growth in all of the epithelial cell lines examined was inhibited by WGFE in a dose-dependent manner (Belford *et al.,* 1995). Thus, WGFE may be an important source of growth promoting factors for the maintenance of mesodermal cells. Furthermore, this growth factor preparation may be able to protect epithelial cells from drug-induced damage in a similar manner to recombinant TGFß.

1.3.4 Actions of WGFE *in vivo*

The cytoprotective qualities of WGFE have been examined in an animal model of intestinal mucositis. In the rat, methotrexate induces morphological damage to both the crypt and villus cells, primarily within the proximal small intestine. Natural recovery from this damage includes a temporary hyperplasia, involving an increase in crypt cell mitotic activity and premature migration of the crypt cells into the villus (Taminiau *et al.,* 1980). Co-administration of WGFE in the diet of methotrexate-treated rats improves histological indices of crypt and villus integrity. Figure 1.2 illustrates the increased crypt area index measured by Howarth *et al.* (1996) in methotrexate-injected rats when WGFE was orally administered for 5 days.

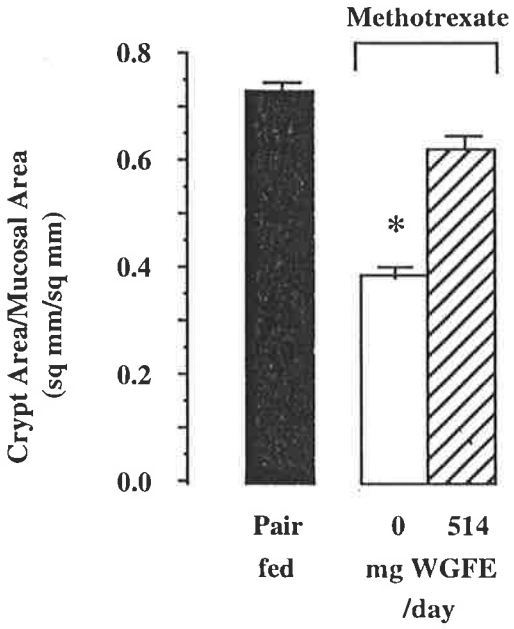


Figure 1.2 Crypt area index in the jejunum of methotrexate-treated rats

WGFE was orally administered to rats for 5 days, beginning at the same time as methotrexate injection (▨). Pair-fed animals (■) were not injected with methotrexate, but had food intake restricted to that of the methotrexate-injected animals not receiving WGFE (□). *, $P < 0.05$, compared with pair-fed data. Data from Howarth *et al.*, 1996.

1.4 AIMS OF THIS THESIS

The application of a whey-based growth factor mixture for the alleviation of mucositis has many obvious benefits. Firstly, there are a number of growth factors present in cheese whey which have previously been shown to reduce damage following chemotherapy treatment. Secondly, the widely embraced perception of milk and whey as natural health-promoting products may aid in patient acceptance of such a form of therapy. This may also be consolidated by the ease of application or consumption of whey-based therapies, anticipated to be via oral exposure or feeding. Thirdly, WGFE may obtain more rapid acceptance from drug administrations due to its origins as a food by-product. And finally, the status of whey as effluent in Australian cheese manufacture reveals this material to be a currently under-utilised source of growth factors. Importantly, the cheap and rapid concentration of growth factor activity from this waste material could potentially reduce the cost of clinical treatment when compared to the application of recombinant proteins.

The utilisation of WGFE to protect against drug-induced cytotoxicity is a recent innovation, originating from the findings of Howarth *et al.* (1996). This study reported that oral administration of WGFE to methotrexate-treated rats partially prevented crypt ablation in the jejunum and reduced bacterial translocation in the small bowel. WGFE feeding also promoted more rapid re-cellularisation of the villi in both the jejunal and ileal regions of the gut. These phenomena were attributed to either the protection of crypt cells against methotrexate-induced damage, or the enhanced regeneration of residual crypts following drug treatment. However, the conclusions able to be drawn from this study were limited to acknowledgments of the cytoprotective properties of WGFE in the small intestinal in reducing methotrexate-induced damage. Any inferences to be made concerning likely mediators of this protection were confounded by the timing of WGFE administration, as the animals retained access to the growth factor preparation both during and after drug exposure. Hence, questions arose concerning the optimal timing schedule for WGFE administration, and whether the growth factor composition of WGFE could be modified in some way to either further enhance cell

protection or vary the timing schedule.

At the commencement of my research, WGFE protection had only been assessed in the rat model of methotrexate-induced mucositis. Methotrexate toxicity may have been attenuated through a general anti-apoptotic mechanism, or perhaps via a more drug-specific effect. With the use of methotrexate, the possibility arose that cell rescue was due to stimulation of the thymidine salvage pathway, as observed with vitamin A (Kosakai *et al.,* 1991). Additionally, the presence of folate-binding proteins in whey, and most probably in WGFE, suggests a potential for cell protection mediated by either an increased bioavailability of folates along the gastrointestinal tract, or reduction in toxicity due to the formation of binding protein-methotrexate complexes. Hence, further studies were considered necessary to investigate WGFE-mediated protection against other drugs known to cause mucositis.

Due to the time consuming nature of adopting or developing new animal models of drug toxicity, I elected to assess the cytoprotective actions of WGFE in a series of *in vitro* experiments. This necessitated the development of an *in vitro* model which would be responsive to a number of toxic stimuli and thus provide preliminary indications as to the likely extent of WGFE-mediated actions *in vivo.*

An *in vitro* model of drug toxicity would also be appropriate for studying the optimal timing of WGFE administration and identification of active WGFE components. The use of recombinant growth factors to identify individual actions certainly becomes a cheaper and more rapid prospect *in vitro* than in an animal model. Furthermore, an cell culture model provides the opportunity for larger scale screening than would be feasible *in vivo*. This screening system permits a more extensive dissection of the WGFE material to be performed, as many WGFE sub-fractions can be assessed in light of different process modification or separation strategies.

Thus, the specific aims of this study were:

1. To develop and characterise an *in vitro* model of drug-induced toxicity

2. To quantify the capacity of WGFE to mediate cytoprotection in this model when applied at various times with respect to drug exposure

3. To assess the effects of WGFE modification on cell survival and optimal timing schedules

4. To determine if the *in vitro* response to WGFE can be entirely attributed to individual growth factors identified within the preparation

5. To compare the responses of the gastrointestinal epithelium and the *in vitro* model following WGFE administration in the absence of drug treatment, and to relate these findings to cell survival outcomes

Chapter Two

Development of an in vitro model

2. *Development of an in vitro model*

Abstract

In epithelial cell populations, exposure to cytokines such as interleukin-11 or transforming growth factor ß (TGFß) reduces the severity of chemotherapy damage. The application of these peptides as chemoprotective agents exploits their ability to transiently arrest cell cycle progression in mid- to late-G_1 phase (Peterson *et al.*, 1996; Laiho *et al.*, 1990). As TGFß is present in the whey-derived growth factor extract (WGFE), and contributes to the overall growth inhibitory action of WGFE in epithelial cells, I employed recombinant $TGFß_2$ to develop a suitable epithelial model for subsequent analysis of WGFE-mediated protection against drug-induced toxicity. In this chapter I have described the selection and characterisation of a cell line derived from mink lung epithelium (Mv1Lu). Mv1Lu cells were extremely sensitive to the growth inhibitory actions of TGFß, with cell number reduced by 39% following a 30 h incubation with 3 ng/ml $rTGFß_2$. This cell line also responded to both etoposide and vinblastine in a dose-dependent manner, showing early indications of drug-induced cell cycle arrest and subsequent induction of apoptosis. In contrast, exposure to a range of concentrations of methotrexate induced growth inhibition, but failed to trigger an apoptotic response. The addition of 3 ng/ml $rTGFß_2$ to Mv1Lu cultures for 48 h, beginning 30 h before the addition of either 1 µM etoposide or 50 nM vinblastine, increased cell survival from 56.1% to 72.3% and from 14.9% to 45.1%, respectively. These results indicate that $rTGFß_2$ is able to enhance epithelial cell survival following chemotherapy drug exposure, and establishes a functional assay for the analysis of WGFE-mediated protection against drug toxicity.

2.1 INTRODUCTION

The toxicity associated with many chemotherapeutic agents is often limited to specific phases of the cell cycle (Bhuyan and Groppi, 1989). Thus, the probability that healthy cells will avoid drug-induced damage may be improved by restricting them from particular cell cycle phases at the time of drug exposure. Strategies for reducing drug toxicity include the induction of cell cycle arrest in G_1 phase, mediated through the growth inhibitory actions of transforming growth factor ß (TGFß), or in G_0 phase by serum-depletion of the culture medium (Weyman and Stacey, 1996). Theoretically, this reduction in cycling will render the cell population less susceptible to the phase-specific actions of many chemotherapy agents. In practice, recombinant TGFß is able to partially prevent the damage caused by agents such methotrexate, 5-fluorouracil, etoposide, cytarabine and vinblastine, all of which target the S, G_2 or M phases of the cycle (McCormack *et al.,* 1997). Conversely, TGFß is less effective in protecting cells from cisplatin-induced death, as this drug is active over the entire cell cycle.

The growth factors present in the whey-derived growth factor extract (WGFE) comprise only a small fraction of total whey proteins, yet they are responsible for the induction of growth arrest in a number of epithelial cell lines (Belford *et al.,* 1995). The presence of TGFß, predominantly in the $TGFß_2$ isoform (Rogers *et al.,* 1996), contributes to the anti-proliferative response generated by WGFE in epithelial cells, and promotes the possibility of exploiting WGFE as a chemoprotective agent.

This study adopts a strategy of transient growth inhibition to protect against drug toxicity in the development of an *in vitro* model. As WGFE contains active TGFß, and mediates its growth inhibitory effects through this peptide, this chapter concentrates upon use of recombinant $TGFß_2$ to establish the model. Prior to the commencement of experimental work, I devised an assay protocol which I believed would form the basis for a model of TGFß-mediated cell survival following exposure to chemotherapeutic agents (Figure 2.1). This protocol took into account a number of considerations.

1. The cell cultures should be incubated for 24 h between seeding and the addition of growth inhibitory treatments to ensure that the cell populations had begun exponential growth. This would maximise the difference in cycling states between treated and untreated cell cultures.

2. The growth inhibitory treatments should be administered to the cells prior to drug exposure to ensure that cell cycle progression was maximally arrested. This required a pre-treatment equal to the length of at least one cell cycle so that every cycling cell could reach the growth inhibitory block. Additionally, growth inhibitory treatments had to remain on the cultures during drug exposure to prevent the cells from re-entering the replicative cycle when they would be most susceptible to drug toxicity.

3. The duration of drug exposure had to be approximately equal to the length of one cell cycle. This requirement guaranteed that if the drug was acting in a completely phase-specific manner, then the majority of cycling cells would transit through that particular phase and accumulate DNA damage (Gray, 1983). The concentration of drug required to induce cell death would be less than that needed to elicit an equal degree of toxicity over a shorter exposure time (Eichholtz and Trott, 1980). By using lower concentrations I could also reduce the likelihood that drug exposure would induce necrosis, rather than the characteristic apoptosis observed in the gastrointestinal tract *in vivo* following chemotherapy treatment (Anilkumar *et al.,* 1992).

4. The survival assay should incorporate an extension period following drug exposure to accommodate the possibility that changes in cell survival may not become evident immediately. If I were to measure survival by monitoring an early marker of apoptosis, such as a change in gene expression levels, then this requirement may be unnecessary. However, I planned to assess cell survival by quantifying the number of cells remaining on the culture monolayer after drug exposure. Desjardins *et al.* (1995) have previously shown that detachment of adherent cells occurs as a late development in the induction of a cell death pathway. Hence, this requirement was included as a safeguard until I could determine if it bore relevance to my model. The inclusion of an extension period after drug exposure also built flexibility into the model so that I could examine other treatment schedules, such as post-drug treatment, at a later

stage.

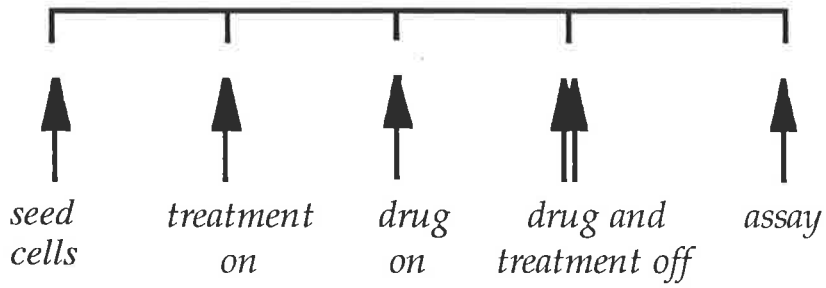


Figure 2.1 Diagram of the proposed survival assay protocol

The proposed timecourse for the survival assay involved seeding cells at a given density and leaving the cultures for 24 h to ensure that they had entered exponential growth. Cultures would then be pre-treated with growth inhibitory agents, followed by the addition of chemotherapeutic agents. After an incubation in the presence of both drugs and growth inhibitory treatments, the cultures would be washed and re-incubated in normal growth medium. Cultures would ultimately be harvested at a later timepoint, and analysed for cell detachment from the culture monolayer.

In order to develop a robust survival assay model, I established a set of criteria to be applied to the selection of an appropriate cell line (Table 2.1). The first two criteria were designed to identify a cell line that would be most likely to reflect epithelial cell responses *in vivo*. Whilst continuous cell cultures may not be truly representative of their *in vivo* counterparts, they were considered to provide a more suitable alternative than the use of primary cell cultures due to their singular phenotype and extensive characterisation. As drug toxicity principally affects actively cycling cells, it was necessary that the cell line to be chosen was able to maintain exponential growth for the duration of the assay. Finally, the candidate cell lines were also required to be susceptible to drug-induced damage, and to respond to TGFß-mediated growth inhibition.

cells should be of epithelial origin, preferably gut-derived
cells should not be virally transformed, but should grow in continuous culture
cells should grow exponentially for as long as possible
cells should be damaged by exposure to chemotherapy drugs
cells should be responsive to growth inhibitory factors, notably TGFß

Table 2.1 Criteria for the selection of a cell line for model development

Having addressed the above selection criteria, I characterised the responses of the selected cell line to three chemotherapy agents. Drug toxicity was assessed in three mechanistically distinct drugs - methotrexate, etoposide and vinblastine, following an 18 h drug exposure. The drug exposure time was chosen to approximate the cell cycle transit time of the chosen cell line, and ensured that the majority of cycling cells passed through the phases specifically targeted by the chemotherapy agents.

The ability of a cytotoxic agent to induce cell death is sometimes dependent upon release of the cell culture from drug exposure. Thus, with Chinese hamster ovary (CHO/AA8) cells it is preferable to intersperse pulses of aphidicolin exposure with periods of drug-free growth, so that accumulated damage can be realised as a loss of cell viability (Kung *et al.*, 1990). In contrast, continuous vincristine exposure merely delays death in this cell line, indicating that re-entry into the cell cycle is not vital for vincristine-induced toxicity. Thus, the incorporation of a 30 h incubation in drug-free medium upon completion of the 18 h drug exposure provided time during which cell death and detachment could become evident. Drug dose response curves were produced only at this final time-point, whilst all other parameters of drug toxicity were measured both immediately following drug exposure and again following the 30 h re-culture period.

Density-dependent drug toxicity effects were also examined, as many *in vitro* cellular responses are influenced by the density of the culture population. Cells cultured at high densities can display a reduced abundance of EGF receptors, and consequently become less responsive to peptide stimulation (Hamburger *et al.,* 1991). Other studies have found that over-confluent cultures of both fibroblast and epithelial cells secrete greater levels of growth inhibitory IGF binding proteins than sparser cultures, suggesting a potential mechanism for regulating cell proliferation (Blat *et al.,* 1994; Kutoh *et al.,* 1995). Of particular relevance to this study, however, was a report by Sasaki *et al.* (1991) which claimed that susceptibility to neocarzinostatin-induced cell death was dependent upon culture density in HeLa epithelial cells. The toxicity of neocarzinostatin appeared to be solely a function of the relative drug dose per cell, not of density-dependent changes in drug sensitivity or cell recovery rates. As I intended to protect epithelial cell cultures from drug toxicity by inducing a transient state of growth arrest, I would have reduced the culture density when compared to control (proliferating) cultures. Therefore, the relationship between culture density and drug-induced cell death was characterised by seeding cultures at different densities and measuring subsequent drug-induced cell detachment. Strategies for minimising the impact of this effect were also examined.

Finally, the cell culture model which I had developed was validated by measuring the cytoprotective potential of growth inhibitory doses of recombinant TGFß$_2$, to ensure that drug toxicity could be reduced in the presence of a cell cycle arresting agent.

For experimental use, trypsinised cell suspensions were washed three times, by repeated centrifugation (325 x *g*, 5 min) and resuspension in fresh growth medium, to remove all traces of trypsin/EDTA. Cells were finally resuspended in 10 ml of growth medium for assessment of cell viability by trypan blue exclusion. An aliquot of the cell suspension was mixed with 2.5 mg/ml trypan blue (CI 23850; Sigma Chemical Company) at a known ratio prior to counting in a haemocytometer. Cell viability by this method was routinely greater than 95%. Suspensions were then adjusted to the desired cell density by dilution in growth medium.

Two hundred microlitre aliquots of cell suspension were sub-cultured into 96-well tissue culture plates (Falcon, Becton Dickinson & Co.) for all studies being analysed by a methylene blue dye binding assay. Cells were added to the inner 60 wells of each plate, with medium being added to the outer wells to minimise the effects of evaporation. Cell cultures for flow cytometric analysis or morphological assessment were seeded in 6-well tissue culture plates (Falcon, Becton Dickinson & Co.) at a cell number/surface area ratio proportional to that used in the 96-well plates. Cultures were maintained under the growth conditions described above.

2.2.2 Characterisation of cellular growth

2.2.2.1 Exponential growth assay

The growth patterns of IEC-6 and Mv1Lu cells were characterised to determine whether they were able to sustain exponential growth for the anticipated duration of a survival assay. All cultures prepared for analysis of exponential growth were sub-cultured into 96-well plates. Cultures were seeded at 1000, 3000, 5000 and, for IEC-6 cells only, 10000 cells/well. Growth medium was changed at 24 h and 72 h after seeding. Cultures were harvested daily, at 24 h, 48 h, 72 h, 102 h, 126 h and 150 h post-seeding, for methylene blue analysis.

2.2.2.2 TGFß-mediated growth inhibition assay

TGFß-mediated growth inhibition was measured in both the IEC-6 and Mv1Lu cell lines, with the intention of optimising the anti-proliferative effects of $rTGF\beta_2$. Cell cultures prepared for

2.2 Materials and Methods

2.2.1 Maintenance of epithelial cell lines

Both the IEC-6 and Mv1Lu cell lines were obtained from American Type Culture Collection stocks. IEC-6 cells (CRL 1592) were originally derived from rat small intestine, displaying immunochemical markers characteristic of an undifferentiated crypt cell type (Quaroni *et al.*, 1979). These cells were used between passages 17-28. Mv1Lu cells (CCL 64) were produced from mink lung epithelium and used between passages 50-65. Both cell lines appeared to retain their original morphology and growth characteristics over the range of passages used for these studies.

Stocks of each cell culture were maintained in 75 cm^2 or 175 cm^2 sterile plastic culture flasks at 37°C in an atmosphere of 95% air, 5% CO_2 and 100% humidity. Experimental cell cultures were grown in sterile multi-well tissue culture plates under identical growth conditions. Both cell lines were cultured in DMEM (Trace Biosciences) containing 2 mM L-glutamine, 20 mM HEPES, 100 IU/ml benzylpenicillin and 0.1 mg/ml streptomycin sulfate (all additives from Sigma Chemical Company). In addition, growth medium was supplemented with foetal bovine serum (FBS) (Trace Biosciences) to a final concentration of 10% or 5% for each of the IEC-6 and Mv1Lu cell lines respectively.

Cell lines were routinely passaged when cultures reached 60-80% confluence. Cells were sub-cultured at ratios between 1:4 and 1:19 in fresh growth medium. Cell trypsinisation was performed using PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.5 mM KH_2PO_4, pH 7.4) containing 1.25 mg/ml trypsin (ICN Chemicals) and 0.5 mM EDTA (BDH Chemicals). Culture medium was aspirated from cell monolayers, and replaced with a trypsin/EDTA wash to remove trace FBS. The cell monolayer was then detached at 37°C using fresh trypsin/EDTA before being neutralised with growth medium. Cell suspensions were then sub-cultured for routine passaging or prepared for experimental use.

analysis of growth inhibition were seeded at the optimal cell densities defined from the studies on exponential growth. Cells were cultured in growth medium for the first 24 h then changed to growth medium containing various dilutions of recombinant $TGF\beta_2$ for 30 h. Cultures were then harvested for methylene blue analysis.

2.2.2.3 Methylene blue dye binding assay

Cellular growth was routinely measured by methylene blue estimation of adherent cell number, using the method of Oliver *et al.* (1989). At the end of each assay, the cell culture monolayers were gently washed twice with 0.154 M NaCl to remove trace medium and fixed with 100% methanol. Cells were then covered with 10 mg/ml methylene blue (CI 52015; Sigma Chemical Company) in 0.01 M sodium tetraborate (pH 8.5) for a minimum of 30 min to ensure complete dye binding. Following stringent washing with 0.01 M sodium tetraborate (pH 8.5) the bound dye was eluted with 0.1 M HCl / 100% ethanol (1:1) and the absorbance of each well was read at 630 nm (A_{630}) using an automated plate reader (MR7000; Dynatech Laboratories). Background subtraction was performed using wells which had contained culture medium but no cells. Data was collected and summarised using Biolinx software (Dynatech Laboratories).

In preliminary studies, IEC- 6 and Mv1Lu cells were seeded in 96-well plates at a range of densities for determination of the relationship between A_{630} values and cell number. Cultures were incubated for 24 h, sufficient for cells to adhere to the plastic without undergoing significant replication. As reported by Kelley *et al.* (1992), these two parameters were linearly related.

IEC-6 cells: $A_{630} = 2.11 \times 10^{-5} \times (\text{cell number/well}) + 1.81 \times 10^{-2}$ $(r^2 = 0.886)$

Mv1Lu cells: $A_{630} = 1.617 \times 10^{-5} \times (\text{cell number/well}) - 1.49 \times 10^{-2}$ $(r^2 = 0.939)$

Additionally, the growth inhibition induced by TGFß does not affect this relationship over the concentration range of its biological activity (Absher *et al.,* 1991).

2.2.3 Preparation of chemotherapeutic agents

Methotrexate and vinblastine (David Bull Laboratories) were obtained as a 25 mg/ml solution in water, and a 1 mg/ml solution in 0.154 M NaCl, respectively. Both drugs were stored at 4°C under sterile conditions. Etoposide (Sigma Chemical Company) was dissolved in DMSO to produce a 20 mM stock solution, and stored in aliquots at -20°C.

2.2.4 Analysis of chemotherapy drug toxicity

Drugs were prepared at 11 x final concentration to be added to cultures in 20 µl/well volumes. Cells were routinely cultured for 24 h after seeding, followed by a further 30 h incubation in fresh medium. The cultures were then exposed to one of the chemotherapy agents for 18 h, washed twice with medium and re-cultured for 30 h.

At the end-point of the assay the cultures were washed, fixed and stained for methylene blue analysis. Absorbance measurements were plotted against drug concentration using Table Curve software (Jandel Scientific) and an LD_{50} value was generated from the resultant logistic dose response curve.

2.2.5 Flow cytometric detection of drug-induced apoptosis

Detection of drug-induced apoptosis in Mv1Lu cells was carried out by flow cytometric analysis of Annexin V-FITC binding to everted plasma membranes, based on the method described by Van Engeland *et al.* (1996). Flow cytometric analysis was performed on cells exposed to chemotherapy agents for 18 h, beginning 54 h after sub-culture into 6-well plates. Cultures were harvested either immediately after the 18 h drug exposure, or following a 30 h re-culture period. Adherent cell monolayers were trypsinised and pooled with their respective culture supernatants to provide total cell yields. Cells were sedimented (600 x *g*, 5 min) and briefly stored on ice for transport to the flow cytometer. Cell pellets were then resuspended in 250 µl of binding buffer (140 mM NaCl and 2.5 mM $CaCl_2$ in 10 mM HEPES pH 7.4) containing 5.0 µg/ml propidium iodide (PI; Sigma Chemical Company). At staggered intervals,

cell suspensions were mixed with 250 µl of binding buffer containing 1.0 µg/ml Annexin V-FITC (Bender MedSystems) and incubated on ice for 15 min. Stained samples were then rapidly analysed for annexin V binding (FITC fluorescence) and cell membrane permeability (PI fluorescence). A minimum of 30000 events were collected using a forward scatter threshold to minimise the inclusion of apoptotic bodies and cellular debris.

Cellular fluorescence was detected using an Epics Elite ESP flow cytometer (Coulter Electronics Inc.) fitted with a 15 mW argon laser. FITC emission was detected with a 525 - 550 nm bandpass filter, whilst propidium iodide was detected with a 550 nm longpass filter. Compensation settings were adjusted to eliminate any overlap of fluorescence signals between the FITC and PI channels. Cell parameters were stored as listmode files and analysed using the Elite software.

2.2.6 Flow cytometric detection of cell cycle phase distributions

The residual cell suspensions from analysis of Annexin V-FITC staining were analysed for changes to the normal cell cycle phase distribution induced by drug exposure. Cell suspensions were fixed in 70% ethanol for a minimum of 30 min at 4°C, then sedimented (600 x *g*, 5 min) and resuspended in 500 µl of Tris-HCl buffer (0.1 M Tris HCl, O.1 M NaCl, pH 7.4) containing 1 mg/ml RNase A (Sigma Chemical Company). RNA was degraded by incubating the cell suspension for 30 min at 37°C. Cell suspensions were again sedimented and resuspended in 500 µl of 0.034 M trisodium citrate containing 50 µg/ml PI. Stained cells were re-analysed, within 3 h of preparation, for their relative DNA content (PI fluorescence). A minimum of 12000 events were measured, at a data rate of 50 events/second, to ensure that at least 10000 singlet events were collected. Events were collected using with a low threshold on the PI channel, to exclude the majority of small apoptotic bodies.

Fluorescent staining of cellular DNA Cell parameters were stored as listmode files and analysed using MulticycleAV software (Phoenix Flow Systems). Doublet events were excluded by software subtraction prior to histogram fitting, with levels of remaining

aggregation and debris estimated by the histogram fitting software.

2.2.7 Statistical analysis

Statistical analysis of the data shown in Figure 2.3 and Figure 2.11 was performed by ANOVA. The data included in Figure 2.6 and Figure 2.8 were analysed with a repeated measures ANOVA following an arc-sine transformation of the percentage data. In all instances, where statistical significance was observed by ANOVA ($P < 0.05$) a multiple comparisons Bonferroni/Dunn post-hoc test was applied.

2.3 RESULTS

2.3.1 Identification of candidate cell lines for model development

A list of potential cell lines was derived from the ATCC catalogue, using search terms which restricted the outcome to cells which met the first two selection criteria. Two of these cell lines, a rat intestinal (IEC-6) epithelial cell line and a mink lung (Mv1Lu) epithelial cell, were then chosen for further assessment.

2.3.1.1 Maintenance of exponential growth

Cellular growth was defined by the production of exponential growth curves (Figure 2.2). Cells were cultured as described in Section 2.2.2.1 and harvested at various times up to 150 h after seeding.

IEC-6 cells were optimally cultured at 3000 cells/well (1.5 x 10^4 cells/ml), having left their lag growth phase by 24 h and growing exponentially until 102 h. Cell cultures seeded at a lesser density of 1000 cells/well exhibited a considerable lag time of 48 h but remained in exponential growth until 150 h. In contrast, cultures seeded at the two highest densities, 5000 and 10000 cells/well, appeared to be growing exponentially by 24 h, but entered the plateau phase of their growth cycle too early in the timecourse.

Mv1Lu cells were optimally cultured at a seeding density of 3000 cells/well (1.5 x 10^4 cells/ml). Cell cultures grew exponentially between 24 h and 72 h, then at a reduced rate until 102 h. A similar growth curve was obtained for cultures seeded at 5000 cells/well, however the transition into the plateau growth phase was more rapid. Mv1Lu cells seeded at 1000 cells/well (0.5 x 10^4 cells/ml) displayed greater variation in their rates of growth. Cultures did not reach their maximal rate of growth until 48 h and began to enter the plateau phase of growth after 72 h. Additionally, cells cultured at this density were especially susceptible to growth inhibition by rTGFß$_2$, exhibiting a prolonged lag phase upon release from growth arrest (results not shown).

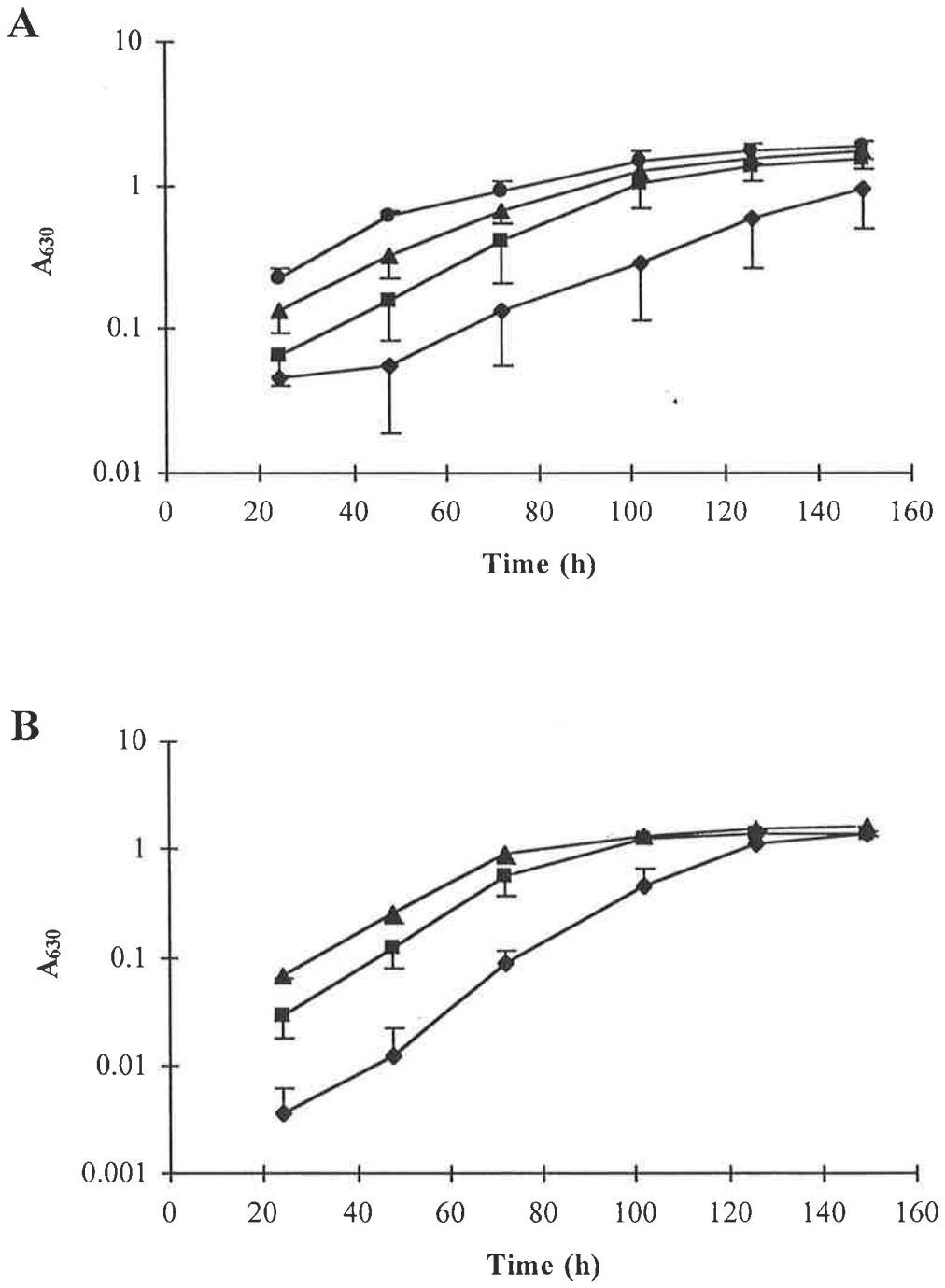


Figure 2.2 Exponential growth of epithelial cell lines

Exponential growth of IEC-6 (**A**) and Mv1Lu (**B**) cell lines, seeded at the following densities:

1000 cells/well (◆)
3000 cells/well (■)
5000 cells/well (▲)
10000 cells/well (●)

Cell number was quantified by methylene blue dye binding, measured at A_{630}. Values are the mean ± SD of three independent experiments (n = 10 replicates/experiment).

2.3.1.2 Dose dependent induction of chemotherapy drug toxicity

Exposure to methotrexate induced a shallow dose response in Mv1Lu cells, with an ED_{50} dose of 0.6716 μM (Table 2.2). The IEC-6 response to methotrexate began at much lower concentrations, with an ED_{50} dose of 0.0313 μM. The IEC-6 response also differed in that there was a very steep gradient between the ED_0 and ED_{100} concentrations, occurring between doses of approximately 0.013 μM and 0.05 μM methotrexate. In contrast, Mv1Lu cells responded to 0.125 μM methotrexate, reaching maximal toxicity with 10 μM methotrexate.

Etoposide exposure induced similar dose-dependent responses in both the IEC-6 and Mv1Lu cell lines, producing ED_{50} concentrations of 0.4001 μM and 0.6964 μM, respectively. Drug sensitivity with both cell lines ranged from a lower limit of 0.1 μM to an ED_{100} dose of 4.0 μM etoposide. Similarly, little difference was observed between the cell lines in response to vinblastine exposure, with ED_{50} values for the IEC-6 and Mv1Lu cultures of 0.0050 μM and 0.0026 μM, respectively. Vinblastine toxicity ranged from an ED_0 of 0.1 nM to an ED_{100} dose of approximately 0.1 μM in both cell lines.

Chemotherapy drug	IEC-6	Mv1Lu
methotrexate	0.0313 ± 0.014 μM	0.6716 ± 0.089 μM
etoposide	0.4001 ± 0.201 μM	0.6964 ± 0.187 μM
vinblastine	0.0050 ± 0.004 μM	0.0026 ± 0.002 μM

Table 2.2 Chemotherapy drug ED_{50} values in IEC-6 and Mv1Lu cells

IEC-6 and Mv1Lu cells were cultured in 96-well plates for 54 h prior to the addition of a range of concentrations of each chemotherapy agent for 18 h. Cultures were washed and re-incubated in drug-free medium for 30 h, then surviving cells were quantified by methylene blue staining of the adherent monolayer. ED_{50} values were calculated as described in "Materials and Methods" and are expressed as the mean ± SD from three independent experiments.

2.3.1.3 Inhibition of cell cycle transit by rTGFß$_2$

Recombinant TGFß$_2$ induced only a minor degree of growth inhibition in the IEC-6 cell line following a 30 h exposure (Figure 2.3). In these cultures, the overall growth inhibitory response was not significant (P = 0.1424). Furthermore, incubation with the highest dose of rTGFß$_2$ (3 ng/ml) resulted in only a 21% reduction in cell number when compared to control cultures.

In contrast, Mv1Lu cell cultures responded to TGFß$_2$ in a more dose-dependent manner. Significant growth inhibition was observed at both 0.3 ng/ml (P = 0.0003) and 3.0 ng/ml (P = 0.0001) concentrations of rTGFß$_2$ when compared to maximally proliferating cultures. In addition, Mv1Lu cells were more sensitive to growth inhibition than IEC-6 cells, falling to 61% of control cell number in the presence of 3 ng/ml rTGFß$_2$.

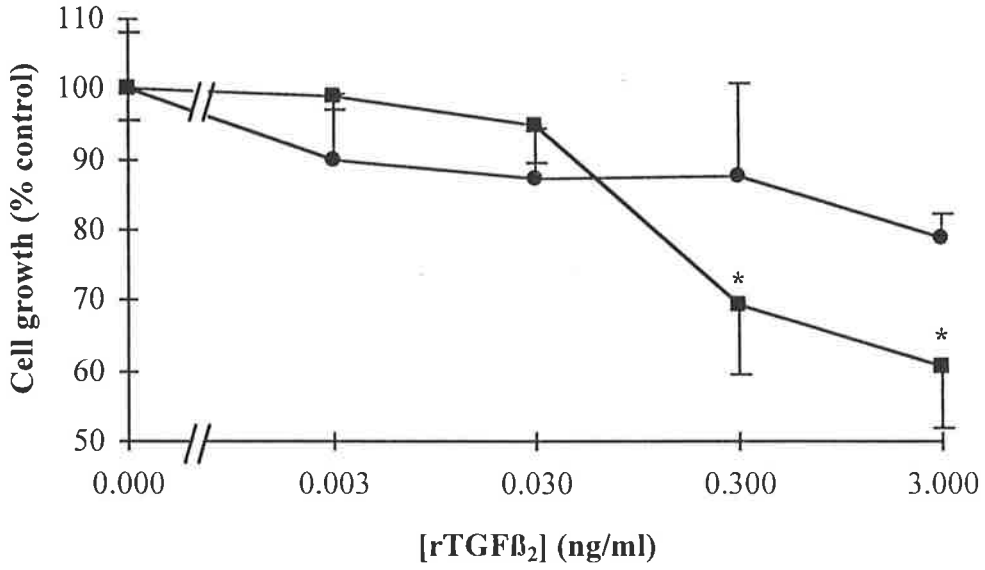


Figure 2.3 TGFß-mediated growth inhibition of epithelial cell lines

TGFß-mediated growth inhibition of IEC-6 (●) and Mv1Lu (■) epithelial cell lines. Cell cultures were incubated with various concentrations of TGFß$_2$ for 30 h prior to quantification of cell number by methylene blue analysis. Values are the mean ± SD of three independent experiments (n = 6 replicates/experiment). *, P < 0.05, compared to 0 ng/ml control.

2.3.1.4 Selection of a cell line for model development

Having addressed the selection criteria listed in Table 2.1 it appeared that the IEC-6 and Mv1Lu cell lines were similar in most respects. Preliminary investigations into the effect of chemotherapy drug exposure on IEC-6 cells indicated that this cell line responded to etoposide or vinblastine exposure in a similar manner to the Mv1Lu cells. However, the IEC-6 cells were much more sensitive to methotrexate-induced toxicity, with an ED_{50} concentration 20-fold less than observed for the Mv1Lu cells. Additionally, the Mv1Lu cell line was considerably more responsive to TGFß-mediated growth inhibition. As TGFß is an active constituent in WGFE, I considered that it was preferable to select the more sensitive Mv1Lu cell line. As there appeared to be no advantage to using the IEC-6 cell line, either instead of or in addition to the Mv1Lu cells, I chose to restrict my assessment of the final selection criteria to the Mv1Lu cell line.

2.3.2 Response of Mv1Lu cells to chemotherapy drug exposure

2.3.2.1 Drug dose response curves

Methotrexate

Methotrexate produced a very shallow dose response curve in Mv1Lu cells, with the highest concentration tested (10 µM) reducing cell number by less than 50% (Figure 2.4 A). The drug had an active concentration range extending from approximately 0.125 µM to at least 10 µM. Drug toxicity was not maximal at this dose, however the response curve had begun to reach a plateau. The ED_{50} concentration for methotrexate was calculated to be 0.6716 ± 0.089 µM.

Etoposide

Etoposide produced a deeper dose response curve than methotrexate, reducing cell number by up to 72% (Figure 2.4 B). Etoposide reduced cell number at concentrations as low as 0.125 µM and reached maximal toxicity between 2 and 4 µM. The ED_{50} dose was calculated to be

0.6964 ± 0.187 μM.

Vinblastine

Vinblastine exposure resulted in the deepest dose response curve of the three drugs tested (Figure 2.4 C). Cell number was reduced to nearly 10% of control levels in the presence of maximally toxic vinblastine doses. Vinblastine was also more toxic than either methotrexate or etoposide when compared on an equimolar basis. Toxicity was detectable at concentrations as low as 0.1 nM, approximately 1000-fold less than the other drugs, and induced maximum cell detachment at less than 0.1 μM. The ED_{50} concentration for vinblastine was calculated to be 0.0026 ± 0.002 μM.

Further investigations into the effect of drug exposure on Mv1Lu cells were restricted to one concentration per drug. These concentrations were chosen with the intention of inducing at least 50% toxicity in the cell population, as assessed from the drug dose response curves shown in Figure 2.4. I elected to use vinblastine at a 100% lethal dose, as the steep gradient of the dose response curve made it difficult to obtain a consistent 50% lethal dose. Therefore, all subsequent experiments in this section relate to studies performed using 10 μM methotrexate, 1 μM etoposide or 0.05 μM vinblastine.

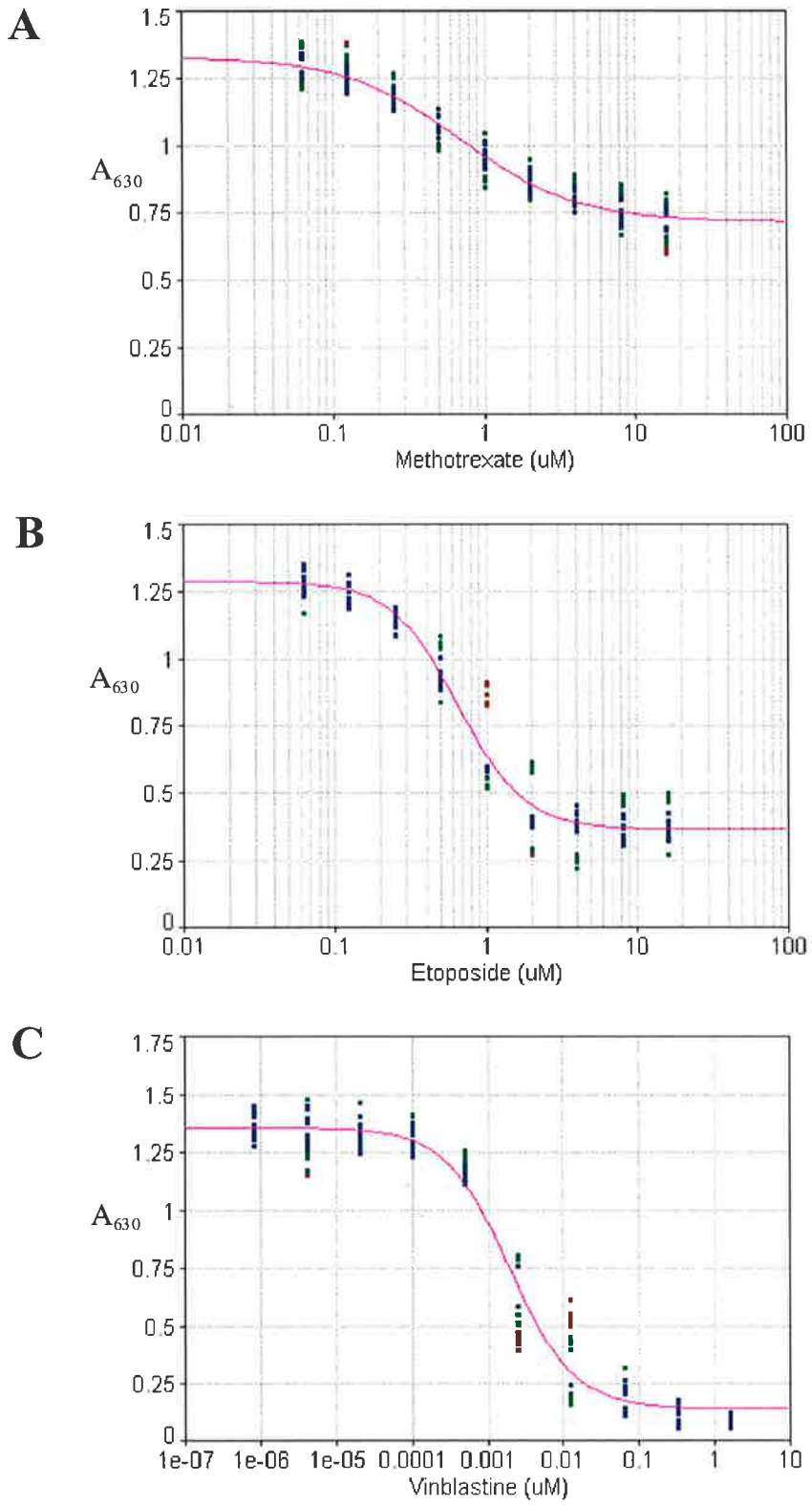


Figure 2.4 Dose-dependent drug toxicity in Mv1Lu cells

Cell cultures were incubated in normal growth medium for 54 h prior to the addition of methotrexate (**A**), etoposide (**B**) or vinblastine (**C**) over a range of concentrations. Drug exposure continued for 18 h, then cells were washed and re-cultured in drug-free medium for a further 30 h. Cell cultures were analysed by methylene blue staining of the adherent monolayer, detected at A_{630}. Each graph depicts the accumulation of data from three independent experiments (n = 6 replicates/experiment).

2.3.2.2 Induction of cell detachment and death

In the absence of chemotherapy drug exposure, a small number of detached cells were detected in the culture supernatant at the time corresponding to the end of the 18 h drug exposure. All of the floating cells appeared viable when assessed by trypan blue exclusion. Following a further 30 h incubation in drug-free medium, no detached cells were found in the supernatant.

Methotrexate

Examination of drug-treated cultures at the end of the 18 h exposure revealed no obvious signs of cell detachment. Surprisingly, however, re-examination of the cultures just prior to harvesting for methylene blue analysis still revealed little evidence of detachment. Instead, exposure to methotrexate seemed to have primarily reduced cell proliferation in the cultures. This observation was confirmed by performing cell counts on culture supernatants collected after exposure to 10 μM methotrexate. At the end of this drug exposure there were no detached cells measured in the culture supernatant. When cell counts were performed again at the end of the assay there was only a slight increase in the total number of detached cells when compared to untreated cultures (Table 2.3). Of the detached cells that were collected, there appeared to be roughly equal numbers of viable and non-viable cells as assessed by trypan blue exclusion.

Etoposide

Etoposide-treated cultures were examined at the end of drug exposure and again at the end of the assay for indications of cell detachment. As illustrated in Table 2.3, there was very little evidence of cell detachment immediately after drug exposure. However, in contrast with methotrexate, etoposide induced a marked detachment of cells from the culture monolayer in the ensuing 30 h re-culture period. After exposure to 1 μM etoposide, trypan blue staining of the detached cell population indicated that approximately 89% of these cells were non-viable.

Vinblastine

Exposure of cells to vinblastine resulted in a similar phenomenon to that observed by Kung *et al.* (1990) with vincristine, whereby cell death was already evident when the drug was removed from the culture medium. Examination of vinblastine-treated cultures directly after the 18 h exposure revealed substantial levels of cell detachment, with further losses occurring during the 30 h re-culture period (Table 2.3). Collection of cell culture supernatants following an 18 h exposure to 0.05 μM vinblastine showed that over 68% of the detached cells remained viable by trypan blue exclusion. When the refreshed culture medium was collected after the additional 30 h incubation, the majority of detached cells (71.9%) were non-viable.

Drug	Detached cells/well after 18 h drug exposure		Detached cells/well after further 30 h re-culture	
drug-free	5000 ± 5000	(100%)	0 ± 0	
10 μM methotrexate	0 ± 0		52000 ± 15300	(42.3%)
1 μM etoposide	3300 ± 5800	(100%)	278000 ± 41900	(11.5%)
0.05 μM vinblastine	423000 ± 69000	(68.6%)	153000 ± 30100	(28.1%)

Table 2.3 Measurement of cell detachment and viability following chemotherapy drug exposure

Mv1Lu cells were seeded in 6-well plates, at 8.775 x 10^4 cells/well, for 54 h prior to the addition of chemotherapy drugs for 18 h. Culture supernatants were collected for analysis immediately after drug exposure, and were replaced with fresh medium whilst the cultures continued for a further 30 h incubation. Supernatants were centrifuged and resuspended in 1 ml volumes for cell counting and assessment of viability by trypan blue exclusion. Values represent the mean ± SD from three replicate wells. Figures in parentheses indicate the mean % viability of the detached cell population.

2.3.2.3 Cell cycle perturbations induced by drug exposure

Cell cycle perturbations were quantified by the deconvolution of histograms which measured the relative DNA content of each cell in the population. These histograms were produced by recording the fluorescent intensity of each cell following re-exposure to an agent such as propidium iodide, which binds to DNA at a stoichiometric ratio (Crissman and Steinkamp, 1987). Fixation of the cell suspension promoted permeabilisation of the plasma membrane, allowing the PI to bind to the cellular DNA rather than act as a viability stain. Thus, cells with normal DNA content (G_1 phase cells) emitted less fluorescence than those with twice the normal DNA content (G_2/M phase cells) or those with an intermediate DNA content (S phase cells).

Figure 2.5 illustrates the effect of exposure to chemotherapeutic drugs on Mv1Lu cell cycle progression, both immediately following an 18 h drug exposure and again following a 30 h re-incubation period in drug-free medium. Overall, exposure to either 50 nM vinblastine or 1 μM etoposide produced significant disruptions to the normal cell cycle distribution ($P = 0.0001$ and $P = 0.0003$, respectively). However, whilst exposure to 10 μM methotrexate altered the normal cell cycle phase distribution, these changes were not significantly different from control-treated cultures ($P = 0.4043$).

Methotrexate

At the end of the 18 h drug incubation, 10 μM methotrexate tended to reduce the number of cells in both G_1 and G_2/M phases, with most of these cells accumulating in the S phase of the cycle (Figure 2.5 A). Quantification of these changes in phase distribution showed that the S phase accounted for 63.3% of the total population in the presence of methotrexate (Figure 2.6). Following a 30 h re-incubation in the absence of methotrexate, the cell population had apparently restored itself towards a normal distribution (Figure 2.5 B). Only 9.9% of methotrexate-treated cells remained in S phase, slightly greater than the 5.0% observed in control cultures (Figure 2.6). Whilst the drug-treated population may have recovered, it was

also possible that the cohort of cells that had previously accumulated in S phase had progressed in a synchronous manner to G_1 phase by the end of the 30 h incubation. Without analysing samples from intermediate time points it was impossible to discriminate between these two possibilities. Regardless, there was certainly no evidence of cell death, which would have manifested as a population with less than G_1 levels of fluorescence intensity.

Etoposide

The presence of 1 μM etoposide induced signs of apoptosis in Mv1Lu cell cultures. A pre-G_1 mass of cells was visible in histograms derived from cultures harvested immediately after the end of the 18 h drug exposure (Figure 2.5 C). These sub-G_1 events comprised 33.6% of the total events measured. The appearance of cells with less than diploid DNA content was attributable to the gradual leaching of DNA fragments from the nucleus of cells in the early stages of apoptosis. In these Mv1Lu cells, it appeared that the culture did not undergo a synchronous induction of apoptosis, as seen in some cell types, but formed a continual progression of healthy cells being degraded to residual debris. Within the population of cells containing between 1 - 2 times diploid DNA content (66.4% of total events measured), there was a marked reduction in G_1 phase cells with a concomitant accumulation in G_2/M phase (Figure 2.6). The G_1 phase fell from 71.6% to 52.4% ($P = 0.0027$), and the S phase from 20.7% to 16.2%, by the end of the 18 h drug exposure. Thirty hours later, the population had begun to return to a normal phase distribution (Figure 2.5 D).

Vinblastine

Exposure to 0.05 μM vinblastine produced the most pronounced cell cycle disturbances of all three drugs tested. At the end of the 18 h drug exposure there was a massive reduction in cells in the G_1 and S phases of the cycle, with many being dispersed to the G_2/M phase (Figure 2.5 E). The G_1 phase fell from 71.6% to 10.4% of the total cell cycle ($P = 0.0001$), whilst the S phase decreased from 20.7% to 7.8% ($P = 0.004$) (Figure 2.6). There were also a large number of dying cells present in the pre-G_1 region of the histogram, although these did not accumulate

into an apoptotic peak. After a further 30 h incubation in the absence of vinblastine, many more cells had lost DNA, and these accounted for a greater proportion of the total cell population (Figure 2.5 F). Of those cells remaining with normal DNA content, there were still significantly fewer G_1 and S phase cells than in drug-free controls ($P = 0.0001$ and $P = 0.0046$, respectively), indicating that the population had not recovered from drug exposure (Figure 2.6).

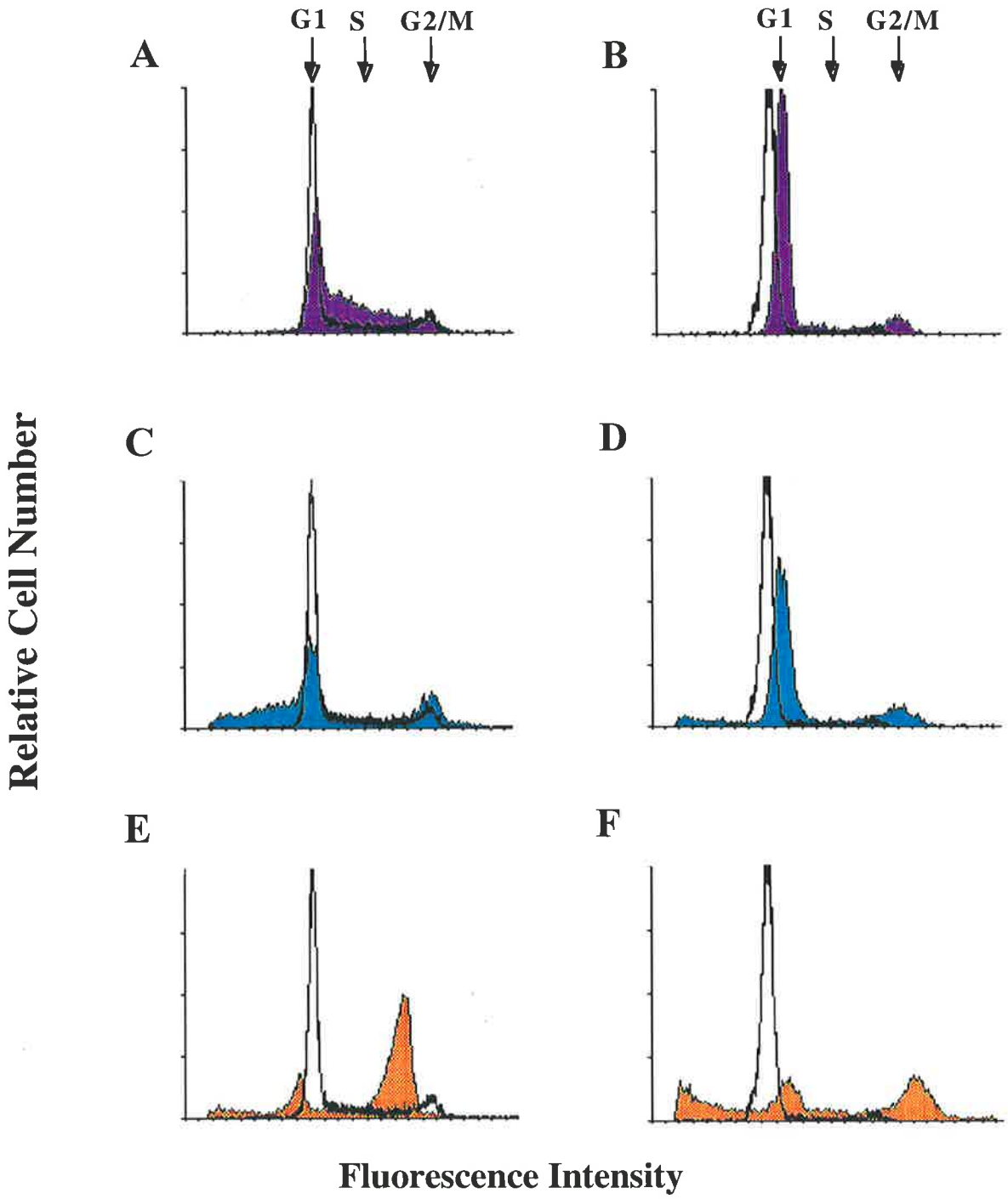


Figure 2.5 Effect of chemotherapy drug exposure on Mv1Lu cell cycle phase distribution

Histograms illustrate Mv1Lu DNA content following an 18 h exposure to 10 μM methotrexate (**A**,**B**), 1 μM etoposide (**C**,**D**) or 0.05 μM vinblastine (**E**,**F**). Cultures were harvested for DNA analysis immediately after drug exposure (**A**,**C**,**E**) or following a 30 h re-culture period (**B**,**D**,**F**). Transparent histogram overlays represent the cell cycle distribution of drug-free control cultures collected at identical times. Histograms are representative of three replicates produced from each of three independent experiments.

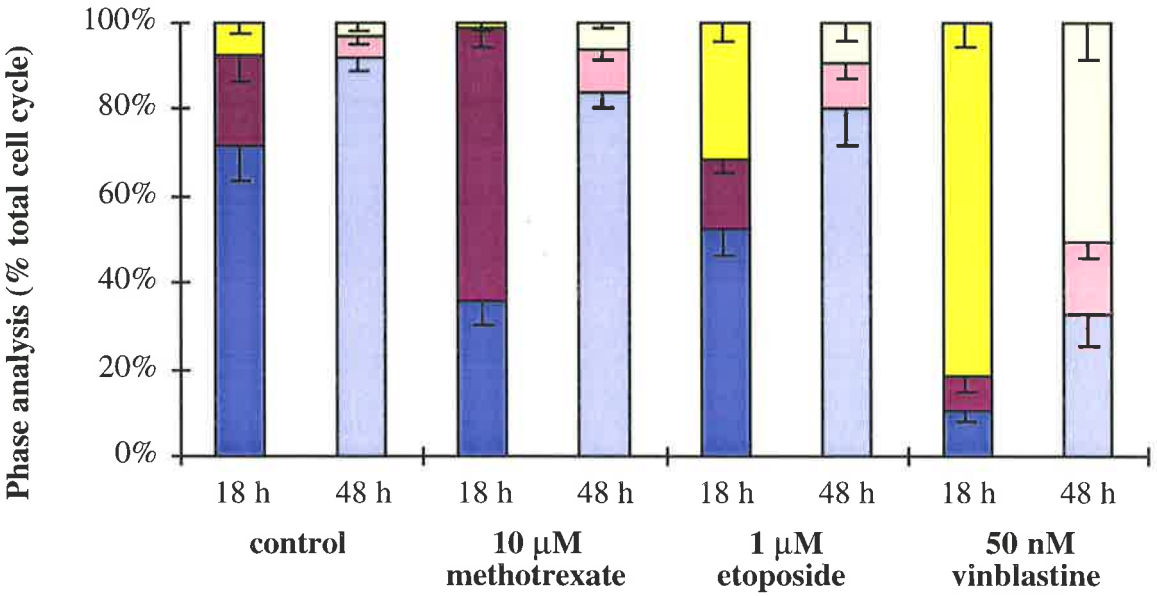


Figure 2.6 Mv1Lu cell cycle phase distribution following chemotherapy drug exposure

Mv1Lu cells were cultured for 18 h in the presence of a chemotherapy agent as indicated above. Cultures were then either harvested (**18 h**) or re-cultured in drug-free medium for a further 30 h before harvesting (**48 h**). Quantification was performed by flow cytometric analysis of cellular DNA content after staining with propidium iodide. DNA histograms were deconvoluted to yield phase distributions for each cell cycle. The total cycling population was comprised of cells in G_1 (■,□), S (■,□) and G_2/M (□,□) phases. Cells with sub-G_1 DNA content were analysed separately. Values represent the mean ± SD from three independent experiments (n = 3 replicates/experiment).

2.3.2.4 Induction of apoptosis following drug exposure

Death induced by many toxic stimuli will result in characteristic changes to the cell, identifiable as either apoptotic or necrotic responses. During the early stages of apoptosis, but not during necrosis, the plasma membrane becomes everted. In some instances, this event precedes both the loss of plasma membrane permeability and chromatin condensation (Martin *et al.,* 1995), whilst in others it occurs shortly after nuclear condensation has begun (Koopman *et al.,* 1994). Annexin V binds to the phosphatidyl-serine residues present on the inner leaflet of the plasma membrane, and can therefore be used as an indicator of the apoptotic status of a cell (Vermes *et al.,* 1995). However, Annexin V can also bind to cells which have lost their membrane integrity, such as those undergoing necrosis, as the molecule can access the phosphatidyl-serine residues via breaches in the plasma membrane. The inclusion of propidium iodide in the staining mixture permits the discrimination of early apoptotic cells from late apoptotic or

necrotic cells (Zamai *et al.,* 1996). Hence, cells staining for neither dye were considered to be viable, those staining only with Annexin V-FITC were early apoptotic, and those binding both dyes were late apoptotic or necrotic. The phenomenon of secondary necrosis arises *in vitro* from ongoing degradation of apoptotic cells in the absence of phagocytic processes, and does not necessarily imply the direct induction of necrosis.

The use of Annexin V-FITC for the detection of apoptosis in adherent cells is complicated by the need to detach the cells from the culture monolayer without disrupting plasma membrane integrity. In contrast to the findings of other authors (Van Engenland *et al.,* 1996), Mv1Lu cells did not accumulate significant membrane damage through the process of trypsinising the adherent monolayer. As seen from the representative scatter plots in Figure 2.7, the majority of the population was located in the lower left (Annexin V^-, PI^-) quadrant after both the 18 h incubation (plot A), and the additional 30 h incubation (plot B). Viable cells accounted for 94.6% and 93.4% of the total population, at the 18 h and 48 h timepoints, respectively (Figure 2.8). Hence, no special precautions were exercised during the collection of adherent cells from the monolayer cultures.

Methotrexate

The representative scatter plots obtained following an 18 h exposure to 10 μM methotrexate were similar to those of the control cells, when examined at both 18 h and 48 h harvest times (Figure 2.7 C and D, respectively). Indeed, quantification of the apoptotic and necrotic cells indicated no overall significant difference between drug-free and methotrexate-treated cultures ($P = 0.1888$). In the 18 h scatter plot a population of cells was observed with an intermediate PI uptake. These cells, which were beginning to lose plasma membrane integrity, were probably in transition towards a state of secondary necrosis. Methotrexate induced a slight decrease in cell viability to 93.2% at the 18 h harvest, and to 88.9% at the 48 h harvest (Figure 2.8). This produced a corresponding minor increase in apoptotic cells (Annexin V^+, PI^-) to 3.2% and 5.5% of the populations present at the 18 h and 48 h timepoints, respectively.

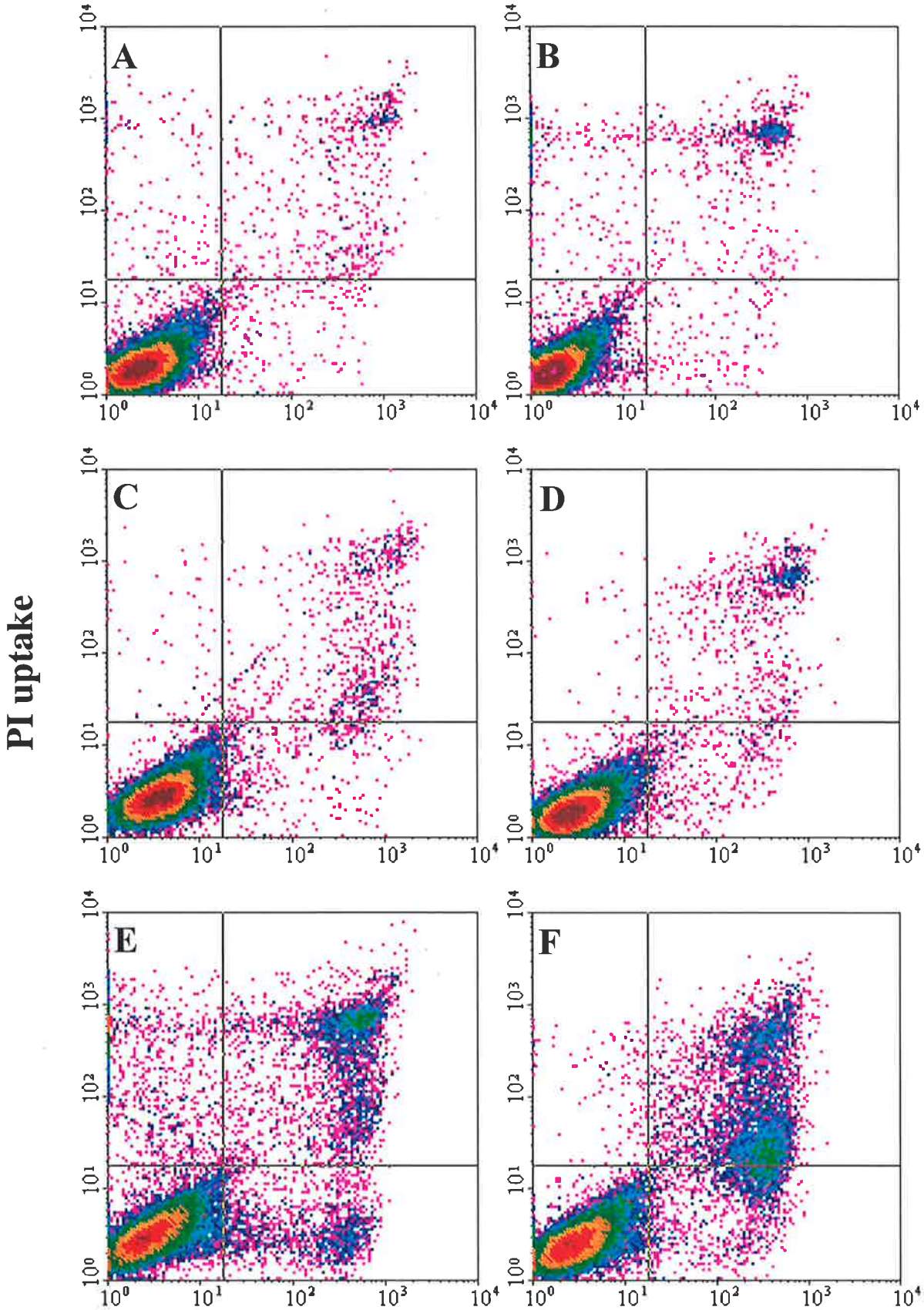
Etoposide

Exposure to 1 μM etoposide for 18 h induced marked changes in the ability of the cell populations to bind Annexin V and incorporate PI, both directly after drug exposure (Figure 2.7 E) and following a 30 h incubation in drug-free medium (Figure 2.7 F). Overall, these changes were significantly different from drug-free values (P = 0.0008). The apoptotic cells accounted for 8.4% of the culture when harvested immediately following drug exposure, and 9.0% when harvested 30 h after drug removal (Figure 2.8). These values suggested that either no additional cells were entering or leaving the apoptotic region, or that there was a steady state progression through the apoptotic quadrant. The scatter plots show that it was a dynamic transition of cells from the viable region through to the late apoptotic/necrotic region, as viable cell number decreased over time. The viable population fell from 79.1% to 66.5%, as cells moved through the apoptotic region to accumulate in the necrotic (Annexin V^+, PI^+) quadrant. The percentage of apoptotic cells was significantly greater in etoposide-treated cultures than in the respective control populations at both the 18 h (P = 0.0016) and 48 h (P = 0.005) harvest times. The late apoptotic/necrotic population also accounted for significantly more cells directly following etoposide exposure (P = 0.0005) and also after re-incubation in drug-free medium (P = 0.0001).

Vinblastine

In cultures treated with 50 nM vinblastine, the overall appearance of apoptotic and necrotic cells was greater than observed in control cultures (P = 0.0002). Levels of apoptosis were similar to those of etoposide treated cultures, with values of 9.5% and 11.9% at the 18 h and 48 h harvest times, respectively (Figure 2.8). Again, this was due to a reduction in cell viability from 83.0% to 42.1% over time, with an increase in late apoptotic and necrotic cells from 6.9% to 44.1% of the population. The representative scatter plots show the early accumulation of cells in the apoptotic (Annexin V^+, PI^-) quadrant (Figure 2.7 G) and their subsequent aggregation within the late apoptotic/necrotic quadrant (Figure 2.7 H). The percentage of apoptotic cells in vinblastine-treated cultures was greater than in control cultures at the end of drug exposure (P =

0.0017) and after the 30 h re-culture period (P = 0.0002). Similarly, there was an increase in the numbers of necrotic cells in the presence of vinblastine, at both the end of drug exposure (P = 0.0286) and after re-culturing cells for 30 h (P = 0.0001).


A
B
C
D
E
F
PI uptake
Annexin V binding

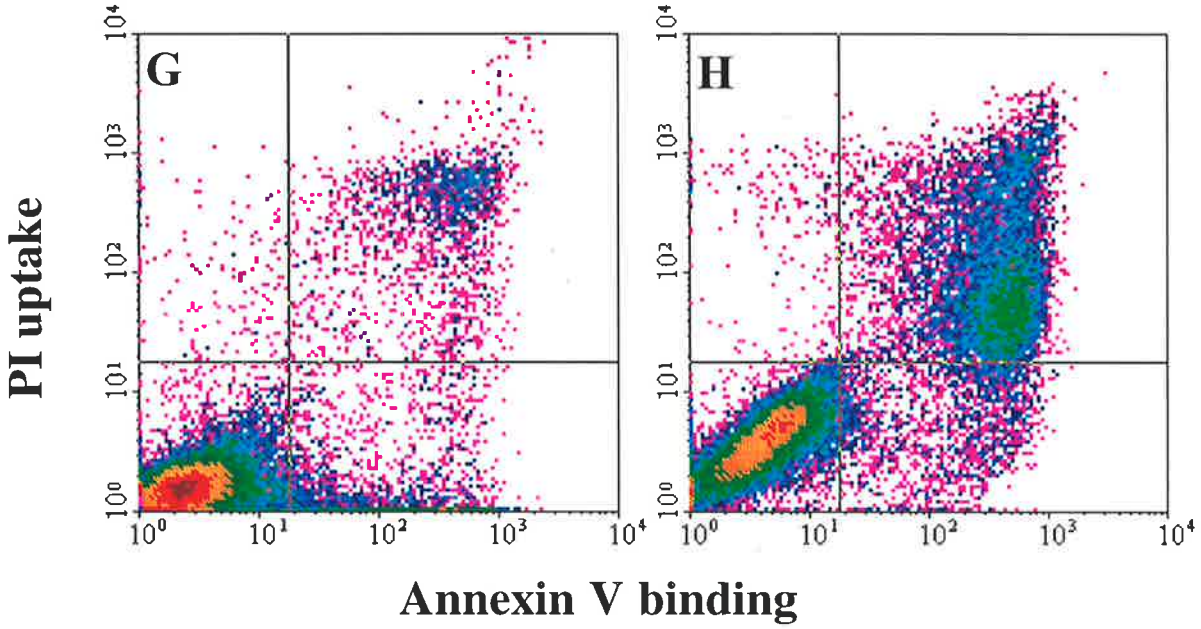


Figure 2.7 Effect of chemotherapy drug exposure on the induction of apoptotic and necrotic markers in Mv1Lu cells

Scatter plots of PI uptake versus Annexin V binding in Mv1Lu cells grown in the presence of control medium (**A,B**), 10 μM methotrexate (**C,D**), 1 μM etoposide (**E,F**) or 50 nM vinblastine (**G,H**) for 18 h. Cultures were harvested immediately (**A,C,E,G**) or re-cultured in drug-free medium for a further 30 h prior to harvesting (**B,D,F,H**). Populations were identified as viable (Annexin V^-, PI^-), apoptotic (Annexin V^+, PI^-) or late apoptotic/necrotic (Annexin V^+, PI^+). Plots are representative of three replicates from each of three independent experiments.

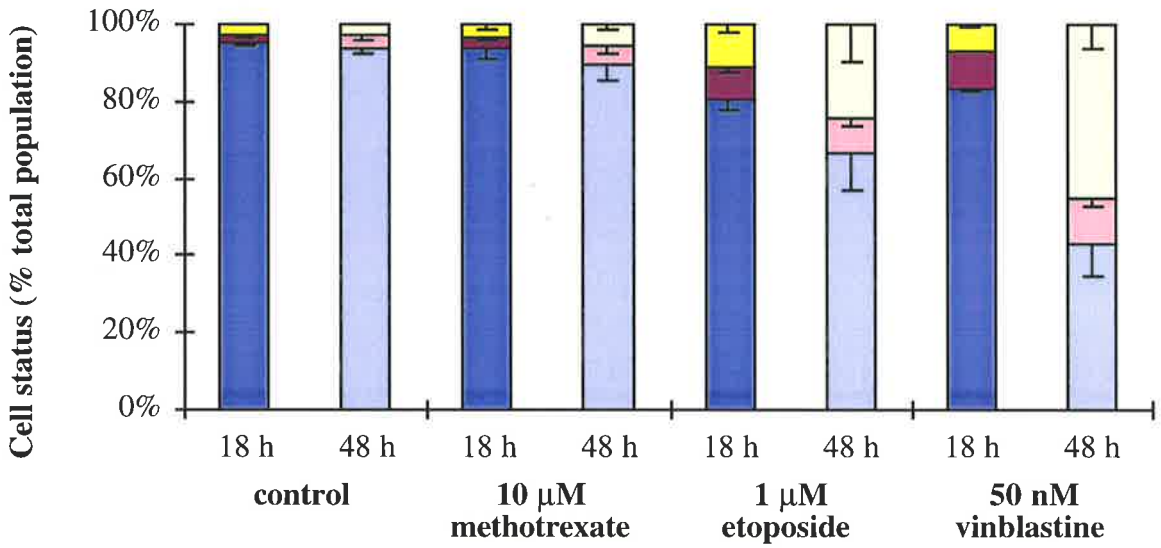


Figure 2.8 Induction of apoptosis and necrosis in Mv1Lu cells following chemotherapy drug exposure

Mv1Lu cells were cultured in the presence of a chemotherapy agent, as indicated above, for 18 h. Cultures were then harvested (18 h) or re-cultured in drug-free medium for a further 30 h before harvesting (48 h). The total population was comprised of viable (■,□), apoptotic (■,□) and late apoptotic/necrotic (■,□) cell sub-populations. Values represent the mean ± SD of measurements from three independent experiments (n = 3 replicates/experiment).

2.3.2.5 Selection of chemotherapy drugs for further studies

Methotrexate failed to induce any significant signs of cell damage, including the induction of apoptosis, detachment from the culture monolayer or a reduction in cell viability. Exposure to 10 μM methotrexate did induce a transient block in progression of cells through the S phase of the cell cycle, but this did not lead to quantifiable drug toxicity within the time constraints of the assay. It is possible that cell damage may have become evident with an extended incubation, however I was restricted to a maximum re-incubation of 30 h following drug removal by the exit of control populations from exponential growth. Thus, I chose to exclude methotrexate from further survival assay developments. Both etoposide and vinblastine had proven effective in inducing drug toxicity and, therefore, investigations with these drugs continued.

2.3.3 Validation of the experimental model

2.3.3.1 Experimental design

The final experimental design chosen for the *in vitro* model was a result of the accumulated findings reported previously in this chapter. The established protocol is illustrated in Figure 2.9, and is based upon the use of the Mv1Lu cell line, seeded at a density of 3000 cells/well in 96-well microtitre plates. The cells were incubated for 24 h to ensure that they had adhered to the plastic wells and entered a state of exponential growth. Cultures were then pre-treated with growth inhibitory agents for 30 h, providing all cells with the opportunity to progress through the cell cycle to the temporal point of growth arrest. This was followed by the addition of chemotherapeutic agents for an 18 h incubation, sufficient to ensure that the majority of cells would have cycled through the phases specifically targeted by the chemotherapy agents. At the time of drug addition, a quality control plate was harvested to measure cell numbers present with each control or growth inhibitory treatment. After continuing cultures were incubated in the presence of both drugs and growth inhibitory treatments for 18 h, the cells were washed and

re-incubated in normal growth medium to allow time for the dying cells to detach from the culture monolayers. Cultures were then ultimately harvested 30 h later, and analysed for cell survival by methylene blue staining of the remaining adherent cells.

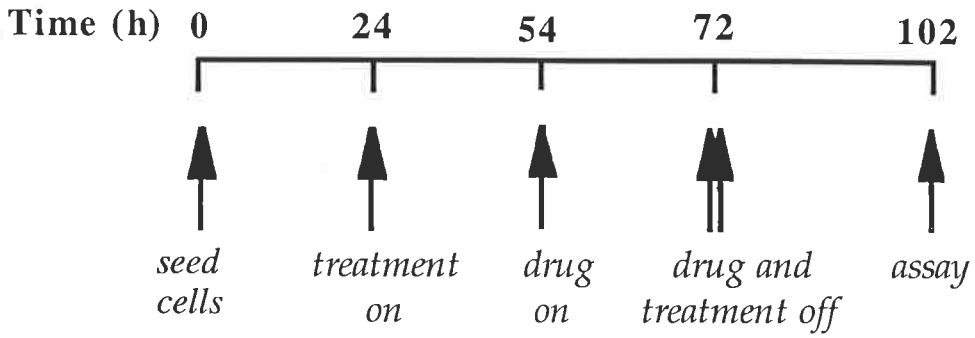


Figure 2.9 Time-course of the pre/concurrent survival assay protocol

2.3.3.2 Culture density-dependence of drug toxicity

Mv1Lu cells became increasingly susceptible to both etoposide- and vinblastine-mediated toxicity in inverse proportion to the number of cells present in the culture at the beginning of drug exposure (Figure 2.10). In cultures exposed to 1 μM etoposide, culture density bore an inverse relationship to cell death up to an A_{630} of approximately 1.10 absorbance units (Figure 2.10 A). Cell death was similarly related to culture density following 50 nM vinblastine exposure (Figure 2.10 B).

For Mv1Lu cells routinely seeded at 1.5 x 10^4 cells/ml, I had previously observed a maximum A_{630} of 0.45 absorbance units in exponentially growing cultures, and a minimum A_{630} of 0.20 absorbance units in maximally growth arrested cultures. Interpolation of the predicted cell loss arising from these extremes of culture density provided an indication of the range of drug toxicities attributable to density effects. Etoposide-mediated cell death could be expected to range from approximately 53% in proliferating cultures, up to 65% in growth inhibited cultures. The differences in percentage cell death expected between cycling and inhibited cultures was less marked following vinblastine exposure, ranging from 85% to 91% cell loss. These

differences in drug toxicity meant that direct comparisons of observed cell survival between control and growth-arrested cultures would be biased towards underestimating the survival mediated by growth inhibitory agents.

In order to compensate for the differences in Mv1Lu culture density apparent at the time of drug exposure, I explored the possibility of seeding cultures at various densities with the expectation that they would equalise. Thus, cultures to be exposed to 3 ng/ml rTGFß$_2$ were seeded at an increased density of 2.5 x 10^4 cells/ml, whilst control cultures were seeded at the established density of 1.5 x 10^4 cells/ml. Such a differential seeding strategy did not produce consistently equal culture densities at the time-point required. Furthermore, it could not be adapted for the examination of intermediate doses of rTGFß$_2$ without dramatically increasing the complexity of the assay.

Instead, I opted to impose quality control limits on the acceptance of each survival assay. All assays performed in this research project included an extra plate to be harvested at the same time as the chemotherapy agents were added to the other plates. This quality control plate contained cultures grown in normal medium as well as any treatments being included in that particular assay. This plate was stained with methylene blue and read at A_{630}, with all values required to fall between 0.2 and 0.45 absorbance units. This ensured that the maximum discrepancy in the estimation of cell survival, when comparing between control and growth arrested cultures, would be 12% for etoposide and 6% for vinblastine.

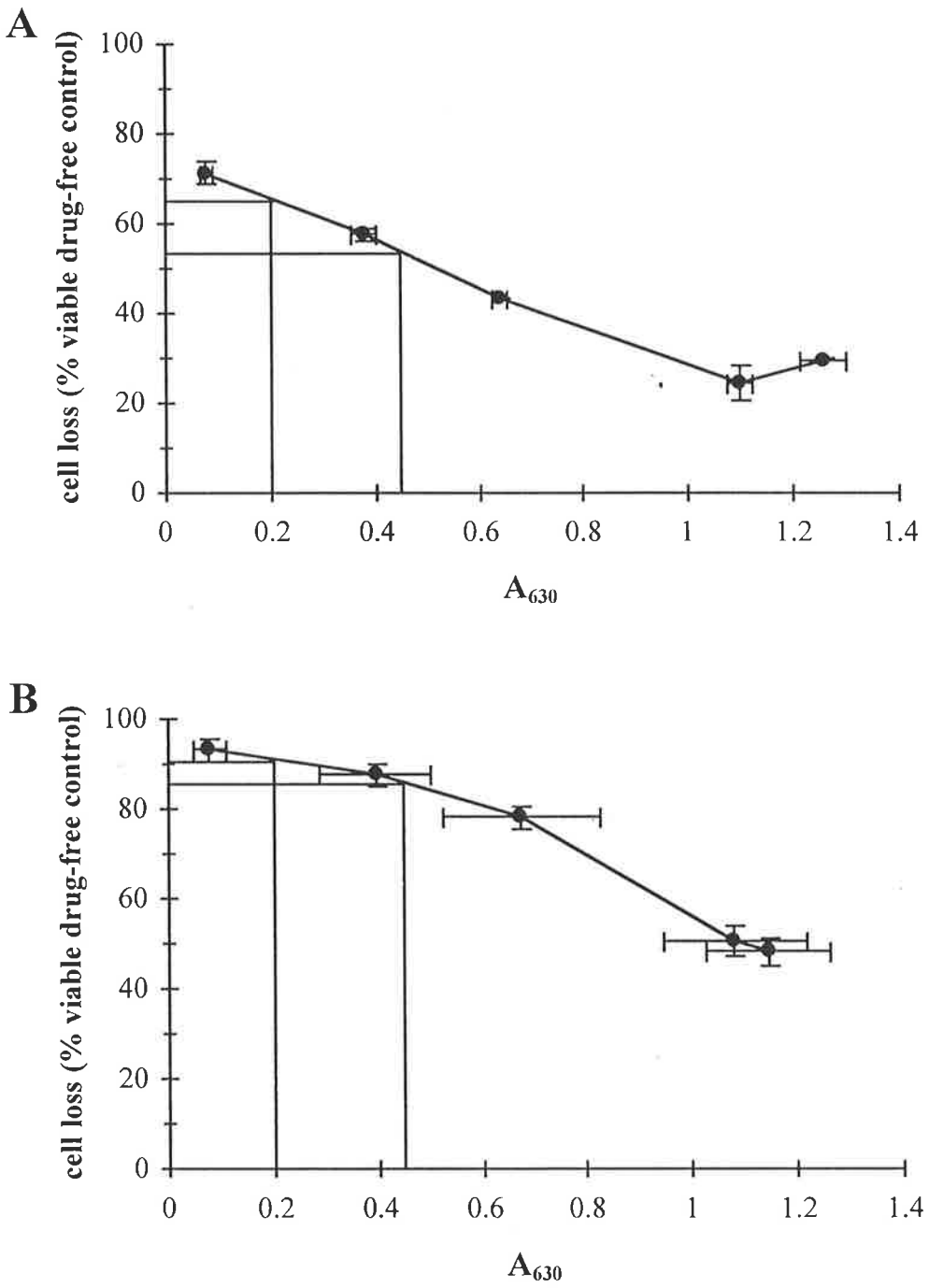


Figure 2.10 Relationship between culture density and drug toxicity

Mv1Lu cells were seeded at various densities and incubated for 54 h before exposure to 1 μM etoposide (**A**) or 50 nM vinblastine (**B**) for 18 h. Culture density at the beginning of drug exposure was quantified by methylene staining of the adherent monolayer (abscissa) and plotted against the percentage cell death observed 30 h after removal of the drug (ordinate). Values represent the mean ± SD of three independent experiments (n = 6 replicates/experiment). Solid lines indicate the range of predicted drug toxicities arising from the extremes of culture density, produced in growth arrested and proliferating cultures of Mv1Lu cells seeded at 1.5 x 10^4 cells/ml.

2.3.3.3 TGFß-mediated protection against chemotherapy drug toxicity

Figure 2.11 illustrates the number of cells remaining on the adherent monolayer following pre/concurrent treatment with $rTGFß_2$ and exposure to etoposide or vinblastine. In the absence of either chemotherapy agent, the growth inhibition induced by $rTGFß_2$ had been overcome and the population had begun proliferating again, as indicated by the increased A_{630} of these cultures when compared to the quality control (54 h) plate. However, the number of cells in the TGFß-treated cultures still lagged behind those in the control cultures.

The presence of 1 µM etoposide induced a 43.9% reduction in control-treated cultures to an A_{630} of 0.74 absorbance units, leaving 56.1% of the population on the monolayer at the end of the assay (Figure 2.11 A). However, cultures treated with $rTGFß_2$ fell by only 27.7% in response to drug exposure, when compared to the $rTGFß_2$-treated drug-free control. Therefore, of the total number of cells present following $rTGFß_2$ treatment, 72.3% remained adherent after etoposide exposure, greater than the proportion remaining in the control-treated cultures.

Exposure to 50 nM vinblastine reduced the final A_{630} of the control-treated cultures from 1.32 units to 0.20 units, leaving only 14.9% of the population on the adherent monolayer at the end of the experiment (Figure 2.11 B). In contrast, 45.1% of the $rTGFß_2$-treated population remained adherent 30 h after removal of vinblastine from the culture medium. As with etoposide exposure, this was a better outcome than observed with the control-treated cultures.

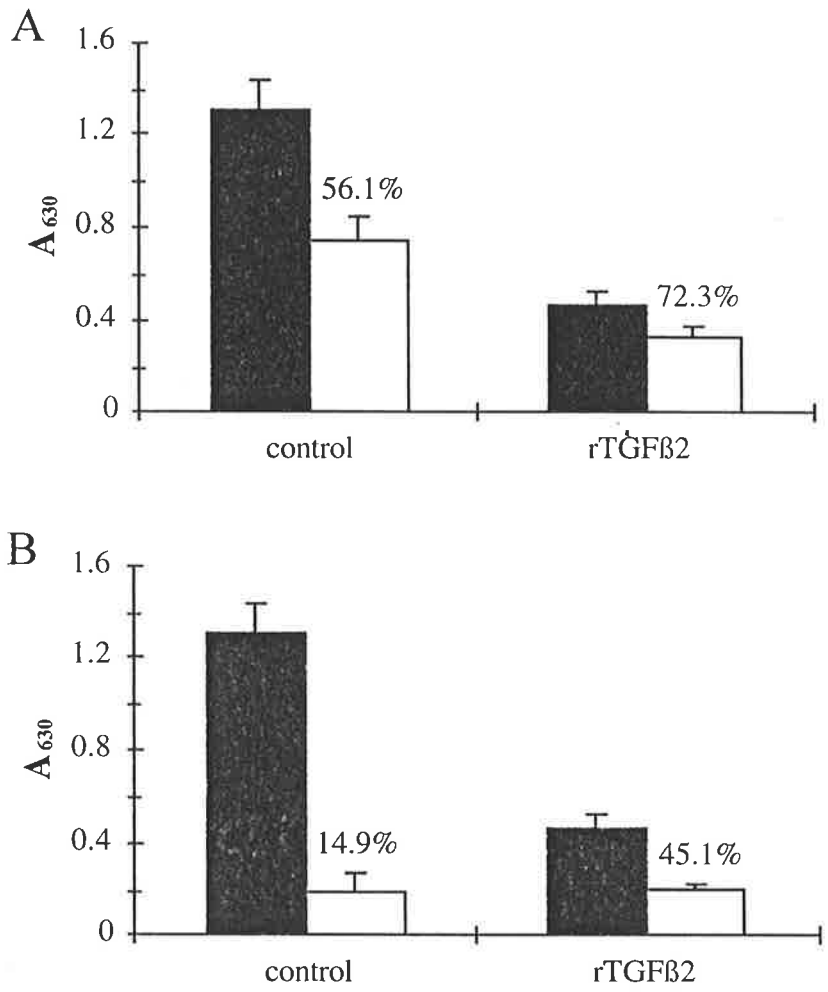


Figure 2.11 Cell survival mediated by 3 ng/ml rTGFß$_2$

Mv1Lu cultures were pre-treated with 3 ng/ml rTGFß$_2$ for 30 h, prior to the addition of 1 µM etoposide (**A**) or 50 nM vinblastine (**B**) for a further 18 h. Surviving cell numbers were quantified by methylene blue analysis (A_{630}), 30 h after removing both rTGFß$_2$ and drug from the culture medium. Bars represent drug-free (■) and drug-treated (□) cultures. Values are the mean ± SD from three independent experiments (n = 6 replicates/experiment). Percentage values indicate cell survival within a treatment group following drug exposure.

2.4 Discussion

This chapter established the necessary foundations for the development of an *in vitro* model of drug toxicity. Development of this model relied upon a detailed assessment of cell growth in two candidate cell lines, under both normal and growth inhibitory conditions. Both the IEC-6 and Mv1Lu cell lines have been reported to synthesise and secrete TGFß-like material under confluent growth conditions (Barnard *et al.*, 1989; Danielpour *et al.*, 1989), although neither study discriminated between latent and active forms of the putative TGFß proteins. Therefore, culture conditions were established to minimise the potential for autocrine growth inhibition of the exponentially growing cultures as they reached confluence, and thus avoid the complication of endogenous survival activity. IEC-6 cells also synthesise functional IGF binding protein 2 in serum-depleted medium (Park *et al.*, 1992) and in response to $TGF\beta_1$ (Guo *et al.*, 1995b), which could mask any potential survival activity of the IGF-I and IGF-II present in the WGFE. Therefore, the selection of the Mv1Lu cell line, with a well established and extremely sensitive inhibitory response to TGFß, provided a more functional basis upon which to examine the effects of WGFE preparations, all of which contain biologically active concentrations of TGFß.

The lack of extensive methotrexate toxicity observed in Mv1Lu epithelial cells may have been caused by repletion of folate levels upon drug removal. Washing methotrexate from the culture medium would result in a rapid shift in equilibrium between free drug and enzyme-bound drug, effectively restoring dihydrofolate reductase activity (Pinedo and Chabner, 1977). An 18 h incubation with methotrexate was sufficient to reduce the existing pools of purine and pyrimidine nucleotides, and possibly even to delay the repair of damaged or mismatched DNA, as an accumulation of cells in S phase was observed at the end of the drug exposure. However, the 18 h drug incubation may not have been long enough to induce apoptosis in these cells, although it is adequate for the induction of DNA fragmentation in other cell lines (Barry *et al.*, 1990), and for the induction of death in the extremely sensitive IEC-6 cells. The presence of 5% serum during methotrexate exposure may also have contributed to Mv1Lu cell survival (Pinedo and Chabner, 1977). Alternatively, the Mv1Lu cultures may have accumulated lethal damage

following methotrexate exposure and simply experienced a drug-induced arrest in G_1 phase, as is suggested by the cell cycle analysis performed at the end of the survival assay (48 h). If these cells were in fact arrested in G_1, either attempting to repair damaged DNA or in the process of initiating cell death, a greater accumulation of apoptotic cells may have become evident with a longer incubation following drug removal. However, there was not enough flexibility in the assay protocol to extend the re-incubation period following drug exposure to determine if this was the case.

The dramatic shift between ineffective and maximally toxic concentrations of methotrexate in the IEC-6 cells suggests that more immediate functional requirements were compromised before DNA synthesis was disrupted in this cell line. This is corroborated by other studies, which indicate that concentrations of methotrexate approximating 1 μM are necessary for inhibiting DNA synthesis *in vitro* (Hryniuk, 1972; Bruce-Gregorios *et al.,* 1991). The lower concentrations at which methotrexate induced cell death in the IEC-6 cell line may be associated with the small pools of endogenous nucleotides contained within these cells, rendering them extremely susceptible to fluctuations in nucleotide supply (Sanderson and He, 1994).

Of the other chemotherapy agents tested, both etoposide and vinblastine appeared to induce cell death in Mv1Lu cells. Death was initiated through an apoptotic pathway, detected as an increased inversion of the asymmetric plasma membrane in apoptotic cells. This mode of cell death was consistent with that observed in crypt cells *in vivo* following administration of methotrexate (Ijiri and Potten, 1987), and therefore posed no particular obstacle to the use of different cytotoxic agents in the development of an *in vitro* model. In fact, the possibility that folate binding proteins would accumulate in the WGFE, by virtue of their basic isoelectric points, argues against the use of methotrexate as a representative drug in this assay. In milk, the majority of folate is complexed to binding proteins, and is present predominantly as 5-methyl tetrahydrofolate (Wagner, 1985). Milk-borne tetrahydrofolate may dissociate from carrier proteins or folate binding proteins at the intestinal cell surface, reducing methotrexate toxicity by introducing an exogenous source of reduced folate (Izak *et al.,* 1972). Therefore, to examine

WGFE-mediated survival I chose to exclude methotrexate from the assay protocols, as I would not have been able to distinguish between folate-related and general survival responses.

Verification of the *in vitro* model yielded similar findings to those of other authors who have employed growth inhibitory peptides to protect against drug toxicity. McCormack *et al.* (1997) and Weyman and Stacey (1996) have reported enhanced cell survival following chemotherapeutic drug exposure, with rTGFß$_3$ and rTGFß$_1$, respectively. However, TGFß$_2$ initiates growth inhibitory signals through a different receptor complex than either of the other two isoforms. TGFß$_2$ requires the presence of the 280 kDa type III TGFß receptor, termed betaglycan, to facilitate binding to the type II TGFß receptor (Rodriguez *et al.,* 1995). In the absence of the type III receptor, TGFß$_2$ is significantly less effective in mediating growth inhibition (Cheifetz *et al.,* 1990). Whilst the anti-proliferative activity of rTGFß$_2$ has previously been measured in Mv1Lu cells there has been no direct link established between growth inhibition and cell survival. Thus, I needed to ensure that rTGFß$_2$ was able to mediate survival in Mv1Lu cells in addition to inducing growth inhibition.

Studies with etoposide showed that treatment of cultures with rTGFß$_2$ increased the proportion of cells surviving drug exposure, but produced an absolute decrease in the methylene blue staining intensity of cultures. With vinblastine exposure, rTGFß$_2$ improved the percentage survival of the population, but had no significant effect on absolute cell number as determined by methylene blue analysis. The increases in percent survival observed with rTGFß$_2$-mediated protection did not take into account the higher predicted toxicity arising from differences in culture density at the time of drug exposure. Thus, the cell fraction protected from drug toxicity by rTGFß$_2$ treatment would actually have been greater than estimated from the control levels of toxicity.

Chapter Three continues to employ the Mv1Lu cell model established in this chapter to characterise the ability of WGFE preparations to mediate protection against etoposide and vinblastine toxicity.

Chapter Three

WGFE-mediated survival in vitro

3. *WGFE-mediated survival in vitro*

Contribution to the work

Tracer IGF-I for the acid gel filtration was kindly prepared by Dr G Robertson, and for the RIA by Dr V Dunaiski. All whey samples were prepared by Bonlac Foods Ltd., Melbourne, Victoria. Whey growth factor extract starting material was produced by CSIRO, Division of Food Science and Technology, Highett, Victoria and by Bonlac Foods Ltd., Australia. The material was further processed by Mr Q Doan to produce the four fractions used for these studies. All other work described in this chapter was performed by myself.

Abstract

Cheese whey contains a mixture of growth factors, some of which are known to mediate protection against chemotherapy drug-induced drug toxicity. This study was designed to evaluate the cytoprotective potential of a whey growth factor extract (WGFE) and extracts modified to enhance particular constituent growth factors. Dose-dependent growth inhibition of Mv1Lu cells was potentiated by transient acidification of the WGFE, which activated latent TGFß present in the material. Separation of this acidified WGFE by filtration through a porous membrane subsequently produced an IGF-enriched permeate fraction and a TGFß-enriched retentate fraction. Each of these WGFE preparations was added to Mv1Lu cultures at a concentration which induced growth inhibition equivalent to that mediated by 3 ng/ml rTGFß$_2$. When added as a pre/concurrent treatment, beginning 30 h prior to the addition of 1 µM etoposide or 50 nM vinblastine, all of the treatments mediated significant increases in cell survival except for the initial WGFE material. In contrast, none of the WGFE fractions was able to stimulate enhanced regeneration of surviving cells when added immediately following drug exposure. Further analysis of the pre/concurrent treatment protocol indicated that the

TGFß and WGFE treatments protected Mv1Lu cells at etoposide concentrations which induced at least 50% toxicity, with a significant increase in protection detected at the highest dose tested (2μM). Similarly, all of the WGFE preparations significantly enhanced cell survival when cultures were exposed to vinblastine concentrations ranging from 2 nM to 50 nM. Serial dilution of each of the WGFE preparations indicated that the acid, permeate and retentate fractions behaved in a TGFß-like manner in mediating protection against drug toxicity. However, the initial WGFE material sustained cell survival at concentrations which did not induce maximal growth inhibition, and at which an equivalent growth inhibitory concentration of $rTGF\beta_2$ had no protective effect. These results suggest that the WGFE starting material contains factors in addition to TGFß which contribute to the protection of Mv1Lu cells against drug toxicity when added as a pre/concurrent treatment.

3.1 Introduction

The previous chapter described the characterisation of an *in vitro* model which could be used to measure TGFß-mediated protection against drug toxicity. This chapter describes experiments in which the model was applied to examine the survival-promoting potential of a whey-derived growth factor extract (WGFE). WGFE contains low levels of biologically active $TGFß_1$ and $TGFß_2$, as well as larger quantities of the latent TGFß complex (Rogers *et al.,* 1996). Recombinant $TGFß_1$ has previously been shown to protect against chemotherapeutic drug toxicity in both epithelial cell culture models and animal models (Grzegorzewski *et al.,* 1994; Weyman and Stacey, 1996). As I have shown that $rTGFß_2$ also possesses cytoprotective properties, I hypothesised that WGFE would mediate a similar protective effect to TGFß, and that the degree of survival would depend upon the extent of the growth inhibition induced by the WGFE preparation.

Of the growth factors known to be present in WGFE, TGFß was expected to mediate cytoprotection. By increasing the concentration of active TGFß, relative to the other WGFE proteins, I believed that I would enhance both the growth inhibitory activity and the survival activity of the WGFE material. Activation of the latent TGFß complexes was achieved by acidification of the WGFE, increasing the specific activity of TGFß when compared to other component growth factors (Rogers *et al.,* 1996). Whilst increased TGFß activity may enhance cytoprotective effects, it would most likely be detrimental to the application of WGFE as a post-drug recovery treatment. In this scenario, mitogenic growth factors such as the insulin-like growth factors (IGFs) would be more likely to enhance the regeneration of naturally resistant cells. To this end, it was desirable to modify the WGFE preparations by depletion of growth inhibitory peptides whilst concomitantly concentrating IGF peptides, such that the preparation would have greater potency following the separation of IGF from complexed IGF binding proteins.

In an attempt to produce sub-fractions of WGFE with enhanced potency, the acidified WGFE

was further modified by filtration through a size exclusion membrane, producing permeate and retentate fractions. The permeate WGFE was enriched in IGF peptides but was surprisingly relatively depleted of TGFß activity, whilst the retentate WGFE contained TGFß but only small quantities of the IGFs. This allowed me to compare the effects of TGFß-enriched and IGF-enriched fractions in a cytoprotective assay.

In this chapter I have defined the anti-proliferative activity of each of these WGFE fractions by standardisation against $rTGF\beta_2$ in a Mv1Lu growth inhibition bioassay. The WGFE fractions were then compared for their relative efficacy in mediating cell survival against drug toxicity using concentrations which produced equivalent growth inhibitory activity to $rTGF\beta_2$. The fractions were also compared for their ability to stimulate cell regeneration when added to cultures following drug exposure. Survival responses were then further defined by the use of a range of drug concentrations, and a range of WGFE dilutions at a single drug concentration.

3.2 Materials and Methods

3.2.1 Preparation of whey and WGFE

The production of WGFE from cheese whey was performed using a modification of the method developed by Francis *et al.* (1995). Bovine cheese whey was obtained as liquid effluent following the coagulation of milk to produce cheese. The whey was pasteurised (15 s at 72°C), delipidated by microfiltration through a 1.4 µm pore size membrane and stored at -20°C until use.

Whey samples were loaded onto a Sepharose Fast Flow-S cation exchange column (Pharmacia) equilibrated in 50 mM sodium citrate (pH 6.5). The majority of the whey proteins (approximately 98%) were collected in the unbound fraction. However the whey-borne growth factors remained bound to the column, by virtue of their basic isoelectric points, and were eluted with 500 mM NaCl to produce the primary whey-derived growth factor extract. The WGFE was concentrated against a 3 kDa membrane, diafiltered to an isotonic salt concentration (150 mM NaCl) and 0.22 µm filter sterilised to produce the final WGFE product. The WGFE was then kept at 4°C for additional processing, or aliquotted and stored at -20°C.

The initial WGFE material (*control WGFE*) was further modified into three additional fractions with different growth factor compositions, first by acidification, then by size exclusion. One litre of control WGFE was acidified to pH 2.5 with 10 M HCl, and stored at 4°C overnight. A 50 ml aliquot was re-neutralised to pH 6.5 with 200 µl of 10 M NaOH, and stored overnight prior to centrifugation at 15000 x *g* for 60 min at 4°C. The supernatant (*acid WGFE*) was pre-filtered (0.45 µm) and sterile filtered (0.22 µm) prior to storage at -20°C.

The remaining acidified WGFE (950 ml) was partitioned against a porous membrane following a 1 h re-circulation step. The membrane retentate was diafiltered with a 20:1 volume ratio of 150 mM NaCl pH 2.5, in approximately 35 sequential additions, and concentrated down to a final volume of 300 ml. This *retentate WGFE* was subsequently re-neutralised and

handled in a similar manner to the acid WGFE fraction.

The membrane permeate (substantially diluted in diafiltrate) was stored in acidic conditions until required for further processing. The material was then re-neutralised to pH 6.0 with 10 M NaOH and concentrated to 1 litre against a 3 kDa membrane. The concentrate was diafiltered against 20 stepwise additions of MilliQ-filtered water (1 litre each) and concentrated back to a final volume of 1 litre. The concentrated *permeate WGFE* was then freeze-dried, dissolved in Dulbecco's modified Eagles medium (DMEM) at 10 mg/ml (dry weight), filter sterilised (0.22 μm) and stored at -20°C.

3.2.2 Protein analysis of WGFE

The protein concentration of each WGFE fraction was estimated using a bicinchoninic acid assay reagent kit (Pierce), based on a protocol developed by Smith *et al.* (1985). WGFE samples were diluted in phosphate buffered saline (pH 7.4), then mixed at a ratio of 1:20 with a reaction substrate containing bicinchoninic acid and cupric sulfate. The reaction mixtures were incubated in a microtitre plate at 37°C for 30 min before the absorbance of each well was measured at 570 nm. Absorbance readings for the WGFE samples were interpolated from a BSA standard curve, prepared in an identical manner to the test samples. The resultant standard curve was linear over a protein concentration range of 0 - 2000 μg/ml.

3.2.3 Quantification of immuno-reactive TGFß$_2$ in WGFE fractions

Immuno-reactive TGFß$_2$ was quantified in each of the WGFE fractions using a human TGFß$_2$ ELISA kit (R&D Systems). Assay validation performed by the manufacturer indicated an intra-assay precision of between 2.7% and 7.1%, and an inter-assay precision of between 4.3% and 5.0%. Recognition of bovine TGFß$_2$ was anticipated as this peptide possesses 100% sequence homology with human TGFß$_2$. One hundred microlitres of assay diluent was added to each microtitre well, which had been coated by the manufacturer with a murine monoclonal antibody against TGFß$_2$. A 100 μl aliquot of a WGFE fraction or TGFß$_2$ standard, diluted in PBS, was then added to the wells. Samples were incubated at 21°C for 2 h, prior to aspirating

and washing the wells. Bound TGFß$_2$ was detected with a polyclonal antibody conjugated to horse-radish peroxidase by incubation at 21°C for 2 h. Unbound antibody was carefully washed from the wells before addition of a colorimetric reagent (tetramethylbenzidine). The reaction mixture was incubated at 21°C for 20 min before the terminating colour development. The absorbance of each well was measured at 450 nm with reference subtraction at 570 nm. Absorbance readings for the WGFE samples were interpolated from the TGFß$_2$ standard curve. The resultant standard curve was linear over a concentration range of 125 - 2000 pg/ml.

3.2.4 Quantification of immuno-reactive IGF-I in WGFE fractions

IGF-I peptides were quantified by radio-immunoassay (RIA) in each of the four WGFE fractions following acid ethanol extraction of complexed binding proteins and high-pressure liquid chromatographic separation, adopting the method of Owens *et al.* (1990). Recombinant human IGF-I (GroPep Pty. Ltd.) was radio-labelled by chloramine-T iodination and used within 60 days of preparation. An aliquot of ^{125}I-labelled IGF-I was diluted to 5 x 10^5 cpm/ml in 1 x mobile phase (see Appendix for preparation). Two hundred microlitres of this radio-labelled peptide was loaded onto a pre-equilibrated Waters Protein-Pak 125 gel filtration column (Millipore Corporation), and eluted with 1 x mobile phase at a flow rate of 1 ml/min. Fractions were collected at 15 s intervals, and measured for radioactivity on a gamma counter to determine the elution time of IGF-I.

WGFE fractions were acidified by thorough mixing with 4 x mobile phase to produce final protein concentrations in the range of 5.0 - 25.0 mg/ml in a final concentration of 1 x mobile phase. After acidification for 10 min at 21°C, the samples were centrifuged (11000 x *g*, 60 s) and the supernatant was filtered though a 0.22 µm membrane.

Two hundred microlitre volumes of each WGFE sample were sequentially loaded onto the gel filtration column and eluted with 1 x mobile phase. Fractions collected between 7.75 min and 12.75 min were divided between four pools - 1. 7.75 - 9.25 min; 2. 9.50 - 9.75 min; 3. 10.0 - 12.25 min and 4. 12.5 - 12.75 min. The IGF-I concentration of each of the pools, from each of

the WGFE fractions, was then measured by RIA.

All pools were assayed without dilution, however pool 3 was also assayed at $^1/_{10}$, $^1/_{25}$, $^1/_{50}$ and $^1/_{100}$ dilutions in 1 x mobile phase. IGF-I standards were prepared over a concentration range of 0 - 2.0 ng/ml, prepared in RIA buffer (see Appendix for buffer preparation). A quality control sample was also included, containing an established assay concentration of 150 - 180 pg IGF-I/tube.

All samples were assayed in triplicate, in a reaction mixture containing the following:

50 µl sample or 1 x mobile phase
200 µl IGF-I standard or RIA buffer
30 µl 0.4 M Tris
50 µl polyclonal rabbit anti-human IGF-I antibody (GroPep Pty. Ltd.); 1/80000 final dilution
50 µl ^{125}-labelled recombinant human IGF-I (25000 cpm)

Blank tubes contained all of the above components except for the IGF-I antibody. Reference tubes contained both ^{125}I-labelled IGF-I and the IGF-I polyclonal antibody, but no competing source of IGF-I. All reaction tubes were incubated overnight at 4°C, then mixed with 50 µl of polyclonal sheep anti-rabbit IgG (1/20 dilution; Silenus Laboratories) and 10 µl of rabbit IgG (1/200 dilution; Dako Corporation). Following a further 30 min incubation at 4°C, 1 ml of ice cold polyethylene glycol solution (9.2 mM polyethylene glycol, 0.154 M NaCl) was added to each tube. Reaction tubes were then vigorously mixed and centrifuged at 2500 x *g* for 20 min at 4°C. The supernatant was carefully aspirated from each tube, and the remaining protein pellet was measured for radioactive content in a gamma counter.

The IGF-I concentration of both the quality control and WGFE pool samples was interpolated from the curve of IGF-I standards. Having confirmed the accuracy of the quality control sample, the IGF-I concentration of each WGFE pool was calculated as ng IGF-I/mg total WGFE protein. IGF-I immuno-reactivity was identified in pool 1, due to the presence of IGF binding proteins, and in pool 3 due to the presence of IGF-I.

3.2.5 Quantification of growth inhibitory activity in WGFE

The growth inhibitory activity of the WGFE fractions was compared to that of rTGFß$_2$ using a previously described method (Absher *et al.,* 1991; Rogers *et al.,* 1996), with slight modifications. Mv1Lu cells were seeded in 96-well plates (20 x 10^4 cells/ml in 0.1 ml volumes) and incubated for 24 h under standard conditions (Section 2.2.1). Growth medium was then aspirated and replaced with fresh medium containing rTGFß$_2$ standards (0 - 10.0 ng/ml), or WGFE fractions prepared from twelve doubling dilutions. Cells were re-cultured for a further 48 h before being washed, fixed and stained for methylene blue quantification of cell number (Section 2.2.2.3). Using this assay, TGFß$_1$ exhibits an ED_{50} concentration of 0.3 ng/ml (Absher *et al.,* 1991). This value is similar to the ED_{50} of 0.5 ng/ml observed with both TGFß$_1$ and TGFß$_2$, using tritiated thymidine incorporation to measure arrest of DNA synthesis (Cheifetz *et al.,* 1987). Consequently, bioassay results were considered acceptable with an ED_{50} value in the range of 0.3 to 0.5 ng/ml, and with variation between triplicate test values of less than 10%.

A logistic dose response curve for the rTGFß$_2$ standards was generated using Table Curve software (Jandel Scientific). The equivalent TGFß activity of each WGFE sample was then interpolated from the linear region of the standard curve and divided by the corresponding WGFE protein concentration to give a value of equivalent TGFß activity in ng TGFß/mg protein.

3.2.6 Analysis of WGFE-mediated effects on cell proliferation

For flow cytometric analysis of cell proliferation, Mv1Lu cells were seeded in 6-well plates at a density of 1.5 x 10^4 cells/ml in a volume of 5.85 ml and cultured for 24 h under standard conditions (Section 2.2.1). Growth medium was then replaced with medium containing a WGFE fraction, diluted to a biologically active equivalent of 3 ng/ml TGFß as determined by growth inhibition bioassay. The inclusion of 5-bromo-2′-deoxyuridine (BrdU; Sigma Chemical Company) in culture dishes, and subsequent detection by immuno-fluorescent staining, was

performed using a modification of protocols described by Dolbeare *et al.* (1983) and Hoy *et al.* (1989). BrdU was added to the Mv1Lu cultures (20 μM final concentration) for the final 2 h of the incubation period. Culture monolayers were then trypsinised (Section 2.2.1) and pooled with their respective culture supernatants to provide total cell yields. Cells were sedimented (600 x *g*, 5 min) and the pellet was resuspended in 250 μl PBS before fixation with 750 μl ethanol. Cell suspensions were stored at 4°C until staining for flow cytometry.

Prior to staining, cells were centrifuged (600 x *g*, 5 min) and resuspended in 500 μl of 1.5 M HCl for a 30 min incubation at 37°C to partially denature the DNA. The cell suspensions were then washed twice with 0.1 M sodium tetraborate (pH 8.2) to restore the pH towards neutral. Incorporated BrdU was detected by resuspension of each cell pellet in 300 μl of FITC-labelled monoclonal anti-BrdU (clone BR-3, Caltag Laboratories), diluted 1/200 in PBS containing 0.5% (v/v) Tween-20 and 75 μM bovine serum albumin, incubated at 37°C for 30 min. Excess antibody was then removed by washing once with PBS, and the cells were counter-stained with 50 μg/ml propidium iodide (PI).

Stained cells were analysed within 3 h of preparation for their relative DNA content (PI channel) and BrdU incorporation (FITC channel). A minimum of 12000 events were measured, at a data rate of 50 events/second, to ensure that at least 10000 singlet events were collected. Flow cytometric analysis was performed as described in Section 2.2.6.

3.2.7 Cell survival assay

Mv1Lu cells were seeded in 96-well tissue culture plates at a density of 1.5 x 10^4 cells/ml in 200 μl aliquots. Cultures were incubated for 24 h, then the medium was replaced and cells were incubated for a further 30 h. Cells were routinely exposed to chemotherapeutic agents, either etoposide or vinblastine, at various doses by addition of an 11 x concentrated stock into the pre-existing culture medium. Drug exposure continued for 18 h, followed by careful washing of the cell monolayers to remove all traces of drug. Cultures were then re-incubated in drug-free medium for 30 h.

Recombinant TGFß$_2$ or WGFE fractions were added to cultures at various concentrations. Treatments were added directly to the cell cultures for a 48 h incubation, beginning 30 h prior to drug exposure (pre/concurrent treatment), or for a 30 h incubation immediately following drug removal (post-treatment). To ensure the consistency of cell densities at the time of drug exposure, and therefore predictable drug toxicity effects in continuing cultures, a quality control plate was harvested at the time of drug addition.

At the completion of every assay, Mv1Lu cultures were washed and fixed for methylene blue analysis (Section 2.2.2.3), which quantified the number of cells remaining adherent on the culture monolayer. Additionally, in some experiments alamarBlue was used as a colorimetric indicator of the metabolic activity of each culture, using a modification of the method described by Page *et al.* (1993). A 20 µl aliquot of alamarBlue (Alamar Biosciences, Inc.) was added to each culture well for the final 4 h of incubation. The metabolic status of each cell population was quantified as the difference between reduced (A_{570}) and oxidised (A_{600}) forms of the dye. The final absorbance values were reported after subtraction of the 600 nm value from the respective 570 nm value ($A_{570-600}$). Background subtraction was also performed using wells which contained culture medium but no cells. Thus, two independent measures of drug toxicity were performed on the same set of cell cultures, providing additional verification of experimental observations.

3.2.8 Statistical analysis

Statistical analysis of the data illustrated in Table 3.3, Figure 3.2 and Figure 3.4 was performed by ANOVA. Values in Figure 3.3 were analysed with a repeated measures ANOVA following arc-sine transformation of the percentage data. Data in Figure 3.5 to Figure 3.9 were analysed by ANOVA. Where statistical significance was reached ($P < 0.05$), a multiple comparisons Bonferroni-Dunn or Fishers protected LSD post-hoc test was applied.

3.3 Results

3.3.1 Protein and growth factor analysis of WGFE fractions

Protein and growth factor profiles for all four WGFE fractions are shown in Table 3.1. The protein concentration of each WGFE fraction was determined by bicinchoninic acid protein estimation. This assay produced similar results to the Lowry protein assay with all WGFE fractions and was more rapid for routine use (results not shown). The growth factor composition of each of the WGFE fractions was tested to determine the relative partitioning of component growth factors likely to contribute to a survival response. Immuno-reactive TGFß was quantified by ELISA using an antibody directed against $TGFß_2$, as over 85% of the TGFß in WGFE has been reported to be present as the $TGFß_2$ isoform (Rogers *et al.*, 1996), whilst immuno-reactive IGF-I was estimated by RIA following the chromatographic separation of contaminating binding proteins.

WGFE fraction	Protein (mg/ml)	$TGFß_2$ (ng/mg protein)	IGF-I (ng/mg protein)
control	30.47 ± 1.05	5.80 ± 0.40	12.10 ± 2.90
acid	23.18 ± 1.20	68.73 ± 11.95	15.84 ± 6.85
permeate	6.99 ± 0.49	6.39 ± 0.54	75.03 ± 6.15
retentate	37.96 ± 3.47	29.15 ± 2.44	6.50 ± 0.99

Table 3.1 Protein and growth factor composition of WGFE fractions

Values are the mean ± SEM derived from a single experiment for protein measurement and IGF-I immuno-reactivity (n = 3 replicates/dilution). Values listed for $TGFß_2$ measurement represent the mean ± SD from two independent experiments (n = 3 replicates/experiment).

3.3.1.1 Protein composition

The protein concentration of the initial WGFE material was 30.47 mg/ml. Following acidification and re-neutralisation of this control WGFE preparation, the protein concentration fell by approximately 24% to 23.18 mg/ml. In part, this was due to the slight increase in volume associated with the addition of acid and alkali, although the use of concentrated HCl and NaOH minimised this effect. The predominant cause of protein loss from the acidified WGFE was the precipitation of lactoperoxidase and other proteins upon reneutralisation. The insoluble material was sedimented by centrifugation and removed from the final acid WGFE preparation. The changes in protein concentration observed with the permeate and retentate WGFE preparations mostly result from the dilution and concentration of the material during membrane filtration. No protein precipitate was collected from the permeate WGFE, which had a final protein concentration of 6.99 mg/ml. There was, however, a loss of protein from the retentate WGFE following restoration of neutral pH, resulting in a final concentration of 37.96 mg/ml.

3.3.1.2 Immuno-reactive TGFß$_2$ in WGFE fractions

The monoclonal antibody used to capture TGFß$_2$ in the ELISA was capable of binding only active peptides. Hence, quantification of TGFß$_2$ in the WGFE fractions was restricted to active forms of the peptide, as latent complexes were not acid activated prior to analysis. The majority of the TGFß$_2$ in the control WGFE fraction was present in latent form, accounting for the low concentration of 5.80 ng TGFß$_2$/mg protein measured (Table 3.1). Irreversible activation of latent TGFß complexes increased TGFß$_2$ immuno-reactivity in the acid WGFE to contained 68.73 ng active TGFß$_2$/mg protein. Separation of acidified WGFE by filtration through a porous membrane resulted in the concentration of active TGFß$_2$ peptides in the retentate fraction. Whilst active TGFß exists as a dimeric peptide of 25 kDa, it was unable to pass through the membrane pores due to secondary membrane effects, yielding a concentration of 6.38 ng TGFß$_2$/mg protein in the permeate WGFE. Thus, the permeate WGFE was depleted of TGFß$_2$ when compared to the acid WGFE, but still contained more TGFß$_2$ per unit protein than the control WGFE. Conversely, TGFß$_2$ accumulated in the retentate WGFE, yielding a

concentration of 29.14 ng TGFß$_2$/mg protein. This concentration was lower than that of the acid WGFE due to the changes in protein concentration following the restoration of neutral pH and the loss of TGFß$_2$ peptides on the filtration membrane.

3.3.1.3 Immuno-reactive IGF-I in WGFE fractions

IGF-I concentrations in the WGFE fractions were measured by RIA following the separation of IGF peptides from binding proteins. The total recovery of radio-labelled tracer IGF-I from the chromatography column was 88%. The peak of radioactivity produced upon elution of the tracer was observed in fractions collected between 10.0 min and 12.25 min.

Transient acidification of the control WGFE did not affect concentrations of immuno-reactive IGF-I, with both fractions containing approximately 12 ng IGF-I/mg protein. Filtration of acidified WGFE through a porous membrane concentrated the IGF-I into the permeate fraction, increasing immuno-reactivity to 75.03 ng IGF-I/mg protein. In contrast, the filtration procedure resulted in a relative depletion of IGF-I in the retentate, with concentrations decreasing to 6.50 ng IGF-I/mg protein.

3.3.2 Growth inhibitory activity of WGFE fractions

As each WGFE preparation represented a complex mixture of growth factors, the overall growth modulatory effect was assessed in a biological system. Whilst the TGFß$_2$ ELISA measured the immuno-reactivity of this peptide, it did not provide an indication of the total growth inhibitory activity of each WGFE fraction. The presence of other growth factors, in particular peptides such as epidermal growth factor (EGF) and basic fibroblast growth factor (FGF), are known to exert antagonistic influences on growth inhibition in biological systems (Garrigue-Antar *et al.,* 1995; Jennings *et al.,* 1988). Furthermore, there may be uncharacterised peptides present in the WGFE fractions which either additively or synergistically enhance the growth inhibition induced by TGFß. As I had hypothesised that the degree of survival mediated

by each WGFE fraction would be directly related to the growth inhibition it induced, it was the biological measure of inhibitory activity that was ultimately of interest in the investigation of cytoprotective activity.

3.3.2.1 Effect of WGFE fractions on cell growth

The growth inhibitory activity of each of the WGFE fractions was measured using a Mv1Lu growth inhibition bioassay. Inhibition was detected following a 48 h incubation and compared to the inhibitory response produced by rTGFß$_2$. Recombinant TGFß$_2$ produced a dose-dependent growth inhibition of Mv1Lu cells, with maximal inhibition occurring in the presence of 2.5 ng/ml TGFß$_2$ (Figure 3.1 A). This dose-dependent response routinely yielded an ED_{50} concentration of between 0.3 and 0.5 ng/ml rTGFß$_2$, with no detectable growth inhibition at 0.1 ng/ml rTGFß$_2$.

Each of the WGFE fractions induced a TGFß-like state of growth inhibition in Mv1Lu cell cultures, although the potency of the WGFE fractions varied considerably (Figure 3.1 B). Thus, the control WGFE exerted half-maximal anti-proliferative effects (ED_{50}) at 148 µg protein/ml, whilst transient acidification reduced this value to 2.54 µg protein/ml in the acid WGFE fraction. The permeate WGFE produced an ED_{50} response at 13.6 µg protein/ml, approximately 5-fold higher than observed with the acid WGFE. However, the retentate WGFE possessed similar potency to the acid WGFE fraction, with an ED_{50} concentration of 3.87 µg protein/ml. The active protein concentration range also differed between WGFE fractions. Growth inhibitory activity in the control, acid and permeate WGFE fractions was eliminated by a 100-fold dilution of protein from the ED_{100} dose. In contrast, a 1000-fold dilution of the ED_{100} concentration was required to abolish anti-proliferative activity in the retentate WGFE.

Interpolation of the growth inhibitory activity present in each WGFE fraction, using the linear region of the TGFß standard curve, yielded the values listed in Table 3.2. For simplicity, growth inhibitory activity was expressed in terms of an equivalent growth inhibitory

concentration of rTGFß$_2$. The control WGFE fraction contained only weak growth inhibitory activity, equivalent to 3.96 ng TGFß/mg protein, consistent with the TGFß$_2$ concentration of 5.80 ng/mg protein obtained by ELISA. Whilst the overall distribution of growth inhibitory activity was similar to that of TGFß$_2$, following acidification and size exclusion modifications, the growth inhibitory values were 3-5-fold greater for the acid, permeate and retentate WGFE fractions. Growth inhibition was potentiated by transient acidification of the WGFE, increasing the activity of the acid WGFE fraction to the equivalent of 229,91 ng TGFß/mg protein. Size separation of the acidified WGFE proteins by filtration through a porous membrane purified the growth inhibitory activity into the retentate WGFE fraction (155.29 ng TGFß/mg protein), consequently weakening the activity in the permeate WGFE fraction (31.13 ng TGFß/mg protein).

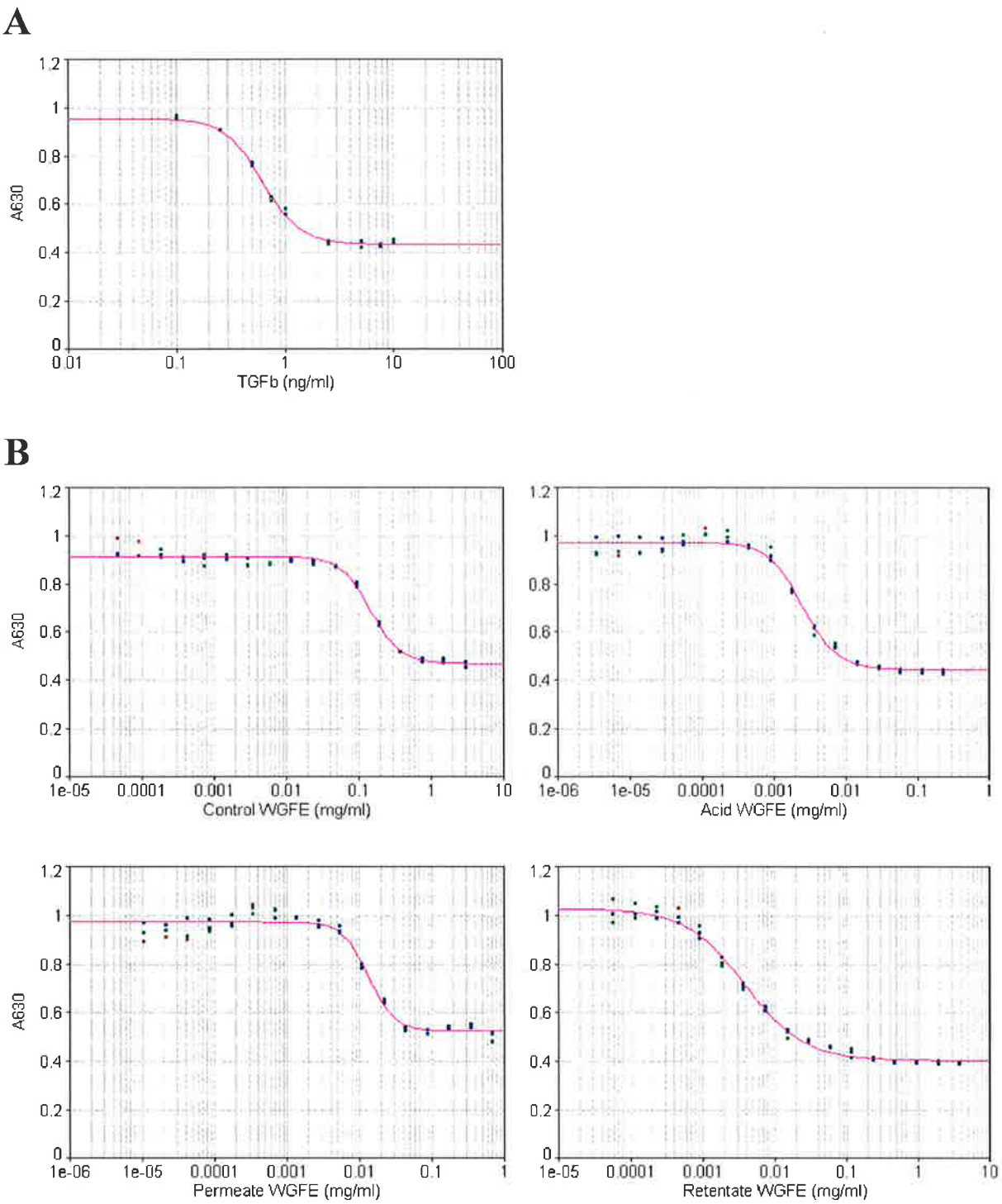


Figure 3.1 Logistic dose response curves to TGFß and WGFE fractions

Dose response curves from Mv1Lu cells exposed to dilutions of recombinant TGFß$_2$ (**A**) or one of the WGFE fractions (**B**) for 48 h in a standard growth inhibition bioassay. Each WGFE fraction is indicated below the abscissa of the relevant graph. Cell growth was quantified at A_{630} following methylene blue staining of the cultures. Values represent individual data points from a single representative experiment.

WGFE fraction	Growth inhibitory activity (TGFß equivalent ng/mg protein)
control	3.96 ± 0.24
acid	229.91 ± 8.84
permeate	31.13 ± 1.79
retentate	155.29 ± 19.02

Table 3.2 Growth inhibitory activity of WGFE fractions

Values are the mean ± SEM derived from 3 sample dilutions located on the linear region of the logistic dose response curve (n = 3 replicates/dilution).

3.3.2.2 Effect of WGFE fractions on BrdU incorporation

When the WGFE fractions were added to Mv1Lu cultures for 48 h, at concentrations producing an equivalent growth inhibitory activity of 3 ng/ml TGFß, they all reduced BrdU labelling to approximately 2% of the cell population, confirming the equivalence established in the growth inhibition bioassay (results not shown).

In contrast, WGFE fractions incubated for only 30 h before analysis of BrdU incorporation induced differing degrees of inhibition of BrdU incorporation into newly synthesised DNA. BrdU was incorporated into the DNA of 42.35% of normally proliferating cells, cultured for 30 h in the presence of serum-supplemented medium, following a 2 h exposure to the label (Figure 3.2). Incubation with $rTGF\beta_2$, however, reduced this value to 4.1% of the cell population. The acid and retentate WGFE fractions also reduced incorporation, to 4.4% and 3.9% respectively. BrdU uptake was also reduced in the control and permeate WGFE fractions when compared to control cultures, although to a lesser extent than observed with the other WGFE fractions. The amount of BrdU incorporation into the DNA of control WGFE treated cultures remained significantly higher than observed in cultures treated with $rTGF\beta_2$ ($P < 0.01$), acid WGFE ($P < 0.01$) or retentate WGFE ($P < 0.01$). Incorporation fell to 20.6% of the cell

population, whilst incubation with permeate WGFE restricted BrdU uptake to 14.6% of the treated cells.

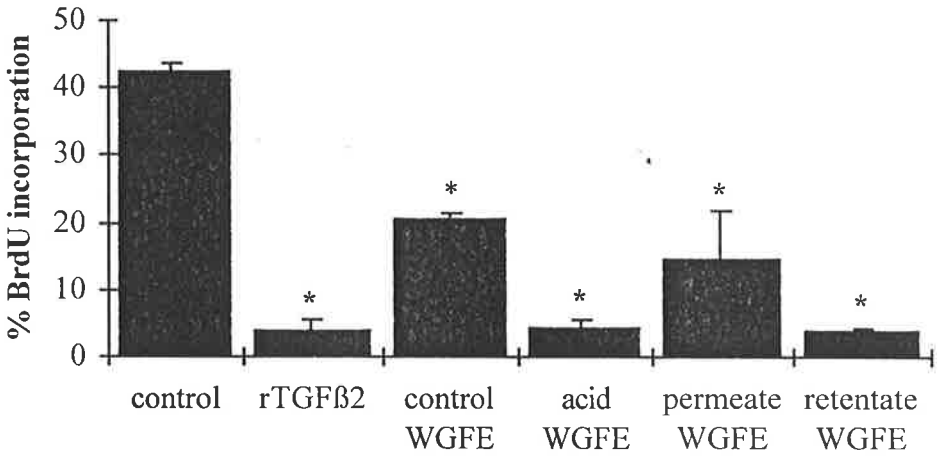


Figure 3.2 Effect of 30 h exposure to TGFß$_2$ or WGFE on BrdU incorporation

Mv1Lu cells were incubated in normal growth medium, or supplemented with recombinant TGFß$_2$ or a WGFE fraction, for 30 h with a final 2 h co-incubation with 20 µM BrdU. All treatments were added at concentrations which produced an equivalent growth inhibition to 3 ng/ml TGFß$_2$ in a standard Mv1Lu bioassay following a 48 h incubation. Values represent the mean ± SD from two independent experiments (n = 3 replicates/experiment). *, $P < 0.05$, compared to control cultures.

3.3.2.3 Effect of WGFE fractions on cell cycle distribution

As described for the BrdU incorporation experiments, a 48 h incubation period produced equipotent G_1 phase arrest with all of the WGFE fractions. In normally proliferating cultures, 64.2 ± 1.8% of cells were detected in the G_1 phase of the cell cycle. Cultures grown in the presence of rTGFß$_2$ or WGFE fractions accumulated between 82.9% and 91.0% of their respective populations in G_1 phase, with concomitant reductions in both the S and G_2/M phase populations (results not shown).

In addition to variable BrdU incorporation, the control and permeate WGFE fractions produced less of a G_1 cell cycle phase arrest than either the recombinant TGFß$_2$, acid or retentate WGFE fractions when incubated for only 30 h (Figure 3.3). Normally proliferating cell cultures

exhibited a G_1 phase content of 57.8% of the total cell population. Incubation with rTGFß$_2$ increased the percentage of cells within the G_1 phase to 89.6% of the population ($P < 0.001$, compared to control cultures), whilst the acid and retentate WGFE fractions induced the accumulation of 89.3% ($P < 0.001$) and 90.4% ($P < 0.001$) of the respective populations into G_1 phase. In contrast, the G_1 phase arrest induced by the control and permeate WGFE fractions was not significantly different from that in proliferating cultures.

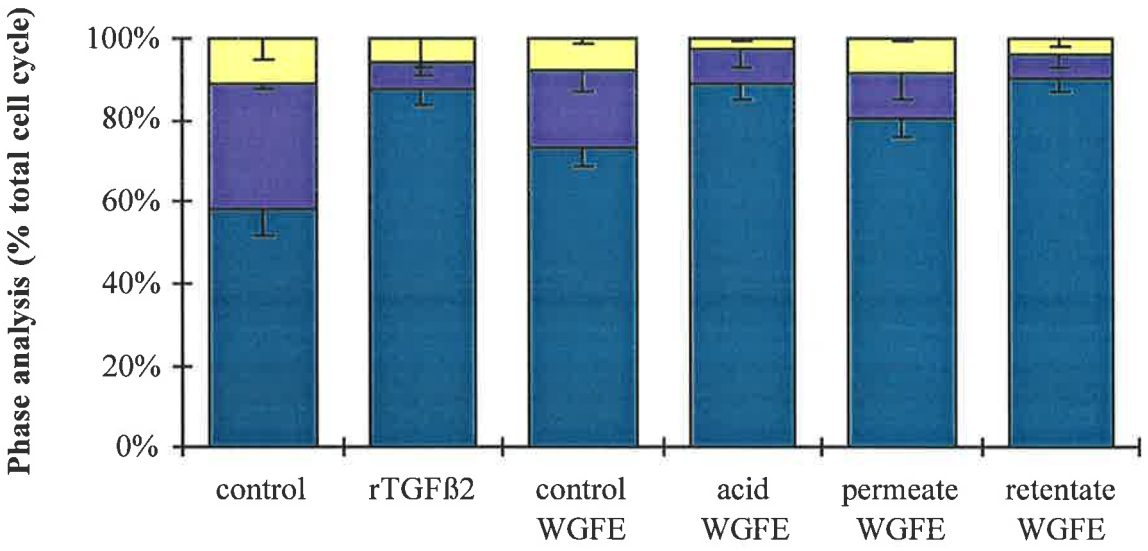


Figure 3.3 Mv1Lu cell cycle phase distribution following exposure to TGFß$_2$ or WGFE for 30 h

Mv1Lu cells were analysed for changes in cell cycle phase distribution following a 30 h incubation with recombinant TGFß$_2$ or a WGFE fraction. All treatments were added at concentrations which produced an equivalent growth inhibition to 3 ng/ml TGFß$_2$ in a standard Mv1Lu bioassay. The total cycling population was comprised of cells in G_1 (■), S (■) and G_2/M (■) phases. Values represent the mean ± SD from three independent experiments (n = 3 replicates/experiment).

Whilst the control and permeate WGFE fractions induced equivalent growth inhibition at the end of a 48 h exposure, they appeared to be less potent than the acid or retentate fractions when cultures were assayed after only 30 h of incubation. Thus, whilst each fraction achieved the same end result, it was not clear that this occurred as a result of a common mechanism of action. As the acid and retentate WGFE fractions induced the most rapid cell cycle arrest, I predicted that they would provide the greatest protection against chemotherapeutic drug toxicity

in the survival assay. Accordingly, I hypothesised that the presence of counter-inhibitory peptides in the control and permeate WGFE fractions would result in sub-optimal levels of cytoprotection when added as a pre/concurrent treatment in a survival assay, and may be more suited to application as a post-drug treatment.

3.3.3 WGFE-mediated survival against chemotherapy drug toxicity

3.3.3.1 Effect of WGFE fractions on cell density at the time of drug exposure

Mv1Lu cultures were harvested after a 30 h incubation with WGFE fractions in order to ensure that similar cell densities were produced in response to these fractions at the time of drug addition. Each WGFE fraction was initially prepared at a protein concentration which produced growth inhibitory activity equivalent to 3 ng/ml rTGFß$_2$ when measured by Mv1Lu growth inhibition. The protein concentration of each WGFE fraction was therefore 0.77 mg/ml, 13.0 µg/ml, 96.4 µg/ml and 19.3 µg/ml in the control, acid, permeate and retentate WGFE fractions, respectively. Each WGFE fraction was then serially diluted 1:4 to produce a dose response curve.

The highest concentrations of each WGFE fraction and rTGFß$_2$ consistently reduced cell number, as estimated by A_{630} values, when cultures were stained with methylene blue after a 30 h incubation (Figure 3.4). Whilst dose-dependent variation in methylene blue staining intensity was detected, comparison between the treatment groups indicated that there was no significant inter-treatment source of variation ($P = 0.190$, 2-way ANOVA). As the culture densities within each equivalent TGFß dose were similar, and remained within the quality control limits established in Chapter 2, the final predicted drug toxicities were directly comparable between treatment groups at the same equivalent TGFß concentration.

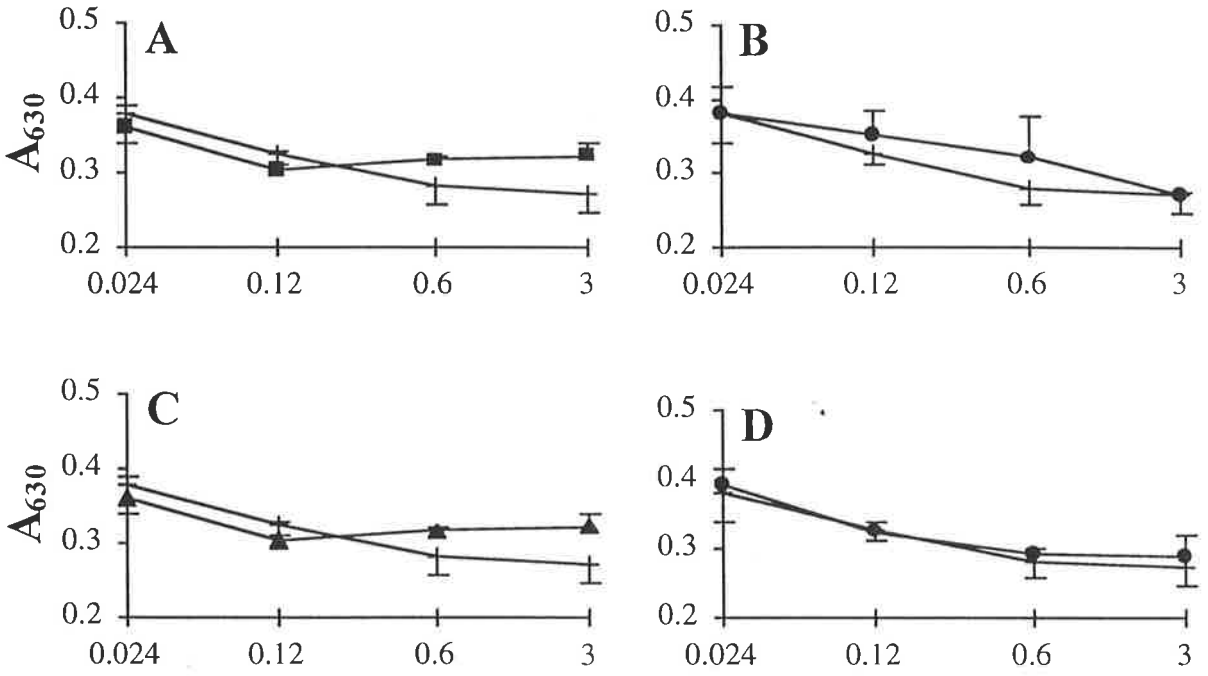


Figure 3.4 Effect of 30 h incubation with WGFE fractions on cell density

Changes in cell culture density were measured by methylene blue analysis of cell number (A_{630}) following exposure to control WGFE (**A**), acid WGFE (**B**), permeate WGFE (**C**) or retentate WGFE (**D**). All WGFE fractions were assayed at protein concentrations which induced growth inhibition in a Mv1Lu bioassay equivalent to the indicated dose of rTGFß$_2$. A_{630} values obtained with rTGFß$_2$ are superimposed on each graph (+). Values represent the mean ± SD from three independent experiments (n = 5 replicates/experiment).

3.3.3.2 WGFE-mediated cell survival in drug-treated cultures

The four WGFE fractions were then compared for their ability to either protect against drug toxicity, when added to cultures before and during drug exposure, or to enhance the regeneration of surviving cells following drug exposure. Each WGFE fraction was assayed at a protein concentration which produced growth inhibitory activity equivalent to 3 ng/ml rTGFß$_2$ when measured by Mv1Lu growth inhibition. The protein concentration of each WGFE fraction was 0.77 mg/ml, 13.0 µg/ml, 96.4 µg/ml and 19.3 µg/ml in the control, acid, permeate and retentate WGFE fractions, respectively. Although the growth inhibitory activity of each fraction was equivalent, the concentration of immuno-reactive IGF-I peptides in each fraction varied considerably, ranging from 9.32 ng/ml and 7.23 ng/ml in the control and permeate

WGFE fractions, respectively, to 0.21 ng/ml and 0.125 ng/ml in the acid and retentate WGFE fractions, respectively.

3.3.3.3 Comparison of alamarBlue and methylene blue analysis of cell survival

Preliminary studies investigated the ability of two stains, alamarBlue and methylene blue, to quantify cell survival. Methylene blue is a non-specific biomass stain which binds to cellular material at a stoichiometric ratio. Hence, the greater the number of cells remaining adherent on the culture monolayer, the higher the absorbance values resulting from elution of the bound dye. Whilst I had previously observed that Mv1Lu cells detached from the monolayer at an early stage in the progression of cell death, I wished to ensure that an increase in survival measured using methylene blue corresponded to an increase in survival using a second measure of cytotoxicity.

The addition of alamarBlue to the cultures for the final 4 h of the survival assay provided a colorimetric indicator of the metabolic state of each culture. AlamarBlue measures proliferative activity *in vitro* by virtue of its inclusion in the oxidation-reduction pathway of the cellular electron transport system. The dye is chemically reduced by the consumption of oxygen, without interfering with electron transfer through the rest of the respiratory chain. The reduction process is then visualised by the inclusion of a non-toxic redox indicator which is red when reduced and blue when oxidised. Thus, the metabolic status of each surviving cell population can be quantified as the difference between reduced (A_{570}) and oxidised (A_{600}) forms of the dye.

AlamarBlue and methylene blue analysis was performed in the same set of cultures, as the alamarBlue dye remained soluble and was non-toxic to the culture for the duration of the 4 h incubation. Following spectrophotometric analysis of the cultures at $A_{570\text{-}600}$ the medium and dye was removed, leaving an undisturbed monolayer for methylene blue staining. Absorbance values collected by alamarBlue analysis ($A_{570\text{-}600}$) provided similar patterns of cell survival to the values collected by methylene blue analysis (A_{630}). In control treated cultures, survival

following exposure to 1 μM etoposide was 68.95% of that observed in drug-free cultures when calculated using alamarBlue absorbance values (Table 3.3). There was a trend towards increased survival in cultures treated with rTGFß$_2$, acid, permeate and retentate WGFE, however the high degree of variability rendered these differences insignificant. When survival was calculated from the methylene blue absorbance values, 56.07% of the control-treated cultures survived drug exposure. Importantly, the rTGFß$_2$ and WGFE treatments produced similar patterns of increased survival as identified from the alamarBlue measurements, however with the methylene blue analysis this increased survival was significantly greater than control levels. Whilst the actual survival values differed between the two staining procedures the trends were similar across treatment groups, confirming that methylene blue analysis was a valid means of measuring cell survival.

Treatment	Staining intensity (% drug-free value)	
	alamarBlue	methylene blue
control	68.95 ± 11.13	56.07 ± 4.17
rTGFß$_2$	90.99 ± 9.68	72.34 ± 2.44$^{\#}$
control WGFE	68.68 ± 15.48	67.40 ± 9.84
acid WGFE	85.56 ± 19.55	70.08 ± 0.25$^{\#}$
permeate WGFE	95.59 ± 11.89	70.82 ± 2.33$^{\#}$
retentate WGFE	103.01 ± 19.51	77.16 ± 5.65$^{\#}$

Table 3.3 Comparison of alamarBlue and methylene blue measurement of cell survival

Cell survival was measured in Mv1Lu cultures following the addition of control medium, 3 ng/ml rTGFß$_2$ or a WGFE fraction for 48 h, beginning 30 h before exposure to 1 µM etoposide. All treatments were added at a concentration which provided equivalent growth inhibition to 3 ng/ml rTGFß$_2$ in a Mv1Lu growth inhibition bioassay. The absorbance of each culture was measured at $A_{570\text{-}600}$, following a 4 h incubation with alamarBlue, then at A_{630} following staining of the same cultures with methylene blue. Values were converted to % survival by calculating the ratio of absorbance values in the drug-exposed versus drug-free cultures for each pre/concurrent treatment. Values are the mean ± SD from three independent experiments (n = 4 replicates/experiment). $^{\#}$, $P < 0.05$, all treatments compared to control.

3.3.3.4 Pre/concurrent WGFE treatment

Cell survival mediated by each of the WGFE fractions was initially determined using either 1 µM etoposide or 50 nM vinblastine as the source of drug toxicity, and a single concentration of each WGFE preparation previously standardised for equivalent growth inhibitory activity in the absence of drugs.

Consistent with earlier studies performed in the absence of drug exposure (Figure 3.1), the addition of 3 ng/ml rTGFß$_2$, or equivalent inhibitory doses of each WGFE fraction to Mv1Lu cells for 48 h resulted in a reduction of cell number at the end-point of the survival assay (Figure 3.5 A). Growth factor treated cultures all exhibited significantly lower absorbance values than the control-treated culture, with values falling from 1.32 units to between 0.4 and

0.6 units. The similarity in cell responses between the cultures treated with $TGF\beta_2$ or WGFE fractions confirmed the equivalent inhibitory activity of these preparations as calculated from growth inhibition bioassays.

In control cultures incubated without pre/concurrent growth factor addition, exposure to 1 μM etoposide for 18 h decreased cell number markedly from 1.32 absorbance units to 0.74 absorbance units. Etoposide also reduced cell number in $rTGF\beta_2$- and WGFE-treated cultures to less than 0.4 absorbance units in most cases. Whilst the difference in absorbance values between the control and treated cultures decreased in the presence of etoposide, methylene blue staining remained significantly lower in all treatment groups when compared to the untreated control.

To determine the extent to which $rTGF\beta_2$ and the WGFE fractions protected against chemotherapy drug-induced cell death, the A_{630} value obtained in the presence of etoposide was expressed as a percentage of the value obtained in the absence of etoposide for each growth factor treatment group. Thus, survival in the absence of TGFß or WGFE addition was reduced to 56.1% by etoposide exposure (Figure 3.5 B). Pre/concurrent treatment of Mv1Lu cultures with each of the WGFE and TGFß treatments induced significant increases in cell survival to at least 67%.

The addition of 50 nM vinblastine to control-treated Mv1Lu cultures reduced methylene blue staining of cell number from 1.32 absorbance units to 0.20 units (Figure 3.5 A). Importantly, no further reductions in cell number were observed with any of the pre/concurrent growth factor treatments. In fact, incubation with acid or retentate WGFE fractions tended to increase methylene blue staining above that observed with the control culture.

The drastic reduction in absorbance values measured in the control-treated cultures following vinblastine exposure translated into a lower proportional survival than was observed with etoposide. Cell survival fell to 14.9% after cultures were exposed to 50 nM vinblastine for 18 h (Figure 3.5 B). As with etoposide exposure, WGFE and $rTGF\beta_2$ treatments significantly

enhanced survival against vinblastine toxicity in all cases except for the control WGFE fraction. However, the magnitude of the protection conferred by these treatments was much greater than with etoposide, suggesting that the extent of the protection partly dependent upon the degree of toxicity, or the mechanism of toxicity, induced by the chemotherapy agent.

3.3.3.5 Post-drug WGFE treatment

Mv1Lu cultures grown without post-drug TGFß or WGFE addition exhibited the same methylene blue absorbance values as observed in pre/concurrent control cultures, demonstrating the consistency of the survival assay procedure (Figure 3.6 A). Incubation with 3 ng/ml rTGFß$_2$, or equivalent inhibitory concentrations of the acid and retentate WGFE fractions, for 30 h slightly reduced absorbance values from 1.34 units to 1.14 units in the absence of drug exposure. In contrast, the control and permeate WGFE fractions did not inhibit cell growth to any measurable extent.

The addition of each of the growth factor treatments for 30 h immediately following 1µM etoposide exposure also reduced methylene blue staining, but had no negative effects on cultures incubated with 50 nM vinblastine. When absorbance values were converted to percentage survival it became evident that none of the growth factor preparations mediated enhanced survival or regenerative responses (Figure 3.6 B).

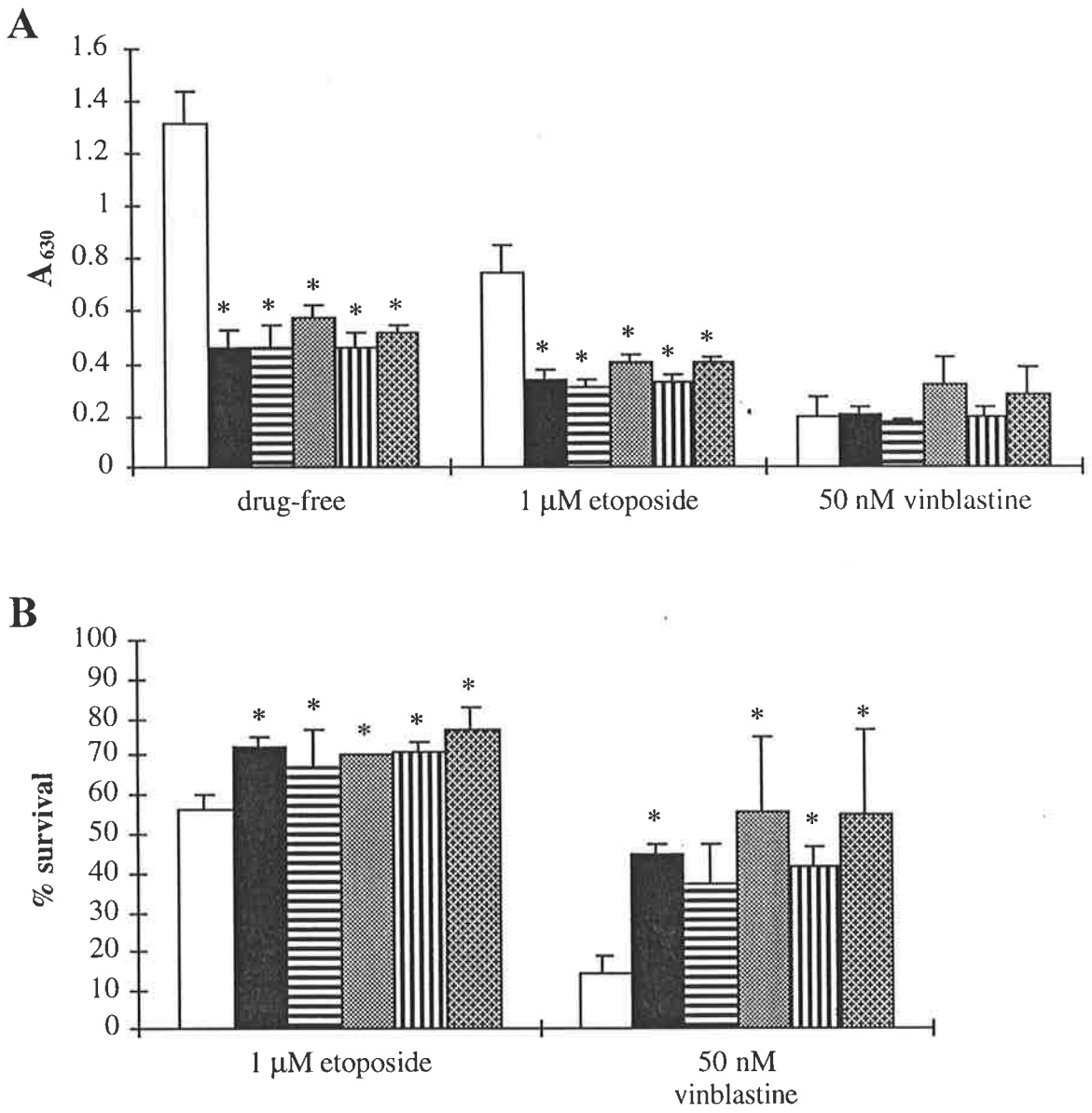


Figure 3.5 Effect of pre/concurrent WGFE treatments on cell survival

Cell survival was measured in Mv1Lu cultures following the addition of control medium (□), rTGFß$_2$ (■), control WGFE (▤), acid WGFE (■), permeate WGFE (▥) or retentate WGFE (▩) for 48 h, beginning 30 h prior to chemotherapeutic drug addition. All treatments were added at a concentration which provided equivalent growth inhibition to 3 ng/ml rTGFß$_2$ in a Mv1Lu growth inhibition bioassay. Survival was assessed by staining fixed cultures with methylene blue, after analysis by alamarBlue. The absorbance of each culture was measured at 630 nm **(A)**. A_{630} values were converted to % survival by calculating the ratio of absorbance values in the drug-exposed versus drug-free cultures for each pre/concurrent treatment **(B)**. Values represent the mean ± SD from three independent experiments (n = 6 replicates/experiment). *, $P < 0.05$; compared to control-treated cultures.

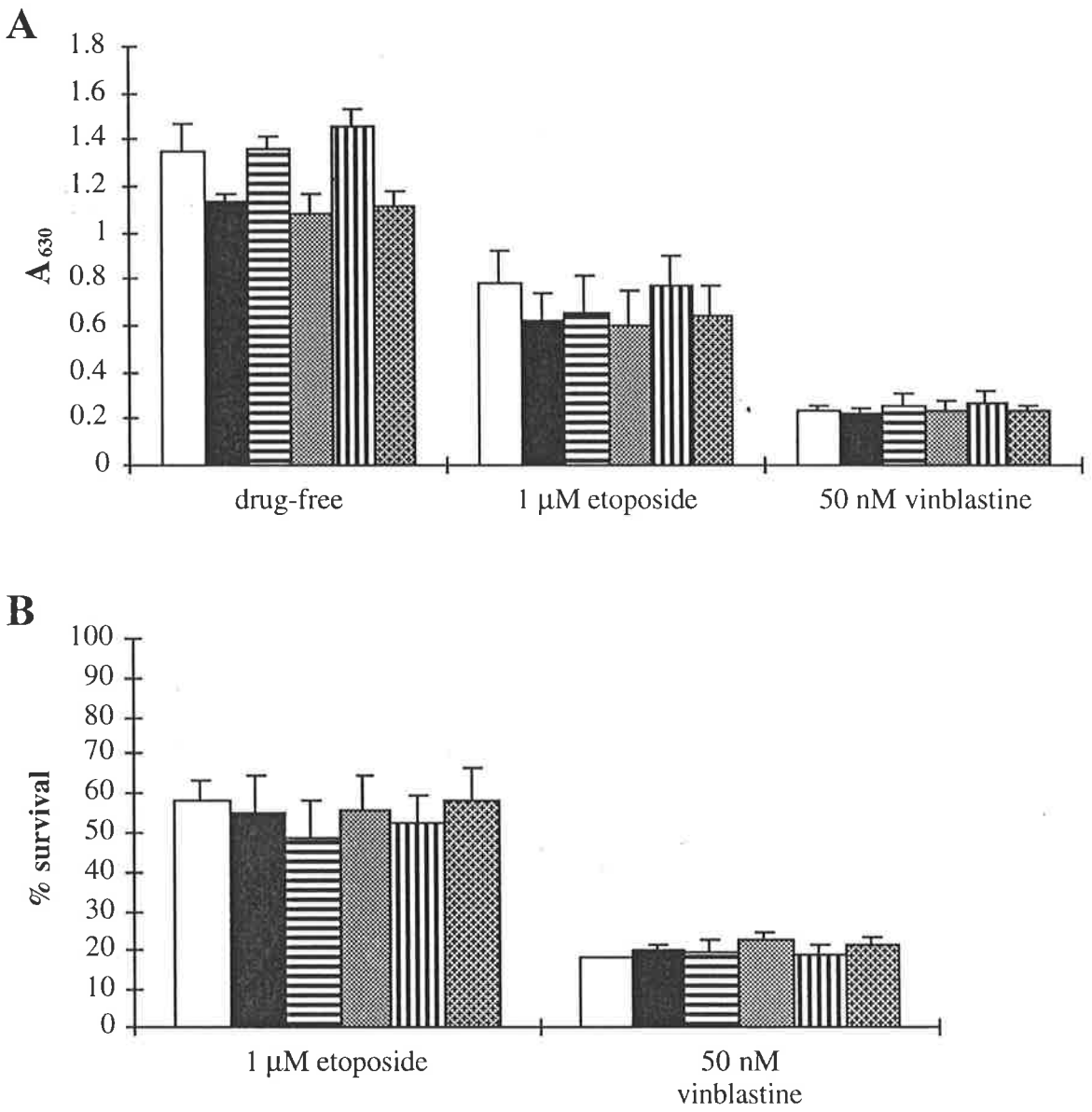


Figure 3.6 Effect of post-drug treatment with WGFE on cell survival

Cell survival was measured in Mv1Lu cultures following the addition of control medium (□), rTGFß$_2$ (■), control WGFE (▤), acid WGFE (▩), permeate WGFE (▥) or retentate WGFE (▦) for 30 h, beginning immediately after removal of chemotherapeutic drugs from the culture medium. All treatments were added at a concentration which provided equivalent growth inhibition to 3 ng/ml rTGFß$_2$ in a Mv1Lu growth inhibition bioassay. Survival was assessed by staining fixed cultures with methylene blue, after analysis by alamarBlue. The absorbance of each culture was measured at 630 nm (**A**). A_{630} values were converted to % survival by calculating the ratio of absorbance values in the drug-exposed versus drug-free cultures for each post-treatment (**B**). Values represent the mean ± SD from three independent experiments (n = 6 replicates/experiment).

3.3.3.6 WGFE-mediated survival at different drug concentrations

Previous studies in this chapter focussed on the protection conferred by WGFE fractions at a single concentration of each chemotherapy agent - 1 μM etoposide and 50 nM vinblastine. As the magnitude of the cell survival response mediated by pre/concurrent growth factor treatment was greater following 50 nM vinblastine exposure than 1 μM etoposide exposure, I predicted that survival would become more evident as drug toxicity increased. To determine whether the protection afforded by rTGFß$_2$ and the WGFE fractions was in fact similar across all concentrations of a given drug, I selected five doses of both etoposide and vinblastine which spanned the drug dose response curves generated in Chapter 2.

Etoposide

Etoposide toxicity was dose-dependent in the absence of rTGFß$_2$ or WGFE treatment, as indicated by the reduction in methylene blue absorbance values from 1.37 units in the absence of drug to 0.44 units in the presence of 2μM etoposide (Figure 3.7 A). At the highest etoposide concentration tested, cell survival fell to 32% of that measured in cultures maintained in drug-free medium (Figure 3.7 B).

Recombinant TGFß$_2$ and all of the WGFE fractions inhibited the growth of Mv1Lu cells cultured without etoposide, as illustrated by a reduction in A_{630} values to approximately 0.5 units in all cases. In contrast to the marked decline in absorbance values seen in control-treated cultures incubated with increasing concentrations of etoposide, cell number fell only slightly in cultures containing rTGFß$_2$ or WGFE fractions. Accordingly, survival was sustained above 50% with all growth factor treatments added 30 h prior to 2 μM etoposide exposure. The protective effect of the acid and retentate WGFE fractions was significantly greater than that of control cultures at a concentration of etoposide of 1 μM or above. All treatments significantly enhanced Mv1Lu cell survival in the presence of 2 μM etoposide. Thus, it was concluded that the TGFß and WGFE treatments protected cultures at etoposide concentrations inducing at least 50% toxicity, with the largest increase in protection observed at the dose of etoposide which

induced the greatest cell loss.

Vinblastine

Vinblastine also induced dose-dependent increases in cell loss (Figure 3.8 A), with maximally toxic concentrations allowing cell survival of less than 10% in cultures containing no growth factor treatments (Figure 3.8 B). As observed with etoposide exposure, the addition of increasing concentrations of vinblastine to cultures containing rTGFß$_2$ or a WGFE fraction resulted in only small reductions in methylene blue absorbance values. Recombinant TGFß$_2$ and all of the WGFE fractions mediated survival greater than that of the control cultures at vinblastine concentrations of 2.0 nM and above. Thus, the ED_{50} concentration of vinblastine was increased by pre/concurrent treatment with rTGFß$_2$ or the WGFE preparations. Protection mediated by these treatments was evident at concentrations of vinblastine which induced at least 60% toxicity, however the magnitude of the protection did not diverge with increasing drug concentration. Instead, it appeared that the differences in cell survival observed between the treated and untreated cultures remained relatively constant at all drug concentrations tested above 2 nM vinblastine.

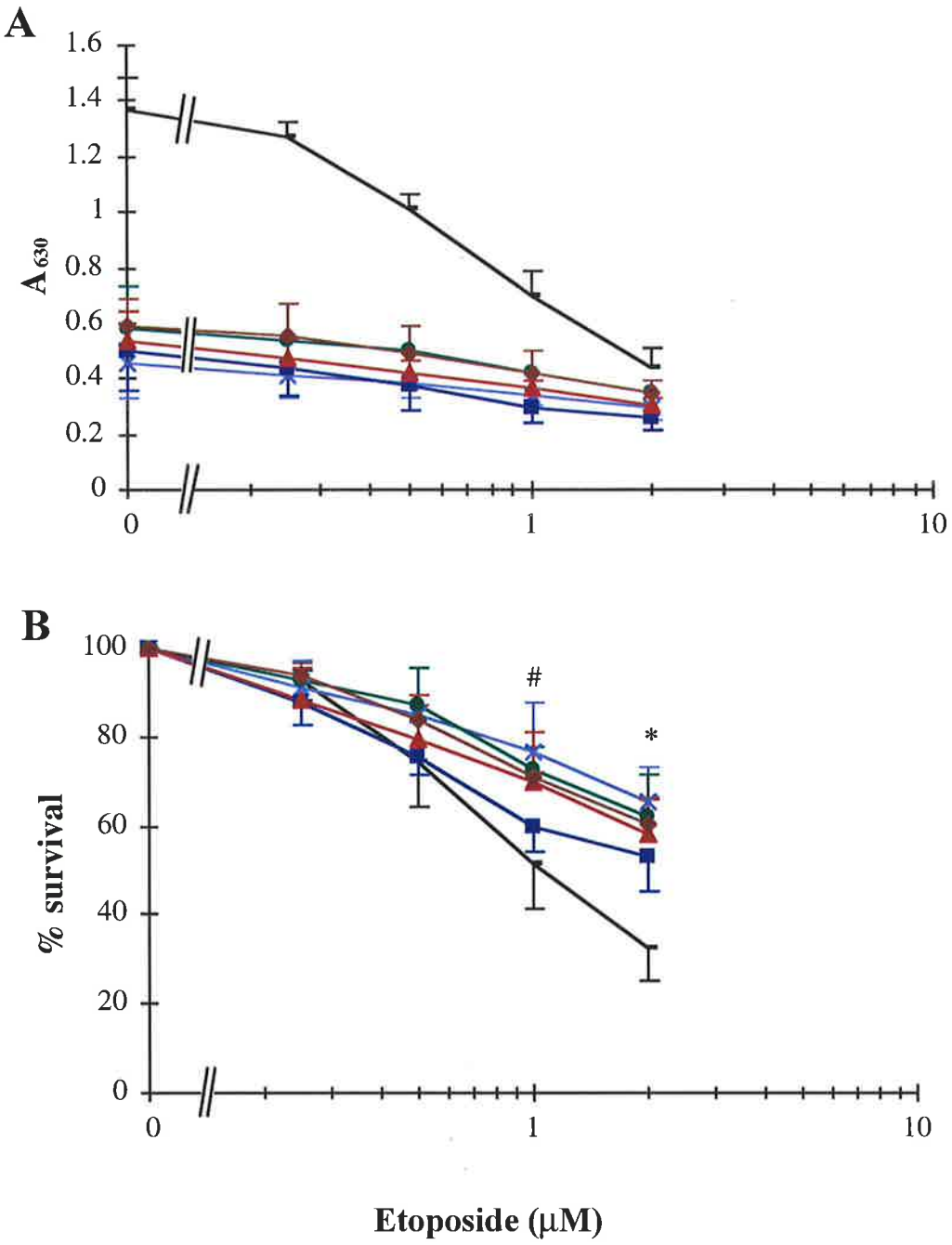


Figure 3.7 Effect of etoposide concentration on WGFE-mediated cell survival

Cell survival was measured in Mv1Lu cultures following the addition of control medium (-), rTGFß$_2$ (✕), control WGFE (■), acid WGFE (●), permeate WGFE (▲) or retentate WGFE (◆) for 48 h, beginning 30 h before chemotherapeutic drug exposure. All treatments were added at a concentration which provided equivalent growth inhibition to 3 ng/ml rTGFß$_2$ in a Mv1Lu growth inhibition bioassay. Etoposide was added for 18 h at the concentrations indicated. The absorbance of each culture was measured at 630 nm following staining with methylene blue **(A)**. A_{630} values were converted to % survival by calculating the ratio of absorbance values in the drug-exposed versus drug-free cultures for each pre/concurrent treatment **(B)**. Values represent the mean ± SD from three independent experiments (n = 6 replicates/experiment). *, $P < 0.05$, all treatment groups compared to control. #, $P < 0.05$, rTGFß$_2$, acid, permeate and retentate WGFE fractions compared to control.

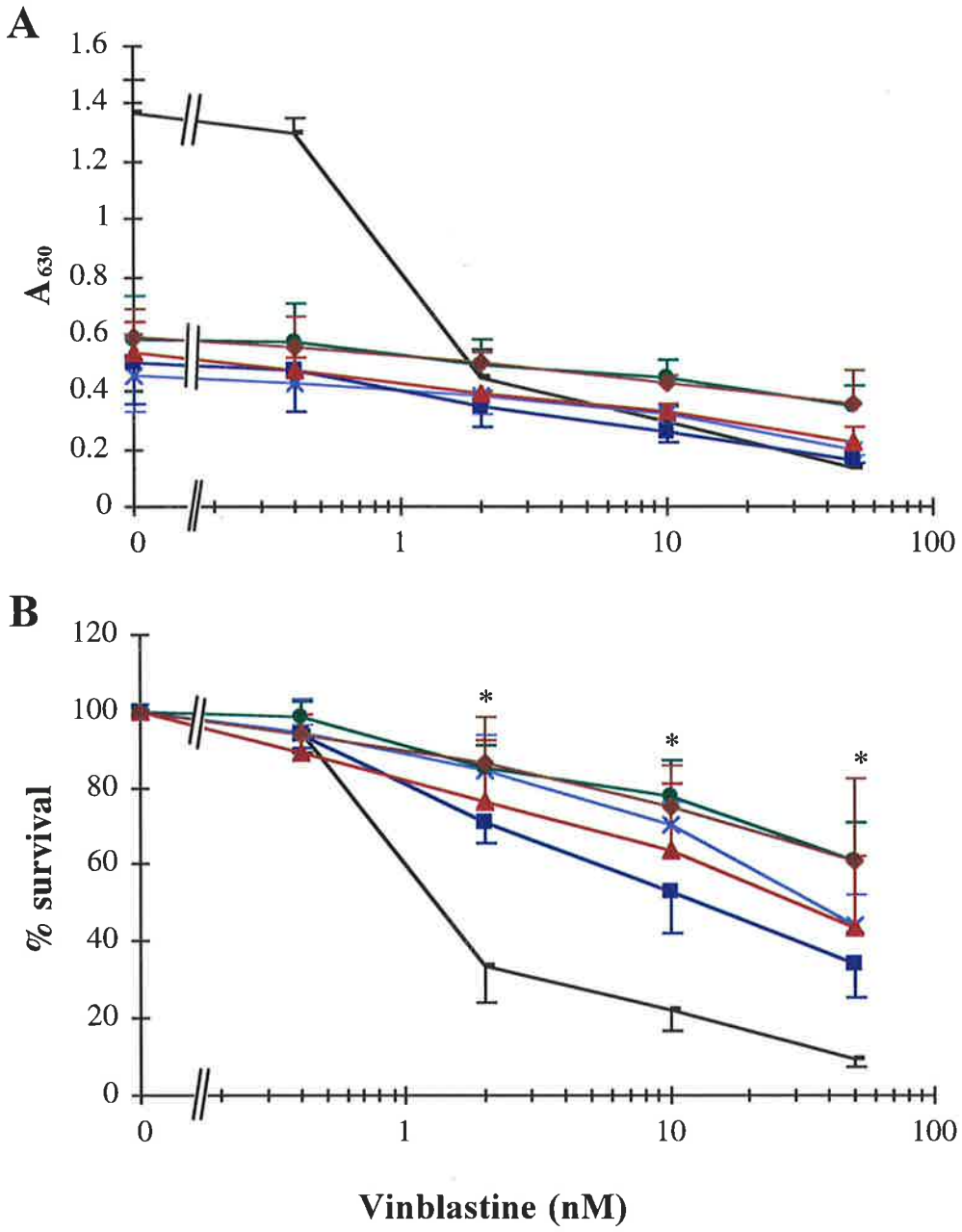


Figure 3.8 Effect of vinblastine concentration on WGFE-mediated cell survival

Cell survival was measured in Mv1Lu cultures following the addition of control medium (-), rTGFß$_2$ (✕), control WGFE (■), acid WGFE (●), permeate WGFE (▲) or retentate WGFE (◆) for 48 h, beginning 30 h before chemotherapeutic drug exposure. All treatments were added at a concentration which provided equivalent growth inhibition to 3 ng/ml rTGFß$_2$ in a Mv1Lu growth inhibition bioassay. Vinblastine was added for 18 h at the concentrations indicated. The absorbance of each culture was measured at 630 nm following staining with methylene blue (**A**). A_{630} values were converted to % survival by calculating the ratio of absorbance values in the drug-exposed versus drug-free cultures for each pre/concurrent treatment (**B**). Values represent the mean ± SD from three independent experiments (n = 6 replicates/experiment). #, $P < 0.05$, acid and retentate WGFE fractions compared to control. *, $P < 0.05$, all treatments compared to control. #, $P < 0.05$, acid and retentate WGFE fractions compared to control.

3.3.3.7 Dose-dependent WGFE-mediated survival at a single drug concentration

WGFE-mediated survival effects were also examined using one concentration of each chemotherapy agent to test the dose responsiveness of Mv1Lu cells to the growth factor treatments. For direct comparison, WGFE preparations were expressed in terms of their TGFß equivalent growth inhibitory activity, as measured in Table 3.2. As in previous experiments, cell survival was expressed as the proportion of methylene blue absorbance values in the drug-exposed versus drug-free cultures for each pre/concurrent growth factor treatment.

Etoposide

Pre/concurrent treatment of Mv1Lu cultures with recombinant TGFß$_2$, beginning 30 h prior to the addition of 1 µM etoposide, induced a dose-dependent increase in cell survival from 52.3% in the absence of rTGFß$_2$ to 74.1% at a concentration of 3 ng/ml (Figure 3.9 A). Several of the WGFE preparations, specifically the acid, permeate and retentate fractions, promoted similar survival responses to rTGFß$_2$ at each of the dilutions examined. However, the control WGFE fraction induced significant increases in cell survival at low TGFß equivalent concentrations, where none of the other preparations showed any cytoprotective effect. At the 3.0 ng/ml TGFß equivalent concentration the control WGFE increased Mv1Lu cell survival from a baseline value of 55.0% up to 68.5%. Similar levels of survival were sustained at all of the lower concentrations tested. The protection mediated by the control WGFE fraction was significantly greater than that observed with equivalent growth inhibitory doses of rTGFß$_2$ at concentrations of 0.024 and 0.12 ng/ml TGFß equivalence. Therefore, the cytoprotective properties of control WGFE did not appear to entirely depend upon growth inhibitory activity, as was clearly the case with the rTGFß$_2$ preparation.

Vinblastine

The addition of recombinant TGFß$_2$ to Mv1Lu cultures, beginning 30 h before exposure to 50 nM vinblastine, induced a dose-dependent increase in cell survival from 11.6% in the absence of rTGFß$_2$ to 45.1% at a peptide concentration of 3 ng/ml (Figure 3.9 B). Only the permeate

WGFE fraction promoted similar survival responses to $rTGF\beta_2$ at each of the dilutions examined. The acid and retentate WGFE mediated TGFß-like protection against vinblastine toxicity at the lower concentrations tested, but displayed a trend towards inducing greater survival at the 3.0 ng/ml TGFß equivalent dose. As observed with cultures exposed to etoposide, the control WGFE fraction mediated distinct increases in cell survival at low TGFß equivalent concentrations. This cytoprotection reached maximal effect at the 0.6 ng/ml TGFß equivalent concentration, inducing 68.0% survival. Whilst the magnitude of the survival response mediated by the control WGFE fraction was attenuated at lower concentrations, the survival remained significantly higher than was produced by equivalent growth inhibitory concentrations of $rTGF\beta_2$. Thus, I concluded that the control WGFE fraction, and possibly the acid and retentate fractions, contained material in addition to TGFß which was capable of stimulating Mv1Lu cell survival against vinblastine toxicity. This material did not appear to depend upon growth inhibitory activity to mediate survival responses against either etoposide or vinblastine, yet was most effective when administered as a pre/concurrent treatment.

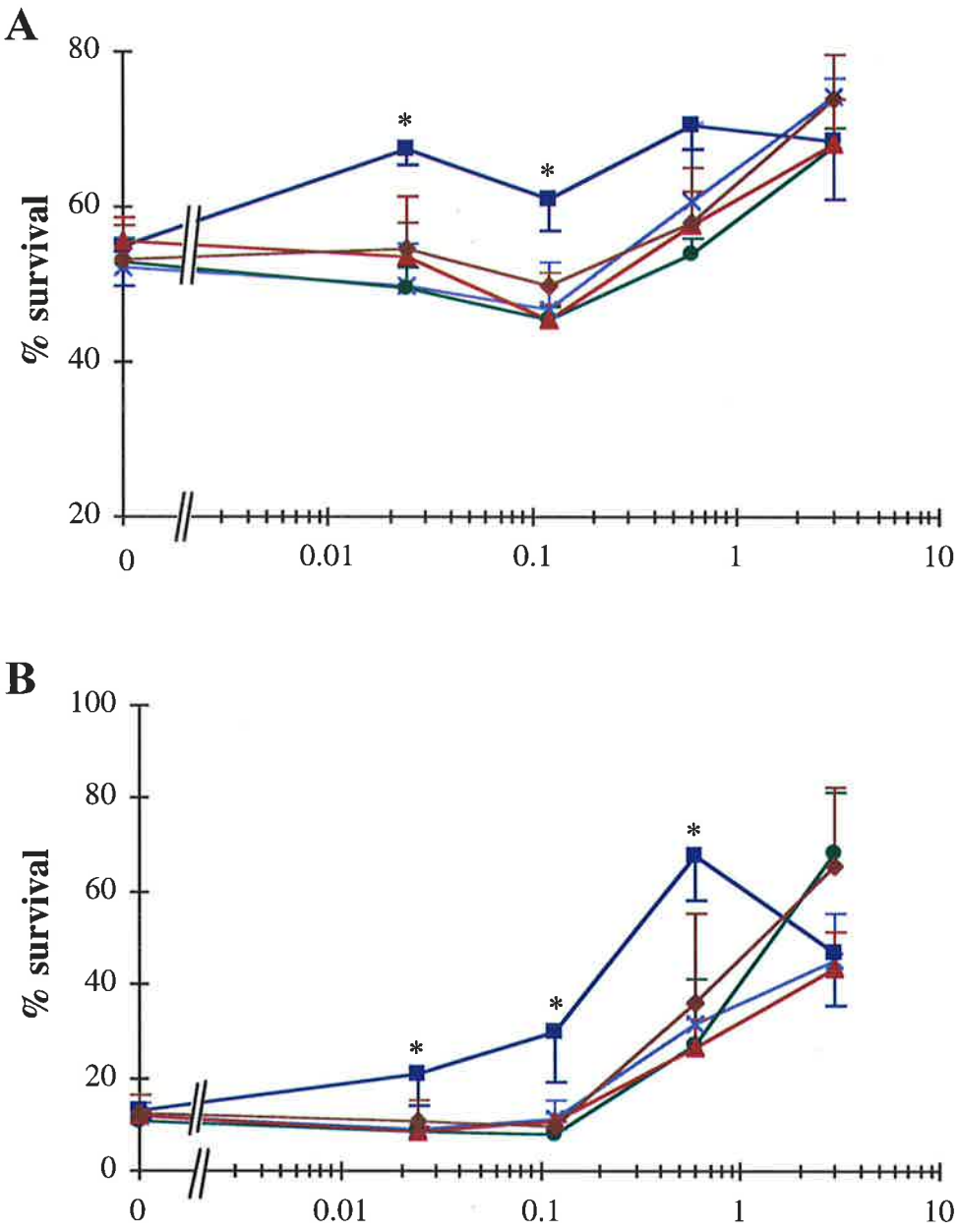


Figure 3.9 Dose-dependent WGFE-mediated protection against drug toxicity

Cell survival was measured in Mv1Lu cultures following the addition of rTGFß$_2$ (✖), control WGFE (■), acid WGFE (●), permeate WGFE (▲) or retentate WGFE (◆) for 48 h, beginning 30 h before exposure to 1 µM etoposide (A) or 50 nM vinblastine (B). All treatments were added at concentrations which provided equivalent growth inhibition to rTGFß$_2$ in a Mv1Lu growth inhibition bioassay. The absorbance of each culture was measured at 630 nm following staining with methylene blue. A_{630} values were converted to % survival by calculating the ratio of absorbance values in the drug-exposed versus drug-free cultures for each pre/concurrent treatment. Values represent the mean ± SD from three independent experiments (n = 4 replicates/experiment). *, $P < 0.05$, control WGFE compared to rTGFß$_2$. #, $P < 0.05$, acid and retentate WGFE fractions compared to rTGFß$_2$.

3.4 Discussion

Protection against chemotherapy drug-induced toxicity has been characterised for a number of growth factors known to be present in WGFE, namely TGFß (McCormack *et al.,* 1997) and IGF-I (Sell *et al.,* 1995). Transforming growth factor ß induces growth inhibition in the G_1 phase of the cell cycle, providing a potential mechanism by which cells can avoid drug toxicity specifically targeted to the S, G_2 and M phases. In contrast, over-expression of the IGF-I receptor promotes an IGF-I-mediated enhancement of cell survival unrelated to phase arrest or mitogenesis (Sell *et al.,* 1995).

Preliminary studies in this chapter characterised the concentrations of $TGFß_2$ and IGF-I peptides present in each of the WGFE fractions. Whilst other growth factors, such as $TGFß_1$, IGF-II and PDGF, may have also contributed to cytoprotective responses, analysis of these two peptides provided an indication of the effects of acidification and membrane partitioning on the WGFE growth factor composition. Thus, the fractions with the greatest concentration of $TGFß_2$ also proved to be the most growth inhibitory. Recombinant EGF has been shown to counteract the effects of TGFß in a Mv1Lu growth inhibition assay (Garrigue-Antar *et al.,* 1995; Kelley *et al.,* 1992). Therefore the EGF-like peptide recently characterised in the permeate WGFE (Dunbar *et al.* 1997), and which is presumably also present in the control WGFE, may antagonise the TGFß-mediated anti-proliferative activity of these fractions. Additionally, the presence of latent TGFß complexes in the control WGFE may also contribute to the reduced inhibitory activity of this fraction.

Whilst the overall pattern of TGFß activation and separation measured by growth inhibition bioassay was similar to that observed with the ELISA, the actual values were 3 - 5-fold higher for the acid, permeate and retentate fractions. Three scenarios may account for this discrepancy between bioassay and ELISA values. Firstly, there may have been non-TGFß material present in the WGFE fractions which possessed growth inhibitory activity in a Mv1Lu bioassay. If this material was activated by acidification, it would have elevated the equivalent TGFß activities of

these WGFE fractions without increasing the concentrations of immuno-reactive $TGF\beta_2$. Secondly, the latent TGFß complexes present in the control WGFE may have consisted of predominantly $TGF\beta_1$ isoform peptides. Thus, acidification of the WGFE fractions would again increase TGFß equivalent activity in a bioassay, but would not have resulted in increased recognition in a $TGF\beta_2$ ELISA. Finally, the presence of recombinant human latency associated peptide (LAP) is known to interfere with $TGF\beta_2$ measurements from cell culture supernatant samples with the particular ELISA kit used. It is therefore plausible that the LAP, released from the TGFß by acidification, may have prevented the accurate quantification of $TGF\beta_2$ peptides. Irrespective of this discrepancy, it was the actual growth inhibitory activity of the WGFE fractions which was of primary relevance, as I had predicted that WGFE fractions which induced equivalent growth inhibition would also induce equivalent survival responses.

The experiments described in this chapter were designed to examine the efficacy of all four WGFE fractions in enhancing Mv1Lu cell survival following exposure to etoposide or vinblastine. Whilst the WGFE fractions were initially compared at concentrations which induced maximal growth inhibition in a Mv1Lu bioassay, these concentrations did not all produce equivalent levels of either G_1 phase arrest or inhibition of BrdU incorporation DNA at an intermediate time point (30 h). The slower G_1 phase arrest induced by the control and permeate WGFE fractions suggested that the reduced ratio of active TGFß peptides to other, possibly antagonistic, peptides in these fractions may contribute to poorer survival responses. Pre/concurrent addition of these fractions to both the etoposide and vinblastine survival assays did indeed promote less survival than either the acid or retentate WGFE fractions. The reduced concentrations of IGF-I in the acid and retentate WGFE fractions thus argued against a primary role for IGF peptides in enhancing Mv1Lu cell survival. In addition, the lack of enhanced survival observed with all post-drug treatments suggested that the WGFE fractions acted primarily as cytoprotective agents, dependent upon the growth arrest induced by TGFß, rather than as regenerative treatments acting to stimulate mitogenesis or repair of pre-existing DNA damage. Similar observations have been reported by Potten *et al.* (1997a), where post-treatment of radiation-induced damage with $TGF\beta_3$ reduced crypt integrity, whereas $TGF\beta_3$ pre-

treatment induced the opposite effect. These results support the hypothesis that TGFß, or preparations acting through a predominantly growth inhibitory pathway, are most effective when given prior to drug exposure, rather than after the accumulation of lethal damage and induction of an apoptotic program.

Concentrations of recombinant $TGFß_2$ which induced growth inhibition in the Mv1Lu bioassay also improved cell survival following chemotherapy drug exposure, suggesting that the G_1 phase arrest induced by this peptide was mechanistically responsible for improving survival. The optimal survival observed when $rTGFß_2$ was added as a pre/concurrent treatment, as opposed to a post-drug treatment, suggests that this mechanism may be associated with the restriction of cells from those phases specifically targeted by the chemotherapy agents. Alternatively, TGFß could act to delay damaged cells at the G_1 cell cycle checkpoint whilst DNA repair was undertaken. However, such a mechanism would be more likely to be operate following drug exposure, once the cell population was released from drug-induced phase arrest in the S, G_2 or M phases.

Extension of the pre/concurrent survival assays to include various drug concentrations indicated that all of the WGFE fractions increased the concentrations of both etoposide and vinblastine required to produce an ED_{50} response. The addition of $rTGFß_2$ or any of the WGFE fractions restricted the proportion of cells dying from drug exposure, as reported previously for $TGFß_3$ treatment (McCormack *et al.*, 1997). In addition, the acid and retentate WGFE fractions reduced the susceptibility of the Mv1Lu cultures at maximally toxic vinblastine concentrations, resulting in an absolute increase in surviving cell number. The differences in the magnitude of enhanced survival observed between the two drugs at equimolar concentrations probably reflects differences in the mechanisms of action of the two drugs, and their true cell cycle specificity. Whilst etoposide and vinblastine are recognised as targeting cells in the G2 and M phases, at high concentrations their effects may not be fully excluded from other phases of the cell cycle (Chow and Ross, 1987; Madoc-Jones and Mauro, 1968).

Dilution of the WGFE fractions in the absence of chemotherapy drugs resulted in the generation of similar dose response curves to $rTGF\beta_2$. Thus, although the cytotoxic actions of etoposide and vinblastine are dependent on the density of the cell culture at the time of drug addition, this factor could not account for the differences in protection mediated by the WGFE fractions and $rTGF\beta_2$. The density-dependent toxicity effects defined in the previous chapter also led to the under-estimation of the survival detected within a WGFE treatment group, as the predicted drug toxicity would have increased in response to a reduction in density caused by growth inhibition.

In the absence of chemotherapy drugs, the permeate WGFE fraction required a longer period of exposure than did the acid or retentate WGFE fractions to induce TGFß-equivalent G_1 phase arrest, possibly due to the relatively high concentrations of IGF-I and other mitogenic factors it contained. However, this fraction behaved in a TGFß-like manner when mediating cytoprotection against drug toxicity, indicating that these mitogenic factors did not enhance the overall survival response. The growth inhibitory activity of the control WGFE fraction was also impaired by the presence of antagonistic peptides, rendering the treated population less cell cycle-arrested at the time of drug addition. In contrast to the permeate WGFE, the control WGFE fraction induced greater survival responses than equivalent growth inhibitory concentrations of $rTGF\beta_2$. This survival activity occurred predominantly at concentrations which produced sub-optimal or no growth inhibition in Mv1Lu cells, and appeared to be unrelated to the dilution of growth inhibitory activity. Importantly, at protein concentrations less than 0.154 µg/ml, the control WGFE fraction was capable of mediating absolute increases in cell number.

The activation and concentration of TGFß in the acid and retentate WGFE fractions resulted in the requirement for only small amounts of protein to produce equivalent growth inhibition to $rTGF\beta_2$. Therefore, if the novel survival factor was not enhanced by transient acidification, less of the peptide would have been present in the acid and retentate WGFE dilutions used. The fact that these fractions did not produce increases in cell survival similar to those observed with low

dilutions of the control WGFE tend to suggest that this unknown factor was not acid-activated. The acid and retentate WGFE fractions did, however, mediate increases in cell survival at concentrations consistent with a TGFß response, confirming the contribution of TGFß to WGFE-mediated survival. There was some indication that these fractions also possessed additional survival activity at highest dilution tested, in that they protected cells against vinblastine toxicity more effectively than the equivalent dose of $rTGF\beta_2$.

Whilst each of the WGFE fractions possessed a different growth factor profile, comparison between the preparations on a functional basis showed that the survival mediated by the acid, permeate and retentate WGFE fractions could mostly be attributed to their growth inhibitory activity. In contrast, the survival activity of the control WGFE preparation could not be accounted for in such a manner. Thus, my hypothesis that WGFE mediated protection against drug toxicity would be greatest in those fractions which induced the most potent growth inhibition following a 30 h incubation was proven to be incorrect. The apparent presence of additional non-TGFß survival activity in the control WGFE fraction thus suggested two main questions. Firstly, was this residual activity dependent upon growth arrest to mediate cytoprotection, and could it be separated from the TGFß component of the control WGFE fraction. Secondly, was the TGFß component of the control WGFE likely to be responsible for the reduced severity of drug-induced damage observed *in vivo*.

Subsequent experimental work was designed to examine WGFE-mediated cell survival *in vitro*, using a battery of recombinant growth factors known to be present in WGFE. Additional strategies involve the use of TGFß immuno-neutralisation and gel filtration chromatography to investigate the novel survival activity. Further studies then detail an assessment of the growth inhibitory activity of a control WGFE preparation in the crypts of the small intestine following an oral administration schedule.

Chapter Four

Investigation of non-TGFß mediated survival against drug toxicity

4. *Investigation of non-TGFß mediated survival against drug toxicity*

Contribution to the work

The preliminary investigation of the neutralising capacity of the anti-TGFß antibody was performed by Ms C Payne. All other work described in this chapter was performed by myself.

Abstract

Transforming growth factor ß (TGFß) has been shown by myself and others to mediate protection against chemotherapy drug-induced toxicity in epithelial cell populations. The control WGFE fraction contains small amounts of active TGFß which may contribute to the survival response observed when this preparation is added to Mv1Lu cultures prior to drug exposure. However, this WGFE fraction appears to contain additional survival activity which does not rely upon the induction of growth arrest to mediate cytoprotective effects. In this chapter I show that immuno-neutralisation of the TGFß component of both the control and acid WGFE fractions, by co-incubation with 15 µg/ml neutralising antibody, completely abolished growth inhibition. Although Mv1Lu cultures then grew normally in the absence of drug, both of the WGFE fractions retained residual survival activity against 50 nM vinblastine. Examination of the cytoprotective properties of a number of growth factors known to be present in the WGFE material failed to account for the additional survival activity. Thus, recombinant IGF-I, PDGF and EGF peptides did not affect cell survival when added to Mv1Lu cultures as a pre/concurrent treatment, although post-drug treatment with 10 ng/ml EGF promoted enhanced mitogenesis. Physical separation of TGFß from the residual survival activity present in the control WGFE fraction was attempted by size exclusion chromatography. Under neutral pH conditions, the growth inhibitory and survival activities partially co-eluted from the column.

Transient post-acidification of neutral pH fractions increased their growth inhibitory activity without inducing a concomitant increase in survival activity. Elution of control WGFE at acidic pH (2.8) resulted in the almost complete loss of survival activity, although growth inhibitory activity remained extremely potent. These studies confirmed the presence of non-TGFß survival factors in the control WGFE preparation, and suggests that their activity is either masked or destroyed by acidification of the WGFE. I also conclude that IGF-I, PDGF and EGF do not contribute individually to the survival response induced by WGFE, although they may synergise with other uncharacterised peptides.

4.1 Introduction

The studies reported in chapter 3 showed that control WGFE mediated a level of survival which could not be accounted for by the estimated contribution of growth inhibitory peptides. Whilst TGFß was able to enhance cell survival following drug exposure, it did so at concentrations which also induced at least 50% growth inhibition. In contrast, the control WGFE fraction was able to confer protection to Mv1Lu cell cultures at concentrations which induced minimal growth inhibitory responses.

Studies in this chapter employed a neutralising antibody directed against a pan-specific epitope of TGFß isotypes 1, 2 and 3 to confirm the presence of residual survival activity. The verification of non-TGFß-mediated survival factors in both the control and acid WGFE fractions then led to an investigation of the cytoprotective activity of a number of growth factors, some of which were known to be present in WGFE. IGF-I is known to enhance cell survival against a number of toxic stimuli, including hypoxic injury (Gluckman *et al.,* 1992) and cytotoxic drug exposure (Sell *et al.,* 1995). PDGF is also able to protect cells against drug toxicity (Harrington *et al.,* 1994), possibly through the induction of increased IGF-I receptor expression (Boyle and Hickman, 1997). Harrington *et al.* (1994) have recently reported that the cytoprotective properties of both PDGF and IGF-I occur independently of mitogenic activity. For IGF-I, this may result from the activation of physically distinct domains within the IGF-I receptor (O'Connor *et al.,* 1997). However, the mitogenic activity of both the IGF-I and PDGF peptides may also contribute towards improving survival responses by modulating colony re-population from stem cells which are able to resist drug damage.

Whilst EGF is not found in bovine milk, a related peptide has recently been characterised in the permeate WGFE fraction (Dunbar *et al.*, 1997). When comparing all of the WGFE fractions on the basis of their equivalent growth inhibitory activity, the permeate WGFE elicited the response most closely resembling that of rTGFß$_2$. Although this suggested that the EGF-like peptide did not contribute to Mv1Lu survival when added as a pre/concurrent treatment, it was

plausible that such a growth factor might promote increased mitogenesis when applied following drug exposure.

The inability of these three peptides to account for the balance of the survival activity not attributable to TGFß prompted further investigation of the properties of the putative survival factor. Control WGFE was therefore fractionated by size exclusion chromatography under both neutral and acidic pH conditions in an attempt to physically separate the TGFß growth inhibitory activity from the non-TGFß survival activity and provide material suitable for the characterisation of novel survival factor(s).

4.2 Materials and Methods

4.2.1 Immuno-neutralisation of TGFß in WGFE fractions

Neutralisation of the TGFß activity in the WGFE fractions was achieved by the inclusion of a pan-specific neutralising antibody directed against TGFß. Mv1Lu cells were seeded in 96-well plates (20 x 10^4 cells/ml in 0.1 ml volumes) and incubated for 24 h under standard conditions (Section 2.2.1). Growth medium was then aspirated and replaced with fresh medium. The fresh medium included 154 µg/ml control WGFE (containing the equivalent growth inhibitory activity of 0.6 ng/ml TGFß) or 13.0 µg/ml acid WGFE (containing the equivalent growth inhibitory activity of 3.0 ng/ml TGFß). An equivalent dose of $rTGFß_2$ was included in each assay for comparison with the WGFE fractions. TGFß-mediated growth inhibitory activity was neutralised by the concurrent addition of a pan-specific mouse IgG_1 anti-$TGFß_{1,2,3}$ antibody (Genzyme) during TGFß or WGFE incubation. An isotypic control antibody directed against the inorganic compound tri-nitrophenol (PharMingen) was also included in each assay. Assays were performed using an 18 h exposure to 50 nM vinblastine, following the pre/concurrent survival assay protocol described in Materials and Methods, Chapter 3. At the conclusion of each assay, experimental analysis was performed by methylene blue staining of the surviving cell monolayer (Section 2.2.2.3).

4.2.2 Screening of recombinant growth factors for survival activity

Recombinant forms of peptides known to be present in the control WGFE material, or with activity similar to those present in control WGFE, were examined for their possible contribution to the uncharacterised survival observed in Chapter 3. Receptor grade recombinant IGF-I was purchased from GroPep Pty. Ltd. EGF and PDGF were obtained from Upstate Biotechnology Inc. All peptides were obtained in lyophilised form and diluted to stock solutions in 10 mM HCl before storage at -20°C. Peptides were subsequently prepared by dilution of the stock solutions directly into culture medium. Survival activity was measured by methylene blue analysis following the survival assay protocols described in "Materials and

Methods", Chapter 3.

4.2.3 Gel filtration chromatography of WGFE

Control WGFE was fractionated by size exclusion chromatography using a Superose 12 HR column (Pharmacia Biotech), under both neutral and acidic pH conditions. Molecular weight markers (Pharmacia Biotech) were also eluted under neutral conditions to establish a range of standards. The column was equilibrated with neutral buffer (50 mM NaH_2PO_4, 150 mM NaCl, 10% (v/v) acetonitrile; pH 7.4) or with acid buffer (150 mM NaCl, 1 M CH_3COOH, 10% (v/v) acetonitrile; pH 2.8).

The WGFE was diluted to a protein concentration of 20 mg/ml in PBS, with 500 µl added to the column. For chromatographic separation under acidic conditions, the control WGFE was acidified to pH 2.5 with concentrated HCl 1 h prior to loading onto the column. WGFE samples were eluted, using either neutral or acid buffer, at 0.3 ml/min with fractions collected at 1 min intervals for 95 min. Fractions were freeze-dried overnight, then grouped into six pools according to the peaks observed in the elution profiles. Fraction numbers were corrected for a 1.3 min delay between absorbance measurement and fraction collection prior to pooling. Grouped fractions were resuspended in a total volume of 2.71 ml/pool with 0.22 µm filtered water. Insoluble material contained in some pools from the acidic pH elution was sedimented (2500 x *g* for 15 min at 4°C) and discarded. Each pool was then sterile filtered through a 0.22 µm membrane and diluted in culture medium to a final volume of 27.1 ml, equivalent to 10 x final concentration. Pools were then aliquotted and stored at -20°C until required.

WGFE samples were also separated under neutral pH conditions to examine survival activity following transient acidification of the neutral separation pools. Eluted fractions were freeze-dried and resuspended in water to 2.71 ml/pool as described above. Each pool was then divided into two equal volumes, with one kept as the neutral pH control and the other transiently acidified. Acidification was performed by the addition of 7 µl of 0.5 M HCl to each 1.355 ml pool volume. Pools remained at pH 2.5 for 2 h before being re-neutralised with 8 µl of 0.5 M

NaOH and clarified by centrifugation (2500 x *g* for 15 min at 4°C). All samples were then sterile filtered through a 0.22 µm membrane and diluted in culture medium to a final volume of 13.55 ml, equivalent to 10 x final concentration. Pools were then aliquotted and stored at -20°C until required.

4.2.4 Assessment of cytoprotective and growth inhibitory activity in chromatographic pools

WGFE pools collected after size exclusion chromatography were tested for their growth inhibitory activity and the ability to protect Mv1Lu cells against vinblastine-induced cell death. For growth inhibition assays, the WGFE pools were diluted 1:1 in culture medium from the 10x concentrated frozen WGFE stocks and added to Mv1Lu cell cultures prepared as described in Section 3.2.3. Recombinant TGFß$_2$ standards (0 - 10 ng/ml) were included with each assay to calculate maximum growth inhibition. Cultures were incubated for 48 h, then washed and fixed for methylene blue analysis of cell number. The reduction in A_{630} values produced with each pool was expressed as a percentage of that produced by 10 ng/ml rTGFß$_2$.

For survival assays, the WGFE pools were diluted 1:9 in culture medium from the 10x concentrated frozen stocks, with further 1:4 serial dilutions. The WGFE pools, each at three dilutions, were added to Mv1Lu cultures which were prepared as described in Section 3.2.6. Cultures were incubated with the pools for 48 h, beginning 30 h prior to the addition of 50 nM vinblastine. Cells were then washed and re-cultured in drug-free medium for a further 30 h. Survival was measured at 630 nm following methylene blue staining of the culture monolayers as previously described (Section 2.2.2.3).

4.2.5 Statistical analysis

Statistical analysis was performed by ANOVA. Where significance was observed ($P < 0.05$), a Bonferroni-Dunn multiple comparisons post-hoc test (1- and 2-factor ANOVA) or specified means comparisons (3-factor ANOVA) was applied.

4.3 RESULTS

4.3.1 Survival mediated by TGFß-neutralised WGFE

4.3.1.1 Characterisation of the neutralising antibody

Preliminary investigations into the neutralising capacity of the anti-TGFß$_{1,2,3}$ antibody were undertaken to determine the minimum amount of antibody required to achieve complete neutralisation of TGFß-mediated growth inhibition. The neutralising capacity of the antibody directed against a TGFß$_{1,2,3}$ pan-specific epitope was tested using a permeate WGFE fraction which contained the equivalent growth inhibitory activity of 10 - 20 ng TGFß/mg protein. In the absence of antibody, a 48 h incubation with the permeate WGFE induced dose-dependent growth inhibition of Mv1Lu cells, evident from a protein concentration of 4.7 µg/ml (Figure 4.1). In the presence of 10 µg/ml neutralising antibody, growth inhibition was prevented in cultures co-incubated with permeate WGFE up to a protein concentration of 149 µg/ml. Higher concentrations of the permeate WGFE contained sufficient growth inhibitory activity to overcome the neutralising effects of the antibody. These results suggested that 10 µg/ml of the anti-TGFß$_{1,2,3}$ antibody was capable of neutralising growth inhibition equivalent to that produced by 1.5 - 3.0 ng/ml TGFß.

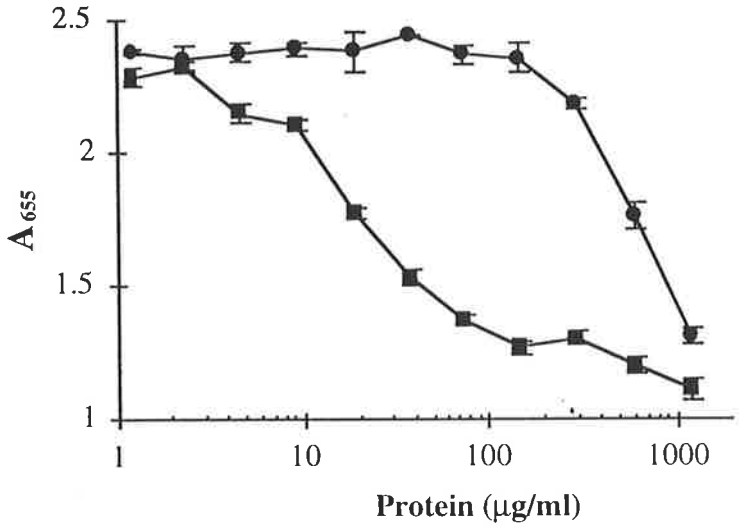


Figure 4.1 Neutralising capacity of anti-TGFß$_{1,2,3}$ antibody

Mv1Lu cells were incubated for 48 h with various concentrations of a permeate WGFE preparation. Cells were simultaneously cultured in the absence (■) or presence (●) of 10 µg/ml pan-specific TGFß$_{1,2,3}$ neutralising antibody. Cultures were assessed spectrophotometrically at A_{655} after being stained with methylene blue. Values are the mean ± SEM from a single experiment (n = 3 replicates).

4.3.1.2 Neutralisation of the TGFß activity in WGFE fractions

A complete analysis of the capacity of the anti-TGFß$_{1,2,3}$ antibody to neutralise growth inhibitory activity was performed using all four of the WGFE fractions characterised for this thesis. Inhibition was measured using a Mv1Lu growth inhibition bioassay, in which cultures were incubated with a concentration of WGFE which produced maximal inhibition, equivalent to 3 ng/ml rTGFß$_2$. Cultures maintained in normal growth medium for the 48 h incubation period produced an A_{630} value of 1.34 units, representing normal proliferative activity and growth during this incubation (Figure 4.2). The inclusion of either 15 µg/ml isotypic control antibody or 15 µg/ml TGFß neutralising antibody had no effect on cell growth in the control cultures.

The addition of 3 ng/ml rTGFß2$_2$ to the Mv1Lu cultures resulted in a significant reduction in final cell number when compared to control cultures. Additionally, cultures incubated with each

of the WGFE fractions were similarly growth inhibited. Co-incubation of all of the cultures with 15 µg/ml isotypic control antibody had no effect on final cell number, indicating that the presence of the antibody did not induce non-specific growth modulation. In contrast, concurrent incubation of the growth inhibited cultures with the pan-specific TGFß$_{1,2,3}$ neutralising antibody resulted in the restitution of proliferative activity equal to that observed in the control-treated cultures. In fact, neutralisation of the TGFß present in the control and permeate WGFE fractions induced slightly greater cell proliferation than was measured in the control cultures, possibly due to the elimination of anti-mitogenic activity.

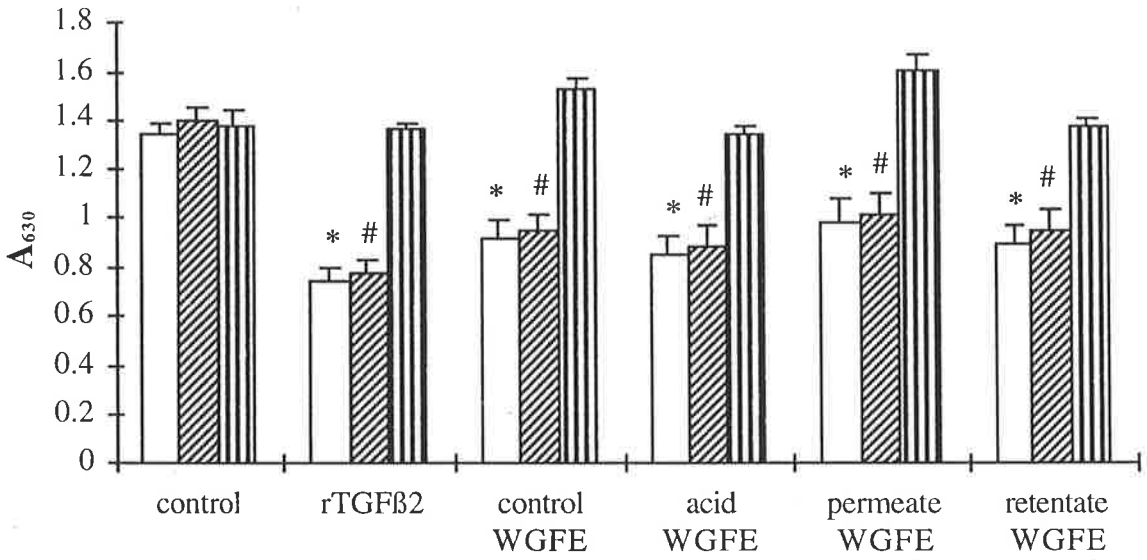


Figure 4.2 Immuno-neutralisation of TGFß-mediated growth inhibition

Growth of Mv1Lu cultures incubated with 3 ng/ml rTGFß$_2$ or equivalent growth inhibitory concentrations of each WGFE fraction. Cultures were concurrently incubated with no antibody (□), 15 µg/ml isotypic control antibody (▨) or 15 µg/ml pan-specific TGFß$_{1,2,3}$ neutralising antibody (▮▮) for 48 h. Growth was measured at 630 nm following methylene blue staining of the culture monolayers. Values represent the mean ± SEM from a single experiment (n = 3 replicates). *, $P < 0.05$, compared to control-treated cultures with no antibody. #, $P < 0.05$, compared to control-treated cultures with isotypic control antibody.

4.3.1.3 Non-TGFß mediated protection against drug toxicity

Control WGFE

The investigation of non-TGFß-mediated survival activity in the control WGFE fraction was carried out at a protein concentration of 0.154 µg/ml. This concentration generated the greatest protection of Mv1Lu cells against 50 nM vinblastine (see Chapter 3), and also produced the greatest difference between the survival observed and that predicted on the basis of TGFß equivalent activity. The addition of $rTGFß_2$ or control WGFE in the absence of 50 nM vinblastine inhibited the growth of Mv1Lu cells (Figure 4.3), as expected from previous experiments. The inclusion of 15 µg/ml isotypic control antibody during control, WGFE or $rTGFß_2$ incubation did not significantly affect cell growth, whilst the neutralising $TGFß_{1,2,3}$ antibody (also at 15 µg/ml) restored the growth of WGFE and $rTGFß_2$ supplemented cultures back to control levels, consistent with studies presented in Figure 4.2.

In the absence of added $rTGFß_2$ or control WGFE, exposure of Mv1Lu cultures to 50 nM vinblastine for 18 h reduced cell number from 1.363 to 0.181 absorbance units, equivalent to the survival of only 10.6 ± 4.2% of the drug-free population. The inclusion of either the isotypic or neutralising antibody did not significantly alter the number of surviving cells. Incubation with 0.6 ng/ml $rTGFß_2$, beginning 30 h before addition of vinblastine, did not increase the yield of cells surviving drug exposure when compared to untreated cultures. As observed with the control-treated cultures, the inclusion of either the isotypic or neutralising antibodies had no effect on survival outcomes. However, due to the reduced population size of the TGFß-treated drug-free cultures, resulting from TGFß-mediated growth inhibition, the percentage of cells surviving drug exposure increased to 17.0 ± 3.9% in the absence of antibody. This slight increase in percentage survival was eliminated by the addition of the TGFß neutralising antibody due to the expansion of cell number in the corresponding drug-free culture.

The presence of 0.154 µg/ml control WGFE restricted the number of cells dying from vinblastine exposure, increasing cell number above that observed with either the control or

rTGFß$_2$-treatments by approximately 3-fold. Absorbance values measured at 630 nm fell from 0.727 units in the absence of vinblastine to 0.438 units following drug exposure, resulting in the survival of 59.5 ± 10.3% of the control WGFE-treated population. Importantly, the inclusion of the neutralising TGFß antibody during control WGFE incubation did not alter the number of cells surviving drug exposure. Nevertheless, the proportion of control WGFE-treated cells surviving drug exposure was reduced to 32.9 ± 9.1% by the addition of the neutralising antibody, due to the expansion of the drug-free population. Although reduced by the inclusion of the neutralising antibody, the proportional survival mediated by the control WGFE fraction was still greater than that observed with the control or rTGFß$_2$ treatments, confirming the presence of non-TGFß mediated survival activity in this fraction.

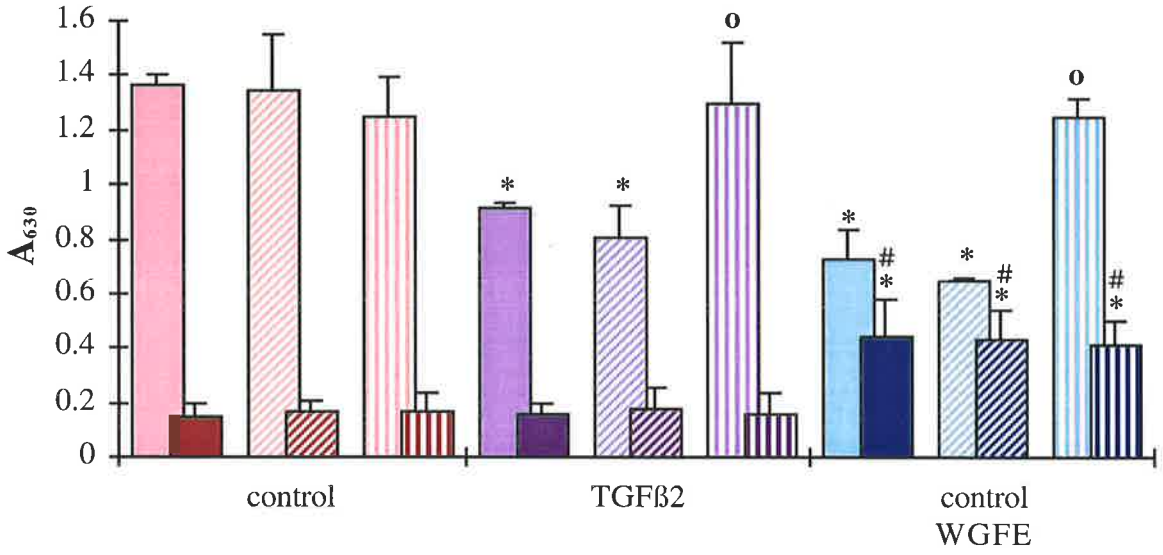


Figure 4.3 Effect of TGFß neutralisation on control WGFE-mediated survival against vinblastine toxicity

Cell survival was measured in Mv1Lu cultures following treatment with control medium, 0.6 ng/ml rTGFß$_2$ or 154.0 µg/ml control WGFE, in conjunction with no antibody (□), 15 µg/ml isotypic control antibody (▨) or 15 µg/ml pan-specific TGFß$_{1,2,3}$ neutralising antibody (▥) for 48 h. Cultures were maintained in the absence of drug (■,■,■), or exposed to 50 nM vinblastine (■,■,■) for the final 18 h of the treatment/antibody incubation. All cultures were then washed to remove treatments and drugs, and re-cultured for a further 30 h. Surviving cell number was quantified at 630 nm following methylene blue staining of the adherent monolayer. Values represent the mean ± SD from two independent experiments (n = 3 replicates/experiment). *, $P < 0.05$, compared to control-treated cultures within the same antibody/drug group. #, $P < 0.05$, compared to TGFß-treated cultures within the same antibody/drug group. o, $P < 0.05$, neutralising antibody compared to no antibody within the same treatment/drug group.

Acid WGFE

Studies of the non-TGFß mediated survival activity in the acid WGFE fraction were performed using a concentration of 13.0 µg acid WGFE/ml. Of the concentrations previously tested in Chapter 3, 13.0 µg/ml acid WGFE mediated greater protection against 50 nM vinblastine, resulting in disparity between the survival observed and that predicted on the basis of TGFß equivalent activity. At this dose, acid WGFE induced equivalent growth inhibition to 3.0 ng/ml rTGFß$_2$ in a Mv1Lu growth inhibition assay. Thus, in the absence of vinblastine, incubation of

Mv1Lu cells with acid WGFE for 48 h, followed by a 30 h re-incubation in normal medium, reduced final cell number to 38.9% of the population size measured in control-treated cultures, or to 35.0% in cultures supplemented with 3.0 ng/ml rTGFß$_2$ (Figure 4.4). The addition of 15 µg/ml isotypic control antibody during the control, acid WGFE and rTGFß$_2$ incubations had no effect on cell growth. Whilst 15 µg/ml neutralising antibody also had no effect on growth of the control-treated cultures, it mediated the full restoration of proliferative activity to cultures treated with the acid WGFE or rTGFß$_2$ preparations.

As in the previous experiment, exposure to 50 nM vinblastine for 18 h reduced cell number in control-treated cultures from 1.206 to 0.122 absorbance units, equivalent to the survival of 10.1 ± 0.8% of the drug-free population (Figure 4.4). The addition of the isotypic or neutralising antibodies did not significantly alter the number of cells protected from drug toxicity. Pre/concurrent treatment with 3.0 ng/ml rTGFß$_2$ only slightly enhanced cell survival following drug exposure when compared to untreated cultures. However, the reduced population size of the drug-free cultures meant that 36.76 ± 5.3% of the TGFß-treated cells survived drug exposure. As observed with the control-treated cultures, the inclusion of the isotypic antibody did not further modify the TGFß-mediated survival response. However, addition of the neutralising antibody reduced A_{630} values to 0.098 units, effectively eliminating any survival activity. The TGFß-mediated increase in percentage survival was also eliminated by the addition of the neutralising antibody, due to both the expansion of cell number in the drug-free culture and to a reduction in cell number in the corresponding drug-exposed culture.

Pre/concurrent treatment with 13.0 µg/ml acid WGFE protected a significantly greater number of cells than treatment with control medium, both in the absence of antibody and the presence of isotypic antibody. In the absence of antibody, A_{630} values increased from 0.122 units with control treatment to 0.296 units with acid WGFE treatment, representing a 2-fold increase in the number of cells surviving drug exposure. Furthermore, as the size of the drug-free cell population was reduced by treatment with acid WGFE, the proportion of cells protected from

drug toxicity increased to 55.7 ± 7.8%. Co-incubation of the neutralising antibody with the acid WGFE did not change the number of cells surviving drug exposure. The A_{630} value of the acid WGFE-treated culture co-incubated with the neutralising antibody was also significantly greater than the values of either the control or rTGFß$_2$-treated cultures incubated in the presence of the same antibody. The proportion of acid WGFE-treated cells surviving drug exposure was reduced to 23.4 ± 1.0% by co-incubation with the neutralising antibody, again due to the expansion of the drug-free population. This proportion was still greater than that observed with either of the control or rTGFß$_2$ treatments cultured with the neutralising antibody, indicating that non-TGFß mediated survival activity was also present in the acid WGFE fraction.

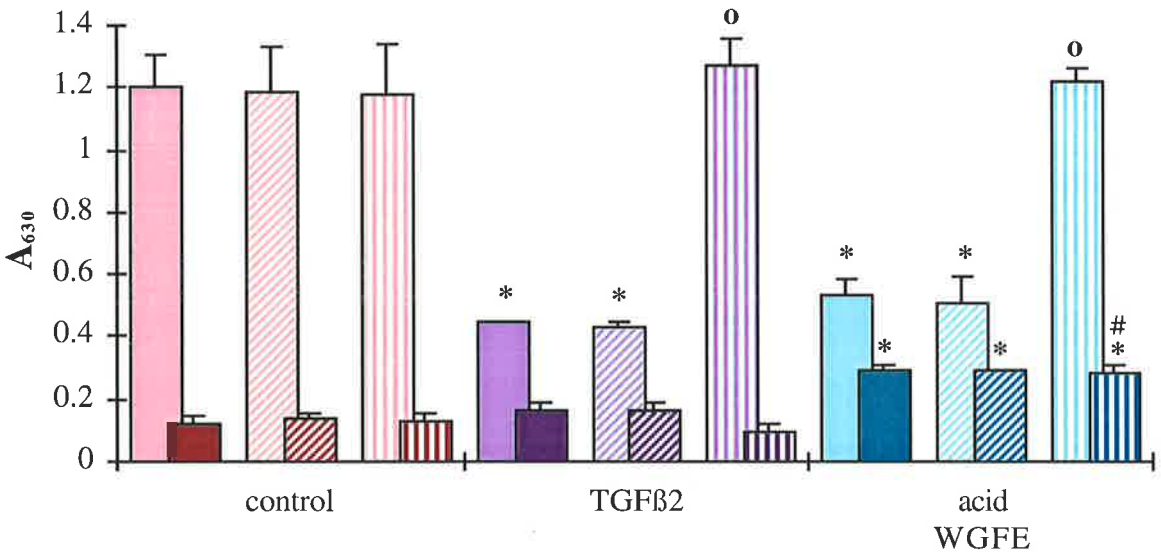


Figure 4.4 Effect of TGFß neutralisation on acid WGFE-mediated survival against vinblastine toxicity

Cell survival was measured in Mv1Lu cultures following treatment with control medium, 3 ng/ml rTGFß$_2$ or 13.0 µg/ml acid WGFE, in conjunction with no antibody (□), 15 µg/ml isotypic control antibody (▨) or 15 µg/ml pan-specific TGFß$_{1,2,3}$ neutralising antibody (▥) for 48 h. Cultures were maintained in the absence of drug (▫,▫,▫), or exposed to 50 nM vinblastine (■,■,■) for the final 18 h of the treatment/antibody incubation. All cultures were then washed to remove treatments and drugs, and re-cultured for a further 30 h. Surviving cell number was quantified at 630 nm following methylene blue staining of the adherent monolayer. Values represent the mean ± SD from two independent experiments (n = 3 replicates/experiment). *, $P < 0.05$, compared to control-treated cultures within the same antibody/drug group. #, $P < 0.05$, compared to TGFß-treated cultures within the same antibody/drug group. o, $P < 0.05$, neutralising antibody compared to no antibody within the same treatment/drug group.

4.3.2 Survival mediated by recombinant growth factors

The cytoprotective properties of IGF-I, PDGF and EGF were investigated using recombinant growth factors applied individually to Mv1Lu cultures at concentrations similar to those measured in the control WGFE preparation. Cell survival was examined following both pre/concurrent and post-drug treatment of cultures with these growth factors. The control WGFE fraction had mediated enhanced cell survival against drug-induced death from a protein concentration of 0.77 mg/ml. The IGF-I concentration in this fraction ranged from 12 to 22 ng/mg, yielding 9 to 17 ng IGF-I/ml in the 0.77 mg/ml control WGFE preparation (see Chapter 3 and Belford *et al.,* 1997). PDGF has been identified in control WGFE at a concentration of 4 ng/mg protein (Belford *et al.,* 1997), yielding 3 ng PDGF/ml in 0.77 mg/ml control WGFE. Without sufficient quantities of the EGF-like peptide available for inclusion in a survival assay, recombinant EGF was substituted at concentrations within the range of its biological activity in Mv1Lu cells (Kelley *et al.,* 1992).

4.3.2.1 Pre/concurrent treatment

IGF-I, EGF and PDGF were added to Mv1Lu cultures over a range of concentrations and examined for cytoprotective properties in cell exposed to 1 μM etoposide or 50 nM vinblastine. Surviving cell number was not affected by the pre/concurrent addition of any of the recombinant proteins before exposure to either 1 μM etoposide (Figure 4.5) or 50 nM vinblastine (Figure 4.6). Additionally, no changes were observed when survival in the drug-treated cultures was expressed as a percentage of the cells surviving in the absence of drug exposure.

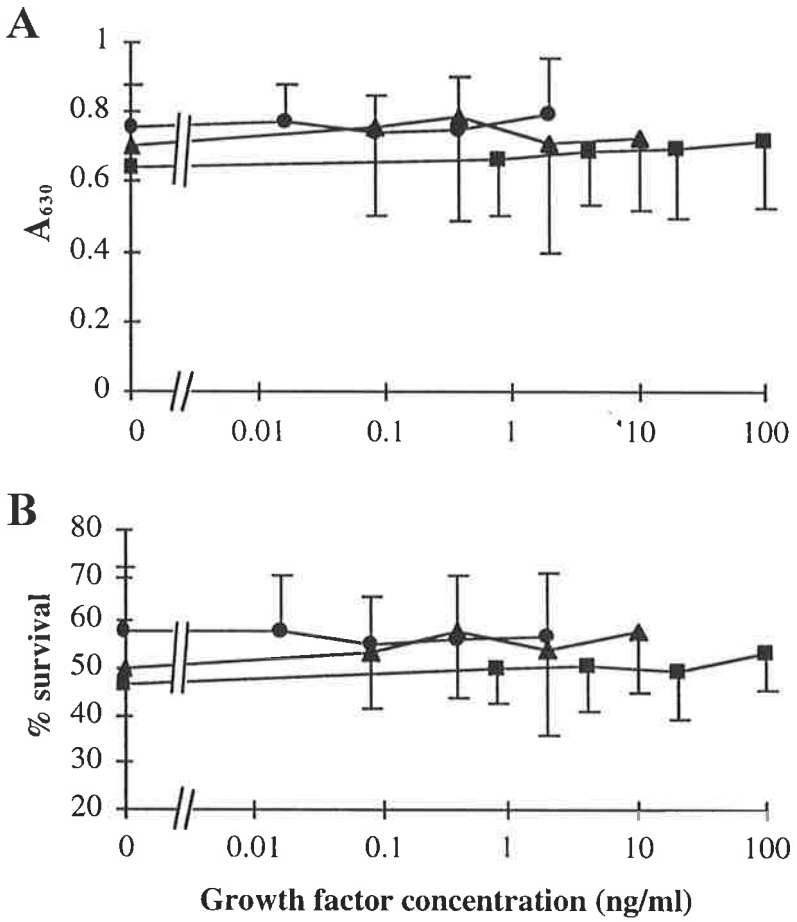


Figure 4.5 Lack of protective effect of pre/concurrent recombinant growth factor treatment against etoposide toxicity

Cell survival was measured in Mv1Lu cultures following the addition of PDGF (●), EGF (▲) or IGF-I (■) for 48 h, beginning 30 h prior to the addition of 1 μM etoposide. Survival was assessed spectrophotometrically at 630 nm following methylene blue staining of drug-exposed cultures (**A**) (A_{630} values obtained for drug-free cultures are not shown). A_{630} values were converted to % survival by calculating the ratio of absorbance values in the drug-exposed versus drug-free cultures for each pre/concurrent treatment (**B**). Values represent the mean ± SD from two independent experiments (n = 4 replicates/experiment).

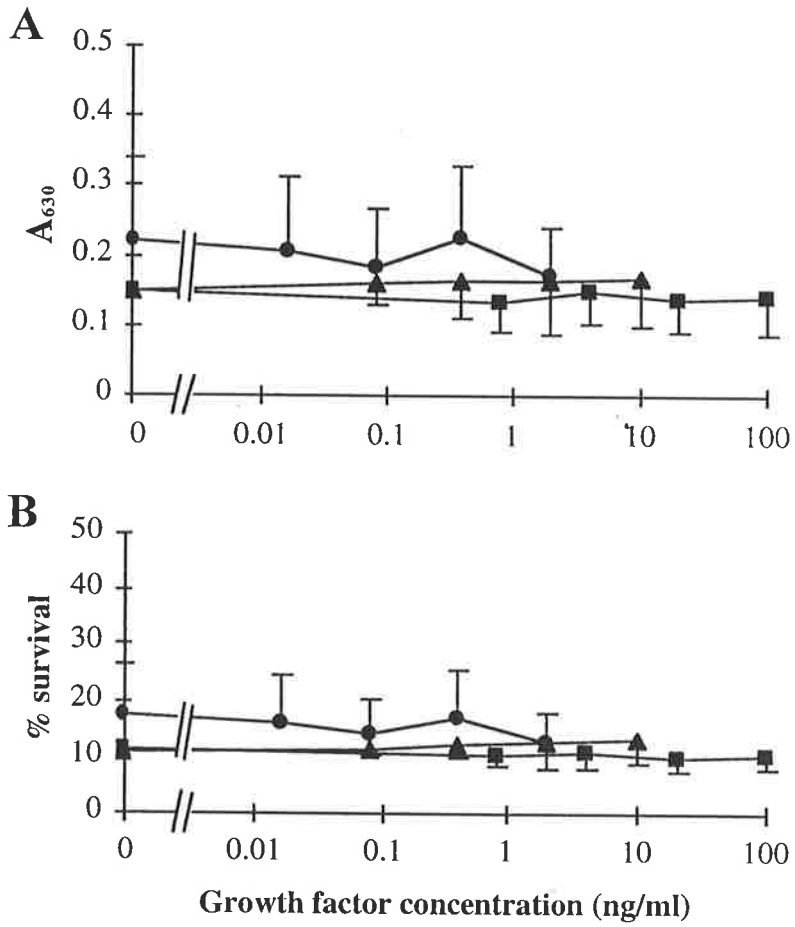


Figure 4.6 Lack of protective effect of pre/concurrent recombinant growth factor treatment against vinblastine toxicity

Cell survival was measured in Mv1Lu cultures following the addition of PDGF (●), EGF (▲) or IGF-I (■) for 48 h, beginning 30 h prior to the addition of 50 nM vinblastine. Survival was assessed spectrophotometrically at 630 nm following methylene blue staining of drug-exposed cultures **(A)** (A_{630} values obtained for drug-free cultures are not shown). A_{630} values were converted to % survival by calculating the ratio of absorbance values in the drug-exposed versus drug-free cultures for each pre/concurrent treatment **(B)**. Values represent the mean ± SD from two independent experiments (n = 4 replicates/experiment).

4.3.2.2 Post-drug treatment

In the absence of drug exposure, incubation with 10 ng/ml PDGF or 50 ng/ml IGF-I for the final 30 h of the survival assay had no effect on final cell number (Figure 4.7 A). The addition of 10 ng/ml EGF, however, induced a significant increase in cell number during this final 30 h period. When these growth factors were added following exposure of the cultures to 1 μM etoposide, PDGF tended to decrease final cell number, whilst EGF increased cell number and IGF-I had no effect. However, none of these treatments induced significant changes when compared to control-treated cultures. Conversion of the A_{630} values to percent survival indicated that an equivalent proportion of the cell populations survived etoposide exposure regardless of the post-drug treatment applied (Figure 4.7 B).

Similarly when cultures were exposed to 50 nM vinblastine, post-drug treatment with PDGF and IGF-I did not further stimulate the regeneration of the surviving cell population (Figure 4.7 A). Incubation of the cultures with 10 ng/ml EGF did induce a slight increase in surviving cell number, however this failed to reach significance. When all post-drug treatments were expressed as a percentage of their respective drug-free cultures, none of the growth factors showed any propensity to modify cell survival (Figure 4.7 B).

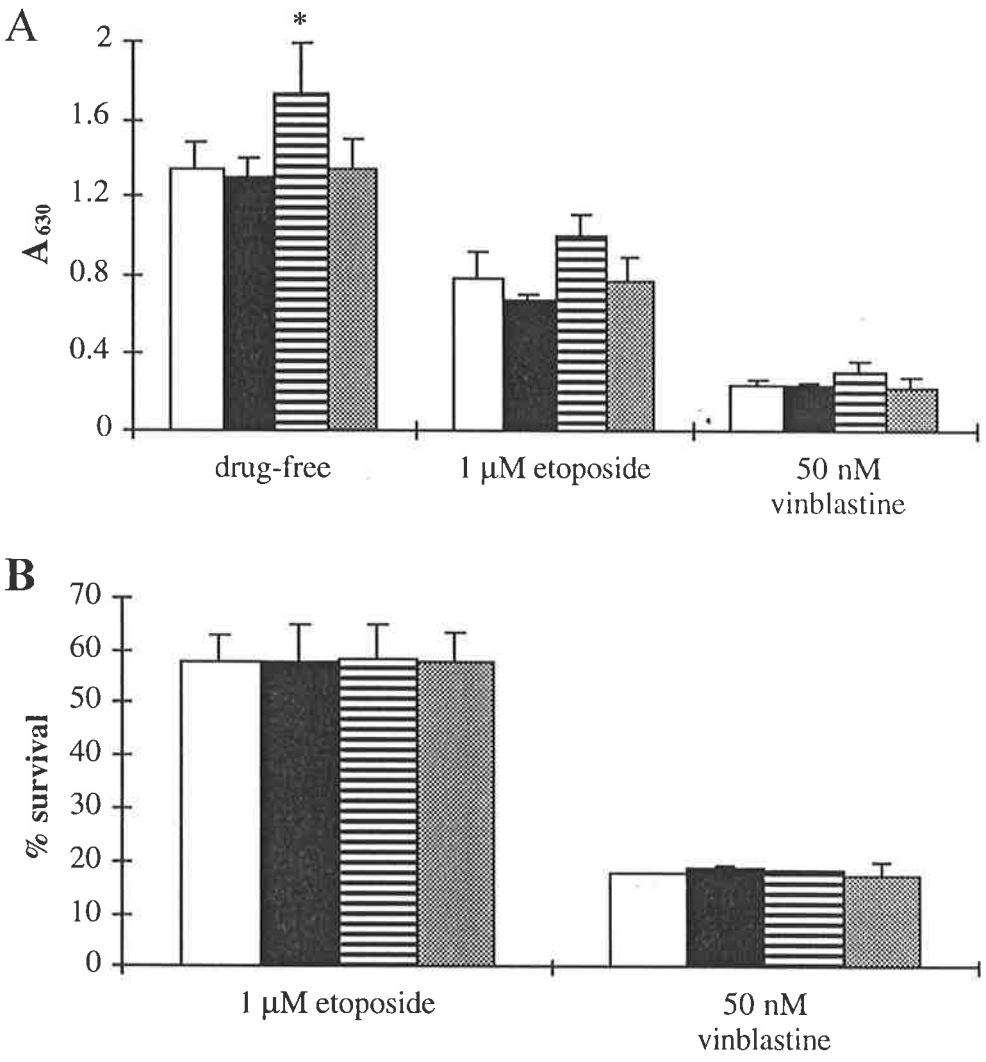


Figure 4.7 Regenerative effect of post-drug recombinant growth factor addition

Cell survival was measured in Mv1Lu cultures following the addition of control medium (□), 10 ng/ml PDGF (■), 10 ng/ml EGF (▤) or 50 ng/ml IGF-I (▩) for 30 h, beginning immediately after removal of 50 nM vinblastine from the culture medium. Survival was assessed spectrophotometrically at 630 nm following methylene blue staining of cultures (**A**). A_{630} values were converted to % survival by calculating the ratio of absorbance values in the drug-exposed versus drug-free cultures for each post-drug treatment (**B**). Values represent the mean ± SD from three independent experiments (n = 6 replicates/experiment). *, $P < 0.05$, compared to control-treated cultures.

4.3.3 Survival and growth inhibition mediated by control WGFE following size exclusion chromatographic separation

4.3.3.1 Elution at neutral pH

Molecular weight markers were eluted under identical conditions to those used for subsequent neutral WGFE separations. Elution times ranged from 3 min for the 2000 kDa blue dextran protein to 48.5 min for the 13.7 kDa ribonuclease A protein (Table 4.1). The calibration curve generated from each Kav versus the logarithmic transformation of the corresponding molecular weight produced an inversely proportional relationship (r = -0.971).

Marker	Molecular weight (kDa)	Elution time (min)
Blue dextran 2000	2000	3
Bovine serum albumin	67	25
Ovalbumin	43	40.5
Chymotrypsinogen A	25	43.5
Ribonuclease A	13.7	48.5

Table 4.1 Elution time of low molecular weight markers from Superose 12 column

Control WGFE (10 mg) was fractionated on a Superose 12 chromatography column at pH 7.4. Eluted protein was continuously monitored at A_{280}, to generate a profile of absorbance readings versus elution time (Figure 4.8). The elution profile showed four major protein peaks, eluting at 28, 38, 45 and 54 min, with minor protein peaks detected between 70 and 95 min. Fractions were collected at 1 min intervals and grouped into 6 pools as listed in Table 4.2.

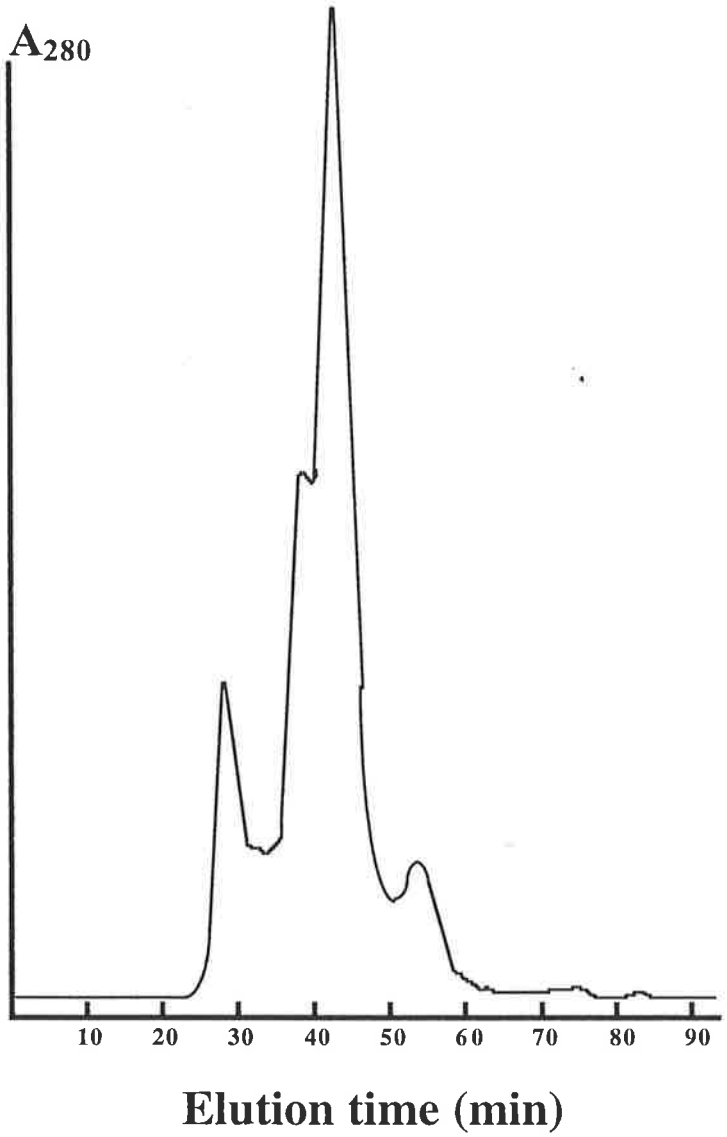


Figure 4.8 Elution profile of control WGFE separated at neutral pH

10 mg of control WGFE was fractionated by size exclusion chromatography on a Superose 12 column at pH 7.4. Separation was performed using PBS containing 10% acetonitrile, at a flow rate of 0.3 ml/min. Eluted protein was continuously monitored at A_{280}.

Neutral pH pool	Elution times (min)
1	24 - 31
2	32 - 38
3	39 - 48
4	49 - 58
5	59 - 67
6	68 - 94

Table 4.2 Pool elution times following neutral pH chromatographic separation

Growth inhibitory activity was quantified in all six neutral pH elution pools following a 48 h incubation with Mv1Lu cultures in the absence of chemotherapy agents (Figure 4.9). Due to the latent state of the majority of the TGFß present in the control WGFE, very little growth inhibitory activity was detected. Growth inhibitory activity eluted primarily in pool 2, earlier than predicted for active TGFß on the basis of its molecular weight of 25 kDa. Growth inhibition in this pool was 25.5% as potent as the inhibition induced by 10 ng/ml $rTGF\beta_2$, whilst pools 3 and 6 induced 16.9% and 14.1% inhibition, respectively.

In contrast to the results obtained with the growth inhibition assay, pools 2 and 6 conferred no survival benefit to Mv1Lu cultures exposed to 50 nM vinblastine (Figure 4.10). Rather, the survival promoting activity was concentrated in pool 3, which protected between 35% and 40% of the cell population at dilutions of 1 down to 1:24 (Figure 4.10). Survival activity was completely eliminated, however, with a 1:124 dilution of pool 3 (results not shown). The survival mediated by pool 3 survival was less than that observed with 30.8 µg/ml unfractionated control WGFE (containing equivalent growth inhibitory activity to 0.12 ng/ml TGFß), but significantly greater than that produced by all of the other pools ($P < 0.05$). Pool 4

also contained some protective activity, evident at the highest concentration tested.

Pools 1 and 5 induced only low level growth inhibition and also lacked survival activity. Bivariate analysis of the growth inhibitory and survival promoting properties of the neutral pH elution pools yielded a correlation co-efficient of 0.176, suggesting that there was minimal interdependence between the two parameters.

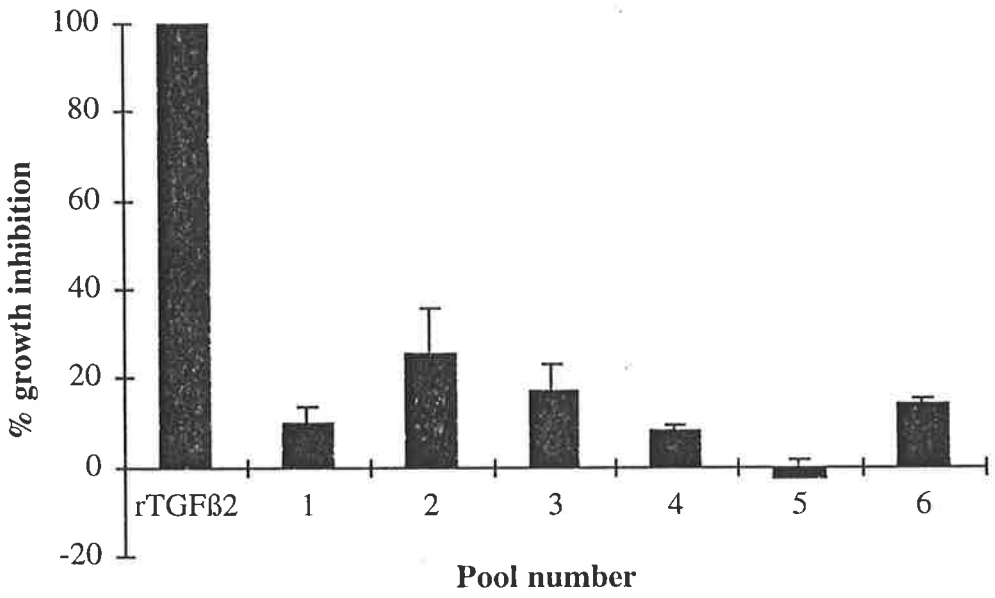


Figure 4.9 Growth inhibitory effects of neutral pH elution pools

Growth inhibitory activity was measured in each neutral pH elution pool using a Mv1Lu growth inhibition bioassay. Each pool was diluted 1:1 in culture medium from 10 x concentrated stocks, and incubated with Mv1Lu cells for 48 h. Growth inhibition was measured at 630 nm following methylene blue staining of the culture monolayers, and expressed as a proportion of the inhibition produced by 10 ng/ml rTGFß$_2$. Values represent the mean ± SD of two independent experiments (n = 3 replicates/experiment).

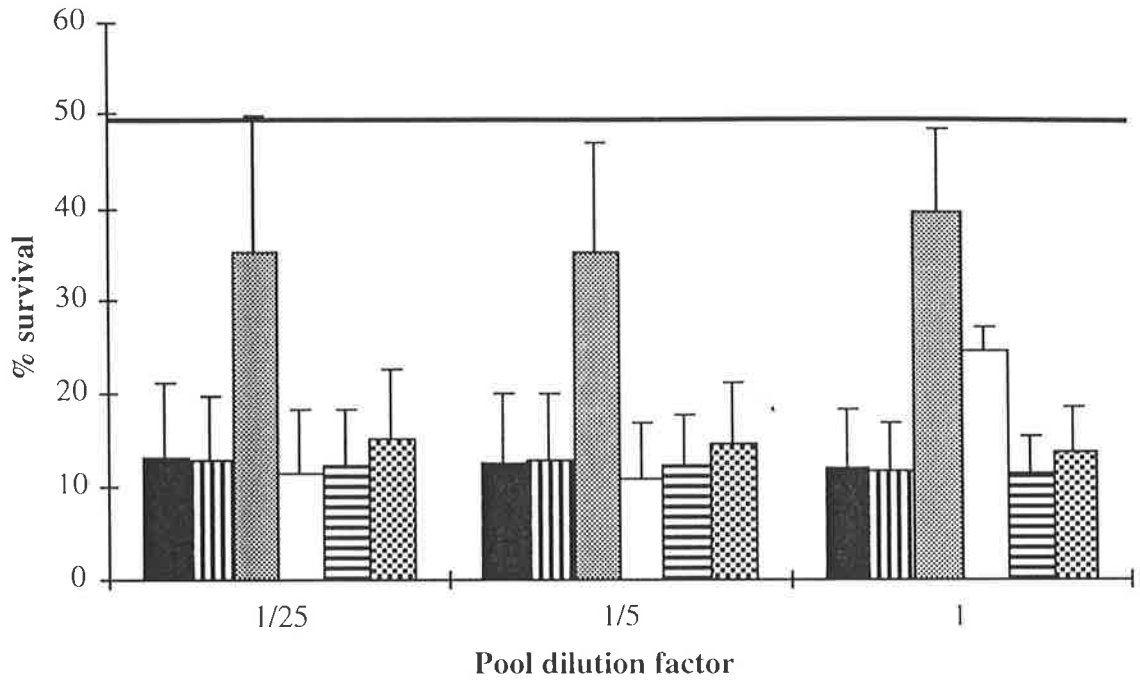


Figure 4.10 Survival mediated by neutral pH elution pools against vinblastine toxicity

Cytoprotective activity was measured in Mv1Lu cells following the addition of neutral pH elution pools for 48 h, beginning 30 h prior to 50 nM vinblastine exposure. Each pool was initially diluted 1:9 in culture medium from 10 x concentrated stocks, with further 1:4 serial dilutions. Pools were 1 (■), 2 (▥), 3 (▩), 4 (□), 5 (▤) and 6 (▦) as listed in Table 4.2. Cell survival was measured at 630 nm following methylene blue staining of the culture monolayers. Survival was expressed as a ratio of the A_{630} values in the drug-exposed versus drug-free cultures within each pre/concurrent treatment. Values represent the mean ± SD of two independent experiments (n = 4 replicates/experiment). -, survival mediated by 30.8 µg/ml control WGFE (producing equivalent growth inhibition to 0.12 ng/ml rTGFß$_2$).

4.3.3.2 Elution at neutral pH with transient post-acidification

Examination of the growth inhibition and cytoprotection mediated by the pools collected from neutral pH fractionation indicated that the pools containing survival activity were not necessarily the same as those mediating growth inhibition. To obtain further evidence for the role of TGFß in the cytoprotective actions of control WGFE, each elution pool was transiently acidified after fractionation at neutral pH to activate latent TGFß. Such an increase in growth inhibitory activity should be reflected through additive increases in survival activity if TGFß was solely responsible for the survival response elicited by control WGFE.

A second sample of control WGFE (10 mg) was fractionated on a Superose 12 chromatography column under neutral pH conditions for transient acidification after separation. The eluted protein was continuously monitored at A_{280}, producing an elution profile identical to that shown in Figure 4.8. Fractions were again collected at 1 min intervals and grouped into 6 pools as listed in Table 4.3. These pools differed slightly from those grouped from the initial neutral pH elution, with 3 fractions from the previous pool 3 being grouped with pool 2, and 2 fractions from the previous pool 2 being grouped with pool 1. I chose to shift these fractions on the basis of the results obtained with the first elution, where it appeared that it may be possible to physically separate the factors responsible for inhibition from those responsible for survival by transferring a small number of pool 3 fractions into pool 2. Following resuspension and pooling of the fractions each pool was divided into two equal volumes, one to be retained at neutral pH and the other transiently acidified to pH 2.5.

Neutral pH pool	Elution times (min)
1	25 - 33
2	34 - 41
3	42 - 48
4	49 - 58
5	59 - 67
6	68 - 94

Table 4.3 Pool elution times following neutral pH chromatographic separation for post-acidification studies

Growth inhibition was quantified in Mv1Lu cultures following a 48 h incubation with each of

the elution pools, from both the control and post-acidified groups. As observed with the original WGFE fractionation, only a small amount of growth inhibitory activity was detected in the non-acidified pools due to the latency of the TGFß peptides (Figure 4.11 A). Growth inhibition was again found predominantly in pool 2 of the non-acidified fractions, producing 27.0% of the level of inhibition mediated by 10 ng/ml $rTGFß_2$. Post-acidification of the WGFE elution pools resulted in the activation of latent TGFß, producing increases in growth inhibitory activity (Figure 4.11 B). As the latent TGFß complex is of a higher molecular weight than the active peptide, I predicted that growth inhibitory activity would increase in pool 1, however this did not occur. Growth inhibitory activity was predominantly activated in pool 2, elevating growth inhibition to 57.1% of that produced by 10 ng/ml rTGFß2. The anti-proliferative activity in pool 3 was also enhanced by the transient acidification process, increasing inhibition from 6.9% to 28.1%. Thus, the latent and active growth inhibitory peptides were collected into the same elution pools, suggesting that the WGFE proteins may not have eluted strictly on the basis of size, or that the inhibitory peptides were partially activated by the cells in the assay.

Each of the acidified and non-acidified elution pools were also tested for cytoprotective activity in an assay of vinblastine toxicity. As in the previous experiments, non-acidified pool 3 contained the most potent protective activity, with non-acidified pool 4 also containing some activity at the highest concentration tested. An obvious difference from the previous experiment was the presence of protective activity in the non-acidified pool 2, probably reflecting the shift in pooled fractions between the two experiments (Figure 4.12 A).

Transient acidification of the neutral pH elution pools did not dramatically alter the profile of protection against vinblastine toxicity. Survival activity remained absent from pools 1, 5 and 6, indicating that there was no latent cytoprotective activity in these pools which could be acid-activated (Figure 4.12 B). Pool 2 mediated the survival of 38.7% of the population at the highest concentration tested. This proportional survival was only slightly higher than was observed in the corresponding non-acidified pool, despite a 2-fold increase in anti-proliferative activity following acid activation of latent TGFß. The cytoprotective properties of the acidified

pool 2 were also evident at the 1:4 dilution, promoting the survival of 28.5% of the Mv1Lu culture. The survival activity of this pool was subsequently lost upon further dilution.

Post-elution acidification of pool 3 did not significantly enhance survival responses when compared with non-acidified pool 3. Survival at the highest concentration was 36.7%, falling to 31.5% and 29.3% with 1:4 and 1:24 dilutions, respectively. As for the corresponding non-acidified pool, further dilution of the acidified pool 3 resulted in a loss of detectable cytoprotective activity (results not shown). The protection mediated by pool 4 was slightly enhanced by acidification, increasing survival from 19.8% to 24.9% of the population. However, this increase was not sustained with dilution of the acidified pool, suggesting that acidification had not enhanced survival activity.

Bivariate analysis of the growth inhibitory and survival promoting properties of the post-acidified neutral pH elution pools yielded a correlation co-efficient of 0.787. This co-efficient was much greater than that observed in the non-acidified pools, suggesting that acidification increased the interdependence between the two variables. The transfer of survival activity to pool 2 during allocation of the eluted fractions would also have contributed to the increased correlation between growth inhibition and cell survival, as the results from the original non-acidified pool 2 had previously argued against such a correlation.

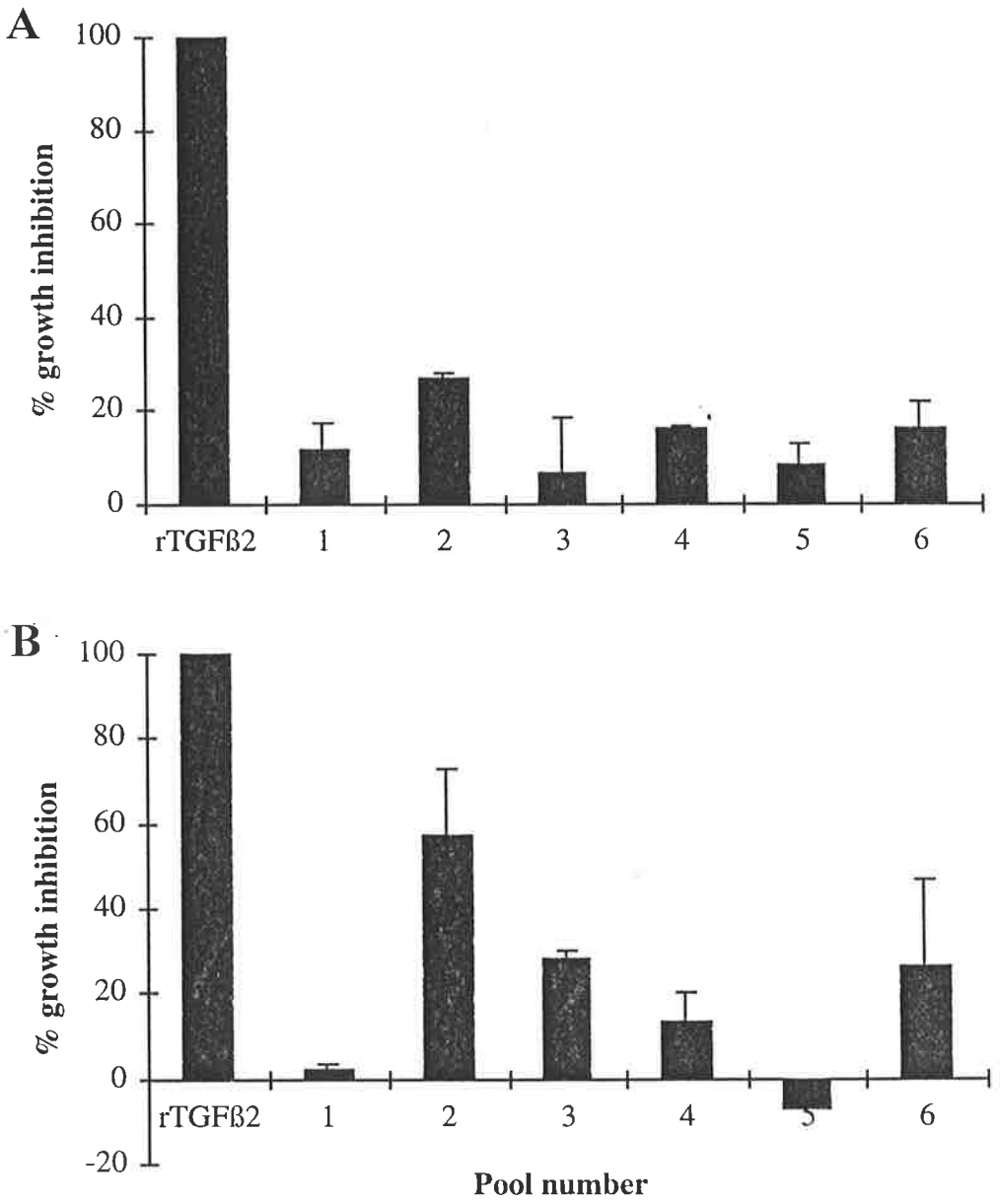


Figure 4.11 Growth inhibitory effects of neutral pH elution pools after transient acidification

Growth inhibitory activity was measured in each neutral pH elution pool, maintained at neutral pH (**A**) or transiently acidified to pH 2.5 (**B**), using a Mv1Lu growth inhibition bioassay. Each pool was diluted 1:1 in culture medium from 10 x concentrated stocks, and incubated with Mv1Lu cells for 48 h. Growth inhibition was measured at 630 nm following methylene blue staining of the culture monolayers, and expressed as a proportion of the inhibition produced by 10 ng/ml rTGFß$_2$. Values represent the mean ± SD of two independent experiments (n = 3 replicates/experiment).

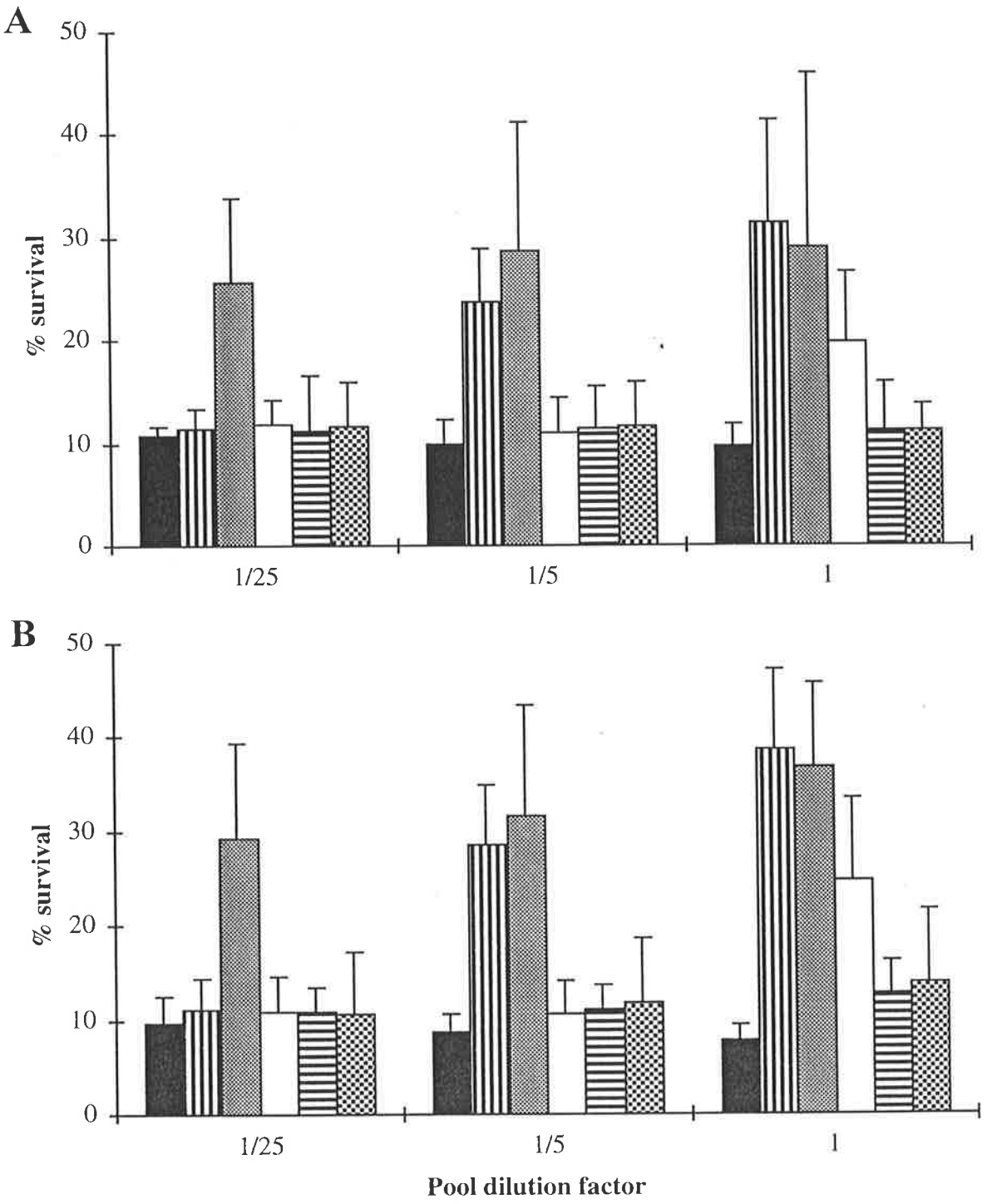


Figure 4.12 Survival mediated by neutral pH elution pools after transient acidification

Cytoprotective activity was measured in Mv1Lu cells following the addition of neutral pH elution pools, maintained at neutral pH **(A)** or transiently acidified to pH 2.5 **(B)**. Pools were incubated with cultures for 48 h, beginning 30 h prior to 50 nM vinblastine exposure. Each pool was initially diluted 1:9 in culture medium from 10 x concentrated stocks, with further 1:4 serial dilutions. Pools were 1 (■), 2 (▥), 3 (▩), 4 (□), 5 (▤) and 6 (▨) as listed in Table 4.3. Cell survival was measured at 630 nm following methylene blue staining of the culture monolayers. Survival was expressed as a ratio of the A_{630} values in the drug-exposed versus drug-free cultures within each pre/concurrent treatment. Values represent the mean ± SD of two independent experiments (n = 4 replicates/experiment).

4.3.3.3 Acid chromatographic separation

As the growth inhibitory and cytoprotective mediators present in control WGFE had partially co-eluted at neutral pH, I re-examined these parameters when the WGFE was eluted under acidic pH conditions. By acidifying the WGFE material prior to fractionation, the majority of the latent TGFß peptides were expected to be activated, thus producing a shift in their elution time. Control WGFE (10 mg) was acidified to approximately pH 2.5 prior to fractionation on a Superose 12 chromatography column at pH 2.8. Eluted protein was continuously monitored at A_{280}, producing a profile of absorbance readings versus elution time (Figure 4.13). The elution profile showed four major protein peaks, eluting at 40, 52, 65 and 73 min, with minor protein peaks detected between 95 and 124 min. Fractions were collected at 1 min intervals and grouped into 6 pools as listed in Table 4.4.

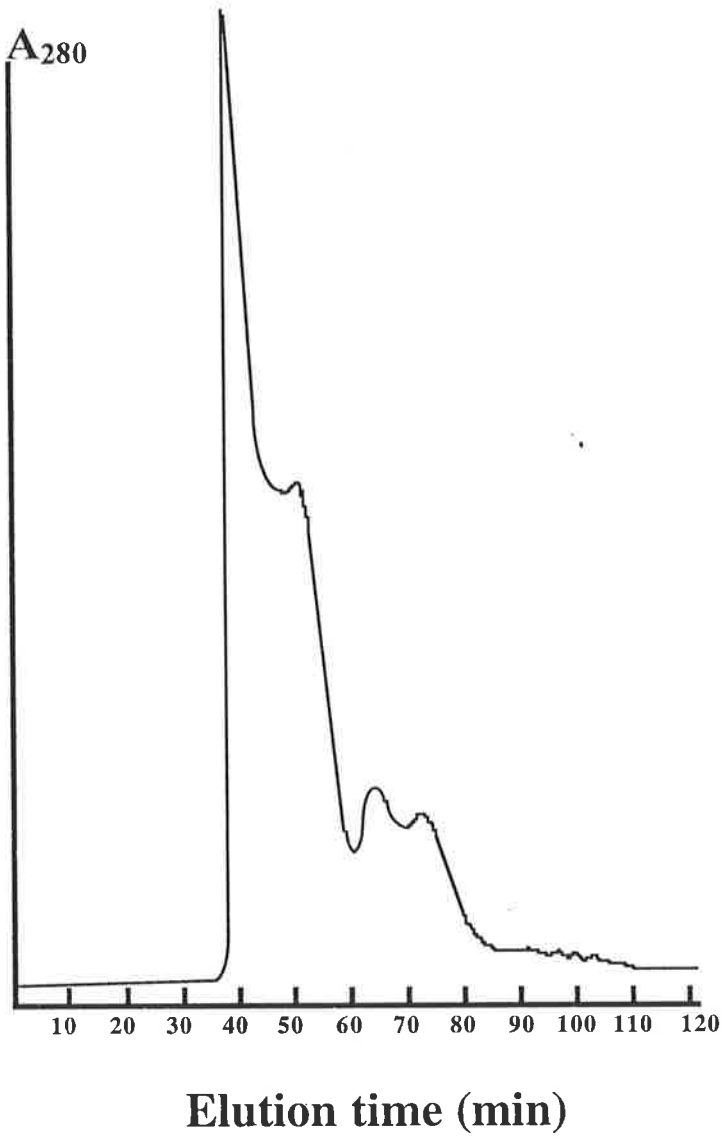


Figure 4.13 Elution profile of control WGFE separated at acidic pH

10 mg of acidified WGFE was fractionated by size exclusion chromatography on a Superose 12 column at pH 2.8. Separation was performed using 150 mM NaCl, 1M acetic acid and 10% acetonitrile, at a flow rate of 0.3 ml/min. Eluted protein was continuously monitored at A_{280}.

Acidic pH pool	Elution times (min)
1	35 - 43
2	44 - 51
3	52 - 60
4	61 - 72
5	73 - 83
6	84 - 95

Table 4.4 Pool elution times following neutral pH chromatographic separation for post-acidification studies

Growth inhibition was measured in all six acid pH elution pools following a 48 h incubation with Mv1Lu cell cultures (Figure 4.14). Fractionation of the WGFE under acidic pH conditions resulted in a shift of the inhibitory activity to pools 5 and 6. Growth inhibition mediated by each of pools 5 and 6 was approximately 120% as potent as the inhibition produced by 10 ng/ml rTGFß$_2$, suggesting that a maximally inhibitory response was evoked.

Under acidic conditions, the WGFE elution pools containing growth inhibitory activity were the same as those mediating protection against vinblastine toxicity. The survival response mediated by pools 2, 3 and 4 under neutral elution conditions was no longer evident (Figure 4.15). Instead, acid elution pools 5 and 6 mediated the survival of 32.8% and 30.3% of the test cultures, respectively. The survival mediated by pools 5 and 6 was less than that produced by the neutral elution pool 3, despite an approximate 7-fold increase in growth inhibitory activity in both of the acid elution pools.

Examination of the growth inhibitory and cytoprotective properties of a seventh pool, prepared in an identical manner to the other six pools, indicated that there was no detectable activity

eluting from the column between 95 and 124 min (results not shown). Bivariate analysis of the growth inhibitory and survival promoting properties of the acid pH elution pools yielded a correlation co-efficient of 0.999, indicating an extremely close interdependence between the two parameters.

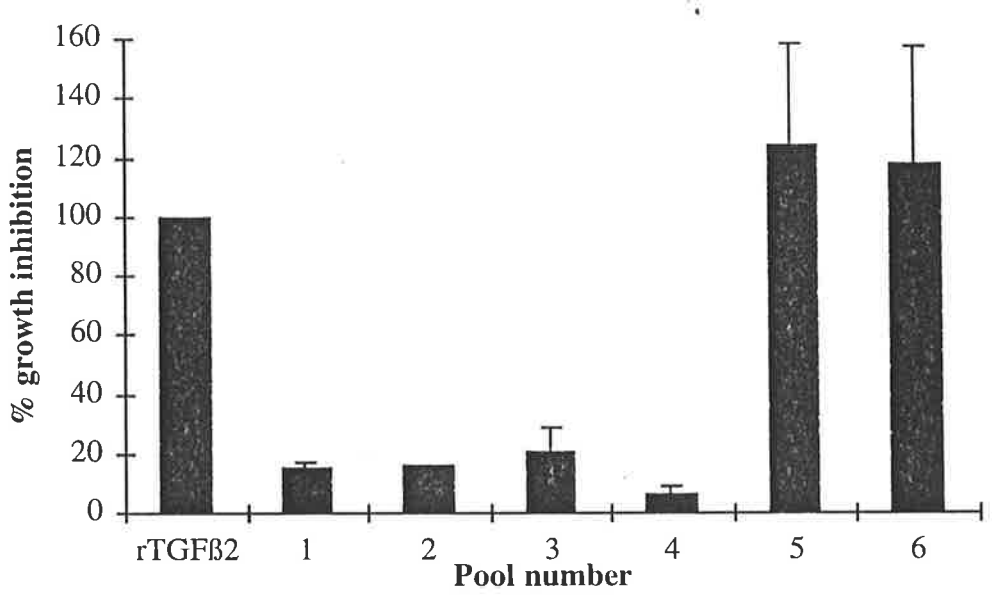


Figure 4.14 Growth inhibitory effects of acidic pH elution pools

Growth inhibitory activity was measured in each acidic pH elution pool using a Mv1Lu growth inhibition bioassay. Each pool was diluted 1:1 in culture medium from 10 x concentrated stocks, and incubated with Mv1Lu cells for 48 h. Growth inhibition was measured at 630 nm following methylene blue staining of the culture monolayers, and expressed as a proportion of the inhibition produced by 10 ng/ml rTGFß$_2$. Values represent the mean ± SD of two independent experiments (n = 3 replicates/experiment).

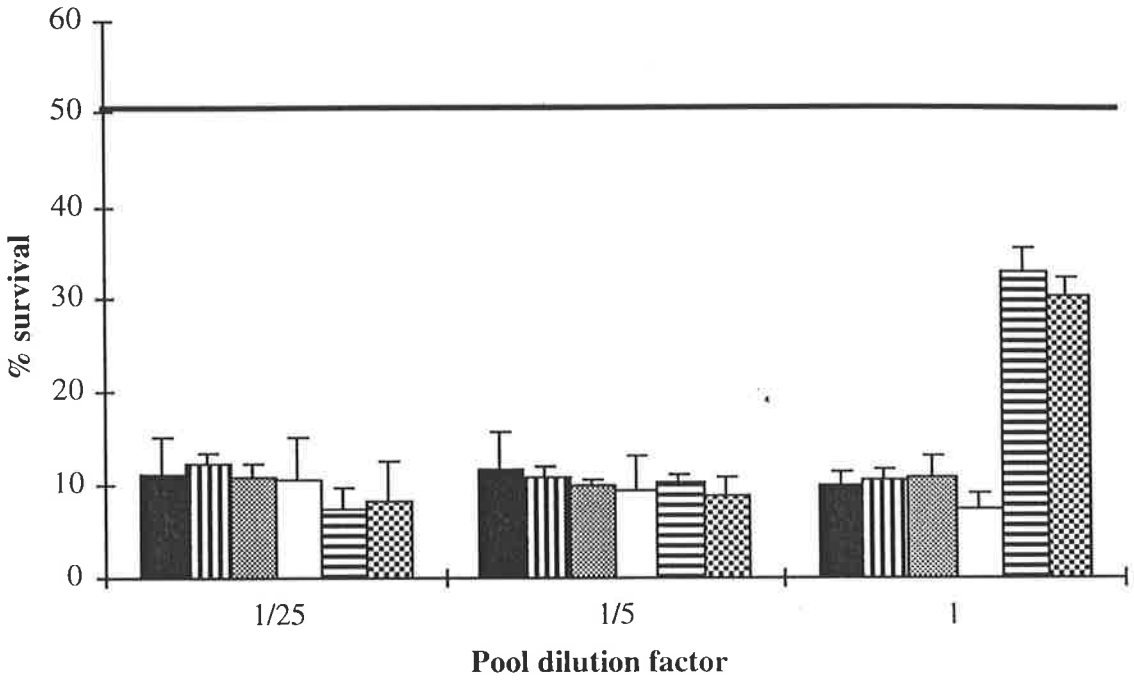


Figure 4.15 Survival mediated by acidic pH elution pools against vinblastine toxicity

Cytoprotective activity was measured in Mv1Lu cells following the addition of acidic pH elution pools for 48 h, beginning 30 h prior to 50 nM vinblastine exposure. Each pool was initially diluted 1:9 in culture medium from 10 x concentrated stocks, with further 1:4 serial dilutions. Pools were 1 (■), 2 (▥), 3 (■), 4 (□), 5 (▤) and 6 (▩) as listed in Table 4.4. Cell survival was measured at 630 nm following methylene blue staining of the culture monolayers. Survival was expressed as a ratio of the A_{630} values in the drug-exposed versus drug-free cultures within each pre/concurrent treatment. Values represent the mean ± SD of two independent experiments (n = 4 replicates/experiment). -, survival mediated by 30.8 µg/ml control WGFE (producing equivalent growth inhibition to 0.12 ng/ml rTGFß$_2$).

4.4 Discussion

4.4.1 Immuno-neutralisation of TGFß in control WGFE

Complete restoration of cellular growth was observed with both the control and acid WGFE fractions when co-incubated with the pan-specific TGFß neutralising antibody. This implied that any factors present in the WGFE fractions which contributed to the growth inhibitory response were mediating their effects through the induction of TGFß synthesis or activation of latent peptides. Neutralisation of the TGFß present in the control WGFE fraction permitted cell growth equal to that measured in the control cultures, confirming that the large quantities of latent TGFß present in the control WGFE were not substantially activated during the incubation period.

Cytokines such as interleukin-1ß, EGF and TGFα, which promote healing of wounded intestinal epithelial cell monolayers *in vitro*, are believed to mediate their effects at least in part through the up-regulation of endogenous $TGFß_1$ synthesis (Dignass and Podolsky, 1993). In the present study, the fact that complete TGFß neutralisation was achieved with both WGFE fractions suggests that any increase in endogenous TGFß production was likely to be minor, and would not contribute to the residual cytoprotection observed. The potential involvement of interleukin 11 (IL-11) is somewhat more complex. *In vitro* studies report that IL-11 inhibits epithelial cell growth (Booth and Potten, 1995) by delaying cell cycle progression from G_1 to S phase (Peterson *et al.,* 1996). The ability of IL-11 to protect epithelial cell populations against drug toxicity *in vivo*, predominantly when administered prior to drug exposure (Potten, 1996), may be related to this inhibitory activity as is proposed with TGFß. In intestinal epithelial (IEC-6) cells, the inhibition mediated by IL-11 is not dependent upon the production of endogenous $TGFß_1$ (Peterson *et al.,* 1996). To date no link has been established between IL-11 and the predominant TGFß isoform synthesised by Mv1Lu cells, $TGFß_2$ (Danielpour *et al.,* 1989). If IL-11 mediates growth inhibition, and presumably therefore also survival activity, indirectly through the induction of $TGFß_2$ synthesis, it cannot account for the non-TGFß survival activity

I have observed. Conversely, if IL-11 induces growth inhibition independently of TGFß it can not be present in the WGFE fractions at biologically active concentrations, as all Mv1Lu growth inhibition was mediated through TGFß activity.

The ability of the control and acid WGFE fractions to sustain absolute cell numbers following drug exposure, irrespective of the proliferative state of the culture, draws into question the role of growth inhibition in mediating the WGFE survival response. In this regard, the studies described in the next chapter compare the growth modulatory and anti-apoptotic actions of orally administered WGFE to ascertain the requirement for growth inhibition in an *in vivo* model.

4.4.2 Protection mediated by IGF-I, EGF and PDGF

The cytoprotective properties of IGF-I, EGF and PDGF were examined in an attempt to account for the non-TGFß-mediated survival detected in the immuno-neutralisation studies. These peptides did not enhance cell survival when applied to cultures as a pre/concurrent treatment, either because they were not individually able to mediate such a response, or because there were already saturable quantities of these peptides present in the serum-supplemented culture medium. However, the fact that the control WGFE enhanced survival above the baseline produced with culture medium alone suggests that this fraction contains one or more peptides not present at biologically active concentrations in 5% serum. Whilst the IGF-I and PDGF peptides may promote the protection or enhanced regeneration of Mv1Lu cells following drug exposure, it is concluded that they do not individually contribute to the non-TGFß survival detected in the control WGFE fraction.

Whilst EGF appeared to stimulate mitogenesis above that promoted by the presence of 5% serum in the culture medium, its overall contribution to culture regeneration as part of a growth factor extract is unclear. The EGF-like peptide identified by Dunbar *et al.* (1997) is known to accumulate in the permeate WGFE fraction. Whilst the permeate WGFE promotes extensive proliferation of fibroblast cultures, it remains growth inhibitory to Mv1Lu cells due to the

presence of TGFß. It is feasible that a permeate WGFE preparation which was further processed to specifically remove the TGFß component may be better suited to application as a post-drug treatment for epithelial cells. It is also possible that the growth inhibitory components of WGFE are preferentially degraded *in vivo* during passage through the stomach and upper small intestine, delivering an essentially mitogenic preparation to the small intestine. Such a preparation might be expected to mediate the rapid recovery of intestinal crypts following drug exposure, as originally observed by Howarth *et al.* (1996). Studies in Chapter 5 investigate such a possibility by examining the effect of orally administered control WGFE on the growth and proliferation of intestinal crypts in normal animals.

4.4.3 Chromatographic separation of WGFE peptides

Post-elution acidification of neutral pH fractions and elution of control WGFE at acidic pH increased the correlation between growth inhibition and cytoprotection, due to both the co-elution of the growth inhibitory and survival activities and the activation of latent TGFß peptides at reduced pH. However when all pools which possessed either inhibitory or survival activity were collectively analysed, regardless of elution pH or post-elution processing, there was a very poor correlation between the two parameters ($r = 0.154$). Thus, the large increases in growth inhibition mediated by particular fractions following acidification did not result in a corresponding expansion of Mv1Lu survival from drug toxicity.

These studies indicate that the best strategy for examining non-TGFß-mediated survival is at neutral pH. Transient acidification of the WGFE tended to mask such activity due to the release of biologically active TGFß peptides. More aggressive acidification, as was employed during acid pH chromatographic separation, resulted in the loss of non-TGFß-mediated survival activity altogether, possibly due to destruction or dissociation into smaller molecular weight components. Extended collection of fractions following acid elution failed to recover this additional survival activity, suggesting that it was either destroyed by the elution buffer or lost during subsequent processing. The presence of insoluble material in some of the vacuum-dried fractions was originally attributed to the precipitation of lactoperoxidase. As this sediment was

removed prior to pooling of the eluted fractions, it is feasible that the non-TGFß survival factor(s) co-precipitated with the lactoperoxidase. Alternatively, the non-TGFß mediated survival may have resulted from the synergistic actions of more than one peptide. Such cooperativity may therefore have been lost if these peptides were not pooled together following the separation of WGFE at acidic pH. The systematic examination of every combination of acid WGFE pools would provide this information, but was beyond the scope of this study.

Instead, other approaches for separating TGFß from the residual survival activity present in WGFE may prove to be more effective. As immuno-neutralisation permits the selective elimination of TGFß activity, without interfering with other peptide interactions, the use of anti-TGFß chromatographic separation is a feasible strategy. Alternatively, TGFß could be specifically extracted from WGFE by affinity binding to the pro-region of $TGF\beta_1$ (latency-associated peptide), as Bottinger *et al.* (1996) have reported that purified latency-associated peptide prevents growth inhibition mediated by TGFß isoforms 1, 2 and 3.

Chapter Five

Effects of dietary WGFE on intestinal cell proliferation

5. *Effect of dietary WGFE on intestinal cell proliferation*

Contribution to the work

The animal tissues used in this study were part of a trial conducted by other researchers in the laboratory investigating the effects of WGFE on apoptosis and proliferation. As such, animal experimentation and tissue collection was performed by Ms L Srpek and Mr B Edwards under the supervision of Dr J Clarke. Gastrointestinal tissues were processed by Mrs J Cool. Sectioning, staining and quantification of growth and proliferative indices was conducted by myself, as was the subsequent collation and analysis of data.

Abstract

The ability of WGFE to reduce chemotherapy drug-induced damage in the gastrointestinal tract has previously been reported (Howarth *et al.,* 1996). In that study, WGFE was administered to rats as a dietary supplement for 5 days, beginning at the onset of drug exposure and continuing for 2 days after the third daily drug injection. Since the protective effect of WGFE fractions against chemotherapy damage in cultured epithelial cells was associated, at least in part, with the anti-proliferative activity of the fractions, it could be extrapolated that similar mechanisms may be responsible for protection *in vivo*. Accordingly, WGFE was administered to rats (in the absence of chemotherapy agents) to determine any effect on crypt/villus growth or crypt cell proliferation. Adult male rats were placed on a diet containing WGFE (3.5 mg/g body weight) for 3 days. The inclusion of WGFE in the experimental diet had no effect on body weight gain, or on the lengths of crypt and villus structures in either the proximal jejunum or proximal ileum. An index of proliferation (BrdU incorporation) in the proximal jejunum remained unaffected by WGFE feeding. Furthermore, uniform patterns of BrdU labelling along the crypt axis indicated that in the drug-free state WGFE did not re-distribute crypt cell proliferation. This

study suggests that WGFE is unlikely to protect epithelial cells *in vivo* by reducing crypt cell proliferation, and instead may be acting either to abort the induction of an apoptotic program and/or to enhance the regeneration of damaged crypts from surviving stem cells.

5.1 Introduction

Howarth *et al.* (1996) originally showed that control WGFE was able to actively reduce the severity of chemotherapy drug-induced mucositis when administered orally, beginning with the first methotrexate injection and continuing for 2 days after the third daily drug injection. This effect was most marked in the jejunum, where the percentages of intact villus surface length and crypt area were greater in WGFE-fed animals than casein-fed controls. However, the nature of the drug exposure strategy in these experiments, which involved daily subcutaneous injections over a 3 day period, meant that damage was accumulating in the intestinal crypts before the drug administration schedule was complete. Furthermore, by feeding the animals with WGFE as a concurrent and post-drug treatment, the authors introduced the additional complexity of not being able to distinguish between preventative and regenerative mechanisms of action.

Other studies involving the alleviation of oral or intestinal mucositis have focused on either a pre/concurrent or post-drug administration strategy, providing some insight into the likely mechanisms by which the degree of mucositis can be modified. Induction of mucositis in the cheek pouch of the Golden Syrian hamster is reduced by prior topical application of $TGF\beta_3$ (Sonis *et al.*, 1994; Sonis *et al.*, 1997), but is exacerbated by concurrent or post-infusion of EGF (Sonis *et al.*, 1992), suggesting that inhibition of epithelial cell cycling is a primary factor in reducing the severity of oral mucositis. However, few studies have investigated the efficacy of orally administered treatments in providing protection or stimulating regeneration from mucositis in the small intestine. Oral administration of monoclonal antibodies directed against doxorubicin, an agent clinically associated with mucositis, reduces intestinal toxicity and mortality following exposure to large drug doses (Morelli *et al.*, 1996). Pre/concurrent dietary administration of a short-chain fatty acid mixture, containing acetate, propionate and butyrate, also minimises the appearance of mucositis induced by cytarabine in an experimental model (Ramos *et al.*, 1997). However these improvements appear to result from a stimulation of glutamine synthesis and gluconeogenesis, rather than any alteration of cell cycling as is induced

by TGFß.

From the *in vitro* experiments described earlier in this thesis I had identified that WGFE was most likely to increase the number of cells surviving drug exposure when added as a pre/concurrent treatment. One mechanism of action which is believed to contribute to the enhanced survival observed *in vitro* is the cell cycle arrest induced by TGFß peptides in late G_1 phase. However, the results outlined in Chapters 3 and 4 indicated that TGFß was not the only survival-promoting agent present in the control WGFE material. In order to determine whether TGFß-mediated growth inhibition was likely to contribute to the improved survival observed *in vivo* by Howarth *et al.* (1996), I examined the effect of orally administered WGFE on intestinal growth and proliferation in the absence of a chemotherapy agent. Parallel studies were conducted by others in the laboratory to identify the extent of apoptosis induced by a single injection of methotrexate and to quantify any changes to this state resulting from the pre/concurrent administration of WGFE as a dietary supplement.

5.2 Materials and Methods

5.2.1 Animals and ethics

Male Sprague-Dawley rats were housed in the animal holding facility at the Women's and Children's Hospital, and weighed approximately 255 g at the beginning of the experiment. Animals were maintained in a controlled environment at 25°C with a 12 h light/dark cycle. All rats had unrestricted access to food and water both before and during the trial procedures.

Ethics approval for the animal experimentation was obtained from the Women's and Children's Hospital Animal Ethics Committee (AE 216b/7/97). Animal tissues obtained as a result of this ethics approval were used by myself with the knowledge and consent of the University of Adelaide Animal Ethics Committee.

5.2.2 WGFE and diet preparation

Control (non-acidified) WGFE was prepared as described in Chapter 3 except that it was dialysed against 150 mM NH_4HCO_3. Vacuum drying of the dialysed WGFE material resulted in the evaporation of the ammonium bicarbonate, leaving a pure freeze-dried powder for inclusion as a dietary supplement. During the experimental period the rats were maintained on either an unsupplemented casein diet or a test diet in which the casein was partially replaced with the WGFE powder. The WGFE was substituted for casein on an iso-nitrogenous basis, comprising 38.7g WGFE/kg diet, and providing 19.6% of the total protein source. This dietary concentration of WGFE was similar to the maximum active dose used by Howarth *et al.* (1996) in their original study.

5.2.3 Experimental design

A generalised protocol of the experimental design for the analysis of growth and proliferative responses is illustrated in Figure 5.1. Before the start of the experiment, rats were transferred to individual Techniplast metabolism cages to permit acclimatisation. All animals were fed a

casein-based diet for the four days of this stabilisation period. Half of the animals were then transferred to the WGFE-supplemented diet, whilst the others remained on the casein diet. Experimental diets were administered for 3 days to provide time for potential growth inhibitory effects to become evident. One hour before sacrifice every animal was injected intra-peritoneally with 5-bromo-2′-deoxyuridine (BrdU) at a dose of 50 mg/kg body weight. Animals were subsequently anaesthetised and blood was collected by cardiac puncture for endotoxin analysis. The rats were then opened via a midline incision, providing access to the gastrointestinal tract for tissue collection.

During the experimental period, food intake was monitored daily to ensure that ingestion of the WGFE-supplemented diet was similar to that of the casein-fed animals. Body weights were also measured daily to record the overall growth of the animals.

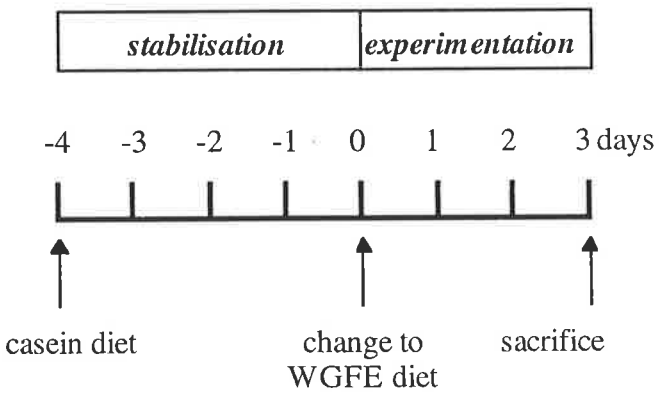


Figure 5.1 Protocol of experimental design

5.2.4 Tissue collection and preparation

Tissues were collected from various regions of the gastrointestinal tract, including the duodenum, proximal and distal jejunum, proximal and distal ileum, and proximal colon and distal colon. All intestinal tissues were preserved in methacarn fixative (see Appendix for

composition) for 2 h, then stored in 70% ethanol until processed for routine paraffin embedding. Samples were embedded using an automated tissue processor (Tissue Tek, Miles Scientific), orientated to produce transverse sections of intestine. A minimum of three segments of each gut tissue were embedded in the same paraffin block to increase the number of samples obtained per section.

5.2.5 Histological analysis

Histological analysis was performed as described by Howarth *et al.* (1996). Briefly, sections were cut at a thickness of 3 µm, then de-paraffinised and hydrated before being stained with haematoxylin and eosin (see Appendix for composition of stains). After staining, the sections were dehydrated and mounted with DePeX medium (BDH Chemicals). Stained sections were examined by light microscopy at 4 x magnification. Images were visualised using a colour video camera connected to image analysis software (Prism View, Dapple Systems). Crypt depth and villus height measurements were directly calculated from digitised images of each tissue following the calibration of the image analysis software.

Measurements of crypt depth and villus height were collected only from selected structures. Crypts were chosen for analysis on the basis that they were transversely sectioned, with the entire length of the crypt evident and a single layer of epithelial cells lining the crypt. Villi were also required to have been cut in full transverse section, with both the tip of the villus and the crypt/villus junction being clearly evident. Preliminary measurements were conducted using tissues from a casein-fed rat to determine the progressive mean and coefficient of variance (CV) of the crypt depth and villus height measurements. Separate measurements were collected for each region of the gut under examination to identify the minimum number of crypt and villus measurements which would yield a CV of less than 10%.

5.2.6 Immuno-histochemical analysis of proliferation

Proliferation was quantified in 3 µm sections of jejunal tissue following preservation in methacarn fixative and routine embedding in paraffin. Proliferative activity in intestinal crypt

epithelial cells was assessed by the immuno-detection of BrdU which had been incorporated into newly synthesised DNA prior to sacrifice of the animals. BrdU was detected using a mouse IgG_1 monoclonal anti-BrdU antibody (clone Bu20a, Dako Corporation) and immuno-peroxidase staining.

Endogenous peroxidase activity was blocked in de-paraffinised sections by immersion in methanol containing 0.3% H_2O_2 for 30 minutes. Sections were then hydrated through a series of graded alcohols into water. To provide accessible binding sites for the anti-BrdU antibody, the DNA within each tissue section was partially denatured by incubation in 1M HCl at 60°C for precisely 8 minutes. Sections were then rinsed and covered with 10% normal horse serum (Vector Laboratories Inc.) in phosphate buffered saline (pH 7.4) for 20 min to reduce non-specific antibody binding. The monoclonal anti-BrdU antibody was then applied to the sections at a 1:99 dilution, prepared in phosphate buffered saline containing 1.32% normal horse serum, and left for 18 h at 4°C. Antibody binding was enhanced by a subsequent 90 min incubation at 37°C. After gentle washing to remove unbound antibody, biotinylated horse anti-mouse IgG (Vector Laboratories Inc.) was applied as a secondary antibody (1:199 dilution) for 30 min. Following further washing to remove the secondary antibody, a complex of streptavidin-peroxidase (Vector Laboratories Inc.) was applied for an additional 30 min. Sites of BrdU incorporation were then labelled by the deposition of a coloured precipitate produced by the reaction of peroxidase with its enzymatic substrate, 3,3'-diaminiobenzidine (DAB), in 0.1% H_2O_2 for approximately 15 min. Sections were then counterstained with haematoxylin, dehydrated and mounted with DePeX.

Measurement of BrdU-labelled cells was performed at 20 x magnification in 30 full-length crypts. Each epithelial crypt cell was assigned a number to indicate its position within the crypt, beginning from the base of the crypt and extending to the crypt/villus junction (Figure 5.2). The presence of BrdU at each cell position was recorded only for the right hand side of the crypt, so as to increase the number of independent measurements collected. The crypt labelling index for BrdU incorporation was then calculated as the proportion of positively labelled cells within each

crypt. For analysis of the topographical distribution of BrdU staining, the labelling index was calculated as the proportion of positively labelled cells at each cell position, with measurements taken from a total of 30 crypts per animal.

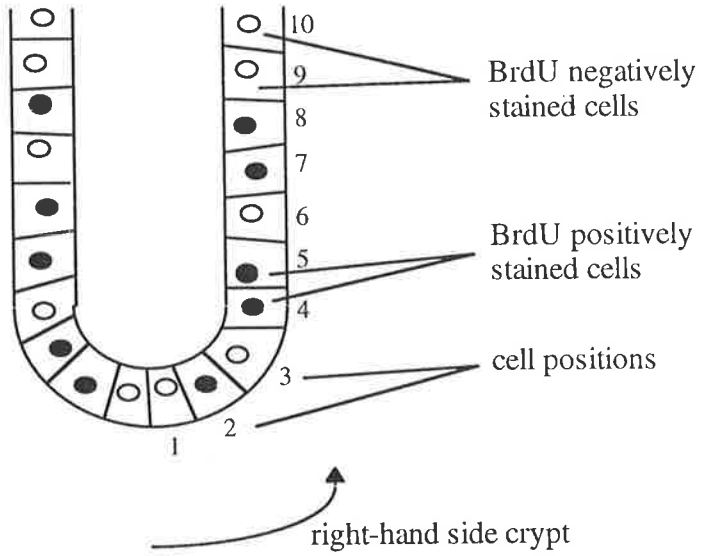


Figure 5.2 Schematic representation of proliferative labelling in an intestinal crypt

Epithelial cell proliferation was measured in the right-hand side of 30 full-length intestinal crypts. Proliferative activity was assessed by immuno-detection of BrdU incorporated into cellular DNA. The BrdU labelling index was calculated as the proportion of positively stained cells in each crypt. The distribution profile of BrdU labelling was also monitored by recording the proliferative status of each cell position along the crypt.

5.2.7 Statistical analysis

Analysis of the effects of dietary treatment on histological and proliferative parameters was performed using an unpaired t-test assuming equal variances. In all instances, the differences between treatment standard deviations were found to be non-significant. Analysis of dietary effects on apoptosis was performed by two-factor ANOVA. Where statistical significance was observed ($P < 0.05$), the inter-group differences were further analysed by direct means comparison.

5.3 Results

5.3.1 Food intake and body weight measurements

The food intake and body weight gain measurements were recorded daily for all rats during the experimentation period to ensure that all rats ingested similar quantities of protein and maintained normal weight gain. The food intake values for rats maintained on a casein diet increased over the 3 day period from 21.68 g to 24.22 g (Table 5.1). Rats fed the WGFE-supplemented diet for 3 days ingested between 22.61 g and 23.35 g of diet per day.

At the beginning of the experimentation period, the average body weights were 254.1 g for rats remaining on the casein diet and 255.7 g for rats switching to the WGFE-supplemented diet (Table 5.2). Body weights steadily increased in both dietary treatment groups, with no significant differences arising between the two. The average daily body weight gain for each of these casein and WGFE diet groups was 9.3 g/day and 9.9 g/day, respectively.

Trial day	casein-fed	WGFE-fed
1	21.68 ± 0.33	22.61 ± 1.04
2	23.74 ± 0.61	23.35 ± 0.74
3	24.22 ± 0.65	23.07 ± 0.72

Table 5.1 Food intake (g) for rats fed casein or WGFE-supplemented diets

Values are the mean ± SEM of 6 animals/diet group.

Trial day	casein-fed	WGFE-fed
0	254.1 ± 4.92	255.7 ± 5.57
1	261.4 ± 5.37	265.9 ± 4.48
2	272.3 ± 5.16	277.2 ± 4.19
3	281.9 ± 5.08	285.4 ± 4.16

Table 5.2 Body weight (g) for rats fed casein or WGFE-supplemented diets

Values are the mean ± SEM from 6 animals/diet group.

5.3.2 Histological indices of intestinal growth

Crypt depth and villus height were measured in the proximal jejunum and proximal ileum regions of the gastrointestinal tract to determine the effect of short-term oral WGFE administration on intestinal growth.

In a preliminary study I calculated the cumulative mean and CV values for both crypt depth and villus height in a casein-fed rat, obtaining 30 measurements for each parameter (Table 5.3). The CVs for both variables, in both regions of the intestine examined, had stabilised after 20 measurements. In all instances except for the crypt length in the ileum, these values fell below 10%. Thus, all subsequent analysis was performed with 20 length measurements per animal for each of the parameters studied.

Intestinal region	Crypt depth (mm)	CV	Villus height (mm)	CV
Jejunum 1	0.111	6.9%	0.440	9.6%
Ileum 1	0.124	12.9%	0.226	7.9%

Table 5.3 Cumulative mean and CV values for crypt depth and villus height

Measurements of crypt depth and villus height were taken from a rat maintained on a casein diet. Values represent the mean length and coefficient of variance accumulated from 30 independent measurements.

Following 3 days of experimental dietary feeding, crypt and villus lengths in the proximal jejunum of WGFE-fed animals were not significantly different from those of the casein-fed animals (Figure 5.3). Crypt lengths in the jejunum were calculated to be 0.115 mm and 0.123 mm in casein and WGFE-fed animals, respectively, whilst villus lengths were 0.491 mm and 0.516 mm. Similar results were obtained from the proximal ileum region of the gastrointestinal tract. The crypt lengths of rats fed either casein or WGFE diets was 0.131 mm and 0.123 mm, respectively (Figure 5.4). Statistical analysis of the crypt lengths indicated that there was no significant difference between these treatment groups ($P = 0.1252$). Villus height measurements in the proximal ileum also showed no significant differences between dietary treatments, with lengths of 0.263 mm and 0.259 mm in casein and WGFE-fed animals, respectively.

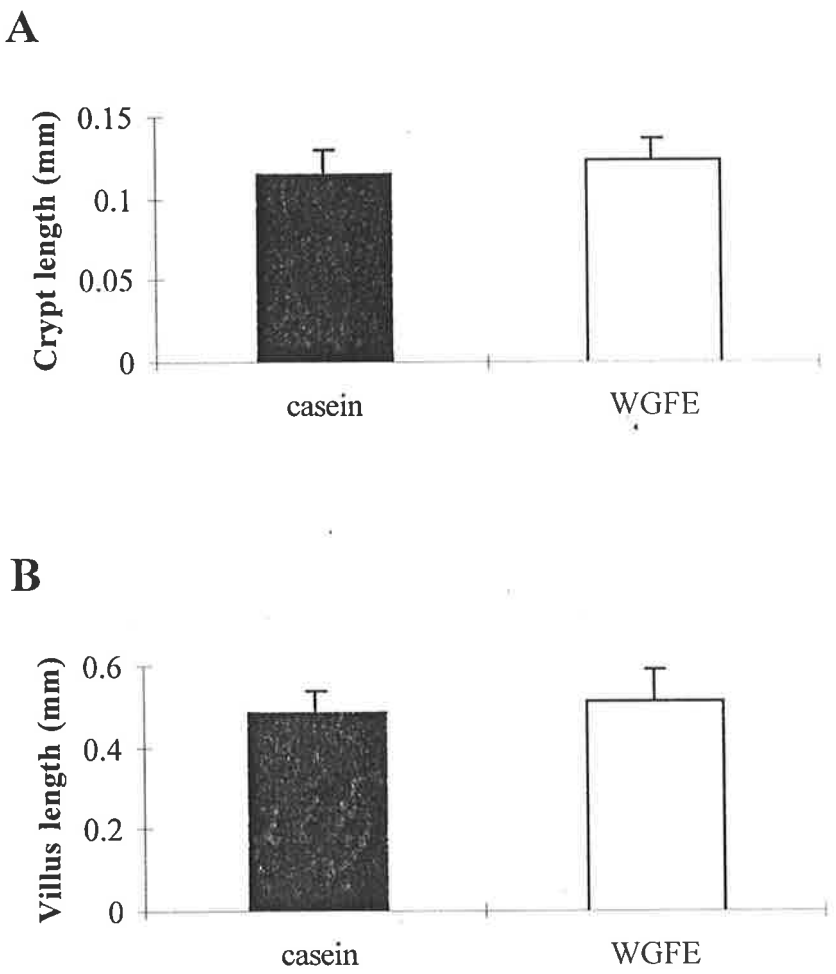


Figure 5.3 Crypt and villus length measurements in the jejunum of rats fed WGFE

Crypt depth **(A)** and villus height **(B)** measurements were determined in the proximal jejunum of normal rats fed a control casein diet or an iso-nitrogenous diet containing control WGFE for 3 days. Values represent the mean ± SD of 20 measurements from each of 6 animals/diet group.

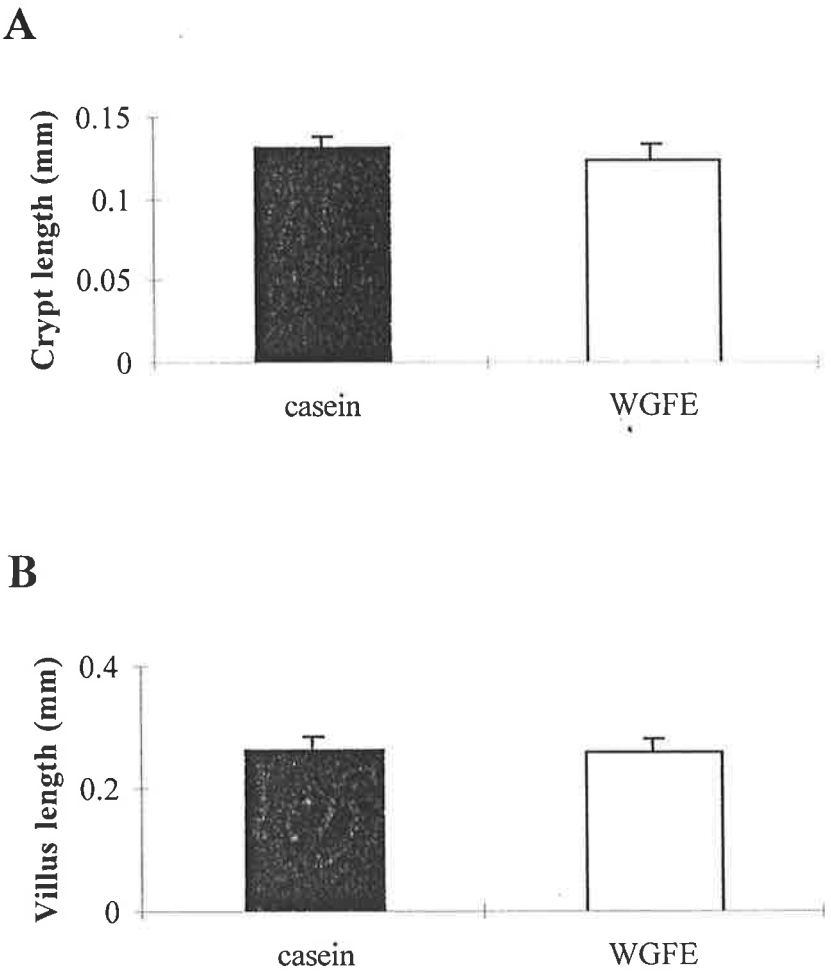


Figure 5.4 Crypt and villus length measurements in the ileum of rats fed WGFE

Crypt depth **(A)** and villus height **(B)** measurements were determined in the proximal ileum of normal rats fed a control casein diet or an iso-nitrogenous diet containing control WGFE for 3 days. Values represent the mean ± SD of 20 measurements from each of 6 animals/group.

5.3.3 Proliferative analysis in the jejunal crypts

The effects of dietary administration of WGFE on crypt cell proliferation were also assessed to examine whether WGFE was able to reduce the amount or the pattern of distribution of proliferation. Immuno-histochemical staining of intestinal tissue sections revealed extensive labelling of epithelial cells in the lower two thirds of each crypt in the proximal jejunum, irrespective of dietary treatment (Figure 5.5). Labelling was not evident in the intestinal villi, consistent with the terminally differentiated status of these cells.

Quantitative analysis of the proliferative labelling indicated that BrdU incorporation was not significantly altered by feeding animals a WGFE-supplemented diet for 3 days. The BrdU labelling index in casein-fed animals was calculated to be 20.95%, whilst in WGFE fed animals positive labelling was observed in an average of 21.60% of crypt epithelial cells (Figure 5.6). Analysis of the positional distribution of the positively labelled crypt cells also failed to distinguish between casein and WGFE-fed animals (Figure 5.7). As previously observed by Potten *et al.* (1997b), the frequency of crypt cell proliferation peaked between cell positions 6 and 13, with position 1 representing the cell at the base of the crypt. Proliferative labelling occurred with a maximal frequency of 42.2% and 45.5% at cell position 7 in the casein and WGFE-fed animals, respectively, and was absent in cells above position 22.

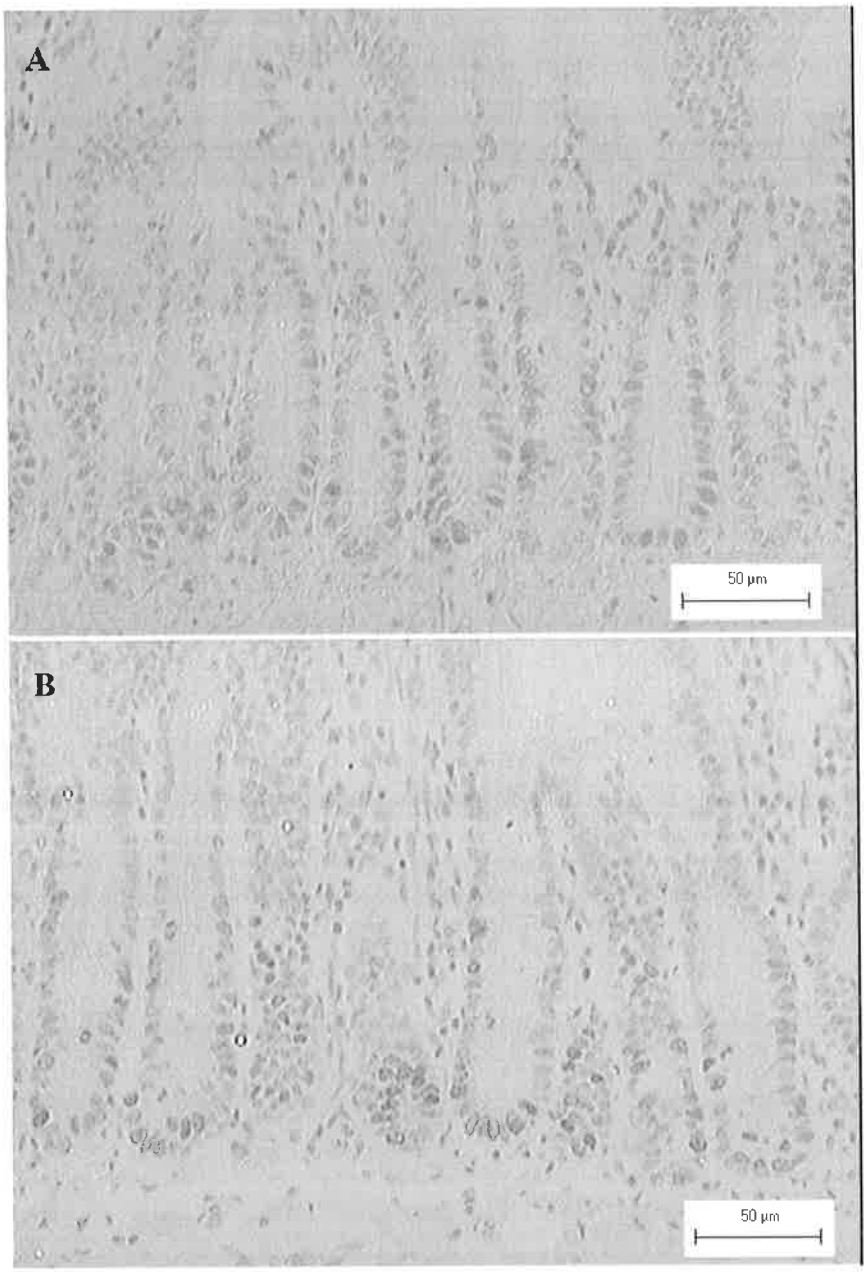


Figure 5.5 Proliferative labelling in crypts of the proximal jejunum

Proliferation was detected by immuno-histochemical staining of BrdU labels in tissue sections from the jejunum of rats maintained on a casein diet **(A)** or fed WGFE **(B)** for 3 days prior to sacrifice. BrdU incorporation is seen as brown staining.

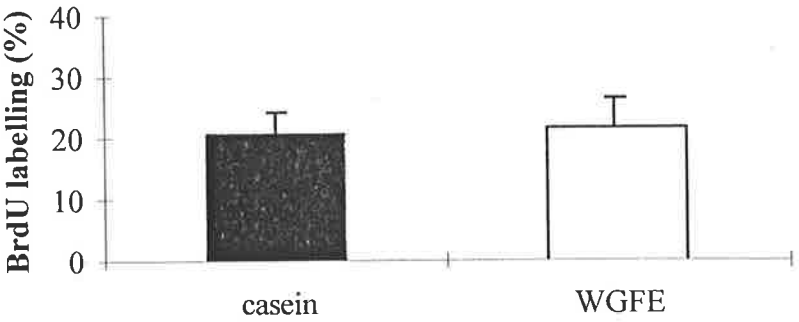


Figure 5.6 Proliferative labelling index of jejunal crypts from rats fed casein or WGFE

Proliferative labelling was detected by immuno-histochemical staining of BrdU labels in tissue sections from the jejunum of rats maintained on a diet of casein, or WGFE-supplemented casein, for 3 days prior to sacrifice. The labelling index was calculated as the percentage of positively labelled cells in each full-length crypt. Values represent the mean ± SD of 30 independent crypt measurements from each of 6 animals/dietary group.

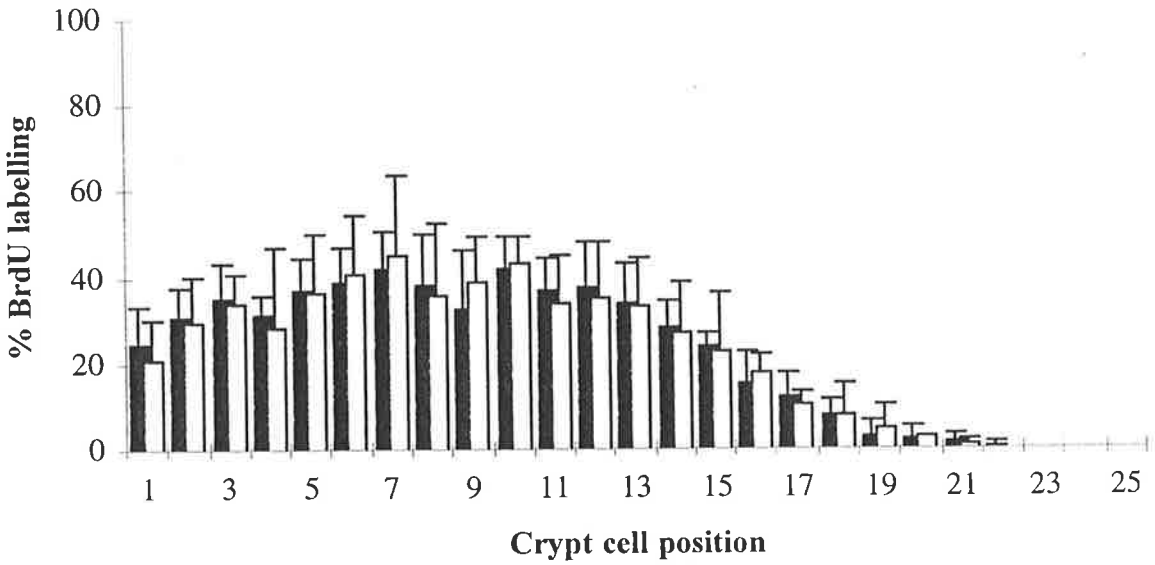


Figure 5.7 Distribution profile of BrdU labelling along jejunal crypts

BrdU incorporation into cellular DNA was detected by immuno-histochemical staining of jejunal sections from rats fed either a casein (■) or WGFE (□) based diet for 3 days prior to sacrifice. The BrdU labelling index was calculated for each crypt cell position by determining the proportion of positively labelled cells measured at that particular location. Cell proliferation was assessed in 30 full length crypts from each animal. Values indicate the mean ± SD of 30 crypts measured from each of 6 animals/dietary group.

5.4 Discussion

The cytoprotective effects of TGFß on epithelial cells *in vivo,* against both chemotherapy drug toxicity and irradiation, are now well established (Sonis *et al.,* 1994; Pierce and Coffey, 1994; Sonis *et al.,* 1997; Potten *et al.,* 1997a). Maximal growth inhibition is achieved by the exposure of an unsynchronised cell population to TGFß for the duration of one cell cycle (Geng and Weinberg, 1993). Thus, most protective strategies have adopted a pre-treatment time of at least 24 h before toxic insult to the gastrointestinal tract, although Pierce and Coffey (1994) have reported a 100% survival rate of animals following a 12 h pre-treatment with TGFß.

The mechanism of TGFß-mediated protection *in vivo* remains unclear, as it does *in vitro.* However, there is a clear association between growth inhibition and parameters of gastrointestinal integrity and animal survival. TGFß has been reported to reduce epithelial cell cycling when applied topically to hamster cheek pouch mucosa (Sonis *et al.,* 1997). Whilst TGFß also reduces crypt and villus height, and crypt cellularity when administered intra-peritoneally (Migdalska *et al.,* 1991), presumably by restricting crypt cell proliferation, there is little evidence in the literature to suggest that TGFß acts on intestinal tissues when given orally, unless protected against acidic conditions and proteolytic degradation (Puolakkainen *et al.,* 1994).

The first section of the chapter examined a number of parameters which I believed would be affected by TGFß if it was able to retain its biological activity following passage through the stomach and duodenum, and could reach the target cells of the jejunal crypts. Measurement of food intake confirmed that both the casein and WGFE-fed animals received equivalent amounts of protein in their diet, thereby excluding variable consumption as a complicating factor. Increases in body weight were also similar between the two dietary groups, indicating no gross alterations in growth resulted from WGFE administration.

Given that the time taken for cells to migrate along the length of a crypt is approximately 20 - 30 hours in an adult rat (Altmann and Enesco, 1967), and that this migration must be counter-

balanced by the production of new crypt cells, a reduction in crypt cell proliferation would reasonably be expected to result in shortening of crypt and/or villus lengths. Following 3 days of dietary supplementation with WGFE, no such alterations in crypt depth or villus height were detectable, indicating that animals maintained on the experimental test diet were able to maintain structural homeostasis.

The absence of proliferative changes in the jejunal crypts, as measured by BrdU incorporation into synthesising DNA, showed that WGFE had no effect on overall levels of DNA synthesis or on the distribution profile of proliferating cells. These results tend to discount the possibility that WGFE reduced the methotrexate damage reported by Howarth *et al.* (1996) by re-distributing the proliferative cell profile away from those cells known to be targeted by methotrexate (positions 11 - 14, (Ijiri and Potten, 1983)). Although not quantified, the reduction in BrdU labelling observed 24 h after a single intravenous methotrexate injection did not appear to be restored by WGFE feeding, suggesting that WGFE was probably not functioning a post-drug regenerative treatment.

Although no changes in intestinal growth were observed following the oral administration of WGFE to rats for 3 days, this test diet induced significant changes in the incidence of apoptotic cells quantified 24 h after exposure to methotrexate (Dr H Beere, unpublished results). The jejunal crypts of WGFE-fed animals contained 26.2% fewer apoptotic cells than their casein-fed counterparts ($P < 0.01$), resulting in a greater number of surviving cells which would be capable of re-populating damaged crypts. Whilst this study suggested that oral administration of WGFE significantly reduced drug-induced apoptosis, a subsequent trial conducted by Dr Beere provided less conclusive results. However, no proliferative measurements were obtained from that trial for comparison with the data reported here. At present, it would appear that WGFE may prevent the induction of apoptosis following methotrexate injection, but further studies are required for verification of these results.

The lack of growth inhibition following WGFE feeding suggests that TGFß is unlikely to be the only cytoprotective component of WGFE, as orally administered WGFE had no effect on

crypt cell proliferation but appeared to be responsible for suppressing methotrexate-induced apoptosis *in vivo*. The ability of WGFE peptides to reduce the incidence of apoptosis further suggests that WGFE does not primarily operate as a post-drug regenerative treatment, but rather contains an anti-apoptotic survival factor. Hence, it is likely that the critical portion of the concurrent/post-drug feeding schedule employed by Howarth *et al.* (1996) was the concurrent treatment period, during which WGFE would be expected to have prevented the induction of apoptosis.

Although TGFß is able to actively reduce drug toxicity *in vitro*, the ability to similarly reduce crypt cell proliferation *in vivo* could be expected to be less straightforward. TGFß is mostly present in the WGFE as part of a latent complex, which may have prevented acidic or proteolytic degradation during passage through the stomach. Indeed, exposure of latent TGFß complexes to the acidic conditions in the stomach may have increased the concentrations of biologically active TGFß reaching the small intestine. Additionally, the presence of a mixture of whey proteins may have helped to protect the TGFß from damage. However, the lack of growth inhibition observed with dietary WGFE suggests that the TGFß was not functionally active in the small intestine, possibly due to peptide degradation by stomach and/or duodenal enzymes before reaching the jejunum and ileum. It is unlikely that the TGFß was simply absorbed into the systemic circulation, as intra-peritoneal administration of TGFß promotes significant growth inhibition in crypt epithelial cells (Migdalska *et al.*, 1991). Alternatively, it is possible that the WGFE-borne TGFß did reach the jejunum in a biologically active form, but was unable to induce growth inhibitory responses due to a lack of appropriate receptors for the $TGFß_2$ isoform. To some extent, the ability to mediate cell cycle arrest in the crypt epithelium may also depend upon the partial loss of gut barrier integrity, providing WGFE with access to $TGFß_2$ receptive cells. During the early stages of methotrexate-induced damage, using a low dose/long term exposure as employed by Howarth *et al.* (1996), it is feasible that intestinal barriers would be compromised sufficiently for $TGFß_2$ to reach such cells.

Despite the fact that $rTGFß_2$ was able to protect Mv1Lu cells from drug damage *in vitro*, the

neutralisation studies performed in Chapter 4 indicated that TGFß neutralisation of WGFE did not reduce the total number of cells surviving vinblastine exposure (only the percent survival fell, due to the expansion of the drug-free population). As TGFß was obviously not essential for the WGFE-mediated protection of Mv1Lu cells *in vitro*, and does not appear to reduce crypt cell proliferation when administered orally, I conclude that there are uncharacterised peptides present in WGFE which are at least partly responsible for the WGFE-mediated survival reported by Howarth *et al.* (1996).

Chapter Six

General discussion

6. General discussion

6.1 Introduction

The prevention or alleviation of symptoms associated with chemotherapy treatment is of clinical importance, not only for improving patient well-being but also for reducing the costs associated with long-term hospitalisation. A number of strategies are presently used in clinical practice to reduce the symptoms of oral and intestinal mucositis, including pain relief, anti-diarrhoea treatments and the application of antiseptics and ice cubes. However, these practices do not address the underlying causes of mucositis and are only partially successful in improving patient well-being and morale. This has several social and clinical implications. Firstly, severe mucositis prevents adequate nutrient absorption by the chemotherapy patient, resulting in the need for hospitalisation and nutrient support, and thereby increasing social costs. Secondly, in instances of extreme mucositis, the doses or schedules of chemotherapy treatment may be altered to allow time for intestinal regeneration, reducing the chances of a successful outcome by providing time for the cancerous cells to recover from drug exposure.

More recently, the focus of efforts directed towards the clinical management of mucositis have shifted from symptomatic relief to preventative therapies. A number of peptides have been identified which reduce the severity of intestinal and oral toxicity observed following chemotherapeutic drug exposure (Pierce and Coffey, 1994; Du *et al.*, 1994). These peptides, TGFß and IL-11, induce a reversible arrest of cell cycle progression in the G_1 phase of intestinal epithelial cells (Ko *et al.*, 1994; Peterson *et al.*, 1996), suggesting a mechanism by which such cells can avoid the toxicity induced by chemotherapy agents which target non-G_1 phases of the cell cycle.

The identification of one of these cytoprotective factors, TGFß, in a growth factor extract derived from bovine cheese whey (termed WGFE) yielded the possibility of exploiting this

extract for therapeutic benefit. Indeed, the oral administration of WGFE to rats receiving three daily injections of methotrexate resulted in a significant reduction in drug-induced damage in the intestinal crypt cells, and helped to preserve intestinal structure and barrier integrity (Howarth *et al.,* 1996). However, the nature of the WGFE feeding schedule, which consisted of administration during and after drug exposure, was inconsistent with the mechanism of cytoprotection proposed for TGFß. Theoretically, cell survival would have been optimised by a reduction in cell cycling prior to and during toxic insult, with a rapid reversal of such growth arrest once the risk of damage had passed (Weyman and Stacey, 1996). By extending the WGFE administration over the concurrent and post-drug period, it was impossible to dissect preventative, anti-apoptotic and regenerative mechanisms. Furthermore, the use of methotrexate as the representative chemotherapy agent introduced other variables, specifically related to its role as an anti-metabolite, including the possible induction of the thymidine salvage pathway or the contribution of whey-borne folates.

The development of an *in vitro* model of drug toxicity provided the opportunity for more extensive investigations into the optimal timing of WGFE administration and the relative contributions of component growth factors within the WGFE preparation. Thus, through this development and subsequent *in vivo* studies, the aims of this thesis were to assess:

- the optimal timing of WGFE exposure in relation to chemotherapy drug exposure
- the effects of growth factor modification on the cytoprotective efficacy of WGFE
- the role of non-TGFß proteins in promoting survival
- the ability of orally administered WGFE to arrest crypt cell proliferation as a potential mechanism of cytoprotection *in vivo*

6.2 Optimal timing of WGFE exposure in relation to drug exposure

Comparison of two timing schedules for WGFE incubation - pre/concurrent or post-drug treatment - showed that all four of the WGFE fractions used in this study promoted enhanced cell survival only when applied prior to and during drug exposure. This treatment schedule is common to a number of chemo-protective agents that induce G_1 phase arrest, namely rTGFß (Weyman and Stacey, 1996; McCormack *et al.*, 1997) and IL-1ß (Potten, 1996). Additionally, the arrest of cells in G_1 phase, by temperature-dependent expression of p53, renders cells less susceptible to phase-specific drug toxicity (discussed in McCormack *et al.* (1997)). Consistent with such a mechanism of action, WGFE induced at least a partial G_1 phase arrest in Mv1Lu cells following a 30 h incubation, and complete growth inhibition within 48 h (Chapter 3).

The lack of a discernible improvement in cell survival when the WGFE fractions were added following drug exposure argues against a role for these preparations in promoting the recovery of damaged cells. Similar findings have been reported by Potten *et al.* (1997a), where post-treatment of radiation-induced damage with $TGFß_3$ reduced crypt integrity, whereas pre-treatment induced the opposite effect. However, it is possible that further modification of the WGFE fractions to eliminate the predominant growth inhibitory effect of TGFß may uncover anti-apoptotic or regenerative activities of other component growth factors.

6.3 Effects of growth factor modification on the cytoprotective efficacy of WGFE

The modification of WGFE by transient acidification and membrane partitioning produced four fractions with distinct growth factor compositions. At maximally inhibitory concentrations of each of the fractions, those with the lowest IGF-I:$TGFß_2$ ratios (acid and retentate WGFE) proved to be the most protective. Although the permeate WGFE remained growth inhibitory (Chapter 3), it exhibited an approximately 50-fold greater IGF-I:TGFß ratio than the acid and retentate WGFE fractions. I had postulated that the presence of such a relatively large

concentration of IGF-I peptides would modify the survival activity of the permeate WGFE when compared to equivalent inhibitory concentrations of the acid and retentate WGFE fractions, or the rTGFß$_2$. Whilst there is no evidence to suggest that IGF peptides alter the growth inhibitory response, they are capable of enhancing cell survival after chemotherapy drug exposure (Sell *et al.,* 1995). However, the fact that the permeate WGFE fraction behaved identically to the rTGFß$_2$ treatment suggests that IGF-I did not contribute to cell survival. This assumption is conditional on a number of points. Firstly, there may have been sufficient quantities of biologically active IGFs already present in the 5% serum which supplemented all culture medium, eliminating any requirement for additional IGF peptides. Secondly, the concentration of IGF-I peptides in each of the WGFE fractions was quantified by radio-immunoassay, and therefore did not estimate the concentrations of IGF-I which were biologically active. It is quite likely that, despite being purified into the permeate fraction, the IGF peptides were partially inhibited by co-purification of IGF binding proteins, and therefore could not contribute to survival responses. The absence of any survival activity in the permeate WGFE which could not be accounted for on the basis of TGFß-equivalent growth inhibitory activity discounts a possible contribution from the EGF-like peptide recently characterised in the permeate WGFE fraction.

The maximally inhibitory concentration of the control WGFE proved to be less effective at mediating cytoprotection than equivalent inhibitory doses of the other fractions. Part of this phenomenon may have been the higher concentrations of protein required to produce growth inhibition with this fraction, necessary due to the low levels of active TGFß per mg protein. However, comparison of the four WGFE fractions at lower concentrations showed that the acid and retentate fractions were not necessarily the most effective. The control WGFE was optimal at concentrations that produced less than complete growth inhibition, and in some circumstances at concentrations which appeared to produce no growth inhibition at all (Chapter 3). These effects were greater than those observed with equivalent concentrations of rTGFß$_2$, suggesting the presence of at least one additional survival-promoting factor. Immuno-neutralisation studies to block the TGFß activity of the control WGFE confirmed this, and even

indicated that TGFß activity wasn't at all required to sustain surviving cell numbers (Chapter 4). Identical experiments with the acid WGFE confirmed the presence of the novel survival activity in this fraction, implying that it was not degraded or precipitated during the acidification procedure, and again suggesting that the biologically active TGFß was not responsible for maintaining cell number following vinblastine exposure.

6.4 Role of non-TGFß proteins in promoting survival

Examination of the effects of IGF-I, PDGF and EGF provided no indication that these growth factors were individually contributing to the additional survival present in the control and acid WGFE fractions. Whilst I know definitively that the residual survival activity is present in both of these fractions, I hypothesise that the survival activity then concentrates into the retentate fraction upon further processing. Without immuno-neutralisation studies of the retentate WGFE, however, my only indication that this is the case comes from the survival assays performed with equivalent growth inhibitory concentrations of each WGFE fraction (Chapter 3). In these experiments the highest concentration of retentate WGFE tested provided slightly more protection than equivalent concentrations of $rTGF\beta_2$, although this failed to reach significance. Additionally, the retentate WGFE behaved almost identically to the acid WGFE over the concentration ranges examined, suggesting that the two fractions contained the same cytoprotective factors. To date, however, all of the growth factors, apart from TGFß, which have been characterised in WGFE seem to concentrate into the permeate fraction. Thus, it is not surprising that the IGF-I, PDGF and EGF (representing the recently characterised EGF-like molecule) did not account for the non-TGFß survival activity.

Although IGF-I and PDGF may have survival activity in epithelial cells, similar to that reported in fibroblastic cells (Sell *et al.,* 1995), it is also unlikely that I would have observed such a response in the presence of 5% serum-supplemented medium. IGF-I or PDGF-mediated survival activity may have been detected in the control WGFE fraction if lower concentrations

of serum had been used, but it was not possible to exclude the serum during the pre/concurrent time period. As the depletion of serum proteins induces cells to enter G_0 phase, it provides a form of protection against drug toxicity similar to that produced by G_1 arresting proteins (Weyman and Stacey, 1996). Hence, serum supplementation was necessary to maintain the cultures in exponential growth and thus to incur damage. Despite this, the survival activity observed with the control WGFE fraction was clearly evident in the presence of serum-supplemented medium, and it was this activity that I was seeking to characterise.

Whilst IL-11 could account for the non-TGFß activity present in the control and acid WGFE fractions, it is improbable. Interleukin-11 induces G_1 phase arrest in IEC-6 cells (Peterson *et al.*, 1996) suggesting a growth inhibitory role for this cytokine. As all of the inhibitory activity present in both the control and acid WGFE fractions was eliminated by co-incubation with a pan-specific $TGFß_{1,2,3}$ neutralising antibody, there is no reason to conclude that other growth inhibitory peptides are contained in these fractions. Of course, there may be differences in the response of IEC-6 and Mv1Lu cells to IL-11, such that this cytokine would not be growth inhibitory in Mv1Lu cells. However, until we are able to confirm the absence of a bovine equivalent of IL-11 in the whey extracts, using a immuno-detection method, or quantify the inhibitory effects of human IL-11 in Mv1Lu cells, there is no way of conclusively excluding this protein as a potential effector of WGFE-mediated survival responses.

Despite being able to provisionally exclude a number of peptides as contributing to the observed non-TGFß-mediated survival, I have not been able to make a positive identification of the unknown factor(s). The neutral chromatographic separation of control WGFE indicates that the unknown survival factor(s) partially co-elute with TGFß, although some separation of inhibitory and survival activities was obtained (Chapter 5). However, under neutral conditions many of the growth factors in WGFE elute in the same range as TGFß (~80 kDa) (Belford *et al.*, 1997). By moving the fractions from pool 2 to pool 3 in the second neutral elution I had hoped to achieve separation of the TGFß from the other survival peptides. Unfortunately, the fractionation used did not produce a highly resolved separation, resulting in the inhibitory and

survival activities combining in the same pool. Under acidic conditions the non-TGFß survival activity was not recovered - possibly due to potentially labile or hydrophobic properties of the survival factor, or to the physical separation of two peptides which interact to produce a survival response. Whilst the chromatography used in this thesis did not result in the isolation of a pure survival factor(s), it did provide evidence that the survival and inhibitory activities may be separated under certain conditions. However, alternative purification strategies would need to be used to completely isolate, and subsequently characterise, the unknown factor(s).

6.5 WGFE-MEDIATED CELL CYCLE ARREST *IN VIVO*

The inclusion of WGFE as a three day dietary supplement was designed to identify potential changes to intestinal crypt cell proliferation. On the basis of the *in vivo* studies reported by Howarth *et al.* (1996), which involved concurrent WGFE feeding during the three days of drug exposure, I had predicted that a three day feeding schedule would be sufficient to observe a reduction in crypt cell proliferation, should such an effect be mediated by the WGFE preparation. The lack of such an effect may have been due to insufficient quantities of biologically active TGFß in the WGFE diet, although similar quantities of WGFE improved gut morphology in the trial conducted by Howarth *et al.* (1996). The reasons for this observed lack of cell cycle arrest are most likely to be that the actions of WGFE only become evident in the presence of chemotherapy drugs, or that the WGFE simply does not mediate crypt cell survival by inducing transient growth inhibition.

Comparable studies performed using a Syrian Golden hamster model of mucositis have indicated that WGFE reduces epithelial cell proliferation ($P < 0.02$) when applied topically to a mechanically irritated cheek pouch (Dr G Regester, unpublished results). The same preparation also reduces the severity of mucositis induced by 5-fluorouracil in the hamster model. In contrast to the *in vivo* study described in this thesis, the growth inhibitory factors present in the topical WGFE preparation could be expected to retain at least short-term activity in the

cheekpouch, whereas they may have been rapidly degraded during passage to the small intestine. As WGFE is able to protect against drug toxicity in both the oral and intestinal mucosa, yet only appears to reduce epithelial cell proliferation when topically applied, it seems unlikely that TGFß is the only active factor mediating protection.

In the *in vivo* model of methotrexate-induced damage used for my studies it is impossible to distinguish drug-induced cell cycle arrest from WGFE-induced arrest using BrdU incorporation alone, as both methotrexate and WGFE reduce BrdU incorporation. Flow cytometric analysis of crypt cell suspensions may prove to be useful in this respect, as DNA analysis would indicate whether the cells were arrested in late G_1 phase (TGFß-mediated) or S phase (methotrexate-mediated). In the absence of such information, and on the basis of my *in vitro* studies, I believe that it is more probable that WGFE does not protect intestinal epithelial cells from drug damage by preventing cell cycle progression.

6.6 Future directions

The major focus of future investigation into the cytoprotective properties of WGFE should centre on the isolation and characterisation of the novel survival factor(s). If the factor(s) are indeed novel, recombinant expression could then be used to obtain sufficient quantities for cell culture and *in vivo* mechanistic studies. If this is feasible, it may then be necessary to re-establish whether a pre/concurrent administration strategy is optimal for the cytoprotective activity of the recombinantly expressed protein(s), as these factor(s) may not be dependent upon a pre-treatment protocol as is the case with growth inhibitory peptides. Such survival factors may be equally as effective in promoting cell survival when applied following drug exposure, once extracted from the predominantly growth inhibitory nature of the WGFE. It is only at this stage that it would be possible to begin examining the mechanisms responsible for increasing Mv1Lu survival. Some studies have recently shed light on the pathways by which growth factors reduce apoptosis, identifying a complex between BAG-1 (a partner of Bcl-2)

and the intracellular domain of the hepatocyte growth factor (HGF) and PDGF receptors which rapidly associates following the induction of apoptosis (Bardelli *et al.,* 1996). Perhaps a similar reaction occurs upon binding of non-TGFß growth factors to their receptors on Mv1Lu cells. This certainly warrants closer examination, as HGF is present in milk from a number of species (Dr N Kallincos, personal communication), and may therefore co-purify into WGFE along with other growth factors.

6.7 Therapeutic applications

The in vitro studies described in this thesis supports the *in vivo* findings of Howarth *et al.* (1996), and may have direct implications for the clinical management of epithelial damage resulting from chemotherapy treatment. The observation that TGFß is not the only survival factor present in WGFE may give rise to a number of therapeutic approaches, depending upon the actions of the unknown factors, including prophylactic and reparative treatments.

The absence of information concerning the ability of $TGFß_2$ to bind to the crypt epithelial cells of the adult human gastrointestinal tract further emphasises the importance of identifying the non-TGFß survival factors present in WGFE. Without positive identification of these unknown survival peptide(s), the control WGFE would appear to be the most promising lead for clinical development. This prediction is based on two points. Firstly, as the non-TGFß survival activity does not appear to be activated by transient acidification, it is likely to present in the highest concentrations in the control WGFE fraction, as no losses will have occurred through protein precipitation and membrane partitioning. Secondly, if $TGFß_2$ is able to induce growth arrest in human intestinal crypt epithelial cells, it is most likely to survive passage through the stomach in latent form, perhaps being acid or proteolytically activated *in situ*.

6.8 Conclusion

From the studies described in this thesis I conclude that TGFß$_2$, as well as at least one non-growth inhibitory peptide contained in WGFE, promote the survival of epithelial cells following chemotherapy damage. The contribution of TGFß to the survival conferred by WGFE appears to be restricted to concentrations at which it induces G_1 phase arrest, consistent with other cytoprotective factors. However, the identification of the residual pro-survival peptide(s) and an understanding of the mechanism by which they operate remains unresolved. It appears likely from the *in vivo* studies that it is this uncharacterised survival factor which is responsible for the protection of crypt cells from drug-induced apoptosis.

Appendix

1. Companies listed in this thesis

Alamar Biosciences, Inc.	Sacramento, CA, USA
American Type Culture Collection	Rockville, MD, USA
Analytical Vision Inc.	Raleigh, NC, USA
BDH Chemicals	Kilsyth, VIC, Australia
Becton Dickinson & Co.	Franklin Lakes, NJ, USA
Boehringer-Mannheim	Mannheim, Germany
Bender MedSystems	Vienna, Austria
Caltag Laboratories	San Francisco, CA, USA
Celtrix Pharmaceuticals Inc.	Santa Clara, CA, USA
Coulter Electronics Inc.	Miami, FL, USA
Dako Corporation	Carpinteria, CA, USA
Dapple Systems	Sunyvale, CA, USA
David Bull Laboratories	Mulgrave, VIC, Australia
Dynatech Laboratories	Chantilly, VA, USA
Genzyme	Cambridge, MA, USA
GroPep Pty Ltd	Adelaide, SA, Australia

ICN Biomedicals Inc.	Aurora, OH, USA
Jandel Scientific	San Rafael, CA, USA
Miles Scientific	USA
Millipore Corporation	Milford, MA, USA
Olympus Optical Co.	Hamburg, Germany
Pharmacia Biotech	Uppsala, Sweden
PharMingen	San Diego, CA, USA
Phoenix Flow Systems	San Diego, CA, USA
Pierce	Rockford, IL, USA
R&D Systems	Minneapolis, MN, USA
Sigma Chemical Company	St Louis, MO, USA
Silenus Laboratories	Hawthorn, VIC, Australia
Trace Biosciences	Castle Hill, NSW, Australia
Upstate Biotechnology Inc.	Lake Placid, NY, USA
Vector Laboratories Inc.	Burlingame, CA, USA

2. *Buffers for quantification of IGF-I in WGFE fractions*

4 x Mobile Phase

0.8 M glacial acetic acid
0.225 M trimethylamine
0.15 M HCl

Immediately prior to use, the buffer was filtered through a 0.22 µm membrane and the pH was adjusted to 2.5. Tween-20 (0.2% v/v) was then added and gently mixed through the buffer.

RIA buffer

30 mM NaH_2PO_4
0.2 g/l protamine sulphate (Sigma Chemical Company)
10 mM EDTA
3.8 mM NaN_3

The buffer was adjusted to pH 7.5, then gently mixed with Tween-20 (0.05% v/v).

3. *Histological fixative and stains*

Methacarn

600 ml methanol
300 ml chloroform
100 ml glacial acetic acid

Mayer's haematoxylin

2 g haematoxylin (CI 75290)
50 g ammonium alum sulphate
0.2 g sodium iodate
1.0 g citric acid
50 g chloral hydrate

Haematoxylin was dissolved in 1 litre of 0.22 µm filtered water using gentle heat. The remainder of the ingredients were then added in sequence. The final solution was stored for at least 4-6 weeks prior to use.

Eosin

5 g eosin (CI 45400)
1 ml of 2% acetic acid

Eosin was dissolved in 100 ml of 0.22 µm filtered water, then filtered through Whatman Grade 1 paper. The acetic acid was then added, along with a few granules of thymol to prevent bacterial growth.

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