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Studies on the mechanism of action of the chemotherapeutic drug bleomycin on cell lines derived from haemangioma and keloid

Clarke, Lisa

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By

Lisa Clarke

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Department of Biological and Biomedical Science

Durham University

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Abstract

Bleomycin has been used successfully in the treatment of haemangioma, keloid and vascular malformation; however the mode of action of this chemotherapeutic drug on these non-malignant dysplasias is not well understood. The aim of this study was to investigate the effects of bleomycin on a range of human primary cell lines to improve our understanding of the mechanism by which bleomycin exerts its effects. This may facilitate the subsequent identification of alternative therapeutic strategies, thereby avoiding the well recognised and potentially life threatening side effects associated with the use of bleomycin. Primary cell lines were isolated from excised lesions using an explant culture technique. The cell lines thus obtained, along with other cell lines previously derived from other tissues, were exposed to a range of concentrations of bleomycin to examine dose response. Results showed that at a dose of 100mU/ml bleomycin, all cell lines underwent a G1 arrest after 48 hours in culture. At higher doses, bleomycin induced a dose-dependent increase in the proportion of cells in sub-G1 with all cell lines treated, a specific marker of apoptosis. Bleomycin induced apoptosis was further confirmed by TUNEL assay and results showed that keloid derived cells had a significantly greater level of DNA strand breaks than foreskin fibroblasts. An antibody specific for cleaved caspase-3 was also used to investigate apoptosis initiation. Both keloid derived cells and foreskin fibroblasts showed a bleomycin-induced cleavage of caspase-3, however the level of caspase-3 cleavage was significantly greater in keloid derived cells. In conclusion, results from this study show that bleomycin induces apoptosis of haemangioma, venous malformation and keloid derived cells in a dose dependent manner. In addition, this study reports for the first time that bleomycin induces a caspase-3 mediated apoptotic cell death in keloid derived cells.

Acknowledgements

Abbreviations

ADC – Analog-to-digital converter

ALK – Activin receptor like kinase

AVF – Arteriovenous fistula

AVM – Arteriovenous malformation

BSA – Bovine Serum Albumin

cdk – Cyclin dependent kinase

CHO – Chinese hamster ovary cell

CO$_2$ – Carbon Dioxide

DMSO – Dimethylsulphoxide

dUTP - deoxyuridine triphosphate

ECM – Extracellular matrix

EGF – Epidermal growth factor

EPC – Endothelial progenitor cell

FasL – Fas ligand

FBS – Foetal Bovine Serum

FITC - fluorescein isothiocyanate

FL – Fluorescence

FSC – Forward Scatter

G1 – Gap 1 Phase

G2 – Gap 2 Phase

HemSC – Haemangioma derived stem cell

HMEC – Human microvascular endothelial cell

HUVEC – Human umbilical vein endothelial cell

IFN – Interferon

kD – kiloDalton

MEM – Minimum essential media

MEM – Minimum essential media

MSC – Mesenchymal stem cell

PBS – Phosphate Buffered Saline

PDGF – Platelet derived growth factor

PG – Pyogenic granuloma

PI – Propidium Iodide

RNA – Ribonucleic Acid

ROS – Reactive oxygen species

RT – Room temperature

S Phase – Synthesis Phase

SSC – Side scatter

Sub G1 – Sub Gap 1 phase

TGF-ß – Transforming growth factor beta

TNF – Tumour necrosis factor

Trypsin-EDTA – Trypsin-ethylenediamine tetra acetic acid

TUNEL – Terminal deoxynucleotidyl transferase dUTP nick end labelling

UV – Ultra Violet

v/v – Volume/volume

w/v – Weight/volume

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Chapter 1

1 Introduction

1.1 Bleomycin

A number of antibiotic cancer therapeutics were discovered in the middle of the 20th century through screening of bacterial filtrates for anti-cancer activity. Examples include neomycin (Waksman et al. 1949), adriamycin (Arcamone et al. 1969) as well as bleomycin. The first report of the isolation of the latter, from the bacterium *Streptomyces verticillus* was published by Umezawa *et al.* (1966). Bleomycin was shown to inhibit the growth of *Escherichia coli* and HeLa cells *in vitro*, and was found to have inhibitory activity against Ehrlich carcinoma, sarcoma 180 and other transplantable mouse ascites tumours (Umezawa et al. 1966; Umezawa et al. 1968; Takeuchi et al. 1968). These studies led to clinical trials evaluating the use of bleomycin in the treatment of a range of human malignancies, including squamous cell carcinomas of the skin, head and neck, oesophagus, lung and cervix, as well as testicular neoplasms, melanoma and Hodgkin's lymphoma.

1.1.1 Pharmacological effects of bleomycin

Bleomycin has been described to have at least two modes of action: direct cytotoxicity by means of DNA degradation (Burger et al. 1981), and cell cycle arrest (Barlogie et al. 1976).

Bleomycin-mediated DNA degradation requires the presence of a redox-active metal such as Fe^{2+} as well as molecular oxygen derived from O_2 (Burger et al. 1981) to form activated bleomycin and Fe^{3+} hydroperoxide. This can then bind to DNA, with the metal binding domain located in the minor groove and the bithiazole group intercalated into the double helix (Sanz et al. 2002). This induces the formation of single and double-strand breaks in a sequence specific fashion.

In addition to DNA cleavage, bleomycin has been shown to block the cell cycle at G2 in a concentration and exposure time-dependent manner (Barlogie et al. 1976) and degrade cellular RNA (Carter et al. 1990). Bleomycin also induces apoptosis, which is though to be another of its antitumour properties. Bleomycin is known to induce apoptosis *in vitro*; Tounekti et al. (1993) demonstrated bleomycin induced apoptosis in Chinese hamster lung fibroblasts identified by fluorescence activated cell cycle analysis, DNA

fragmentation and morphological analysis. Hamilton et al. (1995) found a bleomycin dose-dependent induction of apoptosis in human alveolar macrophages *in vitro*, as determined by increased DNA fragmentation and DNA ladder formation. Vernole et al. (1998) also demonstrated bleomycin-induced apoptosis in human lymphocytes, as detected by cell cycle analysis and detection of DNA strand breaks. Although these studies all detected apoptosis, the exact mechanism by which bleomycin exerts its apoptotic effect remains elusive.

Clinically, the main dose-limiting toxicity of bleomycin is exerted on the lung (Yamamoto 2006). Bleomycin has been shown to produce interstitial pulmonary fibrosis in humans (Weiss et al. 1980; Chandler et al. 1990; Jules-Elyse et al. 1990) as well as in experimental models (Hagimoto et al. 1997). Pulmonary fibrosis is characterised by excessive synthesis and deposition of extracellular matrix (ECM) in the distal airspace (Higashiyama et al. 2007). Although lung fibrosis is the main dose-limiting toxicity, bleomycin appears to have additional effects on the vascular system and cutaneous toxicity has also been described.

1.1.2 Effects of bleomycin on the vascular system

The first report of the antiangiogenic properties of bleomycin was by Oikawa et al. in 1990. Their work using chick embryos showed that bleomycin inhibited embryonic angiogenesis in a dose-dependent manner. These observations were confirmed by Lincun and Gongjja in 2000, using Pingyanmycin, the active component of which is Bleomycin. Their work demonstrated the antiangiogenic effect in chick embryos and on the basis of these findings, proceeded to treat 14 children with haemangiomas by means of intralesional injection. Nearly all patients showed complete regression following less than three topical injections of Pingyanmycin. In 2001, Gao et al. set out to investigate the mechanism of Pingyanmycin sclerotherapy, by injecting the posterior auricular veins of rabbits with the drug. Their work described damage to the vascular endothelium immediately after injection, followed by endothelial and smooth muscle cell proliferation after 7 days, and finally vessel occlusion occurred at 21 days following injection.

Bleomycin is a widely used antineoplastic agent for the treatment of malignant pleural effusions; it's mechanism of action is believed to be as a chemical sclerosant similar to talc or tetracycline (Antunes et al. 2003). It was noticed that in the treatment of pleural effusions, bleomycin caused fibrosis and inflammation. So in 1977, Yura et al. were the

first to try bleomycin solution as a sclerosing agent for the treatment of cystic hygroma. Later work by Tanigawa et al. showed that using bleomycin in the form of microsphere in oil emulsion, improved the confinement of this drug and enhanced its activity in the treatment of cystic hygroma and lymphangioma. Work by Mathur et al. (2005) used bleomycin sclerotherapy as a first line management on ten children presenting with either congenital lymphatic or vascular malformations. After intralesional administration of bleomycin, the size of the lesions reduced by 50% or more in seven patients, with three showing complete or near complete response. In a larger study, involving 95 patients, the effectiveness of intralesional bleomycin injection for the treatment of haemangiomas and vascular malformations was evaluated (Muir et al. 2004). Complete resolution or significant improvement occurred in 80% of all patients treated, with complete resolution observed in almost half of the patients presenting with haemangioma. These data suggest that bleomycin may have a role to play in the control of vascular dysplasias, specifically infantile haemangioma and vascular malformations, but also demonstrate a relative lack of understanding of the mechanism by which it exerts its effect in these lesions.

1.1.3 Cutaneous side effects of bleomycin

Various cutaneous side effects have been described following bleomycin treatment, including flagellate erythema, hyperpigmentation, scleroderma, alopecia, gangrene and nail dystrophy (Duhra et al. 1991; Kerr et al. 1992; Siegel et al. 1990; Elomaa et al 1984; Miller 1984). Bleomycin was shown to be effective in the treatment of keratocanthomas (Sayama et al. 1983) and recalcitrant warts (Shumack et al. 1979). These data prompted a study to investigate the effectiveness of bleomycin treatment for keloid and hypertrophic scars (Bodokh et al. 1996). This French study involving 36 patients; 31 of which had keloid and 5 of which had hypertrophic scars, showed that after administering 3-5 intralesional injections of bleomycin complete regression was observed in 69.4% of the lesions. Another study was published in 2001, involving 13 patients; 6 with hypertrophic scars and 7 with keloids (Espana et al. 2001). In this study bleomycin was dripped onto the scars and multiple punctures were made by a 25-gauge needle. In response to this treatment they observed complete flattening in 6 cases, highly significant flattening in 6 cases and significant flattening in 1 case. In 2005, a study was performed involving 14 patients; 12 keloid and 2 hypertrophic scars, whose

lesions had not responded to intralesional steroid injection. Multiple jet-injections of bleomycin were administered to each lesion and injections were repeated each month. After the treatment 11 lesions showed complete flattening, 1 showed highly significant flattening and two showed significant flattening (Saray et al. 2005). Most recently, a study by Aggarwal et al. (2008) involved 50 patients with keloid and hypertrophic scars, administering bleomycin through the multiple puncture technique. Complete flattening was observed in 44% of cases. Although results from these trials are promising, further double blind, placebo controlled studies would be necessary to confirm the efficacy of bleomycin in the treatment of these scars. Again, as with lymphatic and vascular lesions, the mechanism of action is incompletely understood.

1.2 Vascular Malformations

1.2.1 General

Vascular anomalies were classified on the basis of their endothelial characteristics by Mulliken and Glowaki in 1982, into haemangioma and vascular malformations. Vascular malformations can be arterial, capillary, venous or lymphatic and are a collection of abnormal vessels with normal mast cell count and endothelium (Marthur et al. 2005). Vascular malformations are present at birth, enlarge in proportion to the growth of the individual and never regress spontaneously (Donnelly et al. 2000; Marthur et al. 2005). Vascular malformations can be further divided into high flow or low flow lesions - those involving arterial components are termed high-flow lesions, such as arteriovenous malformations (AVM) and arteriovenous fistulas (AVF). AVMs are characterised by abnormal connections between veins and arteries. Although present at birth, they are often exacerbated during puberty or pregnancy (Kouhout et al. 1998). Low flow vascular malformations include primarily venous, lymphatic and mixed malformations (Donnelly et al. 2000). Lymphatic malformations are abnormally formed lymphatic channels, which can occur in any location, although most are located in the head and neck region. Histologically, they present as thin walled fluid-filled cysts with surrounding connective tissue (Blei et al. 2005). The exact cause of lymphatic malformations is not entirely understood; however it is thought that they arise due to abnormal development during embryonic lymphangiogenesis (Breier 2005).

1.2.2 Treatment of Vascular Malformations

Current therapies for lymphatic malformations include sclerotherapy and surgical resection. Current treatment for vascular malformations include surgery to remove the abnormal vessel tissue or embolisation with various agents such as absolute ethanol, glue or polyvinyl particles. Risk of excessive bleeding during and after surgical excision is high, due to the vascular nature of these lesions. Another draw back of surgical excision is the high recurrence rate of these lesions (Lee at al. 2004). The agents used in embolisation can be toxic, for example if large amounts of absolute ethanol enter the systemic circulation, this can cause damage to the central nervous system. Other major complications associated with ethanol embolisation include infection, acute renal failure and brain infarct; minor complications include skin necrosis, nerve injury and blistering (Do et al. 2005). Intralesional bleomycin injection has been successfully used in the treatment of vascular malformations, with a complete resolution in 32% of cases and significant improvement in 52% of cases of venous malformations (Muir et al. 2004). These preliminary studies suggest that bleomycin is effective; however randomised controlled trials would be required to confirm these effects.

1.3 Infantile Haemangioma

1.3.1 General

Infantile haemangiomas are benign vascular proliferations and are the most common tumour of infancy (Chiller et al. 2002). They occur more frequently in Caucasian infants than other racial groups and female infants are more likely to develop haemangioma than males (Chiller et al. 2002). Typically, they are not present at birth, but present within the first few weeks of life. In contrast to vascular malformations, which grow proportionately with an individual, haemangiomas display 3 unique growth phases. The proliferative phase is characterised by abundant immature endothelial cells (Yu et al. 2004) angiogenesis and clonal expansion of endothelial cells (Boye et al. 2001). The rapid proliferative phase is followed by a slow spontaneous involuting phase, characterised by endothelial-lined vascular channels and endothelial cell apoptosis and finally the involuted phase by an increase in adipocytes and accumulation of fibrofatty tissue (Yu et al. 2006).

The precise cause of infantile haemangioma remains unknown. In 2000, a study by North et al. suggested a relationship between infantile haemangioma and placental

tissue, by identifying GLUT-1 positive staining of infantile haemangiomas. GLUT-1 is not expressed by other vascular lesions, making the identification of this marker a valuable tool for diagnosis. An investigation into cell morphology and protein expression of haemangioma endothelial cells showed that the cells were more characteristic of embryonic microvascular endothelial cells, further supporting the hypothesis that haemangiomas arise from residual angioblast tissue that is undergoing delayed proliferation and remodelling (Dosanjh et al 2000).

Both extrinsic and intrinsic defects have also been proposed as the cause of haemangioma. Bielenberg et al. (1999) proposed that the expansion of endothelial cells in haemangioma was due to an extrinsic defect. Their work found that the epidermis overlying proliferating haemangiomas showed altered expression of angiogenic factors and suggested that the endothelial cells of these tumours were responding to an aberrant exogenous growth signal. The intrinsic defect theory proposes that changes in endothelial behaviour in haemangioma may be caused by somatic mutations. Work by Boye et al. (2001) showed that haemangioma-derived endothelial cells are clonal and exhibit abnormal behaviour. Their findings suggested that haemangiomas are caused by a somatic mutation that led to the expansion of a single endothelial cell. This led to the hypothesis that endothelial progenitor cells (EPCs) could perhaps be precursors of the clonal endothelial cells (Yu et al. 2004). EPC's are precursor cells of vascular endothelial cells, which are mobilised from the bone marrow into the peripheral blood and are thought to promote neovascularisation by differentiating into mature endothelial cells (Zhang et al. 2007). Yu et al. found mRNA encoding CD133-2 (an important marker for EPCs) in proliferating but not involuting or involuted haemangioma, and also identified CD133 and CD34 positive cells by flow cytometry in proliferating haemangiomas. The CD133 gene codes for a pentaspan transmembrane glycoprotein, which is thought to function in maintaining stem cell properties by suppressing differentiation (Entrez Gene PROM1). The CD34 gene codes for a cell surface glycoprotein of the same name, which functions as a cell-cell adhesion factor (Krause et al. 1996). Their work suggested that EPCs may contribute to early expansion of haemangioma.

In 2008, Khan et al. reported the discovery of less differentiated stem cells in haemangioma specimens, which they referred to as haemangioma-derived stem cells (HemSCs). HemSCs were isolated from haemangiomas using anti-CD133 coated magnetic beads. These cells were then clonally expanded *in vitro* and differentiated into

multiple lineages, including adipocytes. The clonal HemSCs were implanted into immunodeficient mice and were shown to produce haemangioma-like lesions consisting of GLUT-1 positive microvessels. Two months after implantation, this HemSC derived lesion was shown to undergo an involutive process by differentiation into adipocytes. Their work suggested that this newly identified HemSC is the cellular origin of infantile haemangioma.

Just as the exact cause of infantile haemangioma is unknown, little is known about the mechanisms that contribute to their spontaneous regression. It was reported that mesenchymal stem cells (MSCs) reside in haemangiomas and that these cells would be likely precursors of the adipocytes found in the involuting phase (Yu et al. 2006). It was proposed that MSCs are recruited to the site of haemangioma from the bone marrow through the circulation or from nearby MSC niches in the dermis and adipose tissue. Once recruited the MSCs would differentiate into adipocytes following specific cues from the microenvironment during the involuting and involuted phases (Yu et al. 2006).

1.3.2 Treatment of Haemangiomas

Most haemangiomas are minor birthmarks that require no treatment; however in approximately 10% of cases of haemangioma, complications occur. These include bleeding and ulceration, functional impairment to functions such as such as vision, hearing and respiration and larger lesions can divert blood supply and cause high output heart failure (Ogino et al. 2001; Haggstrom et al. 2006; Hsiao et al. 2007). Also, the psychosocial effects of cosmetic disfigurement must also be considered. Current treatments for infantile haemangioma include corticosteroids, administered either orally, topically or intralesionally. Oral corticosteroids in the form of prednisolone remains the most common form of treatment for infantile haemangiomas (Chan 2007), however this treatment has numerous potential side effects including stunting growth, gastrointestinal upset, obesity and increased susceptibility to infections. Haemangiomas which have failed to respond to corticosteroid treatment are often treated with subcutaneous Interferon-alpha-2a (IFN-a-2a) injections. IFN-a-2a is a potent angiogenesis inhibitor that acts by blocking the action of basic fibroblast growth factor (Kumar et al. 2002). Adverse effects of IFN-a-2a therapy include fever, nausea, fatigue, malaise, elevated liver enzyme levels, renal failure and bone marrow suppression (Greinwald et al. 1999). Flashlamp-Pumped Pulsed Dye Laser treatment has been found to be somewhat limited for haemangiomas, as the lasers used can only penetrate the upper dermis and are

unable to prevent proliferation of the deep component of haemangiomas (Poetke et al. 2000). The chemotherapeutic drug vincristine is another therapeutic option for complicated haemangiomas that have not responded to corticosteroid treatment. The main dose limiting effect of vincristine is neurotoxicity (Chan et al. 2005), but other potential side effects include fever, headache, gastrointestinal upset and alopecia.

More recently, the use of topical imiquimod cream, which works as an immune-response modifier affecting both the innate and acquired immune system, was shown to be successful in controlling superficial infantile haemangiomas, but had minimal effect on deeper subcutaneous haemangiomas (Ho et al. 2007). Surgical resection is typically considered when haemangiomas are not responding to other medical treatment (Hussain et al. 2008). Lesions which are prone to bleeding and ulceration, or are function-impairing are often considered for removed surgically. Surgery can also be used for cosmetic improvement after involution has occurred, as the lesions often leave cosmetically significant changes including discolouration, redundant skin, fibrofatty tissue and scarring (Chan et al. 2005).

More recently, a number of clinical trials have shown the effectiveness of intralesional bleomycin injection for the treatment of haemangiomas (Sarihan et al., 1997; Kullendorff, 1997; Muir et al., 2004; Omidvari et al., 2005; Pienaar et al., 2006), often eliminating the need for surgery or systemic treatment regimens in 80% of cases (Oak et al., 2006). These studies are as yet not randomised controlled trials.

1.4 Keloid Scars

1.4.1 General

Keloid is an abnormal healing response, characterised by excessive accumulation of extracellular matrix and by overabundant collagen formation (Singer et al. 1999). At present, the mechanism that causes these phenomena is not fully understood. Keloid scars can occur after a variety of cutaneous injuries, including surgery, burns dermal trauma and acne. In contrast to hypertrophic scars, keloids do not regress with time, are difficult to revise surgically and often exceed the boundaries of the initial injury (Ehrlich et al. 1994). Keloids have been linked clinically to the cytokine transforming growth factor-ß or TGF-ß (Lee at al. 1999). TGF-ß is essential for wound healing; however excessive production of TGF-ß can result in increased deposition of scar tissue and fibrosis. Platelet derived growth factor (PDGF) is also known to regulate cell

proliferation and differentiation. TGF-ß was shown to upregulate the expression of PDGF receptors in keloid fibroblasts, but not in normal skin fibroblasts (Messadi et al. 1998).

1.4.2 Treatment of Keloid scars

There is no fundamental treatment for keloid scars. Current treatment is by palliative modalities such as pressure (Brent 1978), intralesional injections of corticosteroids (Kiil 1977), topically applied silicones or gel sheets or surgical excision (Cosman et al. 1972). Surgical excision is usually only temporarily beneficial, due to the high recurrence rate of keloid. More recently the use of bleomycin for the treatment of these scars has proved successful (Bodokh et al., 2006; Espana et al., 2001; Saray et al., 2005; Aggarwal et al., 2008). However these studies were not randomised controlled trials.

1.5 Aims of the thesis.

Bleomycin has been used successfully in the treatment of haemangioma, keloid and vascular malformation, however the mode of action of this chemotherapeutic drug on these non-malignant dysplasias is not well understood. One approach to begin to understand these modes of action is to derive cell lines from these dysplasias so as to allow study *in vitro*. Given that the primary means of action on neoplastic cell types is through apoptosis and cell cycle arrest, these cell lines should be investigated for their sensitivity to bleomycin.

Work described in this thesis reports the isolation of primary cell lines from infantile haemangioma, vascular malformation and keloid. The effect of bleomycin on these and control cell lines was investigated in a staged manner, initially assessing dose responsiveness for a range of cell types *in vitro*, and subsequently focussing on potential mechanisms of action.

Chapter 2

2 Materials and Methods

2.1 Materials

Unless otherwise specified, all materials were sourced from Sigma-Aldrich (Dorset, UK), all procedures were carried out at room temperature and all percentages are expressed as volume/volume (v/v). A list of buffers employed, and their constituent parts are listed in Appendix 1. Manufacturers of equipment and materials employed can be found in Appendix 2.

2.2 Tissue culture

This study has used two sources of cells; primary cells established from tissue specimens of haemangiomas, keloids, venous malformations and lymphatic malformations (2.3.1) in addition to established cell lines. All culture materials were either purchased as aseptic stock or sterilised by autoclaving, unless otherwise indicated.

2.3 Primary cells established from human tissue samples

Tissue specimens were obtained from the James Cook University Hospital, Middlesbrough in accordance with the Human Tissue act. Full details of ethical approval can be found in appendix 3.

2.3.1 Tissue preparation

Tissue specimens were taken immediately after resection and placed on ice. The samples were then washed twice in double strength antibiotic medium (minimum essential media (MEM) containing 20% (v/v) foetal bovine serum (Harlan), 2mM L-glutamine, 100μg/ml gentamycin solution and 500μg/ml amphotericin B). The tissue was cut into small pieces, typically 1cm^3 in size and stored at 4°C for 2-4 hours in a sufficient volume of double strength antibiotic media to cover the sample (typically 25ml). This latter step was performed to reduce the risk of the bacterial and/or fungal contamination of the tissue samples infecting the subsequent cultures. The samples were then placed in explant culture, taken for collagenase digestion or snap frozen for storage.

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2.3.2 Explant culture

A portion of each of the tissue samples were removed from the double strength antibiotic media and cut into approximately $1mm^3$ sized pieces, using a scalpel or surgical scissors. The tissue pieces were then placed on the growing surface of 35mm x 10mm cell culture dishes (Nunc) and left in the absence of media for 5 minutes to allow the tissue to adhere to the surface of the dish. One millilitre of growth medium appropriate for the tissue under study (see Appendix 1) was then added and the samples cultured in a humidified incubator (SHEL LAB) at 37°C and 5% CO_2, for a period of 7-28 days, a period of time dictated by the rate of cellular outgrowth from the explant during culture. The growth media was replaced every 7 days. When the cells which had grown out of the explants were ready to be passaged, the tissue segments were physically removed from the surface of the dishes using sterile forceps. The remaining adherent cells growing on the surface of the dish were then washed twice with sterile phosphate buffered saline (PBS – 10X +calcium +magnesium) (Gibco) and detached by incubation for approximately 5 minutes with 1ml Trypsin-ethylenediamine tetra acetic acid (Trypsin-EDTA). 1ml of growth media was then added to neutralise the trypsin before centrifugation at 300 x g for 5 minutes. The cells were resuspended in 5ml growth media and transferred to a $25cm^2$ cell culture flask (Nunc) and cultured in a humidified incubator at 37°C and 5% CO_2. After passage 2 cells were transferred to a $75cm^2$ flask and these cell lines were cultured up to passage 11 to allow for subsequent experiments.

2.3.3 Collagenase digestion

Tissue specimens were minced with surgical scissors and subsequently digested with approximately 20ml of filter-sterilised 0.1% (w/v) type 1 collagenase (Worthington) in MEM containing 2% FBS at 37°C for 1 hour. The tissue was homogenised with a pestle and mortar and filtered through 100-μm cell strainers (BD Falcon™) to obtain a cell suspension. The cells were centrifuged for 5 minutes at 300 x g. The pellet was gently resuspended in 2ml of hypotonic ammonium chloride solution, and incubated for 10

minutes to lyse any residual erythrocytes. This was pelleted once more at 300 x g and resuspended in 5ml of growth media appropriate for the tissue type under study (see appendix 1). The cell suspension was then transferred into 25cm^2 tissue culture flasks (Nunc) and incubated at 37°C and 5% CO_2.

2.4 Established cell lines

2.4.1 Passaging cells

Cells were passaged when they reached 70-80% confluency. The growing surface of the flask was washed twice with 10mls of PBS before adding 2-4mls of trypsin-EDTA. Cells were then incubated at room temperature (HUVEC) or 37°C (all other lines) for 5 minutes until the cells detached from the surface of the flask. Five millilitres of the appropriate growth media was then added to the flask to neutralise the trypsin. The resulting cell suspension was then centrifuged for 5 minutes at 300 x g. The cell pellet was resuspended in 2mls of growth medium and then split equally between two 75cm^2 flasks. All cells were grown in humidified SHEL LAB incubators at 37°C and 5% CO_2.

2.4.2 Culture of HUVEC cell line

Normal Human Umbilical Vein Endothelial Cells (HUVEC) were purchased from Promocell, Heidelberg, Germany. The cells were grown in Endothelial Cell Growth Medium MV (Promocell), the ingredients of which can be found in appendix 1.

2.4.3 Culture of HMEC-1 cell line

HMEC-1 cell line was kindly supplied by Dr. Noel Carter (Senior Lecturer, Faculty of Applied Science, University of Sunderland). This cell line is an immortalised human microvascular endothelial cell line that retains the morphologic, phenotypic, and functional characteristics of normal human microvascular endothelial cells (Ades *et al.,* 1992). Cells were grown in MCDB-131 growth medium supplemented with 10% FBS,

10ng/ml human epidermal growth factor (EGF), 1μg/ml hydrocortisone, and 50μg/ml gentamycin and 250μg/ml amphotericin B.

2.4.4 Culture of Human Foreskin Fibroblasts

Cultured primary human foreskin fibroblasts were kindly supplied by Dr Rebecca Hill (Durham University). The cells were grown in MEM supplemented with 10% (v/v) FBS, 2mM L-glutamine, 50μg/ml gentamycin and 250μg/ml amphotericin B.

2.4.5 Freezing cells

Cells in culture were detached using trypsin-EDTA (as described in 2.4.1) and combined with an equal amount of growth medium before centrifugation at 300 x g for 5 minutes. The cell pellet was then resuspended in 2ml of freezing solution containing 70% growth medium, 20% FBS and 10% dimethylsulphoxide (DMSO), transferred to freezing vials (Nunc). The vials were then slowly frozen in a -80°C freezer and stored in liquid nitrogen.

2.4.6 Thawing of frozen cells

Vials of frozen cells were thawed quickly in a 37°C waterbath. Defrosted cells were added to 10ml of growth medium and centrifuged at 300 x g for 5 minutes and the pellet resuspended in 1ml of growth medium. Cells were then transferred to a 25cm^2 or 75cm^2 tissue culture flask and grown as described earlier.

2.4.7 Counting cells using a Haemocytometer

To ensure the haemocytometer was clean, it was cleaned with 70% ethanol before each use and the edge of the haemocytometer surface was then moistened to ensure the cover slip was completely affixed. The cells were trypsinised as described (2.4.1). The cell suspension was the aspirated several times using a sterile 1ml pipette to thoroughly mix the cells before removing 20μl of cell suspension. 10μl was added to either side of the dual haemocytometer (Hausser Scientific) and counted using a hand tally counter and the mean cell number was used. The haemocytometer has 2 grids for counting cells,

which measure $1mm^2$ in area x 0.1mm in depth, with a total volume of $0.1mm^3 = 10^4/ml$. All cells were counted under a light microscope at x10 magnification.

2.5 Apoptosis assays

Adherent cells were trypsinised and counted as described in parts 2.4.1 and.2.4.7. For bleomycin dose response experiments, 6 well cell culture dishes were used (Greiner). 1 x 10^4 cells were plated out per well with 2ml of growth media. The cell plates were then incubated for 48 hours, before the addition of bleomycin. This method was used for all apoptotic assays.

2.5.1 Bleomycin treatment

Bleomycin sulphate from Bleo-kyowa, (Slough, UK) is used in the clinic to treat haemangioma. The drug comes in vials containing 15,000IU of lyophilised powder. Five millilitres of sterile PBS was added to a single vial of bleomycin, to make a 3000U/ml stock. The stock solution is stable for 3 months stored at 4°C. Using the stock solution, a range of concentrations 1mU-50,000mU/ml was made up in the relevant growth media. After 48 hours incubation with growth media, the media was removed and replaced with growth media containing varying concentration of bleomycin. Control samples were also set up by replacing the growth media with fresh growth media containing no bleomycin. Cells were then incubated at 37°C for 5 hours, 24 hours and 48 hours, after which the samples were harvested by trypsinisation, and prepared for running on the flow cytometer as described in (2.6.2).

2.6 Principles of flow cytometry

Flow cytometry can measure and analyse multiple physical characteristics of single cells. The properties measured can include size, granularity or internal complexity, and relative fluorescence intensity. The flow cytometer used in this study was a Becton Dickenson FACSCalibur, which can measure five parameters, forward light scatter (FSC), side light scatter (SSC) and three fluorescence parameters, FL1 (filter: 530/30), FL2 (filter: 585/42) and FL3 (filter: >670nm).

A flow cytometer consists of three main systems: fluidics, optics, and electronics (Ochatt., 2006). The fluidics system is responsible for transporting the cell sample through a narrow stream of fluid, allowing the cells to pass individually through a laser beam. The optics system consists of lasers to illuminate the cells in the sample stream. When a laser interacts with the cells, it can be scattered by reflection or refraction, which is indicative of the cells size and granularity, or it can be converted to a new wavelength by cell associated fluorochrome (fluorescence). The optical filters then direct the resulting light signals to the appropriate detectors. The electronics system converts the detected light signals into electronic signals (voltages) by photodetectors. The voltage pulse is assigned a digital value by the Analog-to-Digital Converter (ADC). Once the ADC has converted these voltage pulses to channel numbers, the data is stored and processed by the computer (Robinson, 2006). This results in detailed quantitative information about each cell analysed.

In this study apoptosis and cell death were examined using 3 methods: staining fixed cells with propidium iodide (PI) to look at the integrity of nuclear DNA (2.6.1), DNA damage using the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay (2.7) and detection of apoptosis by intracellular staining using a cleaved caspase-3 conjugated antibody (2.8).

2.6.1 Investigation of cell cycle and apoptosis using flow cytometry

One method that can be used to quantify apoptosis by flow cytometry is the use of propidium iodide (PI). The PI intercalates between bases of double stranded DNA and

produces a highly fluorescent adduct. PI is excited by UV or blue light to emit red fluorescence (Ormerod, 2000). The flow cytometer can then analyse the cells based on the quantity of DNA in each cell. Cells in gap 1 phase (G1) have one complement of DNA. In this phase, the cell is synthesising proteins required for replication. Cells then progress to the synthesis phase (S phase) where new DNA is being synthesised. Once the DNA content in the cell has doubled, the cell has reached gap 2 phase (G2) where the cell is synthesising proteins required for mitosis. Therefore cells in G1 have one complement of DNA, and cells in G2 have double the content of DNA than cells in G1. Cells in S phase have DNA content between that of G1 and G2. Apoptosis is characterised by loss of cell volume, clumping of chromatin and nuclear fragmentation into apoptotic bodies, so the DNA complement is <1 (Nicoletti et al. 1991) and so apoptotic cells are identified as the sub-G1 fraction of the cell cycle.

Since PI can also bind to double stranded ribonucleic acid (RNA), it is necessary to treat the cells with RNAse to degrade the RNA that may bind PI, to optimise DNA resolution. As this method relies on analysing individual cells, cell clumping was avoided by passing cells through a syringe and needle before running the samples through the flow cytometer. A clump of two cells in G1 would have the same DNA content as one cell in G2; however these clumps or doublets can be distinguished by size on the forward scatter versus side scatter plot and can be gated out, so they are not included in the cell cycle measurements.

2.6.2 Sample preparation: PI staining fixed cells

To investigate cell cycle distribution and apoptosis, both treated and untreated cells were harvested at 5 hours, 24 hours and 48 hours. As the cultures in these experiments are of adherent cells, cells in the supernatant of the samples have a higher probability of being apoptotic than do the adherent cells, and therefore the cells in the supernatant were also harvested prior to trypsinisation and spun down with the trypsinised adherent cell layer. The cells were spun at 1500 rpm for 5 minutes to remove trypsin and media. The cell pellet was resuspended in 600µl of ice cold PBS, then fixed with the addition of 1400µl ice cold 100% ethanol. Fixed samples were stored at 4°C. Prior to running on the flow

cytometer, the cells were centrifuged at 1500 rpm for 5 minutes to remove the ethanol and PBS. The cell pellets were resuspended in 400μl of ice cold PBS. 50μl of 40μg/ml PI and 0.1mg/ml RNAse A were added to each sample and incubated for 30 minutes at 37°C.

2.6.3 Flow cytometer settings

To acquire the appropriate settings for analysis of the test samples, a control sample was first run through the flow cytometer. Dot plots showing the side scatter (SSC) versus forward scatter (FSC) and FL2-W versus FL2-A and FL2-height histogram were used to assess the data collected. The G1 peak of the cell cycle should be at approximately 200 FL2-H (one complement of DNA) and the G2 peak at 400 FL2-height (double DNA content of G1). For each test sample 10,000 events were recorded and each experiment was performed in triplicate.

2.6.4 Data analysis

The data was analysed using WinMDI 2.8 software (TSRI flow cytometry core facility, La Jolla, CA). To ensure only single cells were analysed, the cells were gated using the FL2-W versus FL2-A dot plot. The gated data was plotted as an FL2-histogram and to determine the percentage of cells in G1, G2, S and sub-G1, the cells were defined by their individual peaks as M1, M2, M3 and M4. Cells undergoing apoptosis were found in the sub-G1 portion of the histogram (M4).

2.7 Detection of DNA fragmentation by flow cytometry using APO-DIRECT™ Kit

2.7.1 Principles of the TUNEL assay

The APO-DIRECT™ kit is based on the TUNEL assay (Li *et al.*, 1995) and is a single step assay for labelling DNA breaks and total cellular DNA to detect apoptotic cells by flow cytometry. One feature of apoptosis that can be easily measured is the break-up of the genomic DNA by cellular nucleases. Apoptotic cells contain a large number of DNA fragments, which result in a number of 3'-hydroxyl ends of DNA. This kit uses this property to identify apoptotic cells by labelling the DNA breaks with a fluorescein isothyocyanate (FITC) labelled deoxyuridine triphosphate nucleotides (dUTP). Non-apoptotic cells do not incorporate significant amounts of the FITC-dUTP due to the lack of exposed 3'-hydroxyl DNA ends and therefore be identified as a negatively stained population. This kit also contains PI and RNAse A solution for counter staining the total DNA content of each cell.

2.7.2 Fixation protocol

The test cells were harvested as described earlier. The cells were then centrifuged for 5 minutes at 300 x g and the supernatant discarded. The cells were then resuspended in 1% paraformaldehyde in PBS (pH 7.4). The cells were then placed on ice for 30-60 minutes. The cells were then centrifuged for 5 minutes and 300 x g and resuspended in PBS. This wash step was repeated with PBS and the pellet resuspended in 70% ethanol. The cells were then stored at -20°C until all test samples were ready to be analysed by flow cytometry.

2.7.3 Staining protocol

The following procedure describes the method for measuring apoptosis in the positive and negative controls that are provided with the APO-DIRECT™ kit. The same procedure was followed for the bleomycin treated samples.

The cells of the positive and negative controls were resuspended by swirling the vials. 1ml aliquots of the positive and negative controls were removed and placed into separate 1.5ml epindorf tubes. The cells were centrifuged for 5 minutes at 300 x g and the 70% (v/v) ethanol removed by aspiration. Each cell sample was resuspended in 1ml wash buffer and spun for 5 minutes and 300 x g. The centrifugation step was repeated and supernatant removed by aspiration. Each sample was then resuspended in 50μl of staining solution and incubated at 37°C for 60 minutes. At the end of the incubation time, 1ml of wash buffer was added to each of the cell samples and the samples were centrifuged at 300 x g for 5 minutes. The rinse step was repeated with 1ml of rinse buffer and the supernatant was removed by aspiration. The cell pellet was then resuspended in 500μl of PI/RNAse staining buffer and then incubated for 30 minutes at room temperature in the dark. The cells were then analysed in the PI/RNAse solution within 3 hours of staining. Each experiment was performed in triplicate.

2.7.4 Analysis of samples by flow cytometry

The samples were measured on a Becton Dickenson FACSCalibur. The positive and negative controls provided with the kit were analysed first. A dual parameter dot plot and single parameter histogram were used to show the data. To display DNA doublet discrimination, FL2-width was plotted on the X-axis versus FL2-area on the Y-axis. From this display, only single cells are gated. The gated data was then plotted onto a single parameter histogram, showing FL-1 (FITC-dUTP), to identify the percentage of cells positive for DNA strand breaks.

2.8 Detection of Apoptosis by Intracellular Immunostaining for Cleaved Caspase-3

2.8.1 Background

Caspase-3 (CPP32, apoptain, YAMA, SCA-1) belongs to a set of mediators implicated in apoptosis known as aspartate-specific cysteinyl proteases or caspases. Caspase-3 has been identified as a key mediator in mammalian cell apoptosis, as it is partially or totally responsible for the proteolytic cleavage of many key proteins (Nicholson et al. 1997; Cohen, 1997; Woo et al. 1998). The processed form of caspase-3 consists of a small (12kD) and large (17kD) subunits, which associate to form an active enzyme. Cleaved caspase-3 antibody (Alexa Fluor 488 Conjugate) works by detecting endogenous levels of the large fragment of activated caspase-3 resulting from cleavage adjacent to aspartic acid 175.

2.8.2 Sample preparation

Cells were plated out as previously described (2.5) and treated with bleomycin (2.5.1).

2.8.3 Fixation protocol

Test cells were harvested by trypsinisation as previously described. The cells were centrifuged for 5 minutes at 300g and resuspended in 300µl of ice cold PBS. 700µl of ice cold ethanol was added to each tube and the samples placed on ice for 60 minutes or left overnight at -20°C.

2.8.4 Reagent preparation

To make incubation buffer, 0.5g of bovine serum albumin (BSA) was dissolved in 100ml of PBS.

2.8.5 Staining protocol

2ml of incubation buffer was added to each of the samples and centrifuged for 5 minutes at 300g. The cell pellet was resuspended in 90µl of incubation buffer. The cells were incubated for 10 minutes at room temperature in incubation buffer, before the addition of 10µl of the conjugated antibody. The samples were incubated in the dark at room temperature for 1 hour. Cells were rinsed and resuspended in 300µl of PBS.

2.8.6 Analysis by flow cytometry

The samples were measured on a Becton Dickenson FACSCalibur. Control samples were analysed first to acquire the appropriate settings. Dual parameter dot plots showing side scatter on the Y axis, versus FL-1 (alexa-fluor 488) on the X axis were plotted to display the data. The data obtained from the flow cytometer was analysed using WinMDI 2.8. The data was plotted onto a dual parameter dot plot (as before) and gated to determine the percentage of cells which were positive for cleaved caspase-3 antibody.

2.9 Statistical analysis

All data presented are representative of the mean of 3 independent experiments $\pm$ SD. A Student's unpaired two-way t-test was used to compare data between control and test samples. In cases where both time and dose were analysed, these dual variables were also subjected to 2-way ANOVA. Where statistical significance is indicated, this was achieved using both tests unless otherwise indicated. Graphs and statistical analysis were carried out in Excel and SPSS 14.0.

Chapter 3

3 Results

3.1 Explant Cultures

In order to examine the effects of bleomycin *in vitro*, explant cultures were used to derive primary cell lines. Tissue was obtained from surgically excised tissue (that would have otherwise been discarded) from untreated haemangiomas, keloid scars and venous malformations over a period of 10 months and explants cultured for up to four weeks. Single cell cultures were produced from the tissue using collagenase digestion; however no cell lines developed using this technique. From the explant cultures, five cell lines were established (2 haemangioma, 2 keloid, 1 venous malformation) following protocols described in section (2.3.2). After approximately 3-4 weeks, each cell line had grown to confluence in a 35mm x 10mm cell culture dish, at which point the cells were trypsinised and transferred to 25cm^2 cell culture flasks.

The keloid derived cells in culture appeared fibroblastic and homogeneous in size and morphology following the first passage from explant culture. The fibroblast-like morphology was maintained after cell passages and throughout the culture period.

Initially, the cell outgrowth from the haemangioma explants appeared to be heterogeneous, but this soon disappeared with all cells appearing to be of a similar size and morphology (figure 3.1). The passage frequency for these cell lines ranged between 4-7 days. Three of the cell lines derived from the tissue samples (1 haemangioma, 1 keloid, 1 venous malformation) were used for the following experiments along with established cell lines, HUVEC, HMEC-1 and foreskin fibroblasts.
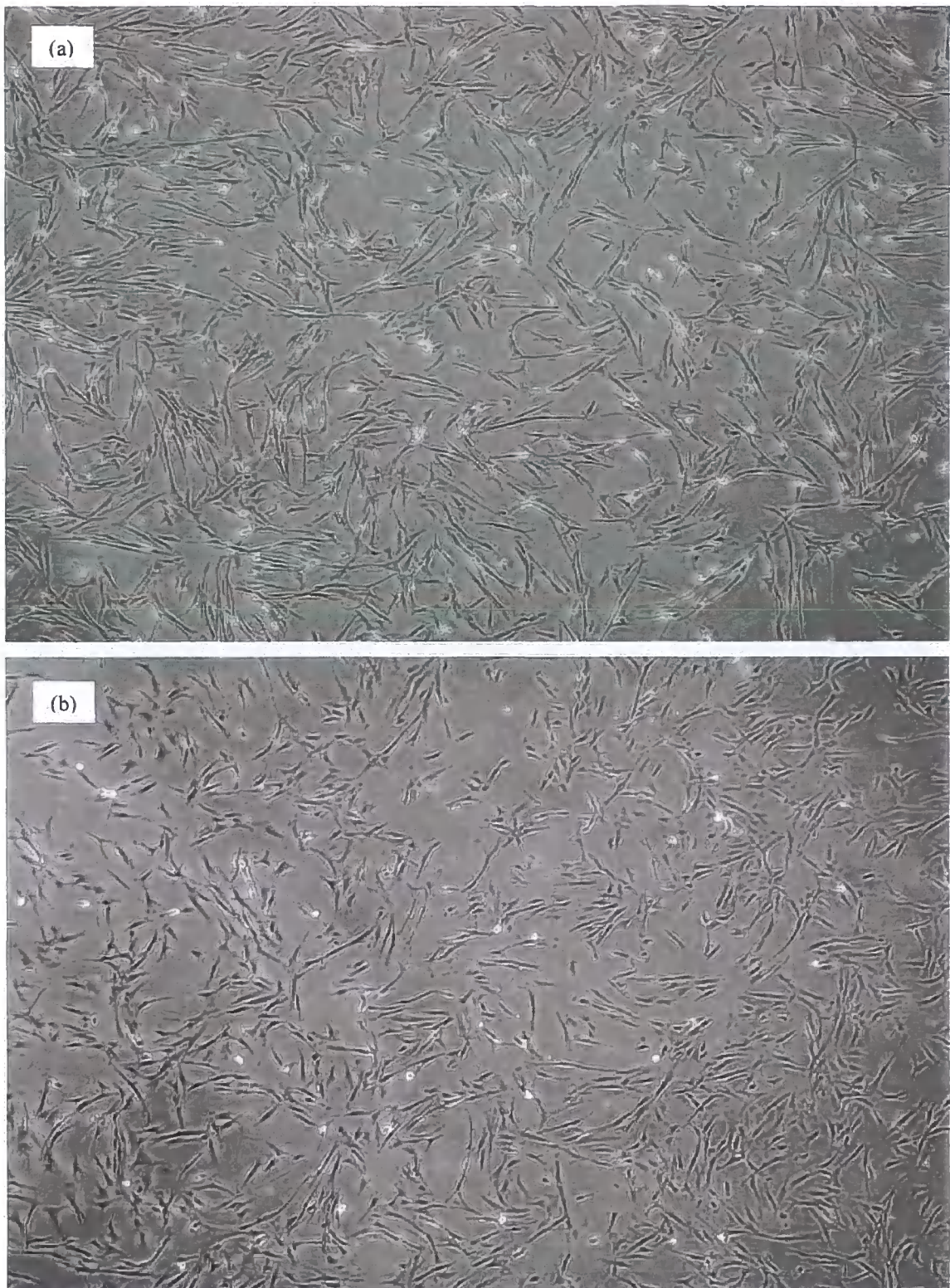
Figure 3.1 Subconfluent monolayer of primary fibroblast-like cells derived from (a) proliferative haemangioma and (b) keloid. Cells magnified using phase contrast microscopy (10 x objective).

3.2 Flow cytometry results

3.2.1 Investigation of cell cycle and apoptosis using flow cytometry

Flow cytometry experiments were performed using the method described in section 2.6. All cells were cultured with increasing doses of bleomycin (1-10,000mU/ml) and assayed at 5, 24 and 48 hours. Apoptosis was measured by propidium iodide (PI) staining to determine the percentage of cells with subdipliod DNA content.

3.2.2 Cell cycle analysis

A representative cell cycle dot plot and histogram for the foreskin fibroblasts are shown on figure 3.2(a&b) to illustrate how the flow cytometry data was obtained.

The G1:S ratio for each cell line before and after treatment with a range of concentrations of bleomycin was compared to that of their untreated controls. G1 arrest was identified by an increase in the proportion of cells in G1 phase and a decrease in the proportion of cells in S phase. The G1:S ratio versus time for each cell line at various doses of bleomycin is shown on figure 3.3(a-d). With the exception of HMEC-1, all cell lines evaluated appeared to show a bleomycin induced increase in G1:S ratio (G1 arrest) at a dose of 100mU/ml after 48 hours, this was statistically significant in the case of foreskin fibroblast, HUVEC and haemangioma derived cells (figure 3.4.a).

Foreskin fibroblasts underwent G1 arrest, with the mean G1:S phase ratio for increasing from 6.13 ± 0.43 in the control, to 9.73 ± 0.58 in the 100mU/ml sample 48 hours post treatment with bleomycin (figure 3.3(a)).

The keloid derived cell line was also shown to undergo G1 arrest in response to treatment with bleomycin at a concentration of 100mU/ml. The G1:S phase ratio increased from 10.76 ± 1.38 in the control, to 17.10 ± 4.13 in the 100mU/ml bleomycin treated sample 48 hours post treatment (figure 3.3(b)).

HUVEC cells underwent G1 arrest, this was observed at a concentration of 100mU/ml at both 24 and 48 hours post treatment with 100mU/ml bleomycin. The initial mean G1:S ratio for the HUVEC control sample was 4.52 ± 0.46, which increased to 19.10 ± 2.32; 48 hours post treatment with 100mU/ml bleomycin (figure 3.3(c)).

Haemangioma derived cells also underwent G1 arrest in response to treatment with bleomycin. This was observed at a concentration of 100mU/ml at both 24 and 48 hours post treatment (figure 3.3(d)). The initial mean G1:S ratio for the haemangioma control sample was 5.57 ± 0.58, increasing to 16.49 ± 6.59, 48 hours post treatment.

The HMEC-1 cells failed to undergo a G1 arrest in response to Bleomycin treatment (data not shown). The mean G1:S phase ratio decreased from 2.61 ± 0.11 in the control sample, to 2.13 ± 0.13, 48 hours post treatment with 100mU/ml bleomycin. The proportion of cells in G2 increased in the 48 hour HMEC-1 sample treated with 100mU/ml bleomycin (the proportion of cells in G1 decreased). This suggests that the HMEC-1 cell line undergo a G2 arrest as opposed to a G1 arrest after treatment with bleomycin.

The increase in G1:S ratio induced by bleomycin is shown on figure 3.4(b). Compared with foreskin fibroblasts, HUVEC, haemangioma derived cells and keloid derived cells all showed a greater increase in G1:S ratio. This was statistically significant in the case of HUVEC and keloid (P= < 0.01; P = < 0.05 respectively).
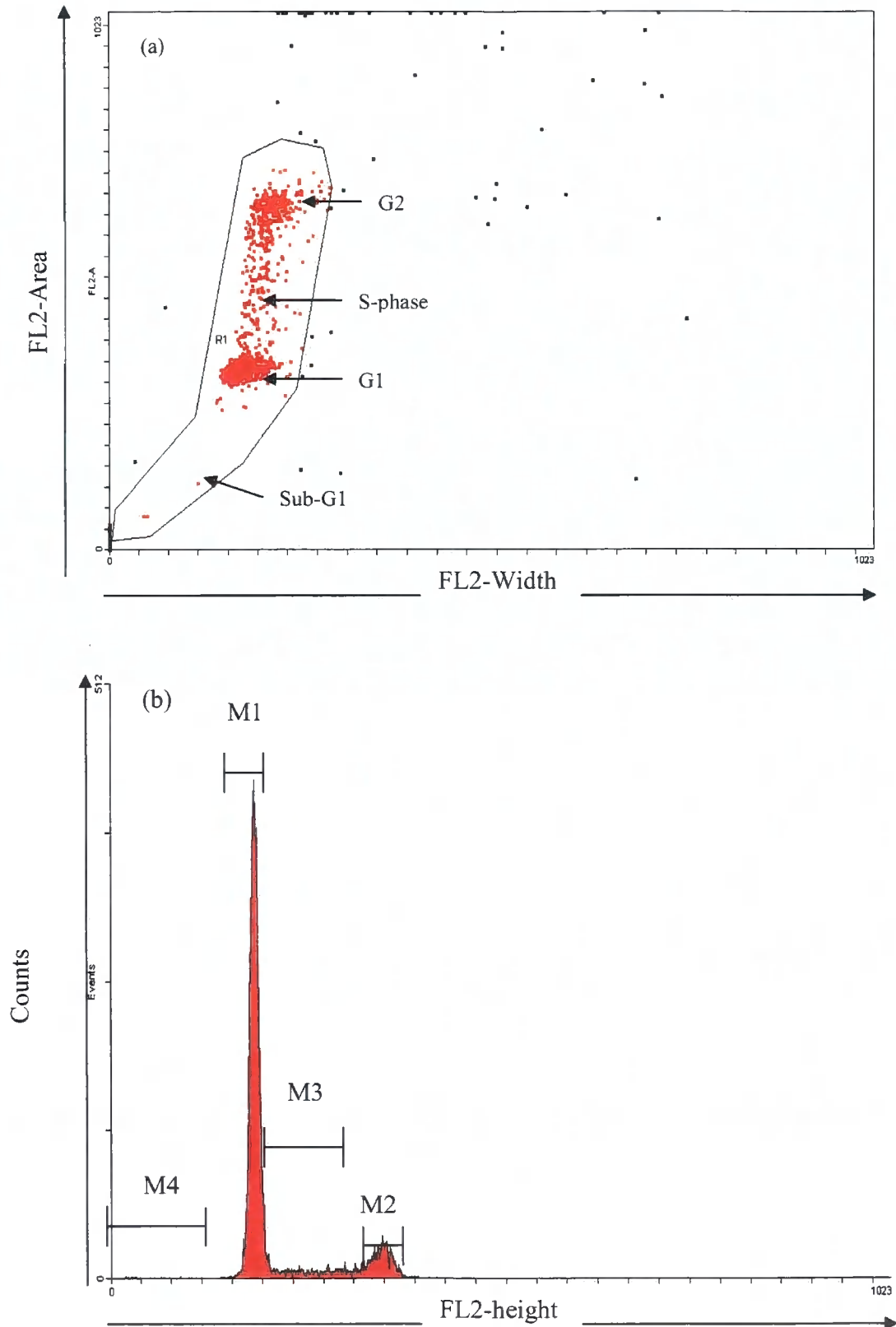
Figure 3.2 Representative width/area dot plot and FL2 histogram analysis of foreskin fibroblasts. (a) shows the gate (in red) used to identify single cells, following the method of Mullen, 2004. **(b)** The cells gated data as in previous figure (a) were plotted as an FL2 histogram. To determine the percentage of cells in G1, G2, S and sub-G1, the cells were defined by their individual peaks as M1, M2, M3 and M4 respectively.

(a) Foreskin Fibroblasts
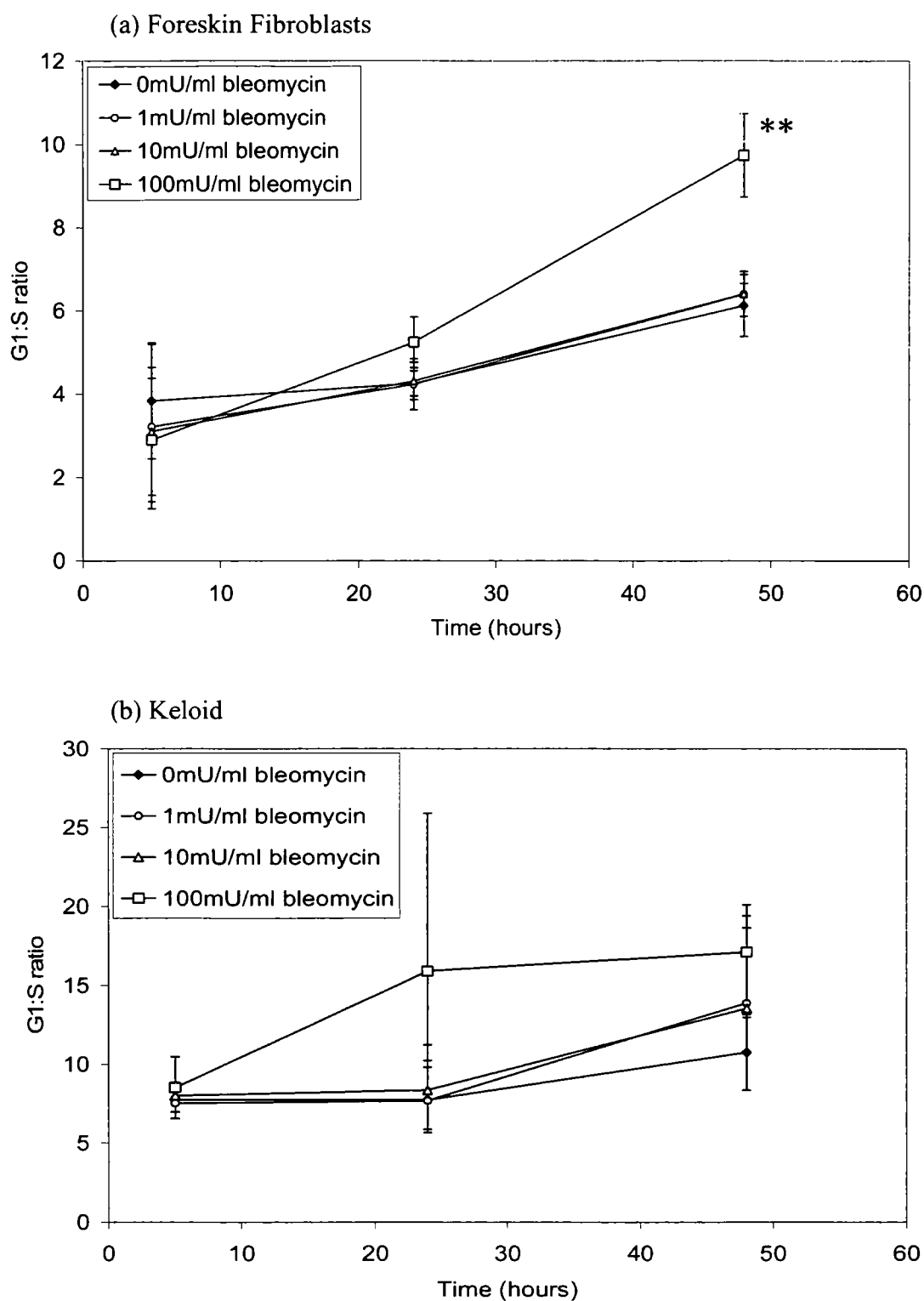
(b) Keloid

Figure 3.3(a&b) Effects of bleomycin dose and time of exposure on G1:S ratio of foreskin fibroblasts (a) and keloid (b). A significant increase in G1:S ratio was observed at a concentration of 100mU/ml after 48 hours incubation with the foreskin fibroblasts. Variance between samples meant that difference observed with the keloid derived cells was not significant Results are presented as the Mean ± SD of 3 independent experiments. ** = P < 0.01 for T-test (NS for 2-way ANOVA)
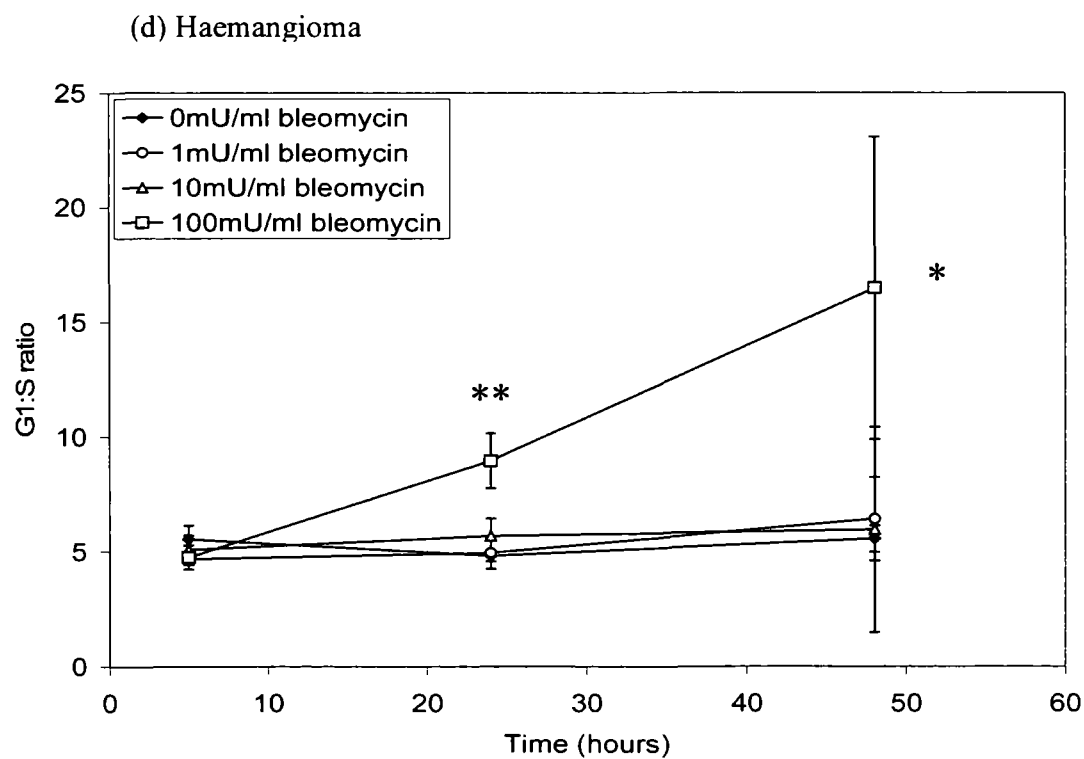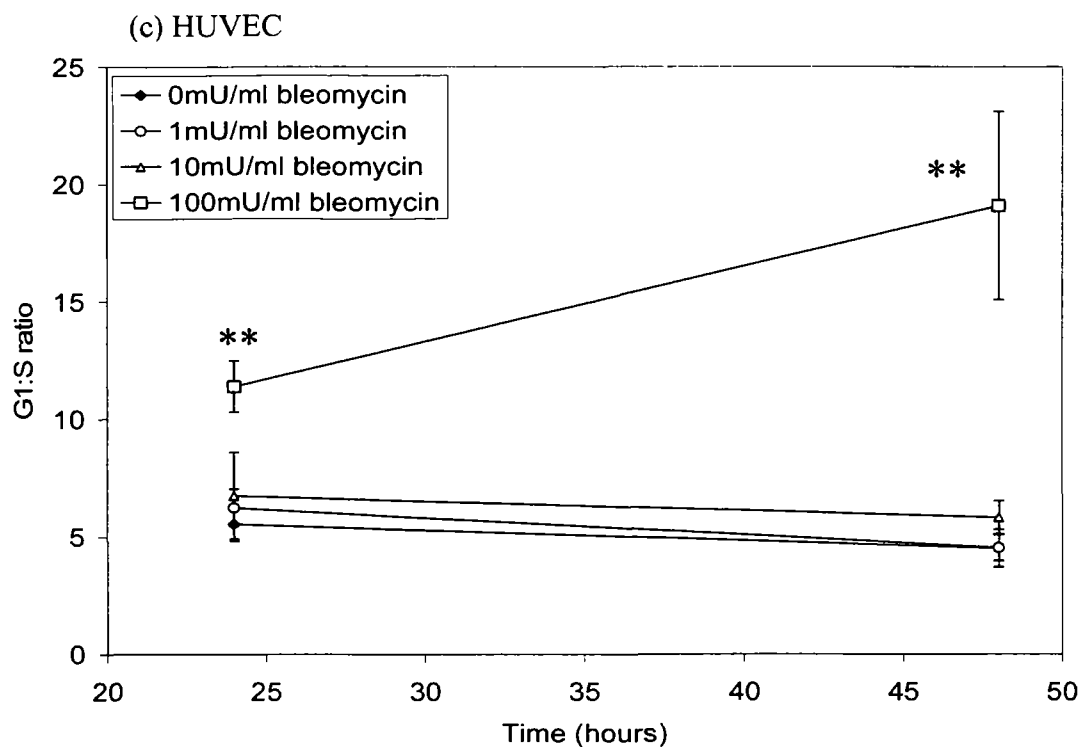
Figure 3.3(c&d) Effects of bleomycin dose and time of exposure on G1:S ratio of HUVEC
(c) and haemangioma (d). A significant increase in G1:S ratio was observed at a concentration
of 100mU/ml after 24 and 48 hours incubation with the HUVEC and haemangioma derived
cells. Results are presented as the Mean ± SD of 3 independent experiments. * = P < 0.05, ** =
P < 0.01
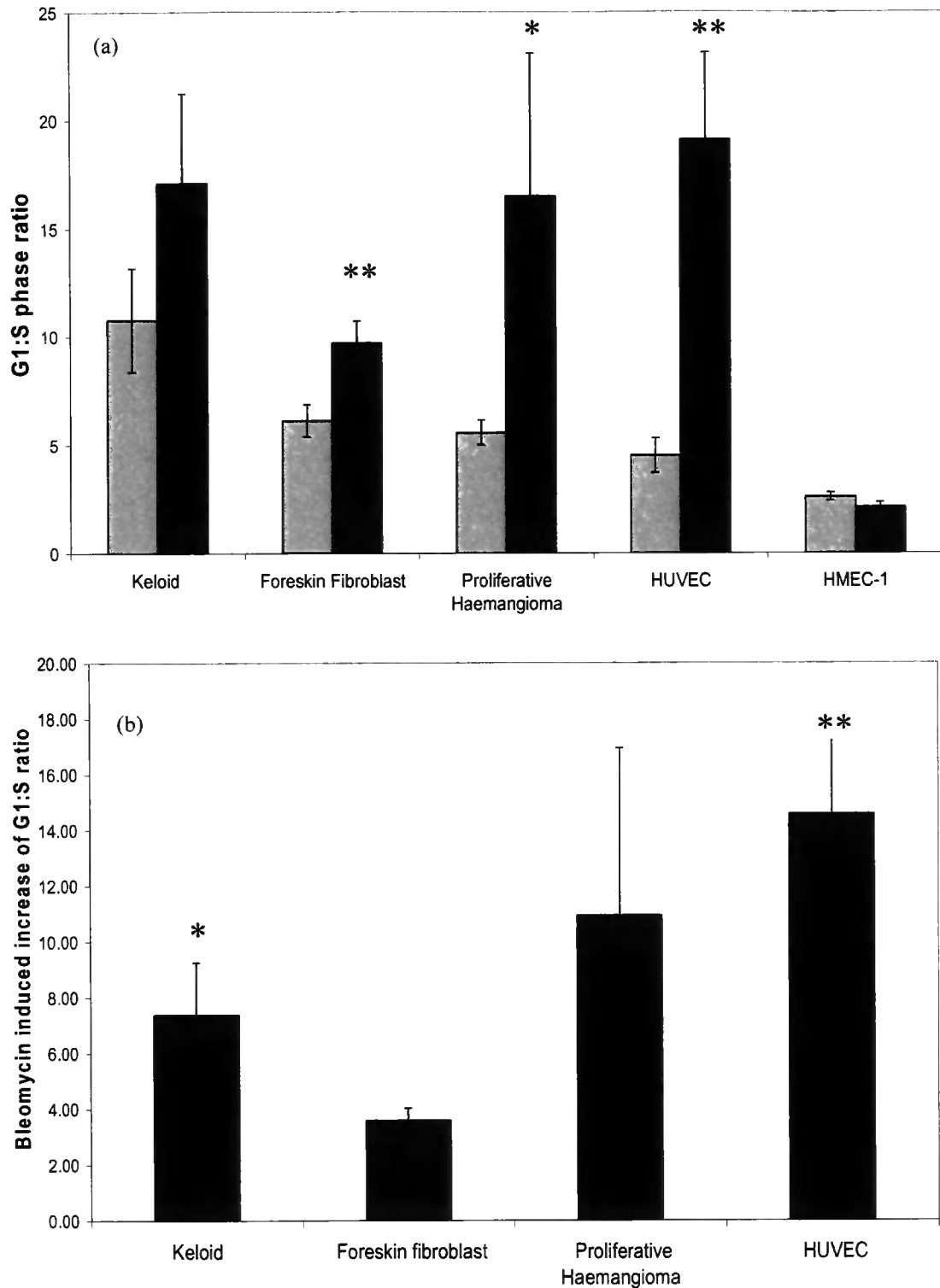
Figure 3.4(a): Cell cycle analysis of cell lines in the presence or absence of bleomycin. Results are expressed as G1:S phase ratios for the 5 cell lines 48hours post treatment with 100mU/ml bleomycin. With the exception of HMEC-1, all cell lines showed a bleomycin induced increase in G1:S ratio **(b) Bleomycin induced increase of G1:S ratio.** The data from (a) is expressed as absolute increase in G1:S ratio. Compared with foreskin fibroblasts, the other three cell lines showed a greater increase in G1:S ratio. This was statistically significant in the case of keloid and HUVEC. * = P< 0.05; ** = P< 0.01. Results are presented as the mean ± S.D.

3.2.3 Sub-G1 analysis

Bleomycin dose response at 24 hours and 48 hours for all cell lines is shown on figure 3.5(a) and 3.5(b) respectively. Cells were fixed and permeabilised then incubated with fluorochrome (PI) as described in 2.6.2. Apoptotic cells were identified as sub-G1 population. The bleomycin induced increase in sub-G1 percentage was calculated as (sub-G1 % in the presence of bleomycin – sub-G1 % in the absence of the drug).

The results demonstrate that bleomycin-induced DNA damage was both concentration and time dependent for all cells; however differences in response to bleomycin was observed between cell lines.

At lower doses of 1, 10 and 100mU/ml bleomycin, the percentage of cells in the sub G1 fraction did not increase significantly in the foreskin fibroblast at both 24 and 48 hours. At the higher dose of 1000mU/ml, no significant difference was observed after 24 hours incubation; however after 48 hours a significant increase in sub-G1% was observed (Table 1).

No increase in sub-G1% was observed at 1, 10, 100 and 1000mU/ml bleomycin in the keloid derived cells after 24 hours incubation (Table 2). At the higher dose of 10,000mU/ml, an increase in sub-G1% was observed, however variance between samples meant that this difference was not statistically significant ($P > 0.05$). After 48 hours incubation, an increase in sub-G1% was observed at 1, 100, 1000 and 10,000mU/ml; this increase was statistically significant in the case of 10,000mU/ml treated sample ($P < 0.05$).

HUVEC and venous malformation derived cells showed a statistically significant increase in sub-G1% at 1000 and 10,000mU/ml at both 24 and 48 hours incubation ($P < 0.01$). The increase observed at lower doses for these to cell lines were not statistically significant (Tables 3&6).

At lower doses of 1, 10 and 100mU/ml bleomycin, the sub-G1% of HMEC-1 cells did not increase significantly (Table 4). At the higher dose of 1000mU/ml, 24 hours incubation did not cause a significant increase in sub-G1%; however after 48 hours incubation at this dose, the increase in the percentage of cells in sub-G1 was statistically significant ($P < 0.01$). At the highest dose of 10,000mU/ml, a statistically significant increase was observed at both 24 and 48 hours ($P < 0.05$, $P < 0.01$ respectively).

None of the increases observed in sub-G1% in the haemangioma derived cells were found to be statistically significant, due to considerable variance between samples (Table 5).

At the higher dose of 10,000mU/ml, all cell lines showed an increase in sub-G1 percentage at both 24 and 48 hours. After 48 hours incubation with 1000mU/ml or 10,000mU/ml bleomycin, HUVEC apoptosis did not increase significantly from the degree of apoptosis that was observed at 24 hours incubation at these doses. In contrast, keloid and venous malformation derived cells, HMEC-1 cells, and foreskin fibroblasts displayed an increase in apoptosis with longer exposure at 1000mU/ml and 10,000mU/ml (see figure 3.6). An increase in the proportion of cells in sub-G1 was observed in the haemangioma derived cell line after longer incubation with 1000mU/ml and 10,000mU/ml of bleomycin; however variance between samples meant that this observation was not statistically significant. This data suggests the effect of bleomycin on HUVEC cells appears to be maximal within 24 hours.

The detection of a sub-G1 DNA peak is a specific marker of apoptosis; cell death by necrosis does not induce any sub-G1 peak (Nicoletti et al. 1991). However, in addition to apoptotic cells, the sub-G1 peak can also represent mechanically damaged cells and isolated apoptotic bodies, resulting in an over-estimation of apoptotic cell percentage. Therefore it was necessary to confirm that the observed increase in sub-G1 cells was indeed due to apoptosis. Apoptosis is characterised by activation of nucleases which produce a large number of DNA strand breaks (Nicoletti et al. 1991). Detection of these breaks was performed using the APO-DIRECT™ kit based on the Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay.
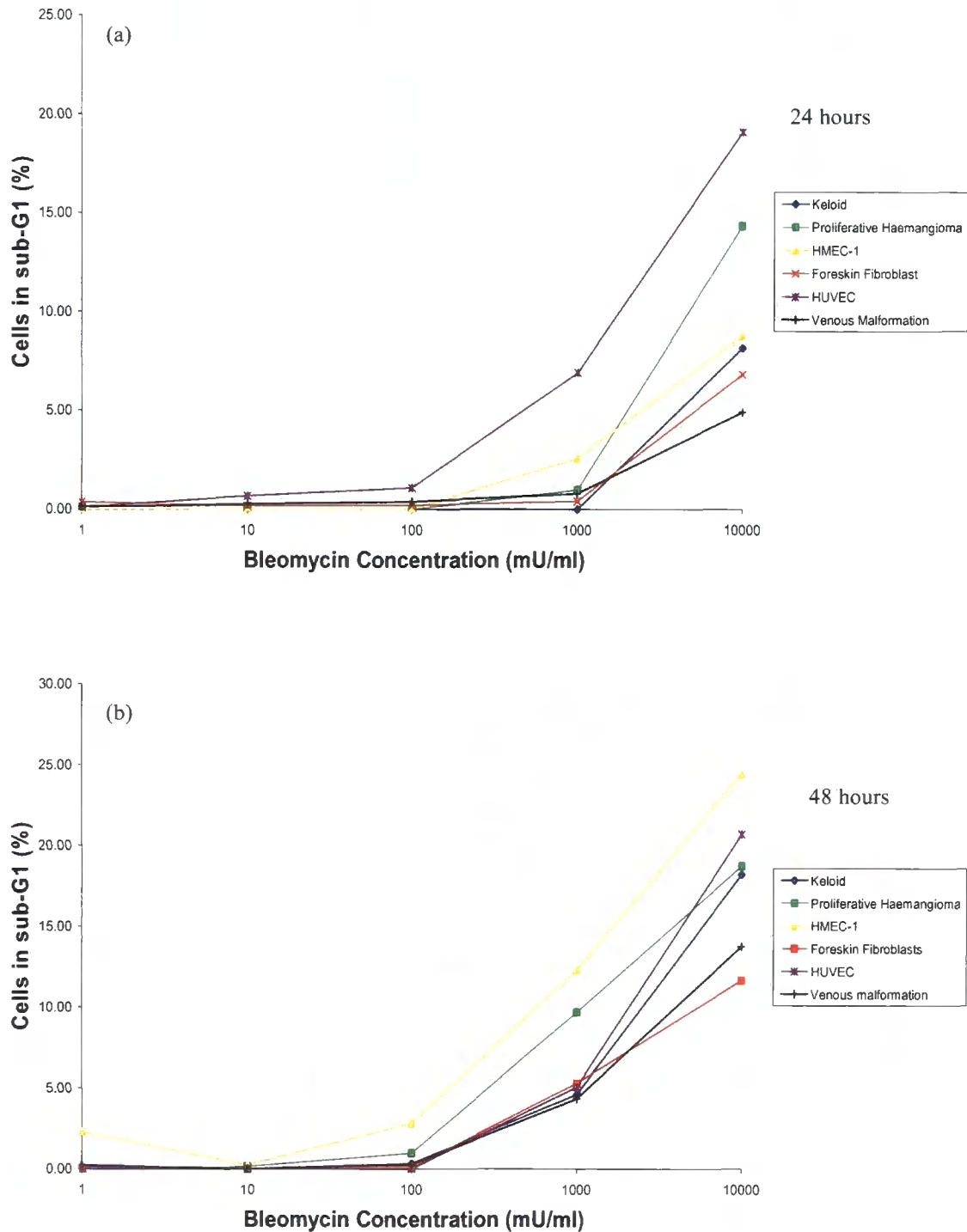
Figure 3.5 Effect of bleomycin treatment on the percentage of cells in sub-G1 fraction. Cells were cultured in the presence or absence of different bleomycin concentrations for 24 hours **(a)** or 48 hours **(b).** Results are expressed as (% sub-G1 in the presence of bleomycin – % sub-G1 in the absence of the drug). Data displayed are the means of 3 independent experiments. SD is not shown in this figure for reasons of clarity.

Table 1: Mean ± SD of bleomycin induced increase in sub-G1% in foreskin fibroblasts

Bleomycin (mU/ml)	Increase in sub-G1 %[1]	
	24 hours	48 hours
1mU/ml	0.36 ± 0.81	-
10mU/ml	0.17 ± 0.60	-
100mU/ml	0.20 ± 0.48	0.11 ± 0.18
1000mU/ml	0.41 ± 0.14	5.31 ± 2.22*
10000mU/ml	6.83 ± 2.74*	11.68 ± 4.11**

[1]Data from three independent experiments

- = No increase observed when compared to untreated control

* = P< 0.05, ** = P< 0.01

Table 2: Mean ± SD of bleomycin induced increase in sub-G1% in keloid derived cells

Bleomycin (mU/ml)	Increase in sub-G1 %[1]	
	24 hours	48 hours
1mU/ml	-	0.14 ± 0.22
10mU/ml	-	-
100mU/ml	-	0.33 ± 0.46
1000mU/ml	-	4.62 ± 3.92
10000mU/ml	8.15 ± 1.06	18.21 ± 4.85*

[1]Data from three independent experiments

- = No increase observed when compared to untreated control

* = P< 0.05.

Table 3: Mean ± SD of bleomycin induced increase in sub-G1% in HUVEC

Bleomycin (mU/ml)	Increase in sub-G1 %[1]	
	24 hours	48 hours
1mU/ml	0.10 ± 0.83	-
10mU/ml	0.67 ± 0.92	-
100mU/ml	1.09 ± 0.99	0.11 ± 0.21
1000mU/ml	6.90 ± 1.04**	5.07 ± 0.93**
10000mU/ml	19.08 ± 1.16**	20.71 ± 1.94**

[1]Data from three independent experiments

- = No increase observed when compared to untreated control

** = P< 0.01

Table 4: Mean ± SD of bleomycin induced increase in sub-G1% in HMEC-1

Bleomycin (mU/ml)	Increase in sub-G1 %[1]	
	24 hours	48 hours
1mU/ml	-	2.28 ± 7.45
10mU/ml	-	0.27 ± 2.43
100mU/ml	-	2.82 ± 2.60
1000mU/ml	2.58 ± 1.20	12.32 ± 2.36**
10000mU/ml	8.80 ± 4.28	24.46 ± 1.62**

[1]Data from three independent experiments

- = No increase observed when compared to untreated control

* = P< 0.05, ** = P< 0.01

Table 5: Mean ± SD of bleomycin induced increase in sub-G1% in haemangioma derived cells

Bleomycin (mU/ml)	Increase in sub-G1 %[1]	
	24 hours	48 hours
1mU/ml	-	-
10mU/ml	-	0.15 ± 0.43
100mU/ml	-	0.97 ± 0.90
1000mU/ml	1.65 ± 2.82	9.69 ± 14.35
10000mU/ml	14.33 ± 12.18	18.74 ± 15.77

[1]Data from three independent experiments

- = No increase observed when compared to untreated control

Table 6: Mean ± SD of bleomycin induced increase in sub-G1% in venous malformation derived cells

Bleomycin (mU/ml)	Increase in sub-G1 %[1]	
	24 hours	48 hours
1mU/ml	0.13 ± 0.14	0.23 ± 0.14
10mU/ml	0.24 ± 0.18	-
100mU/ml	0.38 ± 0.43	0.24 ± 0.09
1000mU/ml	0.79 ± 0.15**	4.32 ± 0.77**
10000mU/ml	4.89 ± 0.85**	13.75± 3.39**

[1]Data from three independent experiments

- = No increase observed when compared to untreated control
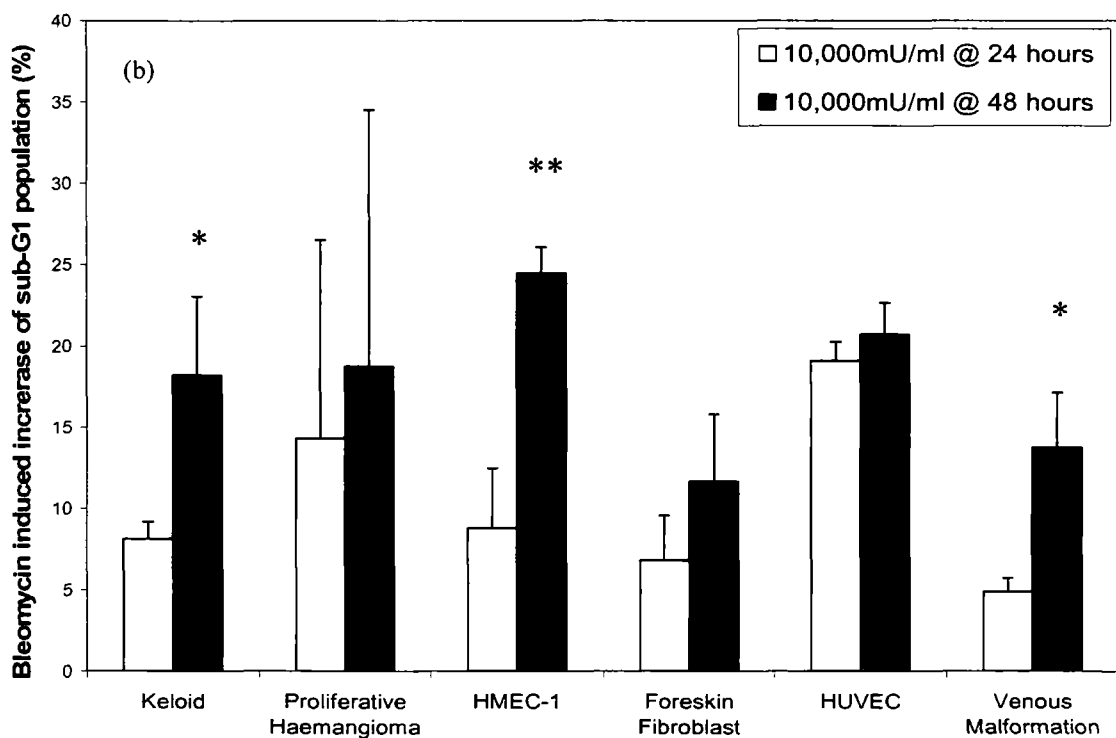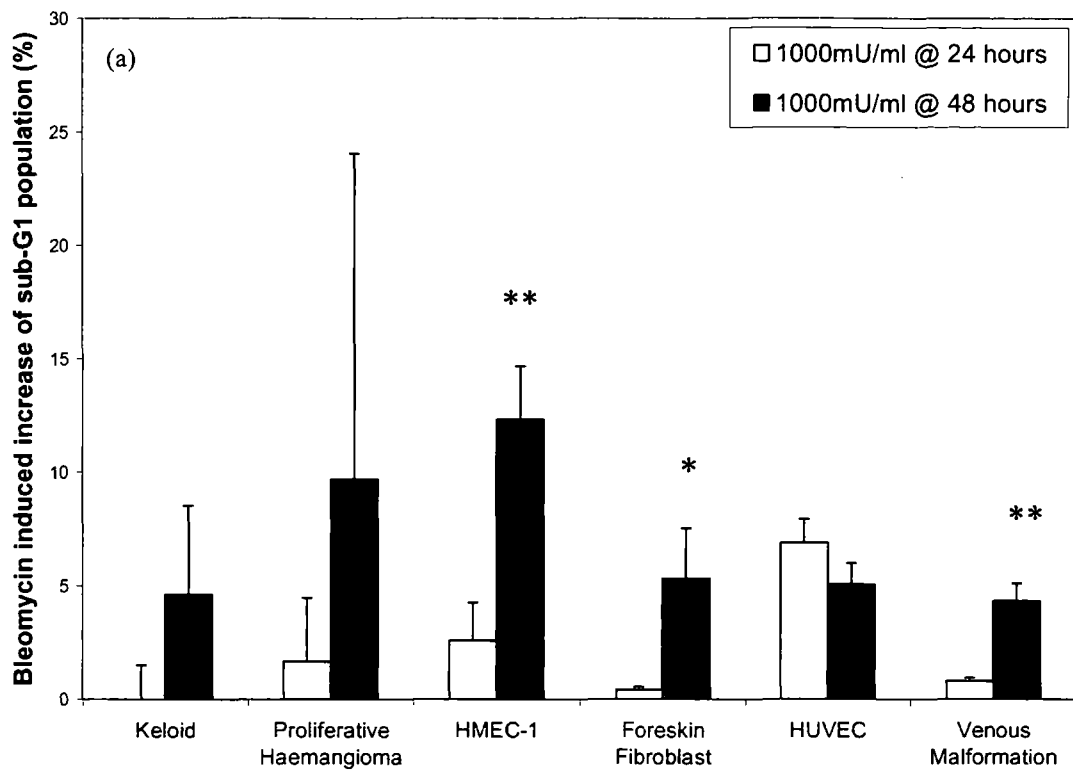
** = P< 0.01

Figure 3.6(a&b) Sub-G1 analysis of cell lines in the presence or absence of bleomycin. This data shows that the effect on HUVEC cells appears to be maximally effective within 24 hours, however all other cell lines show an increase in percentage of cells in sub-G1 fraction 24 hours later.

3.2.4 Detection of DNA fragmentation by flow cytometry using APO-DIRECT™ Kit

Induction of DNA strand breaks was determined using APO-DIRECT ™, by labelling DNA breaks with FITC-dUTP. Non-apoptotic cells were identified as a negatively stained population, whereas apoptotic cells incorporate the FITC-dUTP and are identified as a positively stained population. Following experiment 3.2.3, an increase in sub G1% was observed for all cells at a dose of 10,000mU/ml 24 hours post treatment. Therefore the decision was made to use this dose and time point for the subsequent experiments. All cells were assayed after 24 hours incubation in the presence or absence of 10,000mU/ml bleomycin. Results confirmed the induction of DNA strand breaks in all cell lines after treatment with bleomycin (figure 3.8). An approximately 9-fold increase (1.83 ± 0.53 to 16.90 ± 1.98) in FITC-dUTP-positive keloid derived cells was observed after bleomycin treatment. Haemangioma derived cells showed an approximately 6-fold increase (1.85 ± 0.32 to 11.06 ± 1.10), an approximately 4-fold increase was observed in HUVEC (4.91 ± 0.24 to 21.32 ± 3.56) and foreskin fibroblasts showed an approximately 5 fold increase in FITC-dUTP-positive cells (1.79 ± 0.24 to 21.32 ± 3.56).

Bleomycin induced DNA strand breaks were calculated as (FITC-dUTP-positive cells % in the presence of bleomycin – FITC-dUTP-positive cells % in the absence of the drug). The percentage of bleomycin induced DNA strand breaks for the keloid and haemangioma derived cells and HUVEC cells were compared to that of the foreskin fibroblast (figure 3.8(b)). Both keloid derived cells and HUVEC cells were shown to have a significantly higher induction of DNA strand breaks after treatment with bleomycin than that of the foreskin fibroblasts ($P= <0.01$). Bleomycin induced DNA strand breaks in haemangioma derived cells were not considered to be statistically significantly different to that of the foreskin fibroblast ($P= > 0.05$).

As described earlier (1.1.1), one of the reported mechanisms of bleomycin cytotoxicity is direct DNA binding resulting in single and double-strand breaks. To determine whether the DNA strand breaks detected by the APO-DIRECT™ kit were independent of apoptosis, a further assay was performed using an antibody for cleaved caspase-3, a key mediator in mammalian cell apoptosis.
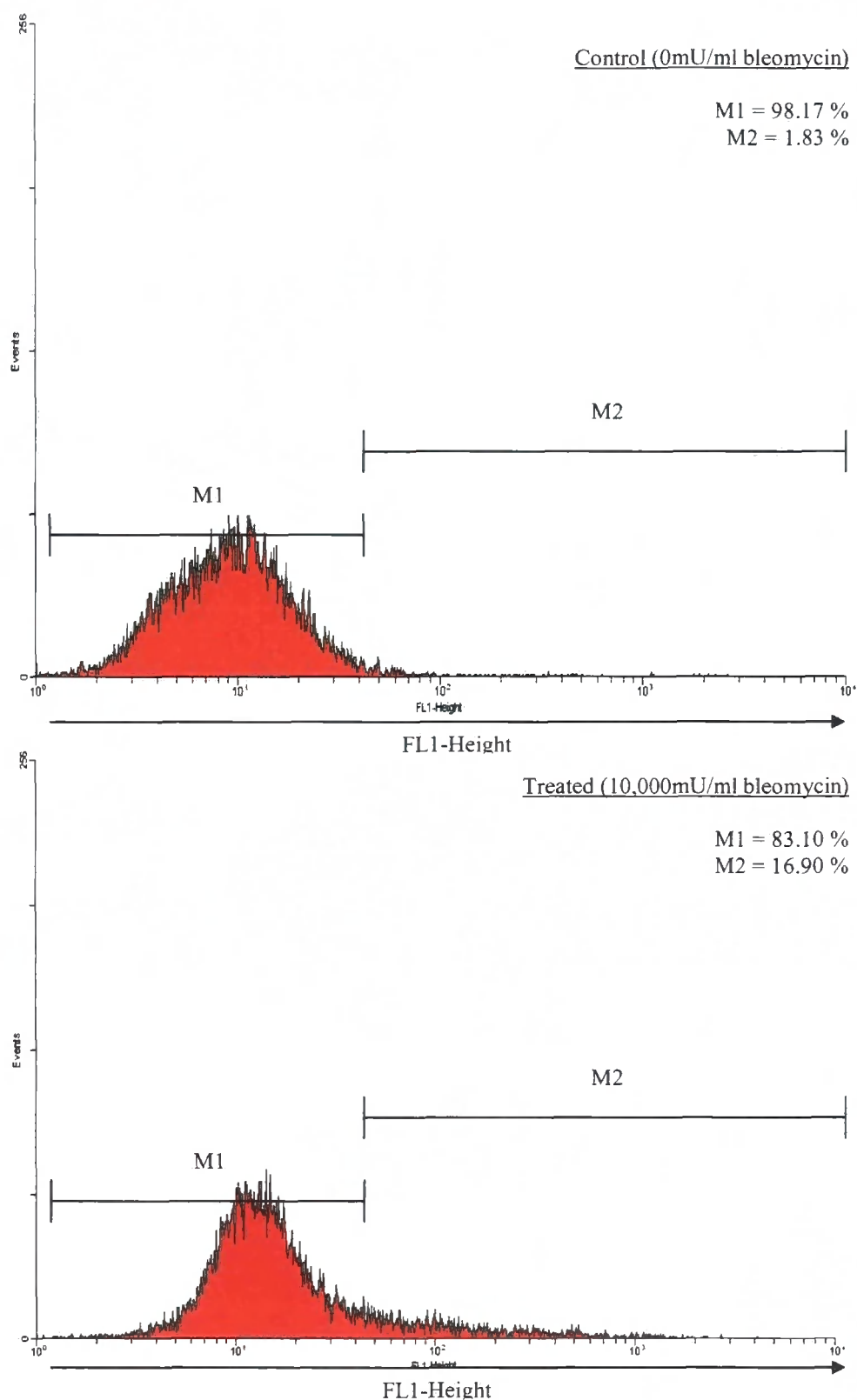
Figure 3.7 Induction of DNA breaks on keloid derived cell line as detected by FITC-dUTP labelling and analysed by flow cytometry. 10,000 cells were analysed per determination. All cells were incubated for 24 hours, in the presence or absence of 10,000mU/ml bleomycin. The data was plotted onto a FL1 histogram and the percentage of FITC-dUTP positive cells was determined. All experiments were performed n=3. Typical flow cytometry histograms in a representative experiment are presented above.
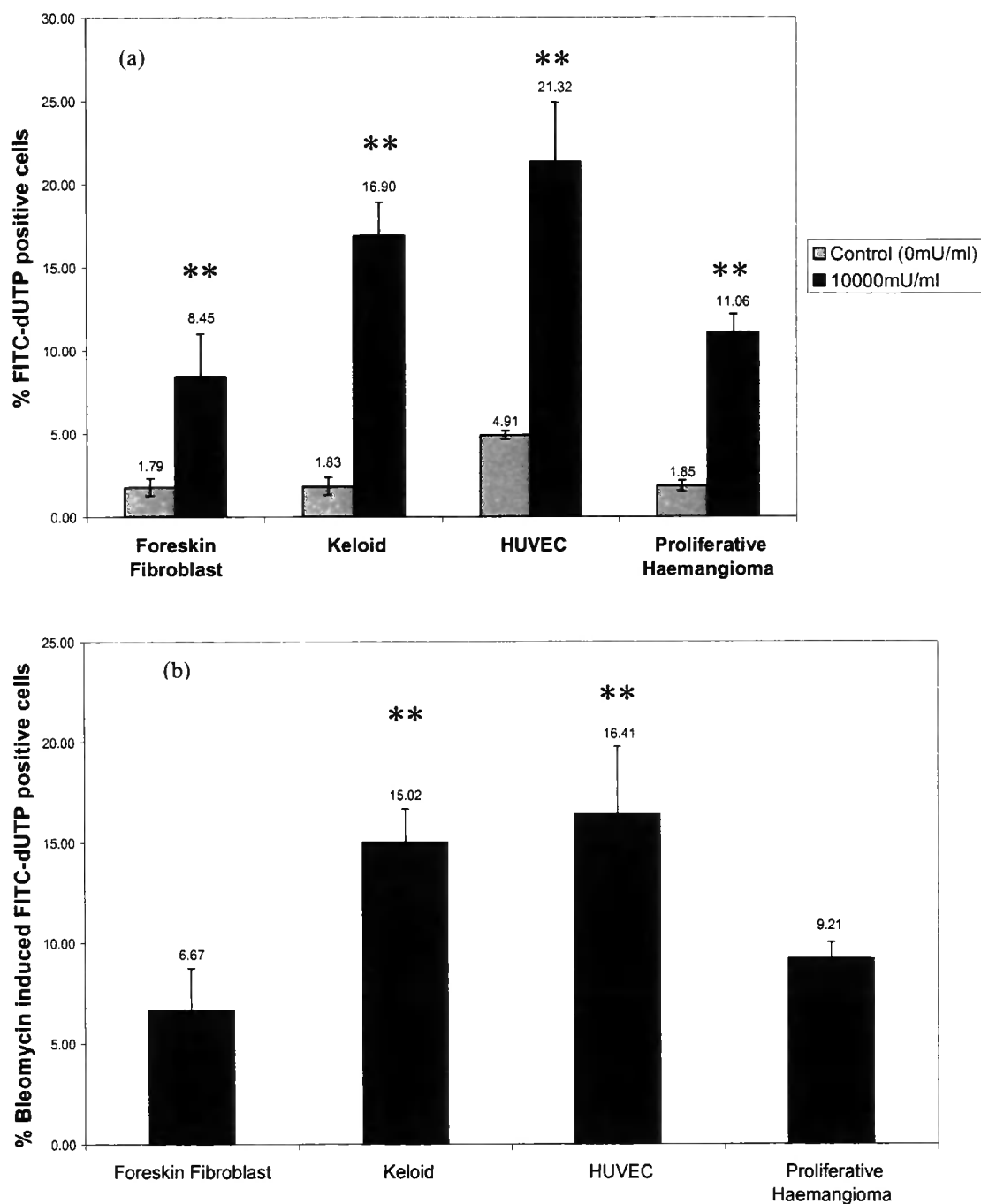
Figure 3.8 Bleomycin induction of DNA strand breaks as determined by FITC-dUTP labelling. Cells were incubated in the presence or absence of 10,000mU/ml bleomycin for 24 hours and assayed by flow cytometry using APO-DIRECT TUNEL method (a) The results are expressed as % of FITC-dUTP-positive cells and are given as the mean ± SD for 3 experiments. **(b) Bleomycin induced increase in DNA strand breaks**. The data from **(a)** is expressed as absolute increase in % FITC-dUTP labelled cells compared with untreated cells. Compared with foreskin fibroblasts, the other three cell types showed an greater increase in DNA labelling, which was statistically significant in the case of keloid and HUVEC ** = P< 0.01.

3.2.5 Detection of apoptosis by intracellular staining using cleaved caspase-3 conjugated antibody

Caspase-3 cleavage was determined using cleaved caspase-3 conjugated antibody. All cells were assayed after 24 hours incubation in the presence or absence of 10,000mU/ml bleomycin. Results confirmed the activation of caspase-3 in the keloid derived cell line after treatment with bleomycin (figure 3.9(a)); an approximately 5-fold increase in cleaved caspase-3 positive cells was observed (5.54 ± 2.04 to 26.53 ± 1.96). The percentage of cleaved caspase-3 positive cells in the foreskin fibroblast cells did not increase significantly following treatment with bleomycin (4.17 ± 0.66 to 5.60 ± 1.02). Bleomycin induced caspase-3 cleavage was calculated as (cleaved caspase-3 positive cells % following treatment with bleomycin – cleaved caspase-3 positive cells % in the absence of the drug). The percentage of bleomycin induced caspase-3 cleavage for the keloid derived cell line was compared to that of the foreskin fibroblast (figure 3.9(b)). Results from this assay showed that keloid derived cells had a significantly higher induction of caspase-3 cleavage following treatment with bleomycin than that of the foreskin fibroblasts (P = <0.01).
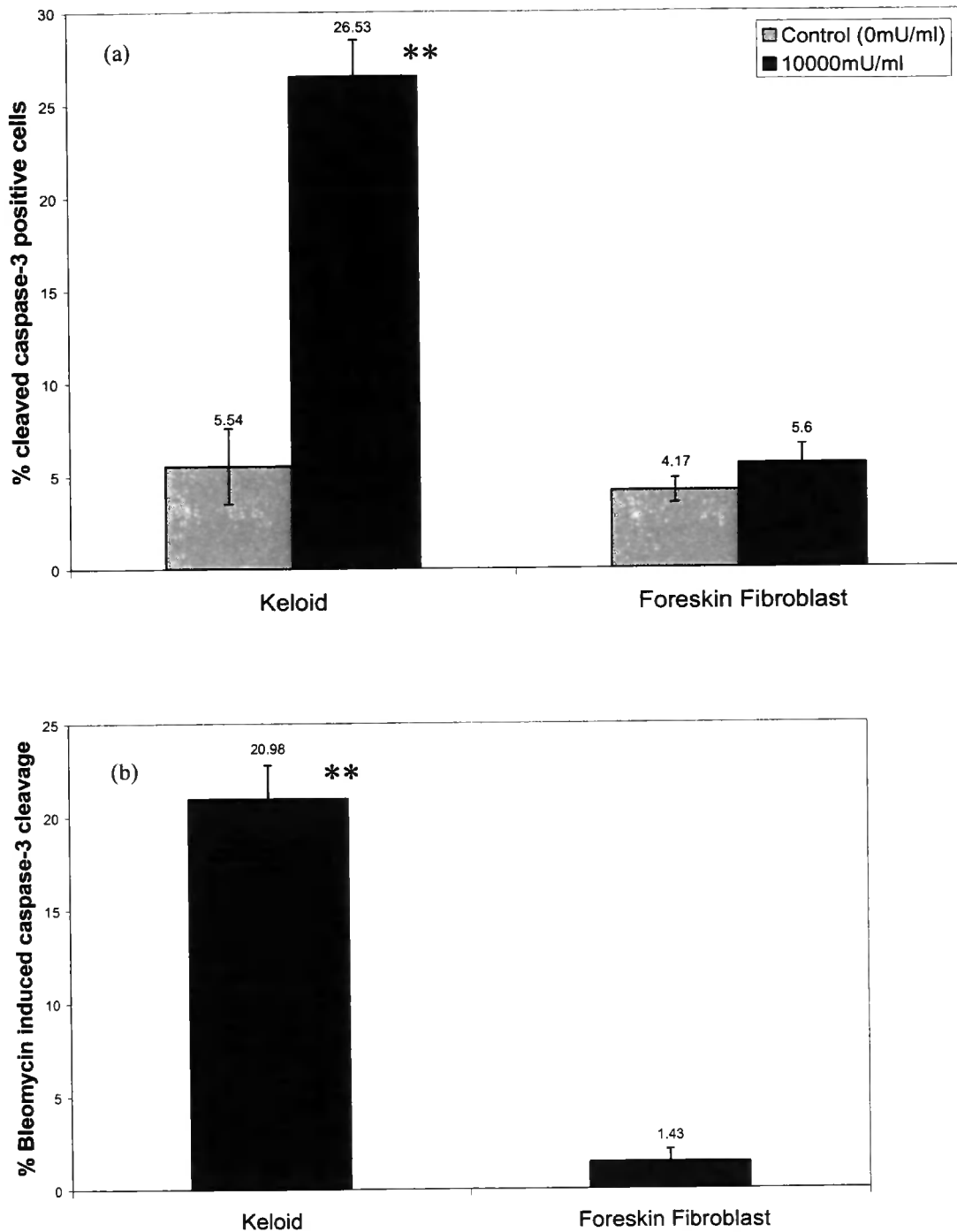
Figure 3.9 Effects of bleomycin on caspase-3 cleavage. Cell lines were incubated in the presence or absence of 10,000mU/ml of bleomycin for 24 hours, and caspase-3 cleavage determined by binding of specific fluorescent antibody. The results are expressed as **(a)** Percentage of cells showing specific antibody binding in the presence or absence of bleomycin and **(b)** the percentage increase following bleomycin treatment. Results are expressed as the mean $\pm$ SD for 3 experiments. ** = P< 0.01 Keloid cells showed a statistically significant greater sensitivity to bleomycin compared with foreskin fibroblasts.

Chapter 4

4 Discussion

This study investigated the effects of bleomycin on a range of human primary cell lines derived from haemangioma, venous malformation and keloid scars. Observations from dose responsiveness of these derived cell lines along with other control cell lines (foreskin fibroblast, HUVEC, HMEC-1) *in vitro* subsequently led this study to focus on possible mechanisms involved in bleomycin-induced cell death in these cells. It demonstrates for the first time that, in keloid derived cells, bleomycin-induced cell death is associated with caspase-3 mediated apoptosis.

The mechanism of action of bleomycin in clinical cases of haemangioma, venous malformation and keloid is not yet described. The aim of this study is to improve our understanding of the mechanism by which bleomycin exerts its effects, which could ultimately lead to the identification of an alternative therapeutic strategy which could avoid the substantial side effects of this powerful cancer chemotherapeutic.

The first component of this was to establish an *in vitro* system. In order to derive the primary cell lines required for this, tissues were cultured as explants of small fragments or as single cell cultures following collagenase digestion. Although the latter approach offers the opportunity for both adherent and non-adherent cell types to grow out, no cell lines developed. However, adherent cell lines were successfully derived from explant cultures of all three tissue types. The cell lines obtained from haemangioma explants had a fibroblast-like morphology, a result consistent with other studies of explant-derived cell types from haemangioma. Yu et al. (2006) isolated cells from both proliferating and involuting haemangiomas using an adherence technique and described these to have a fibroblast-like morphology. Using the same method they also attempted, unsuccessfully, to isolate adherent cells from other skin abnormalities. However, in this study we managed to isolate cells not only from haemangioma tissue, but also from venous malformation and keloid scars. The cells obtained from keloid and venous malformation explants also had a fibroblast-like morphology, which is not surprising as the mode of selection used in this study invites similar cell types out of these tissues. The fibroblast-like morphology of the keloid derived cells described in this work is in agreement with other published works using the explant culture technique (Babu et al. 1989; Chodon et al. 2000; Cheng et al. 2001). Given the similarity in gross cellular

morphology, one possibility is that that an identical cell type has been isolated from all three tissues by virtue of this culture method. If this were the case, then these three cell lines should show a similar sensitivity to bleomycin. This proved not to be the case.

Dose response studies were performed on cell lines derived from tissue specimens (haemangioma, venous malformation, and keloid) along with other cell lines (foreskin fibroblasts, HUVEC, HMEC-1) and showed that at a dose of 100mU/ml bleomycin, all cell lines demonstrated a G1 arrest after 48 hours in culture. Whilst this is in agreement with Gonzales et al. (1998) whose work also described a G1 arrest in response to bleomycin treatment in mouse hepatocytes, in fact most studies into the effect of bleomycin on cells *in vitro* have reported an arrest in the G2 fraction of the cell cycle. Barranco et al. (1971) showed that bleomycin treatment caused G2 arrest in Chinese hamster ovary cells (CHO); this was later confirmed by Tobey (1972) who showed that bleomycin had little effect upon DNA synthesis in CHO cells, but was extremely effective in the irreversible prevention of cells progressing from G2. Barlogie et al. (1976) and Bell et al. (1988) also described a concentration dependent accumulation of human lymphoma cells and human osteosarcoma cells respectively, in the G2 fraction of the cell cycle. Tounekti et al. (1993) also described a G2 arrest in Chinese hamster lung fibroblasts following exposure to low concentrations of bleomycin. More recently, Arai et al. (2004) noted a G2 arrest in a human T-cell leukaemia cell line following treatment with bleomycin; however this study also investigated the effect of bleomycin on HUVEC cell line and reported that bleomycin induced both G1 and G2 arrest in these cells. Future work could include experiments to determine if the G1 arrest observed in the cell lines in this study is reversible, by stopping the treatment after a certain time point and measuring the G1/S ratio after allowing the cells time to recover. Following DNA damage, cells ensure that mutations are not passed on to their daughter cells by either arresting the cell cycle to allow time for DNA repair, or DNA damaged cells which cannot be repaired can be eliminated by apoptosis (Di Leonardo et al. 1994). If bleomycin at a concentration of 100mU/ml was arresting the cells following DNA damage, then it would be fair to assume that treatment with higher doses of bleomycin would lead to greater DNA damage and chromosomal breakup. Results in the present study showed that bleomycin induced a dose-dependent increase in sub-G1 population in all the cell lines treated, which is a specific marker of apoptosis. Dose dependent bleomycin-induced increase in sub-G1 percentage has also been described in

work by Wallach-Dayan et al. (2005) when working with mouse lung epithelial cells and by Chen et al. (2004) when working with human liver carcinoma cells.

Although all cells appeared to have a similar dose-dependent response to bleomycin, further analysis showed that there was a differential sensitivity between cell types to the drug. Endothelial cell lines HUVEC and HMEC-1 appeared to be the most sensitive to bleomycin, showing a greater increase in sub-G1 percentage after 24 hours treatment with bleomycin than the other cell lines treated. However differences between these two cell lines became apparent after a further 24 hours incubation in the presence of bleomycin. While the percentage of sub-G1 cells continued to increase in the HMEC-1 cells following 48 hours in the presence of bleomycin, HUVEC sub-G1 percentage did not increase significantly when incubation time was increased. This suggests that the effect of bleomycin on HUVEC cells is maximal within 24 hours. Differential sensitivity was also observed in the keloid cells when compared to foreskin fibroblasts, with results suggesting that keloid cells were more susceptible to bleomycin treatment. One explanation of these results could be that the differences observed between cell lines are due to differential uptake. It is thought that the action of bleomycin on neoplastic cells is largely dependent on internalisation (Heller et al. 1998). Electroporation is one mechanism that can be used to enhance internalisation of bleomycin. Previous work by Tounekti et al. (1993) has shown that bleomycin cytotoxicity increases *in vitro* following electroporation of Chinese hamster lung fibroblasts. Orlowski et al. (1998) also described a 700-fold increase in toxicity of bleomycin in the same cell line following electropermeabilisation. Further work to this study could include the electropermeabilisation of all the cell lines, to determine whether there are still detectable differences in sensitivity when the same concentration of bleomycin is introduced to the cytoplasm. Another method to assess whether differential uptake is the reason behind the differential sensitivity observed in these cell lines is the use of a bleomycin-specific antibody. Staining cells post-treatment with this antibody could allow identification of the cells which have internalised the drug.

Differential sensitivity to bleomycin has also been linked to a cells ability to inactivate bleomycin. Work by Umezawa et al. (1972) discovered that cells of both the lungs and skin were deficient in their ability to inactivate bleomycin. Bleomycin can be metabolised by a cystein peptidase termed bleomycin hydrolase. Bleomycin hydrolase acts on the carboxamide group of the ß-aminoalanine moiety in bleomycin to render an inactive metabolite (Umezawa et al. 1972). Further studies, including *in vivo* and *in*

vitro work by Lazo et al. indicated that the sensitivity of the lungs to bleomycin induced toxicity could be due to the lack of bleomycin hydrolase activity (Lazo et al. 1983). Yeast strains expressing high levels of bleomycin hydrolase were shown to be more resistant to bleomycin compared with wild-type controls (Kambouris et al. 1992). *In vivo* work using a mouse model that completely lacked any detectable bleomycin hydrolase activity showed that Bh -/- mice were hypersensitive to bleomycin, displaying pulmonary fibrosis or lethality in response to doses of bleomycin which in Bh +/+ mice showed no effect (Schwartz et al. 1999). These results strongly suggest the protective role bleomycin hydrolase plays against bleomycin induced toxicity; therefore it would be interesting to assess the expression of bleomycin hydrolase in the cell lines treated in this study, to determine whether differential expression correlates with cell sensitivity. Both haemangioma and venous malformation derived cells showed a similar dose and time course sensitivity; however there was far greater variance between samples in haemangioma derived cells. Due to the non-selective nature of the derivation of cells from the haemangioma tissue using explant culture, it would not be unreasonable to suggest that the population of cells obtained from these tissue specimens were heterogeneous. From the results obtained, it would appear that there are cells within this population which are uniquely sensitive to bleomycin. Recent work by Khan et al. (2008) identified a haemangioma stem cell (Hem-SC) which when implanted into immunodeficient mice were shown to recapitulate haemangioma-like lesions. From this work they concluded that Hem-SCs were likely to be the cellular origin of haemangioma. One could hypothesise that this stem cell population could be the bleomycin-sensitive cell type seen in the haemangioma derived cell line in this study. One approach to answering this question would be to characterise the cell lines described in this work with the Hem-SC, using cell surface marker expression, secretion of paracrine growth factors and gene expression profile. This data would help address whether the nature of the cells is biologically similar. However, the best approach would be to directly examine the bleomycin sensitivity of Hem-SC in the assays described here.

Apoptosis was detected by the TUNEL method, to determine whether the bleomycin induced sub-G1 population observed in the previous experiments were in fact cells with detectable DNA strand breaks seen in apoptotic cells, and not merely mechanically damaged cell fragments. This assay confirmed bleomycin-induced DNA strand breaks

in all four cell lines treated. As with the previous assay, differential sensitivity was observed between cell lines, with HUVEC displaying the greatest induction of DNA strand breaks following treatment with bleomycin. There also appeared to be a clear difference in bleomycin-induced DNA strand breaks in keloid derived cells when compared to normal foreskin fibroblast cells, with keloid derived cells showing almost double the DNA damage. Although the cells derived from keloid tissue are likely to be fibroblasts; as based on their appearance in culture and the cellular nature of keloid scars, these cells are clearly more sensitive to bleomycin than normal foreskin fibroblasts.

One possible explanation of the results presented in this study is that the difference between these cells lines is genetic; that the individual from whom the keloid scar tissue was obtained is simply genetically more susceptible to the effects of bleomycin. One way to validate this theory would be to obtain normal tissue from patients presenting with keloid scars, as well as from the keloid scars themselves. It would then be possible to assess any differences in response to bleomycin between normal fibroblasts and keloid fibroblasts from the same patient, although performing such a study would carry significant ethical problems, as the aim of most reconstructive surgery is to preserve, rather than resect, normal tissue. Ethical limitations in this study meant that this was not possible, as only tissue from the lesions could be removed. Furthermore, clinical studies to date have demonstrated the near universal sensitivity of these lesions to bleomycin treatment, suggesting an intrinsically higher sensitivity, and whilst there is evidence of bleomycin resistance at a cellular level due to hydrolase expression, it has not been described at the level of the whole individual.

As described earlier in the results (3.2.4), an antibody specific for cleaved caspase-3 was used to determine whether the bleomycin-induced sub-G1 population and DNA strand breaks observed in the previous experiments were due to apoptosis and not simply direct DNA binding by bleomycin resulting in DNA strand breaks. Both keloid derived cells and foreskin fibroblasts showed a bleomycin-induced activation of caspase-3, however the level of caspase-3 cleavage was significantly greater in keloid derived cells. To the best of our knowledge, this is the first report of caspase-3-mediated apoptosis of keloid derived cells induced by bleomycin.

Bleomycin is known to degrade DNA and creates single and double strand breaks in neoplastic cells (Suzuki et al., 1969; Miyaki et al., 1975; Kohn et al., 1975), effects

which are presumed to be responsible for bleomycins chemotherapeutic properties. However there is emerging evidence that bleomycin may act through other pathways. As mentioned earlier, the main dose-limiting effect of bleomycin is pulmonary toxicity, resulting in pulmonary fibrosis. Extensive *in vivo* and *in vitro* research has been performed to try and determine the mechanism by which bleomycin induces pulmonary fibrosis, yielding interesting results. An *in vitro* study by Wallach-Dayan et al. used murine lung epithelial cells to investigate bleomycin-induced apoptosis of lung epithelial cells. Their results showed that bleomycin caused reactive oxygen species (ROS) accumulation, increased caspase activity, apoptosis and increased expression of cell surface Fas/FasL. Apoptosis was shown to be inhibited by glutathione, but not by Fas/FasL pathway antagonists; suggesting that bleomycin functions via ROS-induced mitochondrial cell death and that the Fas/FasL pathway is not predominant (Wallach-Dayan et al. 2006). *In vivo*, treatment with antioxidant amifostine was shown to significantly attenuate bleomycin-induced lung injury in a hamster model, providing further evidence of ROS involvement in bleomycin-induced lung injury (Nici et al. 1998). It is possible that bleomycin may exert its effects on the lesions examined in this study by the same process. Future work could include the assessment of ROS accumulation in these cell lines.

Growing evidence suggests that transforming growth factor- ß (TGF-ß) is important in the process of bleomycin-induced pulmonary fibrosis. TGF-ß pulmonary levels were shown to be elevated in mice (Hoyt et al. 1988) and in rats (Westergren-Thorsson et al. 1993) following intracheal bleomycin installation. Administration of an antibody to TGF-ß (Giri et al. 1993) and a receptor antagonist for this cytokine (Wang et al. 1999) have been shown to attenuate bleomycin-induced pulmonary fibrosis. Inhibition of activin receptor like kinase-5 (ALK5), a type 1 TGF-ß receptor, has also been shown to attenuate bleomycin-induced pulmonary fibrosis (Higashiyama et al. 2006).

An important effect of TGF-ß is induction of apoptosis. This has been demonstrated in human lung epithelial cells by Yanagisawa et al. (1998) when cultured in the presence of TGF-ß, these cells showed a marked induction of apoptotic cell death. TGF-ß has also been shown to enhance Fas-mediated apoptosis of lung epithelial cells via caspase-3 activation (Hagimoto et al. 2002).

Previous work by Kuwano et al. (1999) demonstrated the importance of the Fas-Fas ligand pathway in the development of bleomycin-induced pulmonary fibrosis. Fas

ligand (FasL) is a cell surface molecule belonging to the TNF family. When FasL binds to its receptor Fas, it induces apoptosis of Fas-bearing cells (Kuwano et al. 1999). Bleomycin administration in an animal model for pulmonary fibrosis resulted in an upregulation of FasL mRNA in infiltrating lymphocytes and an upregulation of Fas mRNA in alveolar epithelial cells (Hagimoto et al. 1997). Kuwano and colleagues showed that the administration of a soluble form of Fas antigen prevented apoptosis of epithelial cells and subsequent development of fibrosis.

Caspase activation is required for cell death via the Fas-FasL pathway. This fact led Kuwano et al. to examine the possibility that caspase inhibitors may prevent Fas-FasL mediated bleomycin-induced pulmonary fibrosis. Using the caspase inhibitor (Z-VAD-FMK) Kuwano et al. demonstrated it decreased the number of apoptotic cells, reduced caspase activity in general and decreased the pathological grade of lung inflammation and fibrosis. Similar work by Wang et al. confirmed the effectiveness of the caspase inhibitor Z-VAD-FMK to attenuate bleomycin-induced lung fibrosis by abrogation of alveolar epithelial cell apoptosis (Wang et al. 2000).

Although the Fas-FasL pathway was shown to be required for bleomycin-induced pulmonary fibrosis in mice, some mice deficient in either Fas or FasL developed mild disease; suggesting that other death receptors or death signals such as reactive oxygen species may be involved (Kuwano et al. 2001).

TGF-ß has also been shown to downregulate the synthesis of cyclin-dependent kinase 4 (cdk4), a cdk involved in G1 checkpoint, resulting in G1 arrest in murine lung epithelial cells (Ewen et al. 1995). It is interesting that two of the observed effects of bleomycin in this study (G1 arrest and caspase-3 mediated apoptosis) have been described in previous studies as the subsequent effects of TGF-ß. It may be that bleomycin is acting via TGF-ß signalling in the cell lines examined in this study. Future experiments to assess the involvement of TGF-ß in bleomycin cytotoxity could include the administration of an antibody to TGF-ß, or inhibition of the related effector molecules (caspase-3, Fas) to determine any downregulation in apoptosis. Also expression levels of TGF-ß, p53 and Fas in these cells following treatment with bleomycin would also help determine the signalling pathways involved.

In conclusion, results from this study show that bleomycin induces apoptosis of haemangioma, venous malformation and keloid derived cells in a dose dependent manner. In addition, we also report for the first time that bleomycin induces a caspase-3

mediated apoptotic cell death in keloid derived cells. This work is novel in showing caspase-3 activation in keloid derived cells following treatment with bleomycin, and provides the basis for further investigations, both *in vitro* and *in vivo*, that will confirm whether this mechanism is responsible for the apoptosis observed in the other lesions discussed whilst further elucidating the intracellular processes involved. The use of bleomycin for these common lesions is currently restricted to specialist clinical units and remains limited by its potential toxicity in these young patients. It is, however, very effective and it is only through further understanding of its actions that a less toxic alternative can be found.

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Appendix 1: Chapter 2

Tissue culture

Endothelial cell growth medium MV (HUVEC media)
After adding the attached supplement mix the media contains:
Endothelial Cell Growth Supplement/Heparin: 0.004ml/ml medium
Epidermal Growth factor, human recombinant: 10.0ng/ml medium
Hydrocortisone: 1.0µg/ml medium
Phenol Red: 0.62ng/ml medium
Foetal Calf serum: 0.05ml/ml medium

Growth media for HMEC-1 cells
MCDB-131 growth medium containing 10% FBS, 10ng/ml human epidermal growth factor (EGF), 1µg/ml hydrocortisone, 50µg/ml gentamycin and 250µg/ml amphotericin B.

Growth media for haemangioma, venous malformation and keloid derived cells
Minimum essential media (MEM) containing 20% (v/v) foetal bovine serum, 2mM L-glutamine, 50µg/ml gentamycin solution and 250µg/ml amphotericin B.

Growth media for foreskin fibroblasts
MEM supplemented with 10% (v/v) FBS, 2mM L-glutamine, 50µg/ml gentamycin and 250µg/ml amphotericin B.

Double strength antibiotic Media
Minimum essential media (MEM) containing 20% (v/v) foetal bovine serum, 2mM L-glutamine, 100µg/ml gentamycin solution and 500µg/ml amphotericin B.

Appendix 2: Chapter 2

Manufacturers

BD Falcon

Web address of supplier: *www.bdbiosciences.com*

Gibco

Web address of supplier: *www.invitrogen.com*

Greiner

Web address of supplier: *www.greinerbioone.com*

Hausser Scientific

Web address of supplier: *www.hausserscientific.com*

Nunc

Web address of supplier: *www.nuncbrand.com*

Promocell

Web address of supplier: *www.promocell.com*

Sigma-Aldrich

Web address of supplier: *www.sigmaaldrich.com*

Worthington

Web address of supplier: *www.worthington-biochem.com*

Appendix 3: Chapter 2

Ethical Approval

Bleomycin and the cell biology of benign skin lesions

PROTOCOL

Background:

Juvenile haemangioma is the commonest tumour of childhood, but remains poorly understood. It usually forms a small, pink lesion in the skin and is often considered to be a birthmark. It typically appears a few weeks after birth and grows for a period of time (the proliferative phase), remains unchanged for a variable period of time and then shrinks (regression phase) to subsequently disappear completely. Although the vast majority of cases regress spontaneously as described and cause no permanent problems, a significant proportion, usually due to their size or position, require more intensive intervention.

Treatment with surgery and corticosteroids are the typical treatment options but the results can be disappointing. More recently it has been recognised that bleomycin, a chemotherapeutic agent, when injected into these lesions can cause them to shrink dramatically. This has led to use of bleomycin in other challenging skin disorders, including arteriovenous (AV) malformations and keloid scars, as well as malignancies including metastatic malignant melanoma. Cutaneous AV malformations encompass a range of lesions, including cystic hygroma, which is thought to represent an overgrowth of lymphatic vessels. It is regarded as a benign tumour, but can be challenging to treat. It frequently recurs after surgery, and other therapeutic options are limited, although it does appear to respond well to intra-lesional bleomycin therapy. Keloid is a very common condition, and is a benign, but potentially disfiguring overgrowth of tissue related to a scar. Again, surgery is an imperfect treatment option, with high recurrence rates, and as with the other lesions, there is growing evidence that the injection of bleomycin can lead to regression.

Bleomycin is a well known, frequently used but relatively toxic agent, pulmonary fibrosis being its most significant side effect. It is most often used in the treatment of various lymphomas and testicular carcinomas, where its mechanism of action is probably through DNA cleavage in the dividing cancer cells. However, there is some evidence that it may also act through other pathways; how it acts in these skin lesions is unknown. Given its toxicity, we wish to explore further its action in these lesions at the level of cell signalling, with the ultimate aim of determining whether other, less toxic, compounds, may be able to act in a similar way with the same therapeutic effect.

The plastic surgery unit at the James Cook University Hospital is one of the few in the country where such bleomycin treatment is available. As a tertiary referral centre for these challenging problems, it also receives a substantial number that still require excisional surgery, generating tissue that would otherwise be discarded, that could be used to investigate these conditions further.

Aims and objectives:

1. Establish cell lines from juvenile haemangioma, cutaneous AV malformations and keloid lesions.

2. Determine their relative sensitivity to bleomycin *in vitro*, and in comparison to banked cell lines representative of normal skin components.

3. Determine the mode of action of bleomycin in the cells.

Summary of procedures:

1. Retrieving tissue.

Patients admitted for excision of juvenile haemangioma, AV malformations and keloid, either for therapeutic or diagnostic reasons, will be prospectively identified from waiting lists and at assessment at specialist clinics. They (and their family) will be approached by the consultant plastic surgeon to discuss the study and obtain informed consent. The vast majority of patients with haemangiomas will be under 5 years old, as such, consent to retrieve surplus tissue will be obtained from their parent or legal guardian when consent for the surgical procedure itself is obtained.

Tissue will be obtained at the time of surgery. The majority of specimens will be placed in cold, sterile buffer solution, labelled with the age and sex of the patient, anatomical location of the lesion and the clinical diagnosis. For juvenile haemangioma, the specimen will also be labelled to indicate whether it was felt to be in the proliferative or regression phase at the time of excision (the vast majority are likely to be in the proliferative phase). The specimen will also be labelled to indicate if there has been prior therapy with bleomycin. In some instances, where there is a need to extract RNA or perform particular histological assessment techniques, the lesion will be frozen immediately at the time of excision otherwise they will be refrigerated at 4C.

To further ensure anonymity, the consultant plastic surgeon ordinarily responsible for the care of the patient will maintain a database of patients from whom tissue is obtained. This will enable all specimens to be labelled with a unique identifier, as well as the information outlined above, the database will link the unique identifier with the patient's hospital number, and will be stored only on NHS systems for the duration of the study.

The tissues will be transferred to the laboratories at Durham University within 24 hours of retrieval.

2. Establishing cell lines.

The lesions will be dissected free from normal surrounding tissues and further dissociated by enzymatic treatment. Primary cultures will be established in standard culture conditions, and clonal cell populations derived from these. Similar protocols will be followed for all the lesion types, with variations only in the enzymatic treatment regimens.

Experimental strategies:

1. Characterisation of cell lines.

After the establishment of primary cultures, cell lines thus derived will be characterised by immunohistochemistry and DNA analysis to determine the expression of published markers associated with the pathological lesions. We will also seek to determine if they express markers consistent with stem cell, vascular and other skin phenotypes. Frozen specimens will be used for the extraction of RNA. Their ability to grow in both 2-D and 3-D cultures will be examined and compared to that of cell lines representative of the components of normal skin.

2. Response to bleomycin

Bleomycin will be added to cultures derived from the lesions, as well as cultures of normal dermal cells, epithelial cells, endothelial cells and dermal stem cells. Any effect on cell death (particularly apoptosis) or replication will be examined at varying concentrations of the agent to determine if there is any difference in sensitivity between cell types. Various inhibitors of known apoptotic signalling pathways will be used in an attempt to determine the signalling pathways involved, and the results from these initial experiments will inform further examination of the signalling pathways within the cells.

3. Evaluation of alternative agents

If analysis of the signalling pathways involved suggests other potential therapeutic agents, these will be tested in vitro in a similar manner to the initial protocols. There is no intent to move to the clinical trial stage in this study.

4. Co-culture

In an attempt to further understand the mechanism of development of the lesions, cells derived may be co-cultured with other cell types, including stem cells, in an attempt to clarify any inductive capability of the tumour cell lines themselves.

South Tees Hospitals **NHS**

NHS Trust

Chairman, Northumberland REC
Room 144 TEDCO Business Centre
Viking Industrail Park
Rolling Mill Road
Jarow
NE 32 3DT

Department of Cardiothoracic Surgery,
James Cook University Hospital,
Marton Road, Middlesbrough
TS4 3BW
Tel: 01642 854728
Email: andrew.owens@stees.nhs.uk

19th Nov, 2007

**Reference number 07/H0902/56: A
characterisation of the cell types within
juvenile haemangioma, arterio-venous malformations and keloid, and the cellular response to
bleomycin therapy.**

Mr W Andrew Owens
MB BCh BAO(Hons), MD, FRCSI, FRCS(CTh)
Consultant Cardiothoracic Surgeon and
Honorary Reader

Dear Sir,

Thank you for your letter of the 13th November regarding the recent REC meeting where the above study was discussed.

Two issues requiring further clarification were highlighted in the letter:

1. Anonymity - I can confirm that the patients from whom tissue is obtained cannot be identified by any of the researchers. However, as a record of tissue obtained has to be kept for the duration of the study, and there is a theoretical possibility that the PI could identify the patient from a hospital number, the plastic surgeon performing the procedure will label each sample with a unique identifier. This will be generated by sequential numbering of specimens obtained, the plastic surgeon will maintain a database of the unique identifiers, linking them to hospital number, on the NHS computers that currently hold a database already in existence for these patients. Neither the PI nor any other researchers will have any access to this database. The protocol has been amended accordingly.

2. Question A66 – we apologise that the student undertaking the MSc involved in this work was not properly identified. The candidate appointed to the post is Miss Lisa Clarke, who holds a BSc (2.1) in Biological Sciences. She will be based at the School of Biological and Biomedical Sciences, Durham University, under the supervision of Dr Nicholas Hole, as outlined in the original application.

Further to the instructions of Verity Eggelston, the protocol has been amended and is attached, labeled version 2, 16th November 2007.
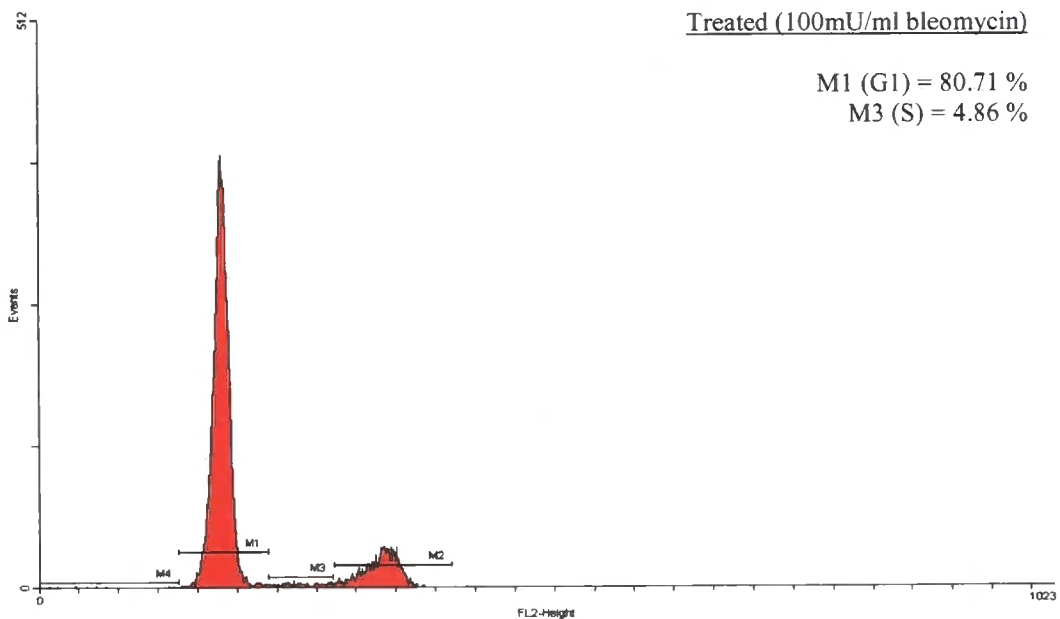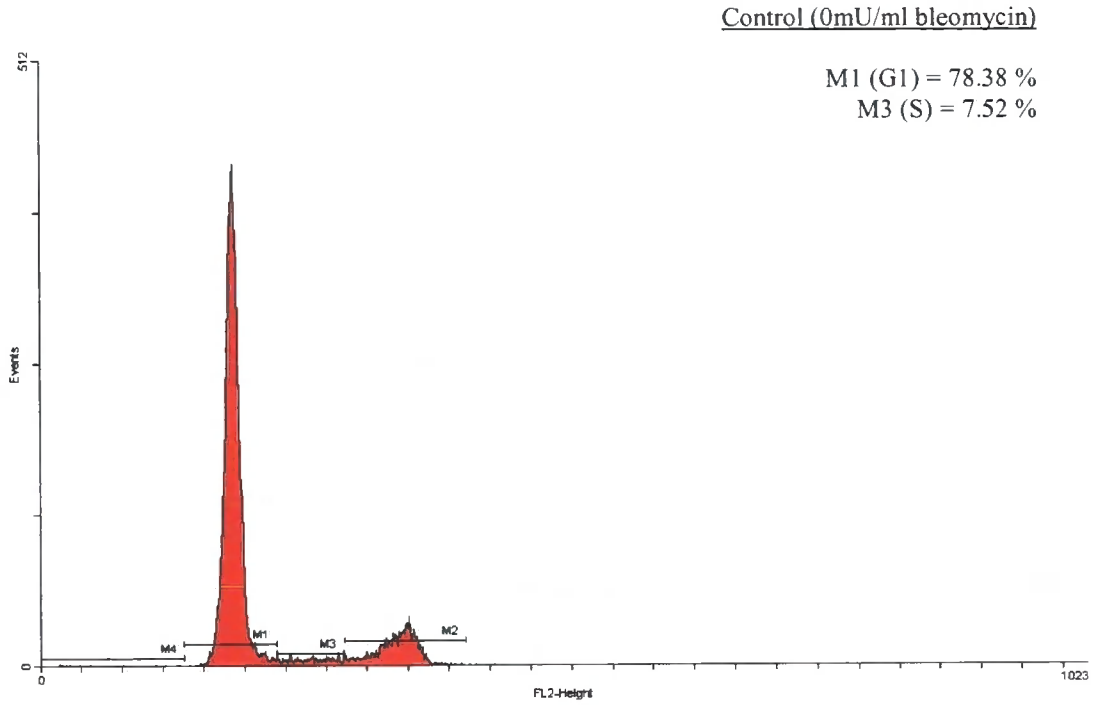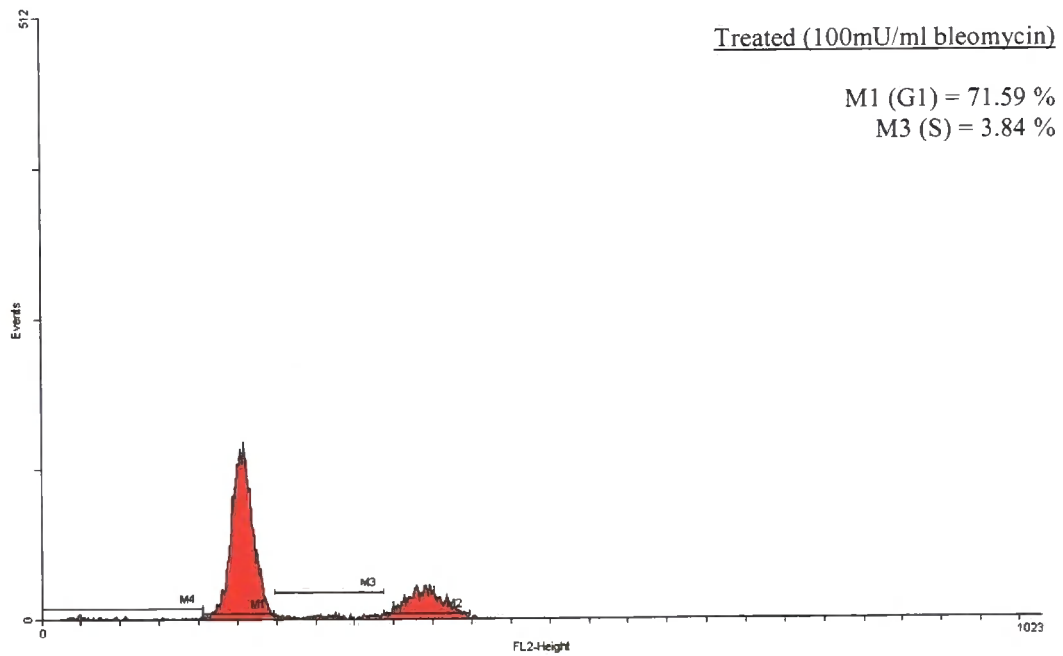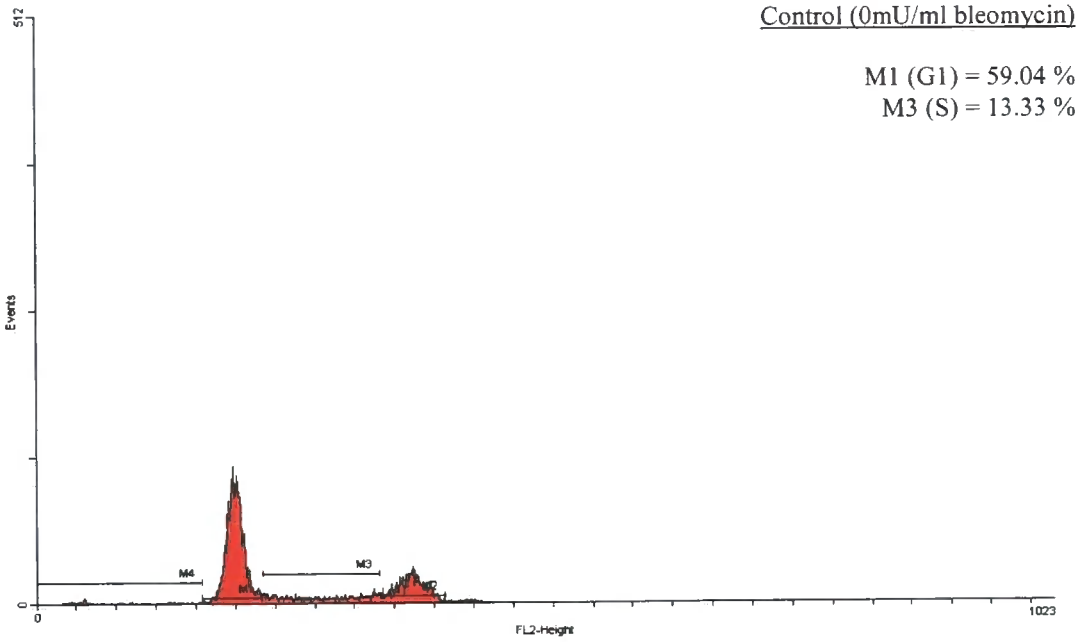
Yours sincerely,

Andrew Owens

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Appendix 4: Chapter 3

Results: Flow cytometry histograms

Control (0mU/ml bleomycin)

M1 (G1) = 78.38 %
M3 (S) = 7.52 %

Treated (100mU/ml bleomycin)

M1 (G1) = 80.71 %
M3 (S) = 4.86 %

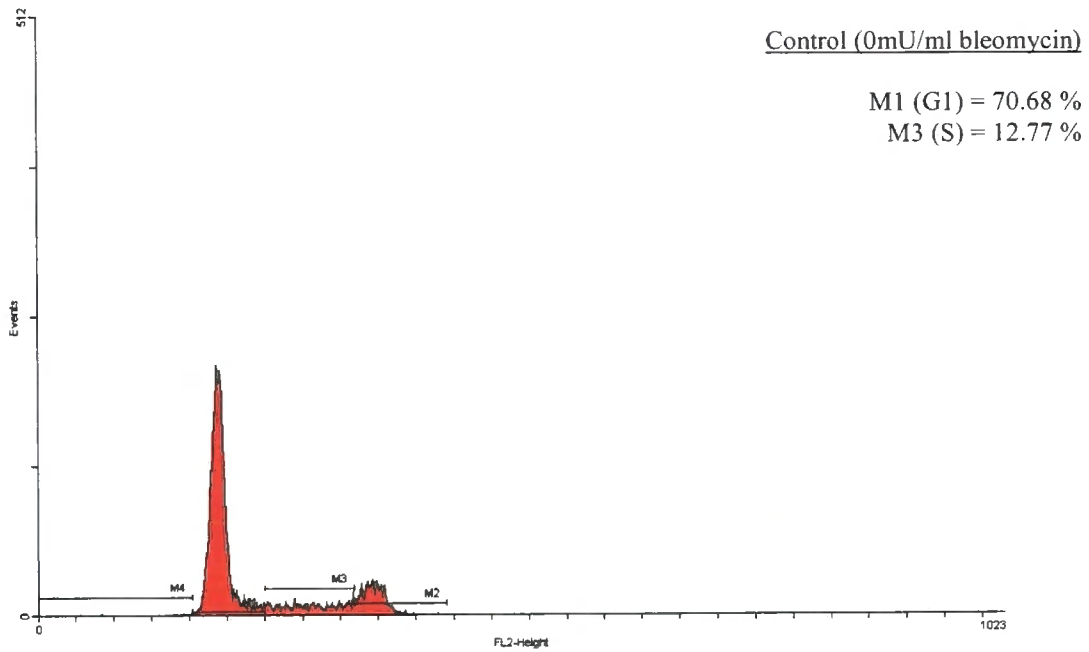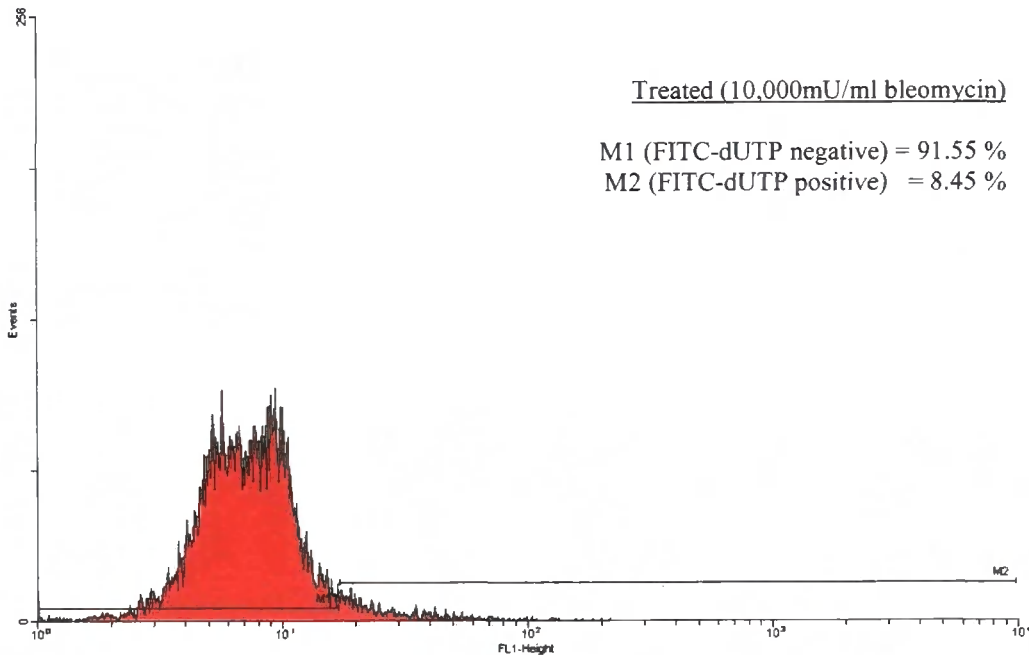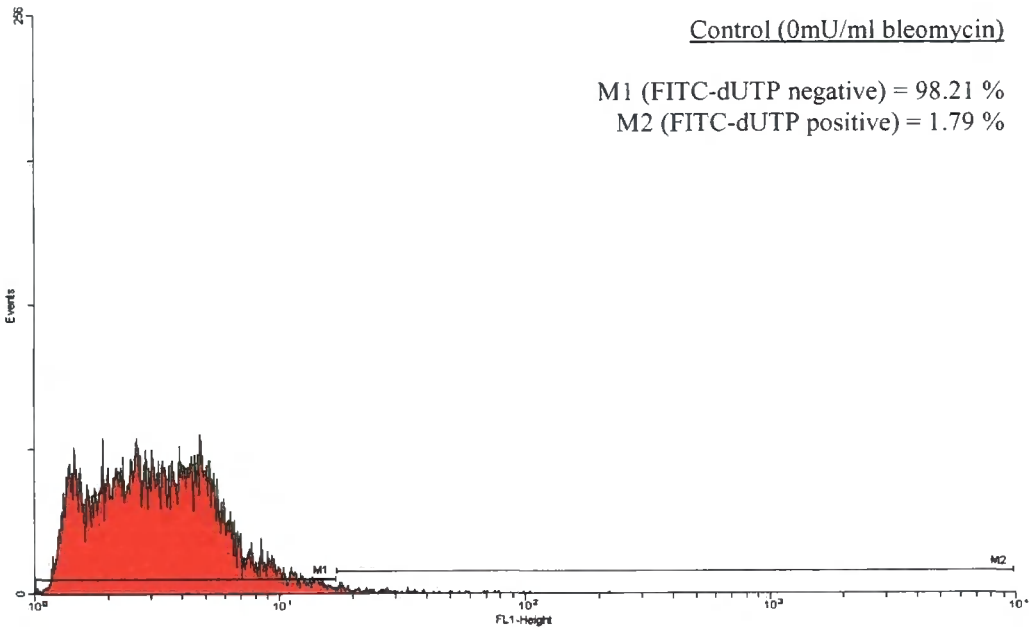**Representative cell cycle histograms of keloid derived cells.** The cells were gated as described in 2.6.4 and the gated data plotted as an FL2 histogram. To determine the percentage of cells in G1, G2, S and sub-G1, the cells were defined by their individual peaks as M1, M2, M3 and M4 respectively

Control (0mU/ml bleomycin)

M1 (G1) = 59.04 %
M3 (S) = 13.33 %

Treated (100mU/ml bleomycin)

M1 (G1) = 71.59 %
M3 (S) = 3.84 %

Representative cell cycle histograms of HUVEC. The cells were gated as described in 2.6.4 and the gated data plotted as an FL2 histogram. To determine the percentage of cells in G1, G2, S and sub-G1, the cells were defined by their individual peaks as M1, M2, M3 and M4 respectively

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Control (0mU/ml bleomycin)

M1 (G1) = 70.68 %
M3 (S) = 12.77 %

Treated (100mU/ml bleomycin)

M1 (G1) = 80.50 %
M3 (S) = 5.37 %
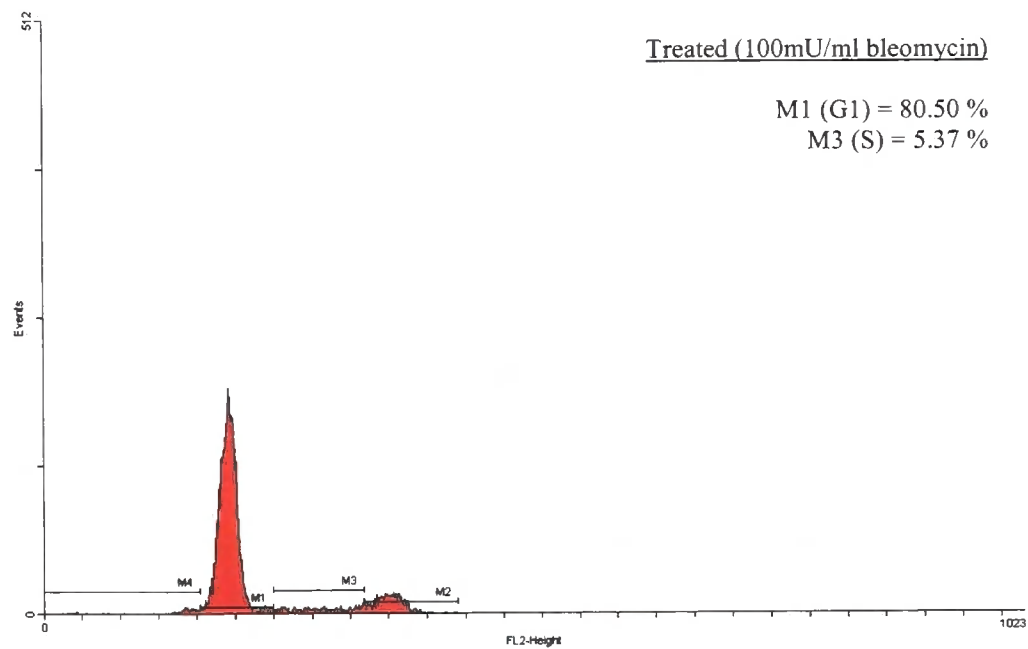
Representative cell cycle histograms of haemangioma derived cells. The cells were gated as described in 2.6.4 and the gated data plotted as an FL2 histogram. To determine the percentage of cells in G1, G2, S and sub-G1, the cells were defined by their individual peaks as M1, M2, M3 and M4 respectively
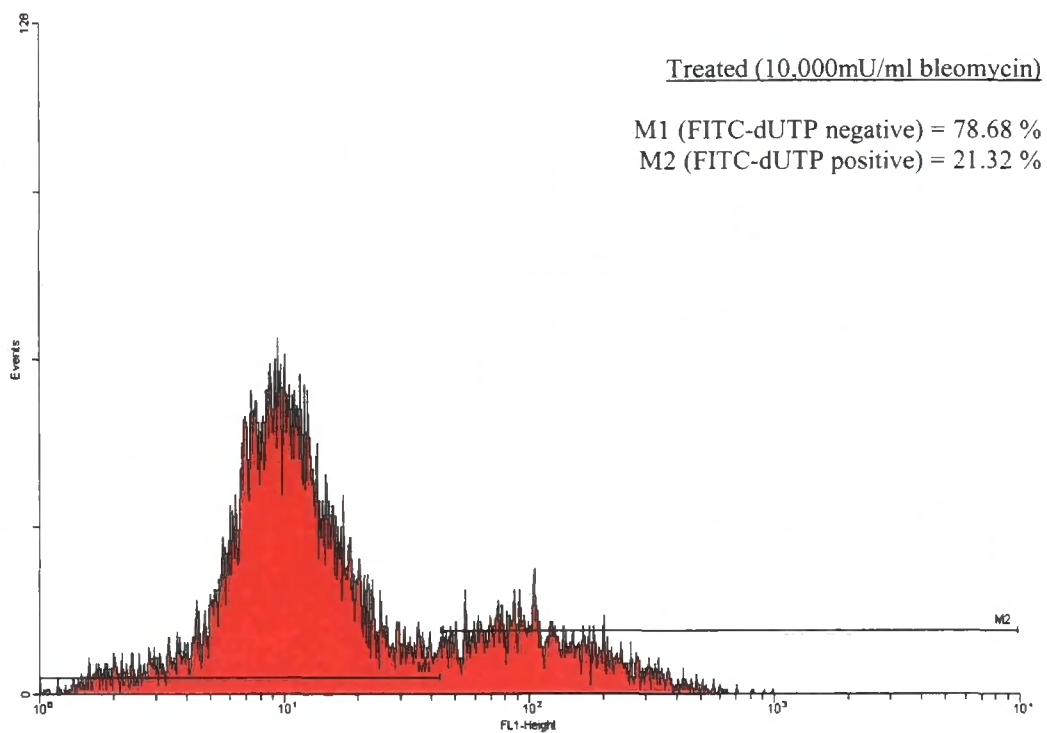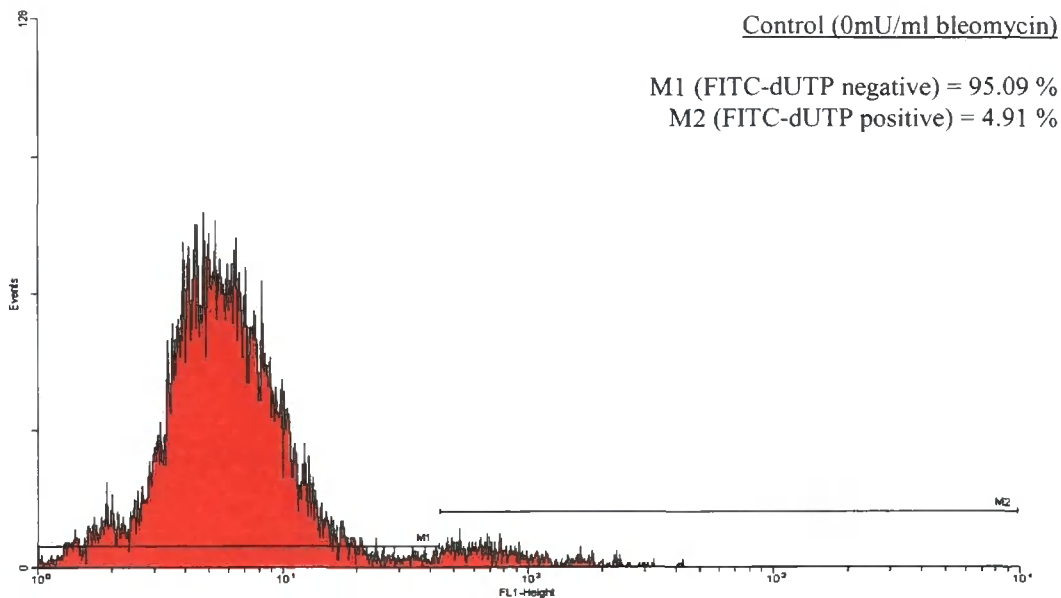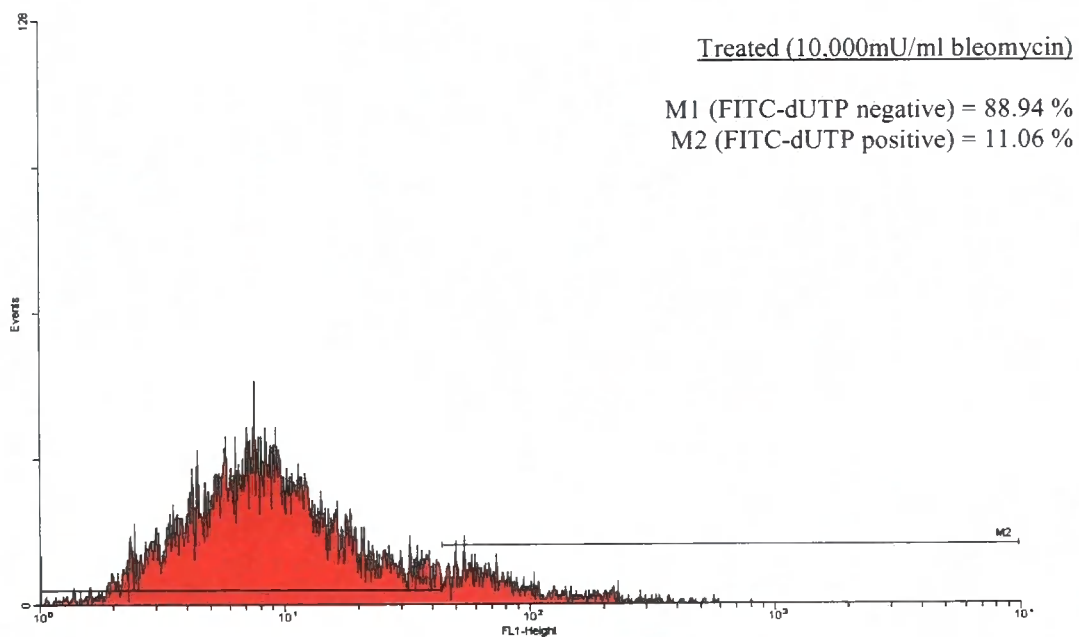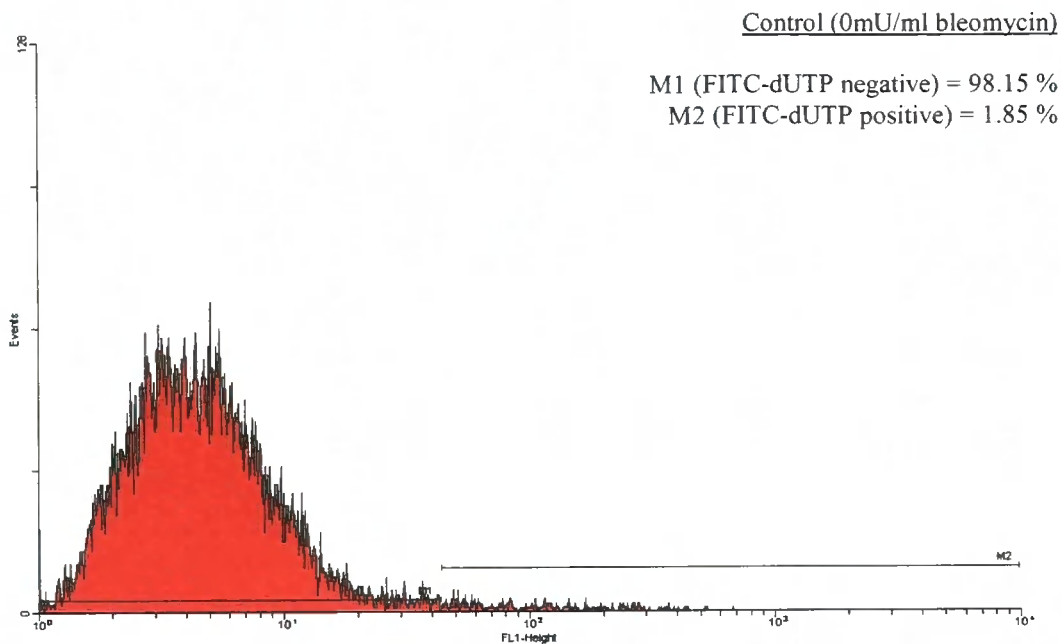
Control (0mU/ml bleomycin)

M1 (FITC-dUTP negative) = 98.21 %
M2 (FITC-dUTP positive) = 1.79 %

Treated (10,000mU/ml bleomycin)

M1 (FITC-dUTP negative) = 91.55 %
M2 (FITC-dUTP positive) = 8.45 %

Induction of DNA breaks on foreskin fibroblast cells as detected by FITC-dUTP labelling and analysed by flow cytometry. 10,000 cells were analysed per determination. All cells were incubated for 24 hours, in the presence or absence of 10,000mU/ml bleomycin. The data was plotted onto a FL1 histogram and the percentage of FITC-dUTP positive cells was determined. All experiments were performed n=3. Typical flow cytometry histograms in a representative experiment are presented above.

Induction of DNA breaks on HUVEC cells as detected by FITC-dUTP labelling and analysed by flow cytometry. 10,000 cells were analysed per determination. All cells were incubated for 24 hours, in the presence or absence of 10,000mU/ml bleomycin. The data was plotted onto a FL1 histogram and the percentage of FITC-dUTP positive cells was determined. All experiments were performed n=3. Typical flow cytometry histograms in a representative experiment are presented above.

Control (0mU/ml bleomycin)

M1 (FITC-dUTP negative) = 98.15 %
M2 (FITC-dUTP positive) = 1.85 %

Treated (10,000mU/ml bleomycin)

M1 (FITC-dUTP negative) = 88.94 %
M2 (FITC-dUTP positive) = 11.06 %

Induction of DNA breaks on haemangioma derived cell line as detected by FITC-dUTP labelling and analysed by flow cytometry. 10,000 cells were analysed per determination. All cells were incubated for 24 hours, in the presence or absence of 10,000mU/ml bleomycin. The data was plotted onto a FL1 histogram and the percentage of FITC-dUTP positive cells was determined. All experiments were performed n=3. Typical flow cytometry histograms in a representative experiment are presented above.

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Appendix 5: Chapter 3

Forskin Fibroblasts Cell cycle and apoptosis @ 48 hours
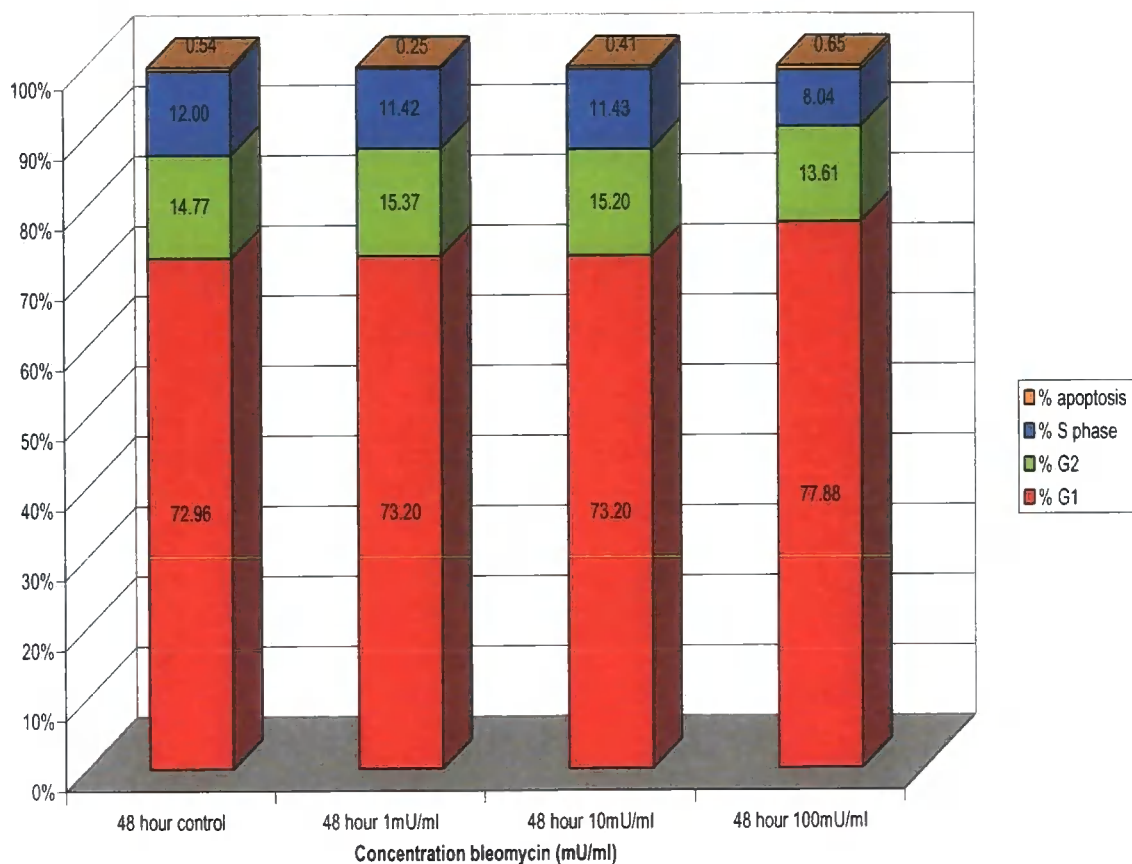
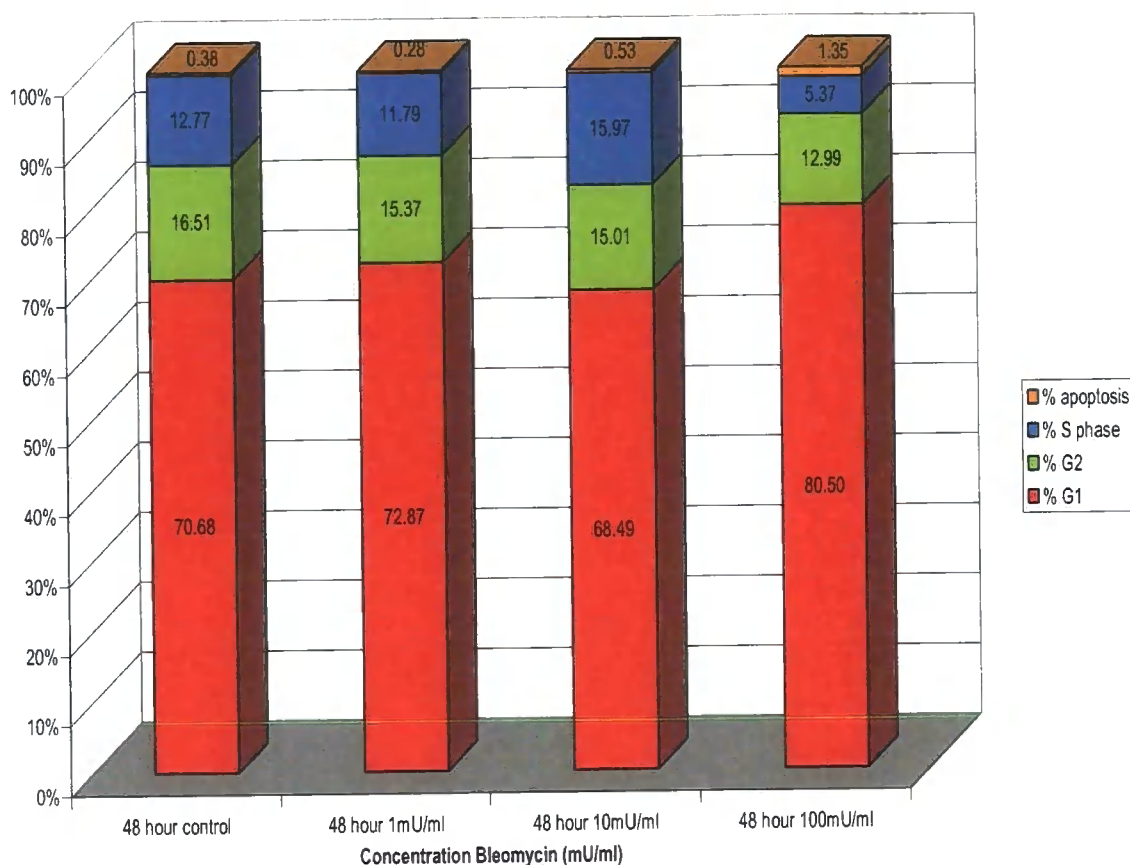| Sample	G1	G2	S phase	Sub-G1
Control (0mU/ml) @ 5 hours	56.96 ± 3.75	25.84 ± 1.71	15.86 ± 4.32	1.65 ± 1.93
1mU/ml @ 5 hours	51.31 ± 7.18	28.34 ± 1.95	19.29 ± 8.13	1.41 ± 1.01
10mU/ml @ 5 hours	51.18 ± 6.86	29.09 ± 2.05	18.47 ± 6.35	1.67 ± 0.50
100mU/ml @ 5 hours	47.32 ± 4.91	31.24 ± 3.31	18.38 ± 6.28	3.47 ± 3.20
Control (0mU/ml) @ 24 hours	65.47 ± 1.07	19.06 ± 1.77	15.40 ± 1.01	0.50 ± 0.47
1mU/ml @ 24 hours	63.93 ± 2.40	20.35 ± 1.07	15.24 ± 1.69	0.86 ± 1.23
10mU/ml @ 24 hours	64.24 ± 1.16	20.47 ±1.85	14.98 ± 1.39	0.66 ± 1.01
100mU/ml @ 24 hours	66.90 ± 2.38	19.85 ± 3.09	12.85 ± 1.44	0.70 ± 0.90
Control (0mU/ml) @ 48 hours	72.96 ± 1.84	14.77 ± 0.24	12.00 ± 1.22	0.54 ± 0.75
1mU/ml @ 48 hours	73.20 ± 0.46	15.37 ± 0.24	11.42 ± 0.43	0.25 ± 0.26
10mU/ml @ 48 hours	73.20 ± 0.62	15.20 ± 1.33	11.43 ± 1.02	0.41 ± 0.53
100mU/ml @ 48 hours	77.88 ± 2.27	13.61 ± 0.89	8.04 ± 0.59	0.65 ± 0.93

Keloid Cell cycle and apoptosis @ 48 hours
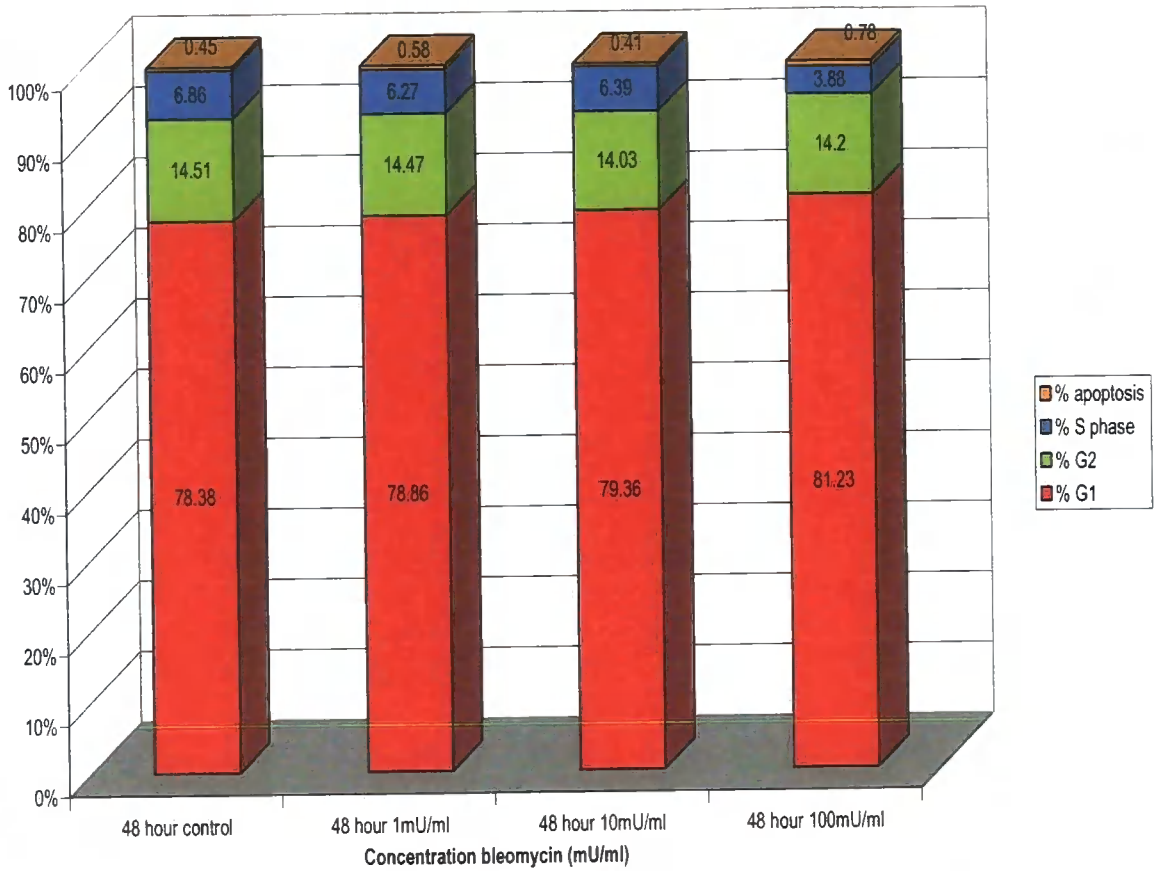
Sample	G1	G2	S phase	Sub-G1
Control (0mU/ml) @ 5 hours	69.74 ± 6.67	20.25 ± 5.30	9.37 ± 2.05	0.84 ± 0.35
1mU/ml @ 5 hours	67.38 ± 1.85	21.95 ± 2.46	9.15 ± 1.75	1.26 ± 0.46
10mU/ml @ 5 hours	66.79 ± 1.85	23.17 ± 2.07	8.40 ± 1.10	1.79 ± 1.25
100mU/ml @ 5 hours	67.43 ± 1.73	23.43 ± 1.87	8.17 ± 1.73	1.08 ± 0.56
Control (0mU/ml) @ 24 hours	72.13 ± 2.22	17.80 ± 2.13	9.69 ± 2.09	0.64 ± 0.56
1mU/ml @ 24 hours	70.64 ± 2.63	19.57 ± 0.35	9.72 ± 2.46	0.34 ± 0.22
10mU/ml @ 24 hours	71.73 ± 2.56	19.11 ± 0.68	9.13 ± 2.38	0.29 ± 0.25
100mU/ml @ 24 hours	76.74 ± 1.36	17.08 ± 1.53	5.97 ± 2.71	0.43 ± 0.31
Control (0mU/ml) @ 48 hours	78.38 ± 2.16	14.51 ± 1.50	6.86 ± 2.56	0.45 ± 0.25
1mU/ml @ 48 hours	78.86 ± 2.76	14.47 ± 1.91	6.27 ± 2.22	0.58 ± 0.44
10mU/ml @ 48 hours	79.36 ± 3.28	14.03 ± 2.21	6.39 ± 2.12	0.41 ± 0.30
100mU/ml @ 48 hours	81.23 ± 1.07	14.20 ± 1.40	3.88 ± 1.87	0.78 ± 0.62

HUVEC cell cycle and apoptosis @ 48 hours
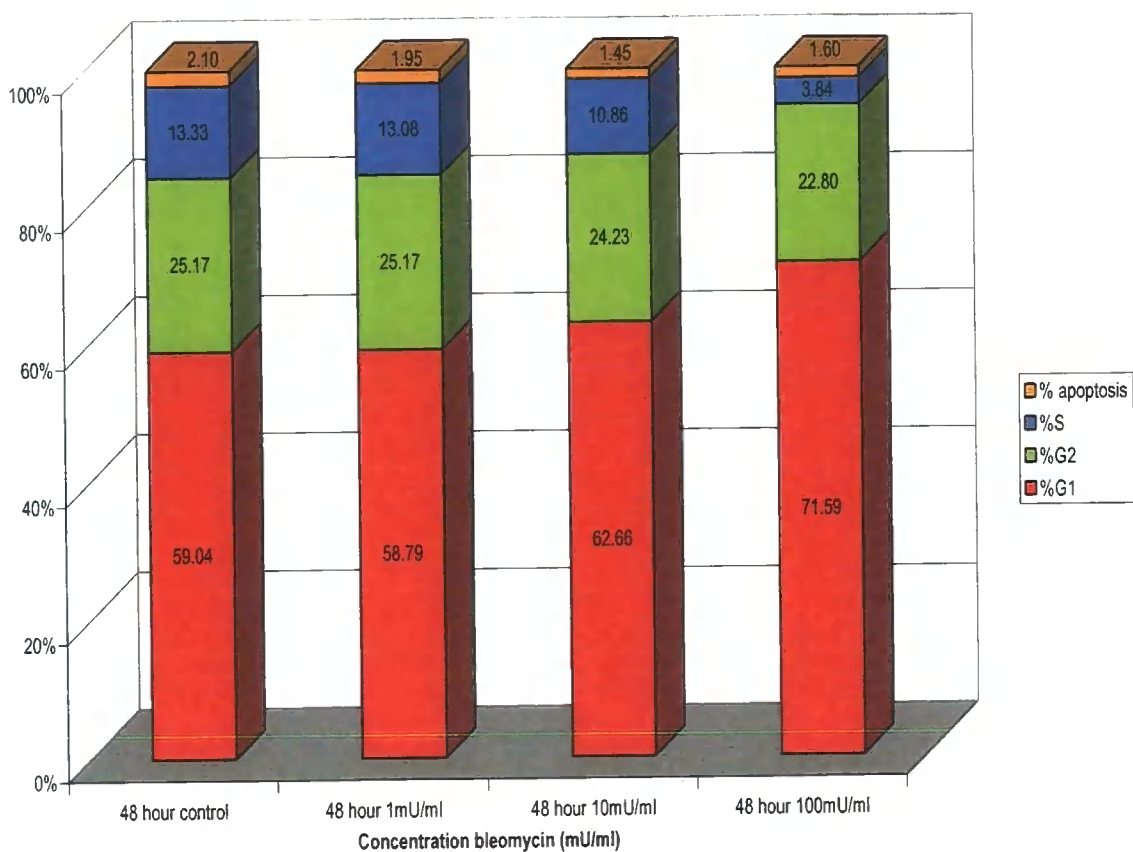
Sample	G1	G2	S phase	Sub-G1
Control (0mU/ml) @ 24 hours	58.98 ± 2.91	27.97 ± 2.23	10.72 ± 1.06	1.50 ± 0.43
1mU/ml @ 24 hours	61.70 ± 0.94	25.02 ± 0.68	9.99 ± 1.21	1.60 ± 0.42
10mU/ml @ 24 hours	60.45 ± 2.56	27.02 ± 0.24	9.32 ± 2.00	2.17 ± 0.50
100mU/ml @ 24 hours	64.06 ± 1.24	27.25 ± 0.77	5.65 ± 0.48	2.58 ± 0.60
Control (0mU/ml) @ 48 hours	59.04 ± 1.28	25.17 ± 1.91	13.33 ± 2.26	2.10 ± 0.30
1mU/ml @ 48 hours	58.79 ± 1.58	25.17 ± 2.33	13.08 ± 1.35	1.95 ± 1.26
10mU/ml @ 48 hours	62.66 ± 1.19	24.23 ± 2.94	10.86 ± 1.40	1.45 ± 0.12
100mU/ml @ 48 hours	71.59 ± 0.98	22.80 ± 0.38	3.84 ± 0.69	1.60 ± 0.21

Haemangioma cell cycle and apoptosis @ 48 hours

Sample	G1	G2	S phase	Sub-G1
Control (0mU/ml) @ 5 hours	68.48 ± 4.61	18.02 ± 5.09	12.38 ± 0.61	1.48 ± 1.23
1mU/ml @ 5 hours	65.74 ± 3.90	19.38 ± 6.06	14.12 ± 1.60	1.31 ± 0.90
10mU/ml @ 5 hours	66.64 ± 2.40	20.06 ± 2.18	13.19 ± 1.25	0.44 ± 0.28
100mU/ml @ 5 hours	64.94 ± 2.54	20.78 ± 2.21	13.71 ± 0.93	0.95 ± 0.54
Control (0mU/ml) @ 24 hours	67.86 ± 2.62	17.07 ± 2.87	14.06 ± 0.52	1.42 ± 1.37
1mU/ml @ 24 hours	67.51 ± 0.40	18.39 ± 1.83	13.76 ± 1.98	0.69 ± 0.19
10mU/ml @ 24 hours	69.64 ± 1.42	17.29 ± 1.94	12.37 ± 1.62	1.07 ± 0.68
100mU/ml @ 24 hours	75.83 ± 1.56	14.43 ± 0.68	8.55 ± 1.08	1.42 ± 0.44
Control (0mU/ml) @ 48 hours	70.68 ± 0.71	16.51 ± 0.81	12.77 ± 1.20	0.38 ± 0.23
1mU/ml @ 48 hours	72.87 ± 3.10	15.37 ± 0.59	11.79 ± 2.45	0.28 ± 0.23
10mU/ml @ 48 hours	68.49 ± 10.53	15.01 ± 1.18	15.97 ± 9.55	0.53 ± 0.43
100mU/ml @ 48 hours	80.50 ± 2.75	12.99 ± 1.05	5.37 ± 1.89	1.35 ± 0.90

Appendix 6: Chapter 3

% Control apoptosis

	24 hours	48 hours
Foreskin Fibroblasts	0.50 ± 0.47	0.48 ± 0.20
HMEC-1	7.23 ± 0.62	6.03 ± 0.69
Haemangioma	1.42 ± 1.37	0.38 ± 0.23
Keloid	0.64 ± 0.56	0.45 ± 0.25
Venous Malformation	0.74 ± 0.09	0.48 ± 0.09
HUVEC	1.50 ± 0.43	2.17 ± 0.40