

**Therapeutic angiogenesis: attempts to influence
the survival of rat epigastric island flaps.**

Cameron Raine

Submitted in fulfilment of the requirements for the degree of MD of the

University of Edinburgh

October 2004



To Morag

Declaration

I declare that the work contained within this thesis is my own and of my composition unless clearly stated otherwise. This work was conducted during a research fellowship in the University of New South Wales Department of Plastic & Reconstructive Surgery in Sydney, Australia and has not been submitted for any other degree.

Acknowledgements

In the course of this work I was lucky to receive the help and encouragement of a great many people who were prepared to offer both their time and expertise in pursuit of this project. I have made reference to all who have helped me within the text that follows however, there are a number of people to whom I would like to express particular thanks. Chris Hicks and Melissa Holmes in the University of New South Wales haematology research laboratory, Leon Vonthethov and Penny Marr, Department of Anatomical Pathology and Ashif, Department of Radiology, The St George Hospital, Sydney and Marcelle and colleagues at The Prince Henry Hospital, Sydney. Thanks also to Jake Lim and Jonathan Clark for their help with the histological preparations and a special thank-you to Poppy Mosses for invaluable support in all manner of ways throughout my fellowship.

Most of all however, I would like to thank my supervisors, Professor Michael D Poole and Dr Rob Lindeman.

Abstract

The targeted stimulation of collateral blood vessel growth in ischaemic tissue, therapeutic angiogenesis, holds great promise as a treatment for chronic vascular disease. It may however, prove equally useful as an adjunct in reconstructive surgery where planned tissue transfers are for the most part limited by native vascularity. The controlled induction of angiogenesis may overcome some of these limitations. Recent work has suggested that therapeutic angiogenesis may be achieved using gene therapy techniques based on the cytokine vascular endothelial growth factor (VEGF). This project will investigate the hypothesis that such techniques may be used to induce angiogenesis in an experimental model of flap ischaemia and improve flap survival.

All members of the VEGF family of cytokines stimulate angiogenesis. This work was based on VEGF beta (VEGFB). Two delivery strategies were investigated: intra-arterial administration of 'raw' plasmid DNA encoding the gene for VEGFB and subcutaneous injection of cultured cells genetically modified to produce the VEGFB cytokine.

The rat epigastric island flap is designed to undergo partial ischaemic necrosis to a predictable extent. In this series mean flap survival was 85% (95% C.I. 82, 89) at one week. Flap vascularity was further characterised histologically and angiographically at two time points: immediately following elevation and at one week when demarcation of the non-viable tissue had occurred. A pattern of physiological angiogenesis occurring within the ischaemic flaps was apparent from the analysis of these results.

Optimal conditions for gene transfer and expression were first investigated *in vitro* using the green fluorescent protein (GFP) reporter system and cultured endothelial cells. Use of the transfection agent DOTAP significantly improved the number of cells expressing GFP at all observations. The substitution of a mammalian expression plasmid containing the VEGFB gene (pEF-BOS-VEGFB) for the reporter plasmid resulted in positive expression of VEGFB by the endothelial cells as detected by immunostaining. Successful transfer of the VEGFB gene to the endothelial cells with exogenous protein production had therefore been achieved. Replication of these conditions *in vivo* using the rat epigastric island flap model however, failed to produce any evidence of either gene transfer to the arterial wall within the flap pedicle or stimulated angiogenesis within the flap itself.

Further experiments were conducted using Chinese hamster ovary cells (CHO) genetically modified to produce VEGFB. In a pilot study, the injection of CHO-VEGFB cells (mean dose 5.9×10^5 cells/cm²) into the axial portion of the experimental flap resulted in a significant increase in mean flap survival to 95% (95% C.I. 89, 100) ($p < 0.05$). A larger, randomised controlled trial designed to test these results however, found no evidence of stimulated angiogenesis when experimental and control groups were compared for flap survival, average microvascular density and angiographic branch point count. Subsequent optimisation of an immunohistochemical protocol for the detection of cellular VEGFB production in culture found fewer than expected CHO-VEGFB cells staining positive. Expression of VEGFB may have declined in the interval between the two studies as the cell population aged.

It is likely that these systems failed as a result of their inability to deliver sufficient active cytokine to the tissues of the flap. In addition, evidence is emerging that therapeutic angiogenesis may best be performed using a combination of agents where additional cytokines are employed in conjunction with a primary angiogenic agent to promote both the stability and function of immature endothelial cells. The optimisation of delivery strategies for the transfer of genetic material to experimental flaps coupled with a greater understanding of the most effective agent, or combination of agents, needed to produce new vessels which are both functional and permanent are essential requirements for the ultimate success of this technology.

Contents

Acknowledgements	4
Abstract	5
Contents	6
List of Figures	14
List of Tables	18
List of Appendices	20
List of Abbreviations	21

Chapter 1 - Introduction

<i>1.1 Growing new blood vessels</i>	23
<i>1.2 Implications for reconstructive surgery</i>	24
<i>1.3 The principles of tissue transfer</i>	
1.3.1 Non vascularised tissue transfer	25
1.3.2 Vascularised tissue transfer	26
<i>1.4 The search for techniques to enhance flap survival</i>	
1.4.1 The delay phenomenon	28
1.4.2 Improving blood flow pharmacologically	30
1.4.3 A potential role for angiogenic cytokines	30

<i>1.5 The control of angiogenesis</i>	32
<i>1.6 The biology of vascular endothelial growth factor</i>	
1.6.1 The VEGF family	36
1.6.2 Vascular endothelial growth factor B	38
<i>1.7 The principles of gene manipulation</i>	
1.7.1 Basic plasmid biology	40
1.7.2 Plasmids as cloning vehicles	42
1.7.3 Plasmids as expression vectors	44
1.7.4 Techniques to improve the efficiency of DNA transfer	45
<i>1.8 Techniques in therapeutic angiogenesis</i>	
1.8.1 The choice of angiogen	47
1.8.2 Delivery strategies	48
<i>1.9 Aims</i>	50

Chapter 2 - Validation of the experimental model

<i>2.1 Introduction</i>	
2.1.1 The model	51
2.1.2 Anatomical considerations	52
2.1.3 Flap design	53
2.1.4 Autocannibalism	55
2.1.5 Data collection	57
2.1.6 Aims	60
<i>2.2 Materials and Methods</i>	
2.2.1 Animals	60
2.2.2 Experimental groups	61

2.2.3 Surgical procedure	61
2.2.4 Immediate postoperative management	64
2.2.5 Daily postoperative management	64
2.2.6 Data collection	64
2.2.7 Radiology	70
2.2.8 Data processing	70
2.3 <i>Statistics</i>	71
2.4 <i>Results</i>	
2.4.1 General observations	71
2.4.2 Flap survival	72
2.4.3 Microangiography	74
2.5 <i>Discussion</i>	76
2.6 <i>Conclusions</i>	80

Chapter 3 –Measurement of average microvascular density in rat epigastric skin

3.1 <i>Introduction</i>	
3.1.1 Overview	81
3.1.2 Aims	88
3.2 <i>Materials and Methods</i>	
3.2.1 Antibodies	88
3.2.2 Reagents	89
3.2.3 Solutions	89
3.2.4 Tissues	91
3.2.5 Immunohistochemistry	93
3.2.6 Quantification of a-MVD	94

3.3 Statistics	95
3.4 Results	
3.4.1 Optimization of endothelial staining technique	96
3.4.2 Measurement of a-MVD	98
3.5 Discussion	104
3.6 Conclusions	107

Chapter 4 –Manufacture of pEF-BOS-VEGFB expression plasmid

4.1 Introduction	
4.1.1 Objectives	109
4.1.2 pEF-BOS mammalian expression plasmid	109
4.1.3 Purification of plasmid DNA	111
4.1.4 DNA sequencing	112
4.1.5 Aims	115
4.2 Materials and Methods	
4.2.1 Acknowledgments	115
4.2.2 Bacteriological reagents and equipment	117
4.2.3 Bacteriological solutions and media	117
4.2.4 Plasmid preparation	118
4.2.5 Sequencing of purified pEF-BOS VEGFB	120
4.3 Results	121
4.4 Discussion	127
4.5 Conclusions	128

Chapter 5 – Transfection experiments with bovine aortic endothelial cells *in vitro*

5.1 Introduction	129
5.2 Materials and Methods	
5.2.1 Acknowledgements	132
5.2.2 Cell culture reagents and equipment	132
5.2.3 Plasmids and transfection reagents	133
5.2.4 Immunohistochemical reagents and antibodies	133
5.2.5 Preparation of cell culture medium and reagents	134
5.2.6 Subculturing of BAECs	134
5.2.7 Estimation of cell concentration	135
5.2.8 Transfection of BAECs	135
5.2.9 pEGFP-N2 plasmid	136
5.2.10 pEF-BOS-VEGFB plasmid	137
5.2.11 Immunohistochemistry	137
5.3 Results	
5.3.1 pEGFP-N2 plasmid	138
5.3.2 pEF-BOS-VEGFB plasmid	145
5.4 Discussion	147
5.5 Conclusions	148

Chapter 6 – Transfection experiments with pEF-BOS and pEGFP *in vivo*

6.1 Introduction	
6.1.1 Overview	149
6.1.2 Aims	152
6.2 Materials and Methods	
6.2.1 Animals	152

6.2.2 Plasmids	153
6.2.3 Experimental groups	153
6.2.4 Transfection mixtures	154
6.2.5 Surgical procedure	156
6.2.6 Data collection	157
<i>6.3 Statistics</i>	158
<i>6.4 Results</i>	
6.4.1 General observations	158
6.4.2 pEF-BOS-VEGFB	158
6.4.3 pEGFP-N2	164
<i>6.5 Discussion</i>	170
<i>6.6 Conclusions</i>	172
Chapter 7 –Experiments with CHO cells genetically modified to produce VEGFB	
<i>7.1 Introduction</i>	173
<i>7.2 Materials and Methods</i>	
7.2.1 Acknowledgements	177
7.2.2 Cell culture reagents and equipment	177
7.2.3 Preparation of cell culture medium and reagents	178
7.2.4 CHO cell culture	178
7.2.5 Immunohistochemical testing	180
7.2.6 CHO-VEGFB cells as agents for therapeutic angiogenesis	182
<i>7.3 Statistics</i>	184
<i>7.4 Results</i>	
7.4.1 Immunohistochemistry	185

7.4.2 Flap survival	187
7.4.3 Microangiography	190
7.5 Discussion	194
7.6 Conclusions	197
Chapter 8 – Randomised controlled trial to evaluate CHO-VEGFB cells as agents for therapeutic angiogenesis	
<i>8.1 Introduction</i>	
8.1.1 Overview	198
8.1.2 Power calculation	198
8.1.3 Randomisation	200
<i>8.2 Materials and methods</i>	
8.2.1 Animals	200
8.2.2 Preparation of CHO cell cultures	200
8.2.3 Surgical procedure	201
8.2.4 Data collection	202
8.3 Statistics	203
<i>8.4 Results</i>	
8.4.1 General observations	203
8.4.2 Exclusions	206
8.4.3 Flap survival	206
8.4.4 Microangiography	207
8.4.5 Average microvascular density	214
8.5 Discussion	218
8.6 Conclusions	220

<i>Chapter 9</i> – Concluding remarks and future work	221
Bibliography	232
Appendices	251

Figures

<i>Figure 1.1</i>	Schematic diagram demonstrating the structure of continuous and fenestrated capillaries	33
<i>Figure 1.2</i>	Schematic diagram illustrating the process of angiogenesis	34
<i>Figure 1.3</i>	Schematic diagram illustrating the principles of cloning using bacterial plasmids	43
<i>Figure 2.1</i>	Epigastric island flap design taken from Padubidri & Brown ...	54
<i>Figure 2.2</i>	Autocannibalism as seen here, was a common occurrence in unprotected flaps	56
<i>Figure 2.3</i>	Protective collar (A) materials and (B) in place on a postoperative animal	56
<i>Figure 2.4</i>	The surgical procedure	63
<i>Figure 2.5</i>	Postoperative record	65
<i>Figure 2.6</i>	Examples of (A) a typical epigastric island flap at one week and (B) a template drawn from the flap at the time of data collection	66
<i>Figure 2.7</i>	Cannulation of the superficial inferior epigastric artery	66
<i>Figure 2.8</i>	(A) Pinning to a plastic template prior to imaging restored the <i>in vivo</i> dimensions of each flap. The resulting angiogram is shown in (B)	69
<i>Figure 2.9</i>	Typical angiogram obtained from group 1 flaps performed immediately following elevation	75
<i>Figure 2.10</i>	Typical angiograms obtained from group 2 flaps performed at one week	75
<i>Figure 2.11</i>	Boxplot of highest angiographic branch point count recorded from the axial pattern flaps of group 1 (immediate flap angiography) and group 2 (angiography at 1 week) animals	76
<i>Figure 3.1</i>	Schematic diagram illustrating the principles of the immunoperoxidase stain technique	85

<i>Figure 3.2</i>	Schematic diagram illustrating the technique devised for the preparation of histological sections from predetermined positions in rat epigastric island flaps	92
<i>Figure 3.3</i>	Immunoperoxidase preparations of sections taken from rat epigastric island flaps	97
<i>Figure 3.4</i>	Boxplot of a-MVD above and below the <i>panniculus carnosus</i> for the random pattern flaps in both groups and the zone of demarcation in group 2 flaps	103
<i>Figure 4.1</i>	The mammalian expression plasmid pEF-BOS-FLAG	110
<i>Figure 4.2</i>	DNA sequencing with dideoxynucleoside triphosphates as chain terminators after the method of Sanger	114
<i>Figure 4.3</i>	Base and amino acid sequence for VEGFB	116
<i>Figure 4.4a</i>	First analysis sequencing data for purified pEF-BOS-VEGFB..	123
<i>Figure 4.4b</i>	Computer generated base and amino acid sequences from the first pEF-BOS-VEGFB analysis	124
<i>Figure 4.5a</i>	Second analysis sequencing data for purified pEF-BOS-VEGFB	125
<i>Figure 4.5b</i>	Computer generated base and amino acid sequences from the second pEF-BOS-VEGFB analysis	126
<i>Figure 5.1</i>	BAEC cultures following transfection with pEGFP-N2	140
<i>Figure 5.2</i>	Bar charts showing the effect of increasing DNA concentration, DOTAP:DNA ratio and incubation time on transfection efficiency	141
<i>Figure 5.3</i>	Bar charts showing the effect of further increases in DNA concentration and incubation time on transfection efficiency using a DOTAP:DNA ratio of 6:1 in all cases	142
<i>Figure 5.4</i>	Line chart showing the effect of increasing DOTAP:DNA concentration on transfection efficiency for two starting concentrations of DNA	144

<i>Figure 5.5</i>	Line chart showing the effect of increased incubation time on transfection efficiency for three starting concentrations of DNA	144
<i>Figure 5.6</i>	Whole BAEC cell preparations stained for FLAG	146
<i>Figure 6.1</i>	Boxplot of percentage flap survival against transfection conditions	160
<i>Figure 6.2</i>	Highest angiographic branch point count/cm ² recorded for the axial and random pattern flaps for each transfection group and controls	162
<i>Figure 6.3</i>	Examples of microangiograms obtained from group 1 animals.	163
<i>Figure 6.4</i>	Boxplots of a-MVD recorded for the random pattern flaps	168
<i>Figure 6.5</i>	Full thickness arterial wall specimens taken from the SIEA's of group 4 animals illuminated with ultraviolet light	169
<i>Figure 7.1</i>	Technique for injection of CHO-VEGFB cells suspended in culture medium into the rat epigastric island flap	184
<i>Figure 7.2</i>	CHO cell monolayers immunostained for the FLAG antigen ...	185
<i>Figure 7.3</i>	Flap section immediately following CHO-VEGFB transfer	186
<i>Figure 7.4</i>	Flap sections stained for the FLAG antigen at (A) 24 hrs and (B) 48 hrs post CHO-VEGFB injection	186
<i>Figure 7.5</i>	(A) Complete survival of one of the experimental flaps receiving the higher dose of CHO-VEGFB cells to the axial portion of the flap. (B) Angiographic appearance of the same flap	188
<i>Figure 7.6</i>	Boxplot of flap survival against experimental group	189
<i>Figure 7.7</i>	Boxplot of HBPC/cm ² recorded from the angiograms of each experimental group and a control group of standard flaps	192
<i>Figure 7.8</i>	Example angiograms from each experimental group	193
<i>Figure 8.1</i>	Examples of epigastric island flaps and microangiograms at one-week post CHO injection: (A) CHO-VEGFB and (B) CHO-BLANK injected into the <i>random</i> portion of the flap	208

<i>Figure 8.2</i>	Examples of epigastric island flaps and microangiograms at one-week post CHO injection: (A) CHO-VEGFB and (B) CHO-BLANK injected into the <i>axial</i> portion of the flap	209
<i>Figure 8.3</i>	Examples of epigastric island flaps and microangiograms at one-week post CHO injection: (A) CHO-VEGFB and (B) CHO-BLANK injected into <i>both</i> the random and axial portions of the flap (whole flap injected)	210
<i>Figure 8.4</i>	Boxplot of HBPC/cm ² for each experimental group	213

Tables

<i>Table 1.1</i>	Angiogenic polypeptides and their related biological activities. Adapted from Folkman and Shing 1992	35
<i>Table 2.1</i>	Survival of rat islanded epigastric flaps expressed as a percentage of final flap area at seven days	73
<i>Table 2.2</i>	Published series of rat epigastric island flap survival	77
<i>Table 3.1</i>	Average MVD for group 1 flaps	99
<i>Table 3.2</i>	Average MVD for group 2 flaps	101
<i>Table 4.1</i>	Plasmid yield and purity	122
<i>Table 6.1</i>	Flap survival at one week for rats receiving 10 µg/ml pEF- BOS-VEGFB with DOTAP transfection agent into the right SIEA	159
<i>Table 6.2</i>	Flap survival at one week for rats receiving 20 µg/ml pEF- BOS-VEGFB with DOTAP transfection agent into the right SIEA	159
<i>Table 6.3</i>	Flap survival at one week for rats receiving 100 µg stat pEF- BOS-VEGFB (no transfection agent) into the right SIEA	160
<i>Table 6.4</i>	Mean HBPC/cm ² recorded for the random and axial portions of each experimental group	161
<i>Table 6.5</i>	Average MVD for rats receiving 10 µg/ml pEF-BOS-VEGFB into the right SIEA	165
<i>Table 6.6</i>	Average MVD for rats receiving 20 µg/ml pEF-BOS-VEGFB into the right SIEA	166
<i>Table 6.7</i>	Average MVD for rats receiving 100 µg pEF-BOS-VEGFB stat into the right SIEA	167
<i>Table 7.1</i>	Inoculation schedule for CHO cells	180
<i>Table 7.2</i>	Mean flap survival expressed as a percentage of final flap area for each experimental group	188

<i>Table 7.3</i>	Mean HBPC/cm ² recorded for the random and axial portions of each flap in each experimental group	191
<i>Table 8.1</i>	Incidence of flap oedema and time to demarcation	204
<i>Table 8.2</i>	Postoperative complications	205
<i>Table 8.3</i>	Median flap survival expressed as a percentage of final flap area and mean cell infiltrate for each group	207
<i>Table 8.4</i>	Mean HBPC/cm ² recorded for the random and axial portions of each flap in each experimental group	212
<i>Table 8.5</i>	Average MVD for rats receiving CHO cells into the <i>random</i> pattern flap only	215
<i>Table 8.6</i>	Average MVD for rats receiving CHO cells into the <i>axial</i> pattern flap only	216
<i>Table 8.7</i>	Average MVD for rats receiving CHO cells into the <i>whole</i> flap.....	217
<i>Table A1.1</i>	Weight : area ratios calculated for exposed radiographic film samples	252
<i>Table A2.1</i>	Calculation of right hemiflap survival for group 2 flaps	254
<i>Table A4.1</i>	Randomisation table for CHO-VEGFB trial	259
<i>Table A4.2</i>	Final operating schedule for CHO-VEGFB randomised controlled trial	259

Appendices

<i>Appendix 1</i>	Weight-area standard for exposed radiographic film	253
<i>Appendix 2</i>	Estimate of right hemiflap survival area	255
<i>Appendix 3</i>	Operating procedure used for the histological preparation of rat skin	257
<i>Appendix 4</i>	Randomisation details for CHO-VEGFB trial	260

List of Abbreviations

<i>A</i>	Adenine
<i>ANOVA</i>	Analysis of variance
<i>a-MVD</i>	Average microvascular density
<i>BAEC</i>	Bovine aortic endothelial cell
<i>bFGF</i>	Basic fibroblast growth factor
<i>BSA</i>	Bovine serum albumin
<i>CHO</i>	Chinese hamster ovary cell
<i>DAB</i>	3,3'-Diaminobenzidine
<i>cm</i>	Centimetre
<i>C</i>	Cytosine
<i>CD</i>	Cluster of differentiation
<i>C.I.</i>	Confidence intervals
<i>DMEM</i>	Dulbeccos modified Eagles medium
<i>DNA</i>	Deoxyribonucleic acid
<i>DOTAP</i>	N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate
<i>FGF</i>	Fibroblast growth factor
<i>G</i>	Guanine
<i>GFP</i>	Green fluorescent protein
<i>g</i>	Gramme
<i>HBPC/cm²</i>	Highest branch point count per square centimetre
<i>HEPES</i>	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
<i>h-MVD</i>	Highest microvascular density
<i>hr</i>	Hour
<i>HRPO</i>	Horse radish peroxidase
<i>Kg</i>	kilogramme
<i>l</i>	Litre
<i>LB</i>	Luria Bertani
<i>m</i>	Milli- (10 ⁻³)

<i>mins</i>	Minutes
<i>mRNA</i>	Messenger ribonucleic acid
<i>n</i>	Number
<i>PC</i>	Panniculus carnosus
<i>PDGF</i>	Platelet derived growth factor
μ	Micro- (10^{-6})
<i>PBS</i>	Phosphate buffered saline
<i>rpm</i>	Revolutions per minute
<i>S.D.</i>	Standard deviation
<i>SDS</i>	Sodium dodecyl sulphate
<i>SIEA</i>	Superficial inferior epigastric artery
<i>T</i>	Thymidine
<i>TGF</i>	Transforming growth factor
<i>TNF</i>	Tumour necrosis factor
<i>TRIS</i>	Tris-(hydroxymethyl)aminomethane
<i>VEGF</i>	Vascular endothelial growth factor
<i>VEGFR</i>	Vascular endothelial growth factor receptor

Chapter 1 - Introduction

1.1 Growing new blood vessels

The targeted stimulation of new blood vessel growth represents an exciting prospect for all clinicians required to deal with consequences of ischaemic disease. The feasibility of such a strategy has now been demonstrated with the results of extensive laboratory studies culminating in the first clinical application of this technology reported by Isner in 1996 (Isner *et al.* 1996). The process has been termed *therapeutic angiogenesis* and can be defined as the ‘clinical use of growth factors to enhance or promote the development of collateral blood vessels in ischaemic tissue’ (Henry DT 1998). In Isner’s paper, gene therapy techniques were used to deliver the angiogenic cytokine, vascular endothelial growth factor (VEGF) to the popliteal artery of a patient with critical lower limb ischaemia. Angiographic and flow data confirmed an increase in collateral vessels and a rise in both resting and maximum arterial flows compared to the preintervention state.

Although the majority of angiogenic gene therapy research has been performed by groups whose primary interest lies in the management of chronic vascular disease, the technique may find equal application in any situation where the augmentation of local vascularity is desirable. Such situations are commonly encountered within reconstructive surgery where the transfer of tissue from one location to another is both made possible by the extent of the native vascularity, and at the same time, limited by it. In this respect, the ability to selectively enhance blood supply by the use of angiogenic agents would find many applications.

1.2 Implications for reconstructive surgery

Techniques in reconstructive surgery have been developed based on a knowledge of the anatomical and physiological properties of living tissue and in particular the recognition of patterns of blood supply. The quality and distribution of this blood supply may however, prove to be a limiting factor in some situations. With therapeutic angiogenesis comes the potential to enhance native blood supply either to increase the chances of success with any given surgical option or to increase the overall number of options available to choose from. An unfavourable set of circumstances may therefore be changed into more favourable ones. In addition, angiogenesis is known to play a key role in normal wound healing and in the vascularisation of grafted tissue elements (Duprez *et al.* 1991; Singer & Clark 1999). Therapeutic angiogenesis therefore, may also be of benefit when physiological wound healing is impaired.

A further consideration in any tissue transfer is the morbidity associated with the donor site. The concept of flap prefabrication is an attempt to address this problem by importing a vascular pedicle into a potentially desirable donor site as a preliminary stage before definitive flap transfer (Erol 1976). The success of the technique relies on communications developing between the pedicle and the vascular channels in the surrounding tissue. Early work has suggested that the use of angiogenic growth factors in conjunction with this technique can increase both the reliability and size of tissue transfer that may safely be undertaken (Bayati, Russell, & Roth 1998).

More innovations will surely follow as the technique of therapeutic angiogenesis undergoes increasing refinement and more novel applications are identified.

One clear application however, is the use of this technique to extend the vascular territory of a given flap and therefore increase the size of tissue transfer that may be reliably undertaken on a given vascular pedicle. Many agents have previously been evaluated for their ability to improve flap survival as will be seen.

1.3 The principles of tissue transfer

The replacement of missing tissue and the restoration of function are the founding principles of reconstructive surgery. To achieve these goals techniques have been developed to permit the movement of tissue from an area of relative surplus to an area of need. To survive however, all living tissues are reliant on an adequate system for oxygen and nutrient delivery and waste product removal in keeping with their metabolic demands. These services are normally provided by an appropriate blood supply.

1.3.1 Non vascularised tissue transfer

Tissue may be moved in two principal ways. For the simplest of transfers, tissue is relocated as a graft. For the graft to survive it must acquire a blood supply from its new surroundings before irreversible ischaemic injury has occurred. A tolerance of ischaemia is therefore a requirement for tissue to be moved by this technique and limits what can be achieved. Similarly the recipient site must be of sufficient

vascularity to provide a foundation from which revascularisation of the graft can proceed. Thus, a balance exists which may be tipped in one direction or the other dependant on factors relating to the nature of the graft but also, and probably more importantly, the vascularity of the recipient site (Wang HJ *et al.* 1997). The most successful grafts are those in which the tissue to be moved is known to be relatively tolerant of ischaemia, has a relatively large surface area to volume ratio and where the recipient bed is vascular.

As the tissue required for transfer becomes less tolerant of ischaemia and as the recipient bed becomes less likely to provide an adequate source of blood vessels to which vascular connections can be made, then the likelihood of a successful tissue transfer proportionately decreases. In these circumstances, an alternative approach is needed.

1.3.2 Vascularised tissue transfer

1.3.2.1 Flaps

A composite of tissue designed for transfer with its circulation intact, or only temporarily interrupted, is termed a *flap*. A flap therefore, contains within its substance a functioning network of blood vessels that ensure its survival independent of the vascularity of the recipient site into which it is placed. The blood enters and exits the flap through a vascular *pedicle*. The maximum dimension to which a flap can be raised with the expectation of complete survival is therefore, limited by the extent to which blood flow can be maintained to all areas of the flap by the vessels of

the pedicle. Knowledge of the local vascular anatomy makes such a prediction possible.

1.3.2.2 The concept of the angiosome

The blood vessels in the skin and superficial fascia form a continuous plexus over the entire body surface however, demonstrable vascular territories do exist (Taylor & Palmer 1987). Such territories are termed *angiosomes*. Using a variety of techniques including ink injection, cadaveric dissection and radiographic analysis Taylor and colleagues have provided an angiosomal map to encompass the entire body (Taylor & Palmer 1987). Angiosomal territories are not however, mutually exclusive and considerable overlap between these territories often exists in life where blood flow characteristics are a reflection of changing pressure gradients (McGregor 1992).

With careful planning however, a block of tissue comprising a given angiosome may be surgically elevated and moved around an axis defined by the position of the feeding vessels, to a new location with the expectation that the circulation will be adequate to ensure complete survival of the flap in its new location. The size and shape of tissue that can be moved in this way is defined in the most part, by the anatomy of the underlying angiosome (Taylor & Palmer 1987).

1.3.2.3 Skin flap failure

Despite careful planning however, flaps still fail. Kerrigan has reviewed the pathophysiological processes implicated in this failure and draws a distinction

between extrinsic and intrinsic factors (Kerrigan 1983). Extrinsic factors include the existence of unfavourable patient comorbidities and adverse local conditions. The author makes the point that in the majority of cases, these factors may be addressed, at least in part, by careful preoperative preparation and postoperative management. In contrast to the variety of extrinsic factors recognised only a single intrinsic factor was identified, namely an inadequate nutrient blood flow. Following a series of experiments using an animal model of skin flap failure Kerrigan draws the simple conclusion that skin flaps fail because of arterial insufficiency (Kerrigan 1983). A more complex explanation is not required.

1.4 The search for techniques to enhance flap survival

The recognition that flap survival was intimately linked to the extent and quality of the nutrient blood supply encouraged attempts at augmenting this blood supply with the hope of extending the described territories and improving flap reliability. Both surgical and pharmacological techniques have been explored with mixed results.

1.4.1 The delay phenomenon

It has long been known that the surgical interruption of a portion of the blood supply of a flap at a preliminary stage before transfer can lead to improved flap survival (Myers & Cherry 1969). The changes within the flap that accompany this procedure are collectively known as the delay phenomenon and debate continues as to the exact mechanism by which the final effect is achieved (Dhar & Taylor 1999). Early work by Braithwaite led him to conclude that the non-lethal ischaemia induced by a delay

procedure conditioned the tissues of the flap to survive with reduced blood flow (Braithwaite 1951). Other authors studying the histological changes in flap vascularity associated with delay have reported increases in both the number and diameter of vessels within the first two weeks following the procedure (Arranz Lopez *et al.* 1995;Jonsson *et al.* 1988). Callegari *et al.* (1992) studied changes in the choke vessels located between perforator angiosomes in a series of skin flaps raised in dogs and noted maximal dilation to occur in these vessels.

It would seem therefore, that vasodilation is an important factor in the success of the delay phenomenon and in particular dilation of the choke vessels located at the boundary between neighbouring angiosomes. Although angiogenesis clearly takes place its contribution to increased blood flow at the time of flap transfer is not certain. Where there is no doubt however, is in the overall effectiveness of the technique in improving flap survival, as demonstrated by many authors (Guba, Jr. 1979;Morrissey, Jr. & Hallock 2000;Pang *et al.* 1986).

Although effective, there are obvious drawbacks with the use of surgical delay as a technique to improve flap survival. Firstly, there is the requirement for an additional surgical procedure with the attendant risks and potential for complications. Secondly, it is of limited use when reconstruction is required urgently making any delay unacceptable. This has fuelled attempts to identify alternative, non-surgical techniques capable of achieving the same effect.

1.4.2 Improving blood flow pharmacologically

The association between the delay phenomenon and vasodilation led to an intensive search for a pharmacological agent that would achieve the same results. Numerous agents have been trialled employing a variety of modes of action. These include sympatholytic drugs (Goshen, Wexler, & Peled 1985), direct vasodilators (Finseth 1979), calcium channel blockers (Pal, Khazanchi, & Moudgil 1991) and prostaglandins (Okamoto, Nakajima, & Yoneda 1993). In general however, the early successes have proved difficult to reproduce (Kerrigan & Daniel 1982). Another approach to mimic the success of the delay phenomenon aimed to improve the flow mechanics of blood either by increasing erythrocyte deformability and flexibility (Takayanagi & Ogawa 1980) or by lowering blood viscosity (Earle, Fratianne, & Nunez 1974). Yet further work has centred on drugs such as dimethyl sulphoxide that offer a degree of protection against ischaemic injury (Grossman *et al.* 1983).

Pang and colleagues offer a detailed review of the successes and failures that have been reported in using these drugs to improve the survival of ischaemic flaps (Pang, Forrest, & Morris 1989). In their closing remarks they conclude that the evidence to support their use is in general weak and often conflicting with inconsistencies in experimental design making the interpretation of results difficult. Consequent to this they have found little place in clinical practice.

1.4.3 A potential role for angiogenic cytokines

Advances in the field of genetic engineering have made possible the large-scale preparation of a wide variety of purified human growth factors including those with a

central role in the control of angiogenesis. The use of such growth factors to improve the survival of ischaemic flaps by the induction of angiogenesis became the focus of much research. Khouri *et al.* were amongst the first to report a significant improvement in flap survival following instillation of 1 μ g recombinant human basic fibroblast growth factor (bFGF) suspended in 2 mg collagen beneath ischaemic dorsal skin flaps raised in rats (Khouri *et al.* 1991). Im *et al.* achieved similar success using bovine bFGF (Im *et al.* 1992) while Ishiguro *et al.* examined the potential benefit of repeated doses of bFGF and found no additional flap survival advantage over a single dose administered at the time of elevation (Ishiguro *et al.* 1994). In a model of flap ischaemia in mice however, Uhl *et al.* reported no improvement in flap survival following subdermal injection of human bFGF unless administered 18 days before flap elevation (Uhl *et al.* 1994). Norrby compared the angiogenic response of bFGF and VEGF using the mesenteric window angiogenesis assay and found VEGF to be the most rapidly active agent (Norrby 1996). A further advantage of VEGF over bFGF is its specificity of action as will be seen later (Ferrara & Davis-Smyth 1997).

In 1996 Padubidri and Browne investigated the action of recombinant human VEGF administered intra-arterially to the pedicle of an ischaemic rat epigastric island flap and reported a significant average increase in flap survival of 21 % compared with controls (Padubidri A & Browne E 1996). The improved flap survival was associated with increased capillary density observed histologically and the authors therefore concluded that the observed survival advantage was due to angiogenesis induced by VEGF.

Although few in number these reports suggest that angiogenic cytokines can be effective in improving the survival of ischaemic flaps. Many such cytokines exist however, together participating in the complex and closely regulated process of angiogenesis. To harness this mechanism for therapeutic ends first requires an understanding of the physiological process.

1.5 The control of angiogenesis

Angiogenesis is the fundamental process by which new blood vessels are formed from existing vascular channels and should be distinguished from vasculogenesis, which describes the formation of blood vessels from angioblasts, a process that occurs in the developing embryo but rarely in adult life (Risau 1997). Normal development, reproduction and wound healing all depend on effective angiogenesis for completion. Regulation is tightly controlled with long periods of inactivity punctuated by periods of intense cellular proliferation in response to an appropriate stimulus.

Capillary blood vessels consist of a single layer of endothelial cells and a basal lamina. The endothelial cells are curved to form a tube just large enough to allow the passage of red blood cells in single file. In cross section the walls of the tube may be formed by a single cell or as many as three cells. Capillaries may be classified as *continuous* or *fenestrated* (Figure 1.1). Fenestrated capillaries contain circular pores ranging in diameter from 80 – 100 nm spanned by a diaphragm with a central thickening. Pericytes may be found in relation to either subtype and when present are enclosed by a further basal lamina that is continuous with that of the

endothelium. The exact role of the pericyte is not clear however, it is thought to represent a relatively unspecialised cell capable of differentiating into other cell types (Ross & Reith 1985). These two cell types carry all of the genetic information to form tubes, branches and complete capillary networks.

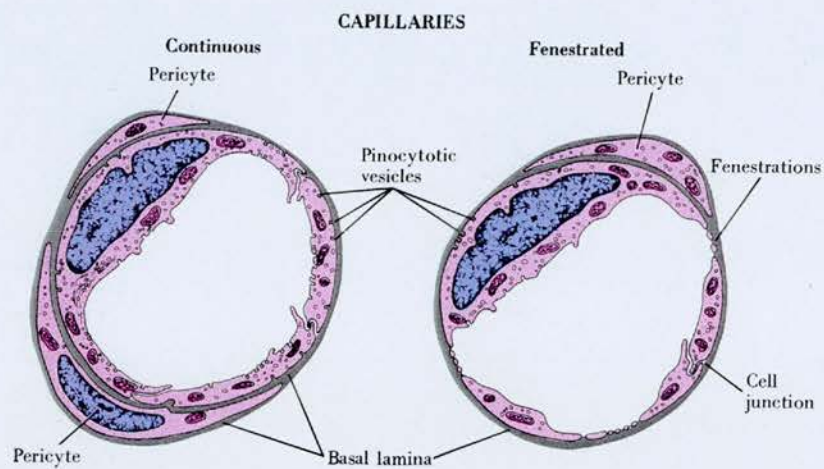


Figure 1.1 – Schematic diagram demonstrating the structure of continuous and fenestrated capillaries. Both may have associated pericytes. Taken from Ross and Reith 1985.

The principal steps involved in the process of angiogenesis can be summarised as: release of angiogenic cytokines, endothelial cell binding, activation and proliferation, directional migration within a remodelled extracellular matrix followed by tube formation and stabilisation with the arrival of pericytes. These steps are shown schematically in Figure 1.2.

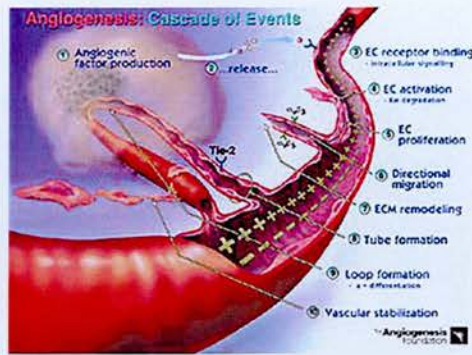


Figure 1.2 – Schematic diagram illustrating the process of angiogenesis. Adapted from the Angiogenesis Foundation 2001 (www.angio.org).

Specific angiogenic molecules can initiate this process whilst specific inhibitory molecules can stop it (Hanahan & Folkman 1996). These molecules with opposing functions appear to act continuously to maintain a dynamic balance resulting in a great stability of the endothelial cell population where cell turnover is measured in thousands of days (Iruela-Arispe & Dvorak 1997). The same endothelial cells however, can undergo bursts of mitotic activity when the need arises. Similarities can be seen between this system and that which operates in the control of coagulation although the protein interactions in the latter are better understood (Folkman & Shing 1992).

Many factors have been demonstrated to initiate angiogenesis both in health and in disease (Kuwano *et al.* 2001). Folkman and Shing have tabulated some of the more important ones (Table 1.1) (Folkman & Shing 1992). These polypeptides differ greatly in their biochemical and biological properties. The VEGF family for example, are highly specific mitogens for endothelial cells whereas by contrast, the FGF's are pleiotropic, that is, they stimulate the growth of several cell types

including endothelial cells, smooth muscle cells and fibroblasts as well as acting as a differentiating factor for nerves (Togari *et al.* 1985).

Table 1.1 – Angiogenic polypeptides and their related biological activities. Adapted from Folkman and Shing 1992.

Angiogenic factor	Molecular weight (kDa)	Angiogenic-related biological activities
VEGF	45	Increases vascular permeability, highly specific endothelial cell mitogen
FGF (a and b)	16.4 & 18	Stimulate migration of endothelial cells and tube formation, embryonic activity
PDGF	45	Stimulates endothelial cell DNA synthesis and chemotaxis, proliferation not reported
TGF- α	5.5	Transformation of normal cells, binds to EGF receptor
TGF- β	25	Enhances extracellular matrix production, chemotactic for monocytes
Angiogenin	14.1	Stimulates endothelial cells to secrete prostacyclin, has unique RNA activity essential for angiogenesis
TNF- α	55	Induction of bFGF in endothelial cells and enhances its secretion, chemotactic for monocytes, activates macrophages

Knowledge of the precise biological properties of these cytokines and the mechanisms by which they interact to control angiogenesis remains incomplete however, most authors agree on the pivotal role played by the VEGF family of polypeptides (Ferrara & Davis-Smyth 1997). These proteins have been the subject of intense research and consequently VEGF is one of the best characterised of all angiogenic growth factors (Ferrara 1999). This work has highlighted a number of important properties of relevance to the use of VEGF as an agent for therapeutic angiogenesis.

1.6 The biology of vascular endothelial growth factor

1.6.1 The VEGF family

VEGF was the first of what is now recognised to be a family of angiogenic polypeptides to be described (Lobb *et al.* 1985) and is often referred to as VEGF-A* to distinguish it from the other family members (VEGF-B, VEGF-C, VEGF-D and placenta growth factor). VEGF-A is a 45 KDa glycoprotein that binds to the VEGFR-1 (Flt-1), VEGFR-2 (Flk-1, KDR) and neuropilin-1 receptors of vascular endothelial cells and illicit a profound angiogenic response seen in a wide variety of *in vivo* models (Leung *et al.* 1989). It is however, mostly devoid of any mitogenic activity for other cell types (Ferrara & Henzel 1989). Production is by a variety of cells including macrophages, T-cells, smooth muscle cells and keratinocytes (Klagsbrun & D'Amore 1996).

As well as acting as a stimulant for endothelial cell division and migration, VEGF is also known to increase vascular permeability and was initially known as vascular permeability factor (VPF) (Senger *et al.* 1983). The conversion of continuous capillaries to the fenestrated phenotype under the influence of VEGF leading to protein extravasation is thought to be the primary mechanism by which permeability is increased (Roberts & Palade 1997). Dvorak *et al.* believe that the increase in microvascular permeability to proteins is a crucial step in angiogenesis allowing the formation of an extravascular fibrin 'gel' into which new vessels sprout (Dvorak *et al.* 1995b). Other authors dispute this (Eliciciri *et al.* 1999).

* VEGF will be used synonymously with VEGF-A in this work.

The gene encoding for VEGF has been assigned to the locus p21.3 on chromosome 6 spanning approximately 14 kb and comprising eight coding regions (exons) interrupted by seven non-coding regions (introns). Alternative splicing of the resultant mRNA leads to the generation of four different species of VEGF in man (VEGF121, VEGF165, VEGF189, VEGF206). The shorter of the four isoforms (VEGF121 and VEGF165) are freely diffusible proteins whilst VEGF189 and VEGF 206 are almost completely sequestered in the extracellular matrix (Park, Keller, & Ferrara 1993). VEGF165 is the most biologically active (Ferrara 2001).

Although VEGF expression can be induced by a variety of hormones, growth factors and cytokines one of the most important regulators of expression is hypoxia. Low oxygen tension has been demonstrated to increase VEGF mRNA expression experimentally by as much as ten fold *in vitro* (Plate & Warnke 1997). Secreted VEGF exerts its effect via high affinity receptors located on the surface of endothelial cells which when bound activate nitric oxide synthase leading to cellular proliferation (Morbideilli *et al.* 1996). Binding sites for VEGF are thought to be restricted to these cells explaining the high target cell specificity of the active protein. VEGF has been shown to provide a potent stimulus to angiogenesis both *in vitro* and *in vivo* (Connolly *et al.* 1989; Shweiki *et al.* 1993).

Two other properties of VEGF are noteworthy with respect to its use in therapeutic angiogenesis. Firstly, maximal stimulation of endothelial cells has been demonstrated to occur at very low concentrations, in the region of 22pM solutions (approx. 1 –4 ng/ml) (Ferrara N *et al.* 1991). Very little cytokine is therefore

required to achieve biological activity. Secondly, VEGF is known to exert a dose dependant vasodilatory effect by relaxing vascular smooth muscle (Ku *et al.* 1993) and therefore will tend to increase blood flow to the local tissues.

All members of the VEGF family are structurally related and all stimulate angiogenesis (Ferrara & Davis-Smyth 1997). Patterns of activity and tissue distribution however vary between species. This work is based on VEGF-B.

1.6.2 Vascular endothelial growth factor B

VEGF-B is a relatively recent addition to the VEGF family (Olofsson *et al.* 1996). The gene has been mapped in humans to the q13 locus of chromosome 11 and is approximately 4Kb in length comprising 7 coding exons. Alternative splicing yields two polypeptide forms, VEGF-B167 and VEGF-B186 with molecular masses 42 kDa and 60 kDa respectively with the 167 homologue being the most biologically prevalent (up to 90 % mRNA transcripts in adult mice) (Olofsson *et al.* 1999).

Although a full characterisation of the protein has yet to be performed, some aspects of its biological behaviour are known. A close structural similarity with VEGF-A (approximately 44 % identical), with which it is often coexpressed, exists and a wide tissue distribution has been recorded although it is most abundant in heart and skeletal muscle as well as pancreas. The myocyte appears to be the principal cell of production and the secreted VEGF-B protein remains, in the most part, bound to cells or to the extracellular matrix (Olofsson *et al.* 1996). Like VEGF-A, VEGF-B is a powerful endothelial cell mitogen and its ability to promote angiogenesis *in vivo* has

been well documented (Silvestre *et al.* 2003). Unlike VEGF-A however, it would appear not to be significantly regulated by local tissue oxygen tension (Enholm *et al.* 1997).

VEGF-B appears to be secreted by the majority of human tumours and may be of particular importance in the angiogenesis associated with ovarian cell tumours (Sowter *et al.* 1997). Although the exact role of VEGF-B in the regulation of angiogenesis remains unclear, it is known that VEGF-B exerts its effect by binding to the VEGFR-1 and neuropilin-1 receptors of endothelial cells and therefore it is likely that the cellular signals resulting from the binding of both VEGF-A and VEGF-B, at least in part, overlap (Olofsson *et al.* 1999).

Based on this overlap we believed that VEGF-B may show similar therapeutic activity to that reported for VEGF-A in the exogenous induction of angiogenesis (Takeshita *et al.* 1994c). In addition, novel techniques have recently been described for delivery of the angiogen to the target tissue in the genetic form rather than the active cytokine itself (Takeshita *et al.* 1997). One major advantage of this approach is the relative stability and improved handling properties of DNA in comparison to the purified polypeptide. The techniques that permit the movement of genetic material from one location to another and achieve its expression in this new location have their foundation in the field of genetic engineering. The development of such techniques has occurred only in the very recent past.

1.7 The principles of gene manipulation

The first genetically engineered drug, human insulin, was marketed in 1982. Since this time the techniques used to sequence, copy, amplify and achieve expression of a given genetic sequence have undergone continual refinement. A core feature of these techniques is the ability to isolate stretches of DNA from their host organism and propagate them in the same or a different host. A technique known as *cloning* (Old & Primrose 1995a). In order for a cloned segment of DNA to achieve propagation in its new location with subsequent cell division it must contain an origin of DNA replication, or *replicon*. Constructions of DNA that act as replicons for the purposes of gene manipulation are known as cloning vehicles. Many such constructs are now available permitting the efficient transfer of genetic material into a host cell, facilitating expression of the target gene and ending with the production of a desired protein (Bendig 1988). The original cloning vehicle however, was the bacterial plasmid (Itakura *et al.* 1977).

1.7.1 Basic plasmid biology

Bacterial plasmids exist in the main as circular, double stranded, extrachromosomal, DNA molecules and are widely distributed amongst prokaryotes. They vary in size from less than 1×10^6 to greater than 200×10^6 daltons and the information they encode is generally expendable in terms of the survival of the organism as a whole. Phenotypes they confer on their host include attributes such as antibiotic resistance as well as antibiotic and enterotoxin production amongst others. They exist in the cytoplasm as single or multiple copies and may be transferred directly from one

organism to another following cellular contact (conjugative plasmids) (Brock & Madigan MT 1994).

For researchers interested in the transfer and expression of genetic material, plasmids possess a number of important and potentially useful characteristics. One of the most important of these is they all contain an origin of replication, allowing replication to proceed independent of nuclear DNA (i.e. integration into the host genome is not required for propagation of the plasmid DNA). In this way plasmids exhibit stable inheritance in an extrachromosomal state (Hartl 1994). In addition, well established methods exist for the isolation and reintroduction of intact plasmid DNA from and to cells in culture, providing a powerful means by which genetic material may be moved from one location to another (Sherratt 1979).

These properties have made bacterial plasmids the starting point for much that has been achieved in the field of gene manipulation (Old & Primrose 1995b).

1.7.2 Plasmids as cloning vehicles

If bacterial plasmids are to be employed as transport vehicles for DNA, then a number of technical difficulties must be overcome. Old and Primrose (1995a) summarise these as:

- 1) Purification and cutting open of the plasmid (vector) DNA
- 2) Insertion of passenger DNA into the vector molecule to create an artificial recombinant
- 3) Joining the ends of the new DNA molecule
- 4) Transformation* of the artificial recombinant into a suitable host cell.
- 5) Amplification of the whole system by expansion of the host cell population.

The stages involved are illustrated in Fig. 1.3.

Initially the majority of successful transformations were carried out in *Escherichia coli* however, since then other species of bacteria (Ehrlich 1977), yeasts (Hinnen, Hicks, & Fink 1978), plants (Fraley *et al.* 1983) and also animal cells (Schaffner 1980) have all been successfully transfected using plasmids.

* The term 'transformation' as used here denotes the transfer of cloned DNA into a host cell (by the process of *transfection*) and should be distinguished from *growth transformation* meaning the sudden change in properties of an animal cell associated with malignancy.

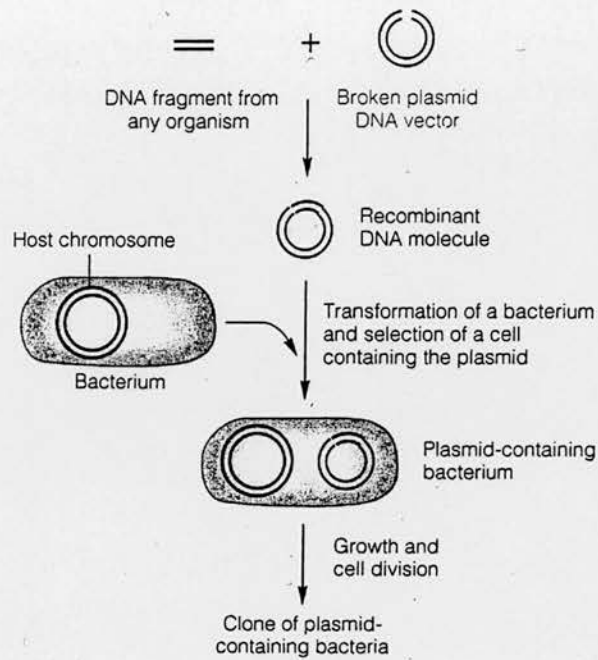


Figure 1.3 – Schematic diagram illustrating the principles of cloning using bacterial plasmids. A fragment of DNA from any organism is joined to a cleaved plasmid. The recombinant plasmid is then used to transform a bacterial cell, and, thereafter, the foreign DNA is present in all progeny bacteria. Taken from *Genetics* by Daniel L. Hartl 1993.

The first experiments were conducted using naturally occurring plasmids, for example Chang and Cohen successfully used the pSC101 plasmid of *Salmonella panama* to achieve expression of *Staphylococcus aureus* genes in *E coli*. (Chang & Cohen 1974). Since this time many other naturally occurring plasmids have been similarly used however, with increased understanding of the genetics involved, it became possible to design and construct plasmids *in vitro* for the sole purpose of cloning (Denhardt *et al.* 1988).

Useful features were included in the design, such as antibiotic resistance (to allow selection), multiple unique restriction enzyme cleavage sites (permitting insertion of DNA at chosen locations) while maintaining a low molecular weight making the final construct less prone to shear damage. The plasmid pBR322 was one of the first to be constructed in this way and has since proved a highly successful cloning vehicle (Balbas *et al.* 1988). The first genetically engineered human protein, somatostatin, was produced from *E coli.* using pBR322 as the cloning vector (Itakura *et al.* 1977).

1.7.3 Plasmids as expression vectors

The primary aim in cloning experiments is to achieve the successful transfer of a target gene from one location to another, the final quantity of gene product that results from the transfer is of less significance (Ehrlich 1977). If instead however, protein production is the goal, optimal conditions for expression of the new genetic material become important (Friebs & Reardon 1993).

To achieve expression of cloned DNA using a plasmid vector a gene *promoter* sequence must be incorporated into the plasmid upstream from the coding region. The ‘power’ of this promoter sequence is one of the most significant factors in determining the level of expression of the target gene and therefore the magnitude of protein production (Kobayashi, Kurusu, & Yukawa 1991). Promoter efficiency varies considerably and the choice of promoter is therefore, an important consideration when selecting or constructing a plasmid for protein production (Jensen & Hammer 1998). Similarly, the promoter must be appropriate for the

chosen host cell, for example, if the target is the bacterium *E. coli* then one of the promoters known to be effective in *E. coli* should be selected (Harley & Reynolds 1987).

Plasmids that have been optimised for protein production are referred to as *expression* vectors. The plasmid expression vector selected for use in this project is known as pEF-BOS and will be described in detail later.

1.7.4 Techniques to improve the efficiency of DNA transfer

Most of the early genetic engineering experiments were performed using *Escherichia coli* as the host organism (Chang & Cohen 1974). This came about because initially, plasmids and bacteriophages* suitable for use as vectors were only known to exist in *Escherichia coli*. To begin with the efficiency with which transformation of *E. coli* could be achieved was disappointingly low however, the results of subsequent experiments resulted in the identification of several important factors, which if present, could result in a significant improvement in the level of success attained. Such factors included the presence of calcium and other metal ions in the transfection mixture and a low initial temperature with a subsequent heat-shock (Hanahan 1983).

An alternative method, which is effective in the transformation of bacterial cells and can also be used in the transfection of mammalian cells, is that of electroporation.

This technique is based on the observation that cell plasma membranes can be

* Bacteriophages are viruses that infect bacteria.

induced to fuse when subjected to a brief electric current (Zimmermann & Vienken 1982). Further work led to the discovery that cells exposed to an electric impulse will also take up exogenous DNA from solution, possibly through holes momentarily created in the cell membrane. A proportion of these cells may then become stably transformed (Dower, Miller, & Ragsdale 1988). Transformation efficiencies with *Escherichia coli* using this technique have been found to equal those using the calcium chloride technique however, electroporation has the added advantage of achieving successful transfection in a wide variety of plant and animal cells and has achieved success with some cell types that had proved resistant to other approaches (Chu, Hayakawa, & Berg 1987).

The transfection of mammalian cells can also be effected by the use of small unilamellar (single bilayer) vesicles prepared from cationic lipids known as *liposomes*. This technique depends on the observation that DNA in solution spontaneously and efficiently, complexes with these vesicles which in turn bind to the cell membrane of animal cells (Felgner *et al.* 1987b). It has been suggested that liposomes achieve their effect by fusing with the plasma membrane of the target cell wall creating a temporary gap through which the exogenous DNA can pass (Pagano & Weinstein 1978). The use of liposomes to achieve enhanced transfection efficiency in this way is termed *lipofection* and the cationic lipid is referred to as a *transfection agent*.

Numerous transfection agents have been described however, the basic mode of action is the same.

1.8 Techniques in therapeutic angiogenesis

Any given technique for therapeutic angiogenesis essentially consists of two main components: an active angiogenic agent, or combination of agents, and a delivery strategy. The most effective agent and the optimal method of delivery however, have yet to be clearly defined.

1.8.1 The choice of angiogen

Although a number of agents are known to play a role in the stimulation of angiogenesis *in vivo*, only a relative few of these have to date undergone evaluation for their ability to stimulate angiogenesis exogenously. In addition to VEGF, acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF) and platelet derived growth factor (PDGF) have provided the focus for much of the published work. A comparative analysis of the relative effectiveness of each of these agents has yet to be performed however, all have been used successfully to stimulate angiogenesis *in vivo* (Machens *et al.* 2002; Pu *et al.* 1993b; Rinsch *et al.* 2001a). The majority of therapeutic angiogenesis experiments have so far employed VEGF as the active agent (Henry 1999). The popularity of VEGF reflects a favourable biological profile namely endothelial cell specificity, a local vasodilatory effect and enhanced activity in ischaemic tissue due to an upregulation of VEGFR-1 receptors (Brogi *et al.* 1996). In addition, some evidence exists to suggest that VEGF is a more potent stimulator of angiogenesis than bFGF under similar circumstances (Lee, Peters, & Mooney 2003).

Initial work was invariably conducted using the VEGF-A165 homologue however, recognition and characterisation of other members of the VEGF family has resulted in encouraging work using these species also (Rissanen *et al.* 2003). At the time of this study, no reports existed on the use of VEGF-B167 for therapeutic angiogenesis.

1.8.2 Delivery strategies

Many different ways of delivering angiogenic agents have been investigated. At present there is no consensus as to the superiority of any one technique relative to the others and thus, at the present time at least, all would appear to be equally valid.

1.8.2.1 Purified cytokine

Purified polypeptide administered intraarterially or subcutaneously has been shown to stimulate angiogenesis in a number of experimental models. Ishiguro *et al.* injected 20 µg bFGF beneath caudal ischaemic skin flaps in rats and reported an average increase in flap survival of 13 % at seven days (Ishiguro *et al.* 1994). As previously noted Padubidri and Browne recorded similar success using 5 µg VEGF administered intraarterially to rat epigastric island flaps (Padubidri A & Browne E 1996). The disadvantage of this approach is the complexity of the purification processes resulting in an expensive final product that also has a relatively short half-life. These limitations have resulted in increased interest in the alternative approach of gene therapy.

1.8.2.2 Unmodified 'naked' DNA

Genetically engineered expression plasmids have also been used successfully to induce angiogenesis in ischaemic flaps. Taub *et al.* report their experience with a plasmid encoding the gene for VEGF-A. Significant improvements in flap survival were noted when this agent was administered intraarterially at a dose of 7 µg to a series of rat epigastric island flaps (Taub PJ *et al.* 1998). Although having the advantage of being easier to work with than purified cytokine, plasmids when used alone have the disadvantage of relatively low rates of gene transfer. In Taub's series the transfection agent lipofectamine was used in conjunction with the plasmid to improve transfection efficiency. An alternative approach is to increase the dose of plasmid DNA used as reported by Takeshita *et al.* who recorded evidence of stimulated angiogenesis when 400 µg plasmid DNA was administered in a rat model of lower limb ischaemia (Takeshita *et al.* 1997).

1.8.2.4 Viral vectors

Viral vectors have been developed to enhance the efficiency of gene transfer. The genome of recombinant viral vectors is modified to carry a copy of the angiogenic gene and all such vectors are rendered 'replication deficient' in their host cell (Safi, Jr. *et al.* 1997). The adeno virus has been used most commonly in this respect for *in vivo* transfections (Poliakova *et al.* 1999). Although highly effective in transferring their genetic material to a target cell the generation and use of live viruses is technically demanding and not without hazard. Nevertheless these vectors have been used in phase I clinical trials without complication (Rosengart *et al.* 1999).

1.8.2.5 Genetically modified cells

A further technique that has been trialled with reported success is the use of cultured cells genetically modified to secrete angiogenic protein as vectors for therapeutic angiogenesis. Machens *et al.* performed *ex vivo* transfection of syngeneic rat fibroblasts to produce cells expressing the platelet derived growth factor gene. Reintroduction of these cells beneath rat epigastric island flaps one week prior to flap elevation resulted in a significant survival advantage ($84 \pm 9\%$ v $57 \pm 7\%$; $p < 0.01$). The authors also comment on an increase in capillary numbers throughout the thickness of the flap when compared to controls.

1.9 Aims

My aim in this project was to determine whether I could use gene therapy techniques based on the angiogenic cytokine VEGF-B, to stimulate angiogenesis in the rat epigastric island flap model of tissue ischaemia and in so doing, influence flap survival. Two delivery strategies would be investigated. Firstly, a bacterial expression plasmid encoding the gene for VEGF-B and secondly, cultured cells genetically modified to secrete VEGF-B.

Chapter 2 - Validation of the experimental model

2.1 Introduction

2.1.1 The model

To evaluate the proposed techniques for the induction of angiogenesis *in vivo*, an animal model of tissue ischaemia was required. A review of the literature identified the laboratory rat as being commonly used (Pang, Forrest, & Morris 1989).

Advantages of the rat over other laboratory animals included local availability, ease of handling, disease resistance and low cost both in terms of acquisition and maintenance (Dunn & Mancoll 1992). Having decided to use a rat model we identified our requirements for an experimental ischaemic flap as being:

- 1) Suitable for the collection of data related to changes in vascularity
- 2) Of sufficient size to allow the planned experimental work
- 3) Founded on reliable anatomy
- 4) Technically easy to raise

The rat epigastric island flap, as first described by Finseth and Cutting, appeared to satisfy these requirements (Finseth F & Cutting C 1978). This flap incorporates the two major ventral angiosomes of the rat integument and comprises the *panniculus*



carnosus muscle layer and all soft tissue superficial to it. In their paper they observe that:

If an island flap of the complete abdominal wall skin of a rat is based on both sets of neurovascular bundles (arising from each groin), it remains entirely viable and heals without necrosis. If the neurovascular bundle in one groin is divided, the corresponding half of the abdominal wall skin that it serves becomes dependent on the opposite half of the abdominal wall skin for its vascular supply. Conceptually, therefore, a random pattern flap is now attached to an axial pattern flap. It is this arrangement in our experimental model that produces a consistent, reproducible pattern of surviving flap area across the midline which interfaces with an area of skin flap necrosis. The area of surviving flap across the midline is, in essence, the bioassay for the nutritional cutaneous blood supply.

(Finseth & Cutting, 1978)

2.1.2 Anatomical considerations

The vascular anatomy of the ventral skin and *panniculus* of the rat, providing the basis for this flap, is well described (Petry & Wortham 1984). The femoral artery and vein emerge from beneath the inguinal ligament and pass directly into the hind limb, deep to the extensor compartment of the hip. In the cleft between this compartment and the muscles of the abdominal wall, the femoral artery gives origin to the superficial inferior epigastric artery (SIEA).

From this point the SIEA turns superiorly and enters a more superficial plane although remaining deep to the *panniculus carnosus*. Here it divides into a medial and larger lateral, branch. The former remains on the ventral surface of the animal travelling cephalad and ending by collateralising with the terminal branches of the

internal mammary vessels descending from their origin in the chest. The latter extending laterally to collateralise with vessels descending from the lateral chest wall in the region of the mid lateral line (Petry & Wortham 1984). Both branches give supply to the full thickness of the overlying integument (skin, superficial fascia and *panniculus carnosus*) via a combination of musculocutaneous and direct cutaneous vessels, with the former thought to predominate (Dunn & Mancoll 1992).

Several perforating vessels arising from the deep epigastric system pass through the muscles of the abdominal wall and reinforce this network. The venous drainage follows a similar pattern with the emergence of the superficial inferior epigastric vein in the groin of the animal, draining into the femoral vein at a point just medial to the origin of the artery. This anatomy is constant and not prone to significant anatomical variation.

2.1.3 Flap design

In the original description, Finseth and Cutting highlighted the difference between the ventral and dorsal rat skin. On the ventral surface the skin is relatively thin and supple when compared to the coarse, and more adherent, dorsal skin. They identified the transition as occurring between 4 and 5 cm from the midline in animals weighing between 350g and 400g. Flap dimensions of 9x9cm were therefore recommended. Petry and Wortham pointed out the variability in survival patterns of these flaps dependent on whether the lateral branch of the SIEA was included in the flap, or not (Petry & Wortham 1984). Padubidri and Browne therefore preferred to base flaps solely on the medial branch of the SIEA and proposed 8 x 8 cm as the dimensions

most likely to facilitate retention of this branch whilst allowing for the successful identification and interruption of the lateral branch (Fig 2.1) (Padubidri & Browne E Jr 1997).

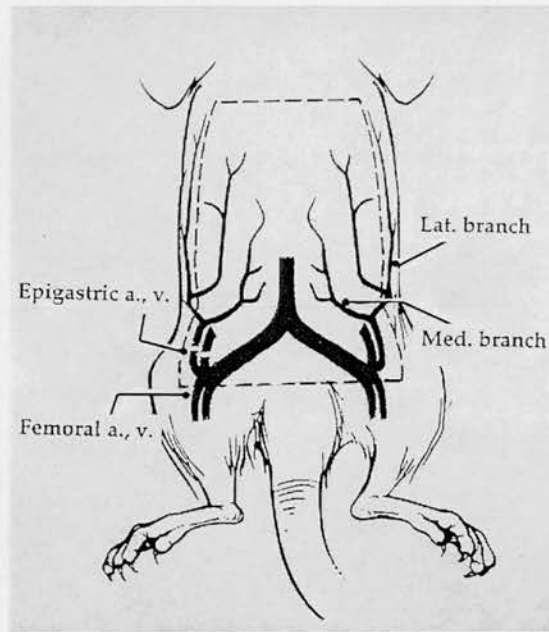


Figure 2.1 – Epigastric island flap design taken from Padubidri & Brown, 1997.

In my preliminary work with this model I found that for rats weighing 350 – 450 g, flaps raised with dimensions 8x8 cm invariably included the lateral branch of the SIEA. Since I did not want to risk damage to the main vascular pedicle by attempting to interrupt the lateral branch within the flap itself, I decided to base my work on a flap design that comprised an 8x8 cm skin island incorporating both medial and lateral branches of the SIEA.

2.1.4 Autocanabalism

A flap designed in this way is, in the main, insensate and the tendency for the animal to devour its own tissue (autocanabalism) is well reported (Brindle, McCarthy, & Bell 1999). It is surprising therefore that neither Finseth and Cutting nor Padubidri and Browne make any reference to the problem. My own experience with this model was that unprotected, I could expect to lose between a third and a half of flaps as a result of autocanabalism (Fig. 2.2). Various strategies have been proposed to combat this problem. The most obvious manoeuvre is to shield the flap from the animals teeth either directly by means of a protective vest, or indirectly, by use of a collar (Bayramicli, Yilmaz, & Numanoglu 1998b). Alternatively the animals teeth themselves may be shortened as advocated by Komorowska-Timek *et al.* (Komorowska-Timek *et al.* 1999). I preferred the use of a protective collar since it avoided direct pressure on the flap and did not interfere with flap observation. Suitable collars were made from unexposed x-ray film (Fig. 2.3). The design was such that the rats' ability to feed and drink was in not impeded.



Figure 2.2 – Autocannibalism as seen here, was a common occurrence in unprotected flaps.

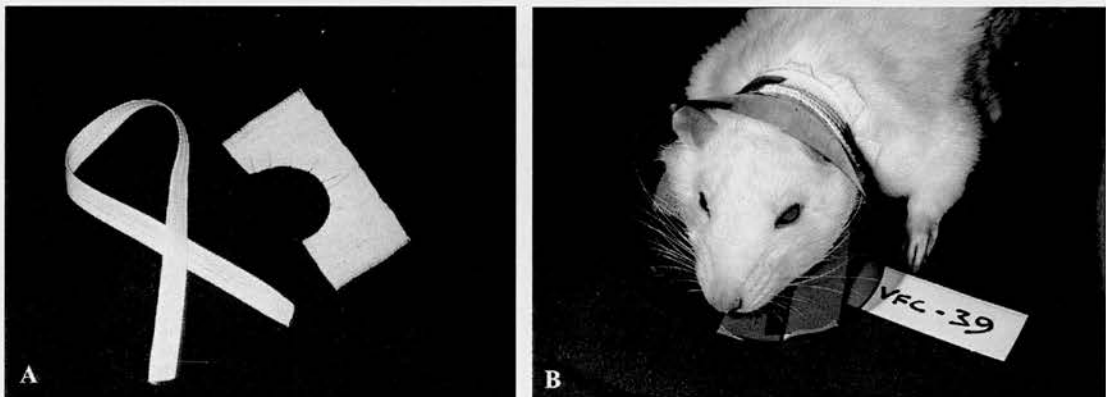


Figure 2.3 – Protective collar (A) materials and (B) in place on a postoperative animal.

2.1.5 Data collection

2.1.5.1 Overview

Methods of data collection were chosen for their ability to provide objective evidence on the extent to which flap vascularity had been influenced by therapeutic interventions. The techniques chosen were: (1) measurement of the area of the surviving portion of the flap after demarcation of the ischaemic portion, and an assessment of the surviving flap vascularity using (2) microangiography and (3) histological analysis. Only the first two systems will be considered here since the histological evaluation of experimental skin flaps forms the basis of the next chapter.

2.1.5.2 Measurement of flap areas

A number of important factors must be considered when selecting a technique for the measurement of skin flap areas. In particular, Bayramicli *et al.* highlighted the tendency for the flap to undergo contraction during the period of demarcation. In a series of 15 epigastric island flaps, they reported the mean final flap area to have reduced to 54% of the starting area when measured at one week (Bayramicli, Yilmaz, & Numanoglu 1998a). Therefore, measurements of surviving area expressed as a percentage of initial area will be misleading (Akyurek *et al.* 1999). A more representative value was obtained by Chow *et al.* by expressing flap survival as a percentage of the final flap area, once demarcation had taken place (Chow, Chen, & Gu 1993).

The method by which the area will be measured must also be considered. Published techniques include template based methods(Finseth F & Cutting C 1978;Roberts, Cohen, & Cook 1996), the use of area grids(Taub PJ *et al.* 1998), or rely on the capture of digital images with subsequent analysis performed by computer software(Nichter *et al.* 1984;Padubidri & Browne E Jr 1997).

The convexity of the rodent abdomen suggested to us that the use of digital images taken directly from the animal would introduce error as the three dimensional surface is compressed into a two dimensional image. This error would be most marked where the surface of the flap was at its most convex, i.e. the edges of the flap where the abdomen joins the flank, exactly the position where necrosis is expected. We therefore preferred the template technique proposed by Roberts (Roberts, Cohen, & Cook 1996). In addition, since the viable portion of the flap is known to undergo less contraction than the necrotic area, we elected to construct each template from the surviving area of the whole flap, thus giving us a figure for percentage flap survival rather than percentage flap necrosis (Bayramicli, Yilmaz, & Numanoglu 1998a). We also anticipated that the majority of the flap would survive and therefore the error incurred in the construction of the template would be less significant than for the smaller necrotic area. To minimise this error further, we elected to use multiple templates with the final analysis based on mean values for each series.

2.1.5.3 Microangiography

Accurate imaging of the vascular architecture of experimental flaps is made possible by the technique of microangiography where barium sulphate and lead oxide are the

most commonly used contrast agents (Quinodoz *et al.* 2002;Rees & Taylor 1986). The experimental flap may be infused directly with contrast agent through the feeding artery, or alternatively, a central artery may be cannulated and the entire arterial system imaged including that of the flap. Both methods achieve acceptable results (Nishikawa, Manek, & Green 1991;Padubidri *et al.* 1997). The use of mammography film is preferred in preference to standard X-ray film when performing the angiogram since it provides for improved vascular definition and is a further refinement in the technique (Quinodoz *et al.* 2002).

Various techniques have been proposed to extract objective data relating to flap vascularity from such angiograms. In their work investigating an anatomical basis for the delay effect, Cederna *et al.* performed angiograms of abdominal cutaneous island flaps in a series of thirty rabbits and counted the number of vessels, over 0.5mm, crossing the midline (Cederna *et al.* 1997). Takeshita *et al.*, also working with rabbits, preferred the use of an angiographic score calculated from vessel intersections with a standard grid (Takeshita *et al.* 1994c). Since I was most interested in detecting differences in overall numbers of blood vessels, I considered the technique of counting the number of vascular branch points in a given area to be most suited to my work (Cheng, Chan, & Wu 2001). All such techniques however, rely on consistent filling of the vascular network during angiography, if meaningful comparisons are to be made.

With particular reference to the rat epigastric island flap, angiographic data reported in previous studies often takes the form of a subjective assessment of vascularity if

reported at all (Akita *et al.* 2002). Similarly, a search of the literature found no reference to the angiographic appearance of normal abdominal rat skin.

2.1.6 Aims

The aim of these experiments was to evaluate the rat epigastric island flap as a model of ischaemic flap necrosis and to characterise the physiological changes in vascularity that occur within the flap over an experimental period of one week by means of microangiography.

2.2 Materials and Methods

2.2.1 Animals

Sixteen male Sprague-Dawley rats, weighing between 355 and 455g were used in this project. Animals were supplied from, and housed at, the animal house facility of the University of New South Wales based at Prince Henry Hospital, Little Bay, New South Wales, Australia. The 'Australian code of practice for the care and use of animals for scientific purposes' was closely followed for all experiments. The procedures were undertaken in an operating theatre equipped for the task and all instruments and disposables were used sterile. The Animal Care and Ethics Committee of the University of New South Wales granted full ethical approval for this work.

2.2.2 Experimental groups

Two experimental groups were created comprising eight rats each. The first group would provide data on the native vascularity of the flap whilst data from the second group would provide information on the extent and consistency of flap necrosis and any accompanying changes in flap vascularity resulting from normal physiological processes. Identical epigastric island flaps were planned and raised in all animals.

2.2.2.1 Group 1 (n=8)

In this group angiography was performed prior to flap elevation. The animals were killed while under anaesthesia using 1ml intracardiac Lethobarb (Arnolds Pty Ltd, Baronia, Victoria, Australia). The flaps were then excised and subjected to data collection as described below.

2.2.2.2 Group 2 (n=8)

These animals were allowed to recover from anaesthesia following surgery and observed for one week to permit demarcation to occur between the viable and non-viable areas of the flap. Before reversal of anaesthesia they were fitted with plastic collars to prevent autocannibalism

All rats underwent elevation of the experimental flap in the same manner.

2.2.3 Surgical procedure

General anaesthesia was achieved using 2.7ml/kg of a solution comprising hypnorm (0.315 mg/ml fentanyl and 10 mg/ml fluanisone, Janssen-Cilag) 1 part; midazolam 5

mg/ml (Roche Products Ltd) 1 part and water for injection 2 parts, administered intraperitoneally. Halothane 2-3% with oxygen (2 l/min) was given via facemask if backup anaesthesia became necessary. The abdomen was shaved and the outline of the proposed 8 x 8 cm flap marked on the skin. The operative field was then painted with aqueous iodine solution and protected with a perforated sterile drape. All operations were performed under loupe magnification (x3.5).

All flaps were raised on the left superficial inferior epigastric artery and vein. The dissection began cephalad and moved caudally. The distal ends of the right and left SIEA's were first identified leaving the flap and coagulated with bipolar diathermy and divided. The multiple musculocutaneous perforating vessels encountered entering the deep surface of the flap were similarly dealt with as the flap was separated from the abdominal muscles. The right superficial inferior epigastric neurovascular bundle was then identified, ligated and divided. The dissection finished with the completion of the skin incisions in the left groin and identification of the flap pedicle. No attempt was made to skeletalise the vascular pedicle and the associated nerve was left intact. On completion, each flap was islanded entirely on a single neurovascular bundle in the left groin with all other soft tissue connections having been divided. The flap was then returned to its bed and secured with a continuous 4/0 prolene suture (Ethicon) across the base to avoid damage to the pedicle and skin clips (Autosuture) to the remaining edges (Fig 2.4).

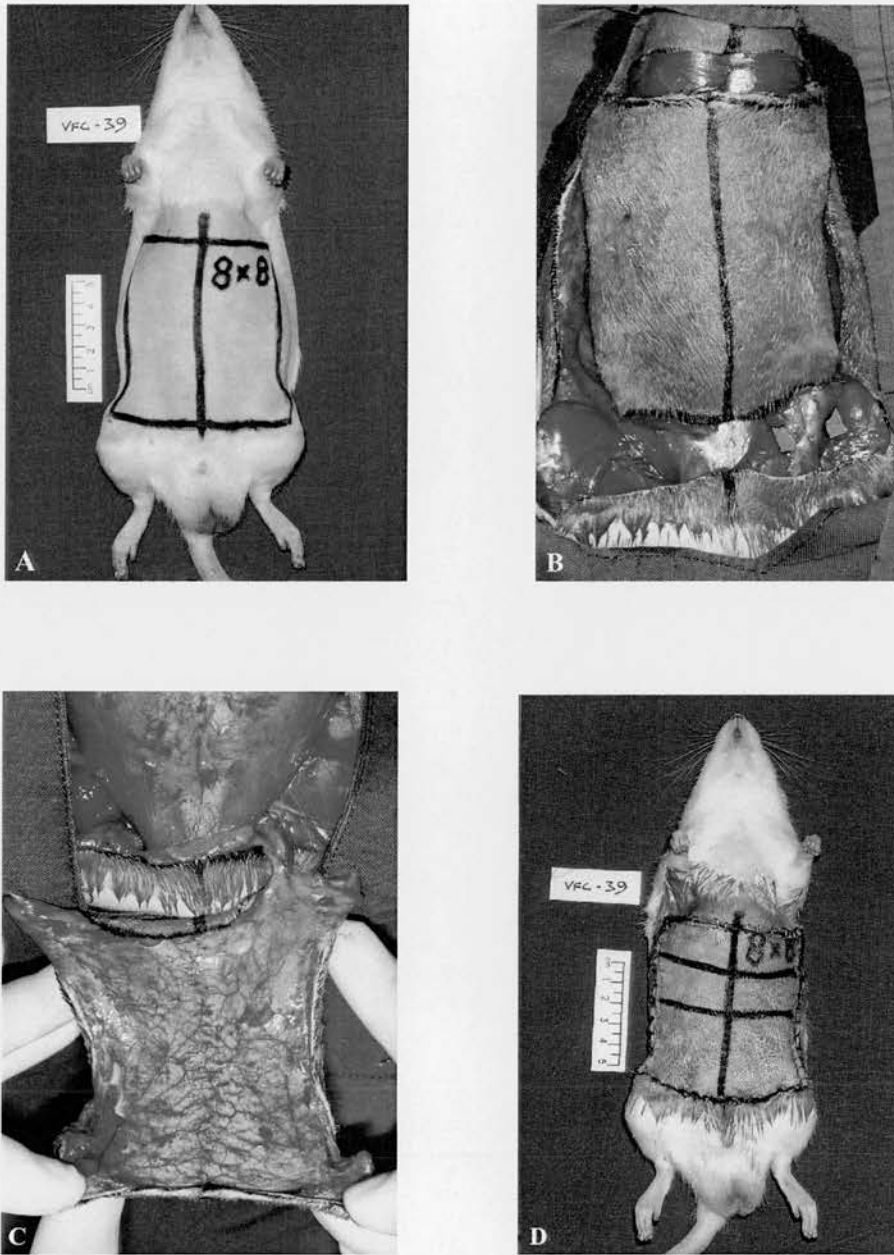


Figure 2.4 – The surgical procedure. (A) The outline of the proposed flap is marked on the abdominal skin. (B) and (C) Intra-operative views of a typical epigastric island flap. The left superficial inferior epigastric vascular pedicle is seen highlighted by a blue background. (D) An example of the experimental flap on completion of surgery. The transverse lines mark the positions where histological sections will be taken (see Chapter 3).

Analgesia (Buprenorphine (Reckitt & Colman) 0.05 mg/kg) and fluid (5ml normal saline) were given at a site away from the flap on the animals back. Anaesthesia was then reversed using subcutaneous naloxone (F.H. Faulding) at a dose of 500 µg/kg.

2.2.4 Immediate postoperative management

Animals were recovered in cleaned rat cages containing fresh bedding (wood shavings) and drinking water. Once fully recovered they were provided with food pellets and transferred to the postoperative rat room adjacent to the operating theatre. Each was housed individually and all animals were reviewed at the end of the days operating and examined for postoperative complications and signs of distress.

2.2.5 Daily postoperative management

All animals were reviewed daily and assessed in terms of their appearance, behaviour, appetite and water intake. The wound was examined for evidence of infection, dehiscence, demarcation and autocannibalism. All data were recorded on postoperative observation charts (Fig. 2.5).

2.2.6 Data collection

The animals were killed humanely on day seven postoperatively in a carbon dioxide euthanasia chamber. The skin clips were removed and the outline of the flap was traced onto clear exposed x-ray film to produce accurate templates (Fig 2.6). Three tracings were taken of each flap. A further tracing was made and put to one side

THE ST. GEORGE HOSPITAL
DEPARTMENT OF PLASTIC AND RECONSTRUCTIVE SURGERY
ANGIOGENESIS TRIAL

Operative Record			
Date:	Animal Identification:	Animal Weight:	Operative Time: start: finish:
PROCEDURE	VENTRAL FLAP 8X8CM	BASED ON	LEFT SIEA RIGHT SIEA
Wound Closure:			
COMPLICATIONS			
Anaesthetic:			
Surgical:			
NOTES:			

Postoperative Record								
OP	DRUGS	APPEARANCE	BEHAVIOUR	APPETITE	WATER INTAKE	CLINICAL ASSESSMENT	WOUND	
							HEALING	DEMARICATION
DAY 1								
DAY 2								
DAY 3								
DAY 4								
DAY 5								
DAY 6								
DAY 7								

Comments:

Figure 2.5 – Postoperative record. Each animal was examined daily and the data recorded.

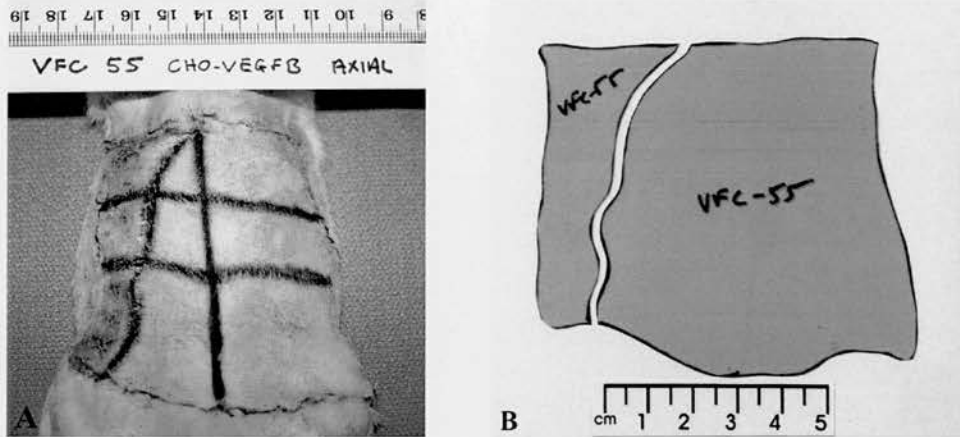


Figure 2.6 –Examples of (A) a typical epigastric island flap at one week showing partial necrosis of the random pattern component and (B) a template drawn from the flap at the time of data collection for use in the calculation of percentage flap survival.

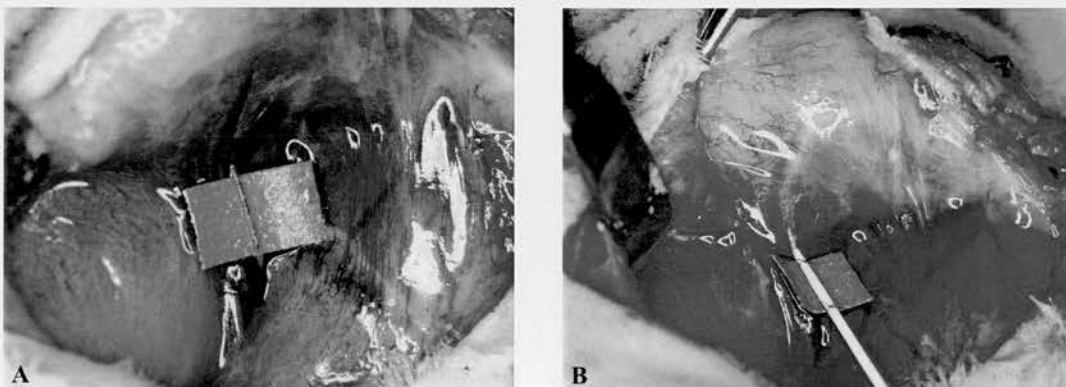


Figure 2.7 – Cannulation of the superficial inferior epigastric artery. In (A) the left groin has been opened and the SIEA identified and prepared prior to (B) cannulation and infusion with barium solution. Two 7/0 prolene ligatures have been used to secure the cannula in place.

Microangiography was then performed. Initial experience with the model confirmed direct cannulation of the SIEA in the groin as the optimal route for infusion of contrast medium into the flaps. This technique proved more reliable and provided for more control than central cannulation of the arterial system via the carotid artery in the neck and was therefore adopted in all experiments.

The left groin was opened outside the flap margin and the SIEA identified at its origin from the femoral artery. Using an operating microscope, the SIEA was separated from the accompanying vein and dissected to expose the vessel wall over a length of approximately 1 cm. Lignocaine 1% was applied as necessary to abolish vasospasm.

A suitable point to perform a proximal arteriotomy was identified and the adventitia at this point excised. A transverse arteriotomy was then performed. The vessel opening was gently dilated to allow cannulation with a 24 gauge intravenous catheter (Terumo Medical), which was advanced and secured in place using two 7/0 prolene ligatures. The cannula was flushed with 0.5ml saline to ensure freedom of flow and the absence of leaks. The flap was then infused with 0.2ml of barium sulphate contrast medium (Liquid C) (Fig 2.7). A single 7/0 prolene ligature placed distal to the tip of the catheter prevented escape of contrast material following removal of the cannula.

The experimental flap was then excised taking a margin of surrounding tissue and washed in clean water. To restore the in vivo dimensions, each flap was then pinned

out on the previously prepared plastic template drawn from the flap whilst *in-situ* on the abdominal wall. A length of fuse wire was secured to the template, passing across the flap at the point of demarcation between viable and non-viable skin, allowing visualisation of this zone radiologically (Fig 2.8). The specimen was transferred to 10% formalin solution prior to transfer.

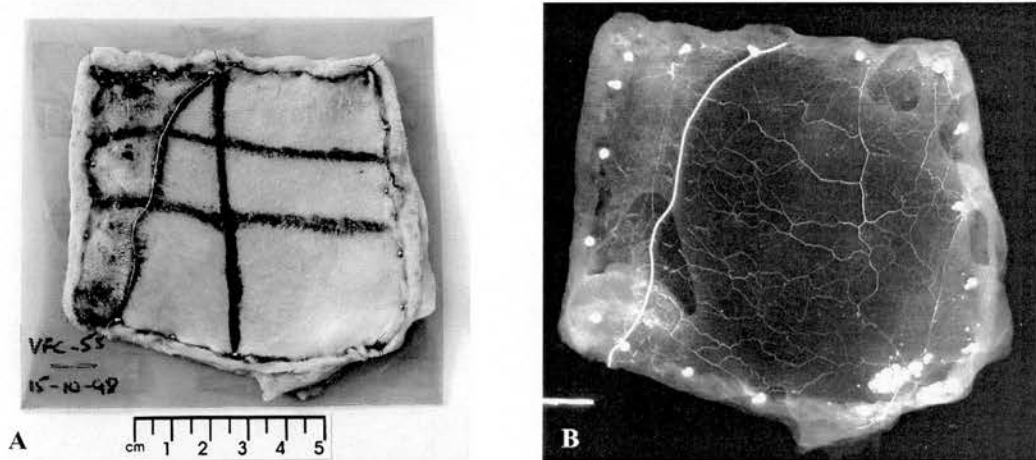


Figure 2.8 – (A) Pinning to a plastic template prior to imaging restored the *in vivo* dimensions of each flap. A wire marker indicates the line of demarcation. The resulting angiogram is shown in (B). This specimen is unusual in that a number of vascular markings are seen within the necrotic skin.

2.2.7 Radiology

All angiograms were exposed using the Giotto Mammography Unit housed at The St George Hospital, Kogarah. I am grateful for the help of Ashif, senior radiographer in the department of radiology who operated the machine in all cases. Kodak Min R 2000 film was loaded into a Kodak Min-R2 cassette with C-1N window and min-R screen. Following a series of trial settings the optimal exposure was achieved with the machine set at 24 kV and 4 mAs with a non-grid bucky. All flaps were imaged in this way.

2.2.8 Data processing

2.2.8.1 Percentage flap survival

Flap templates were drawn from exposed x-ray film in all cases. Prior experiments had confirmed that this material was of an acceptably uniform weight –area ratio to be used for this purpose (Appendix 1). Percentage flap survival was calculated for each animal using flap template weights according to the formula:

$$\text{Percentage flap survival} = \frac{\text{weight of surviving-flap template}}{\text{weight of total-flap template}}$$

All weights were recorded from a single analytical balance accurate to two decimal places. Results were expressed as percentage survival of the whole flap with respect to the final flap area at the time of angiography.

2.2.8.2 Angiographic evaluation

Highest branch point counts per square centimetre (HBPC/cm²) were obtained from microangiograms in the following way: Each angiogram was placed flat on a standard photographic light box and viewed with a photographic loupe (x4 magnification, Nikon). A screen made from black card containing a central aperture cut to one square centimetre was used to define the area to be analysed. The three areas judged to be of highest vascularity for each hemiflap (random pattern and axial pattern) were identified and the number of clear, vessel branch points per square centimetre counted for each hemiflap in each of the three separate locations. The highest of these values gave the final result for each hemiflap for a given animal. The calculation of mean values for an experimental group (mean HBPC/cm²) allowed statistical comparisons to be made.

2.3 Statistics

Confidence intervals are presented at the 5% and 95% levels. Differences in the angiographic scores between the two groups were analysed using the Mann-Whitney U test, $p < 0.05$ was considered to represent statistical significance.

2.4 Results

2.4.1 General

There were no intra-operative complications in either group. All animals in experimental group 2 recovered from anaesthesia without event and remained well

during the study period. There were no incidences of autocannibalism or of flap infection however, one rat required replacement of the plastic collar on the first postoperative day and a further rat required resuturing of a minor dehiscence of the left lateral aspect of the flap on day 2. Both of these procedures were performed under general anaesthesia (Halothane 3 % with 2 l/min oxygen). All animals survived to seven days.

Mean operating time was 55 minutes. The earliest recorded evidence of demarcation between the viable area of the flap and the ischaemic area, detected as a discolouration of the involved skin, was on day two of the follow up period. This was noted in five of the eight flaps in group 2. In all flaps, the zone of demarcation was clearly visible by the third postoperative day.

2.4.2 Flap survival

The flap survival data for each animal in group 2 are given in Table 2.1. The mean flap survival for this group was 85.0 % (C.I. 81.4, 88.6) at one week. The mean area of the flaps had decreased to 78.8 % (C.I. 75.6, 82.1) of the starting area by the seventh postoperative day.

Table 2.1 – Survival of rat islanded epigastric flaps expressed as a percentage of final flap area at seven days.

<i>Animal reference</i>	<i>Animal weight (g)</i>	<i>W1 (g)</i>	<i>W2 (g)</i>	<i>Flap survival (%)</i>
P1	420	1.25	1.05	84
P2	450	1.22	1.01	83
P3	415	1.29	1.19	92
P4	455	1.23	1.01	82
P5	430	1.42	1.29	91
P6	420	1.31	1.11	85
P7	440	1.27	1.06	83
P8	355	1.29	1.03	80
			Mean	85.0
			95 % C.I.	81.4, 88.6

W1=Mean weight of 3 templates taken of the whole flap

W2=Mean weight of 3 templates taken of the surviving flap

2.4.3 Microangiography

Typical angiograms for both groups are given in Fig. 2.9 & 2.10. The gross vascular patterns were markedly different. In the flaps subjected to angiography immediately after elevation (group 1), the vascular markings appeared much finer and very little contrast medium was seen to cross the midline. Where this did occur it was most commonly noted very proximal in the flap (3 of 5 flaps) (Fig. 2.9). In angiograms performed on flaps at one week vascular markings were more substantial than in the immediate harvest group consistent with dilation of the feeding vessels and in all cases contrast medium was seen to travel freely across the midline. In addition, an area containing increased vascular markings was consistently seen adjacent to the zone of demarcation indicated by the wire marker and to a lesser extent around the perimeter of the viable flap (Fig 2.10).

The highest branch point counts (HBPC) for each experimental group were collated and mean values calculated. The mean of the highest branch point counts for the axial pattern hemiflaps in group 1 (n=8) was 11.1 branch points/cm² (C.I. 9.1, 13.2), whilst in group 2 (n=8) it had risen to 16.9 branch points/cm² (C.I. 13.8, 19.9) and this difference was statistically significant (p<0.01) (Figure 2.11). Imaging of the random pattern hemiflap was uniformly poor in group 1 flaps as noted above and not suitable for further analysis. In group 2, the mean HBPC for the random pattern hemiflaps was 16.1 branch points/cm² (C.I. 13.1, 19.2). No significant difference existed between mean HBPC recorded from the axial and random pattern hemiflaps in group 2 animals (p=0.56) i.e. the vascularity, by this measure, was the same on either side of the mid-line.

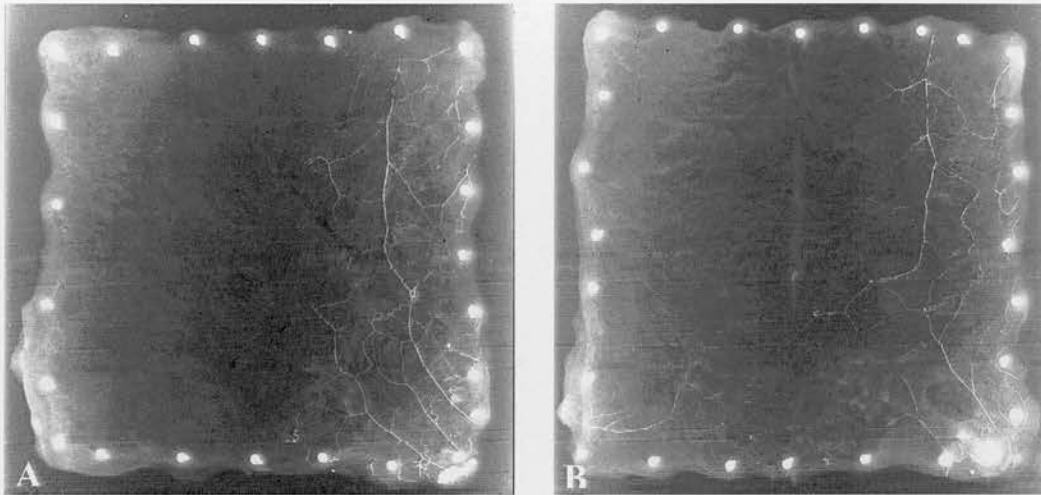


Figure 2.9 - (A) Typical angiogram obtained from group 1 flaps performed immediately following elevation. On the occasions where contrast medium was observed crossing the midline this invariably occurred proximally as seen in **(B)**.

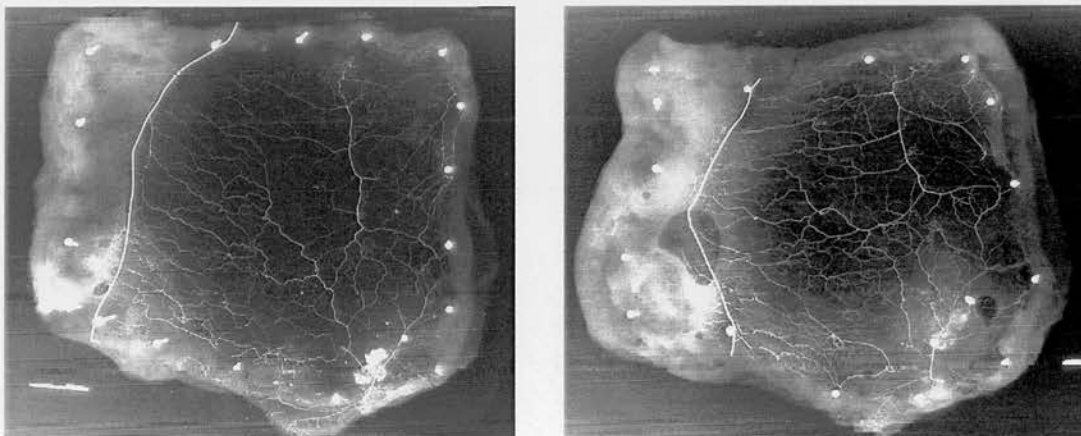


Figure 2.10 – Typical angiograms obtained from group 2 flaps performed at one week. These angiograms consistently exhibited increased vascular markings within the zone of demarcation indicated by the wire marker and in all cases contrast medium was seen to cross the midline.

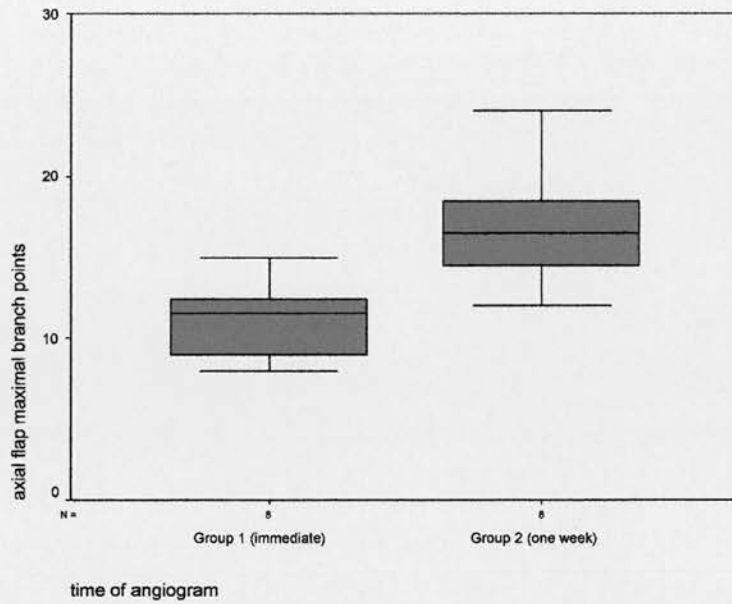


Figure 2.11 – Boxplot of highest angiographic branch point count recorded from the axial pattern flaps of group 1 (immediate flap angiography) and group 2 (angiography at 1 week) animals. The difference between the means was statistically significant ($p < 0.01$).

2.5 Discussion

We were encouraged by these results. The 95 % confidence interval for our flap survival data was narrow (7.2 %), suggesting the extent of flap survival, and therefore necrosis, was likely to be reproducible and predictable. The techniques employed to raise the flap, monitor the animals and retrieve data appeared efficient and effective and well tolerated by the rats.

Comparisons with other reported experimental series of the rat epigastric island flaps are made difficult by the variability both in the size of flaps used, and in the way in

which the results are reported. In their original series of 10, 9x9cm flaps, Finseth and Cutting measured only the area of the flap that survived across the midline. To do this they used single paper templates weighed against an area-weight standard. By this method they reported the area of surviving flap across the midline to be $10.4 \pm 6.2 \text{ cm}^2$ at seven days. The same figure when calculated for my series was $17.7 \pm 2.0 \text{ cm}^2$ (Appendix 2). To perform this calculation however, requires an assumption that flap contraction is uniform, which we know not to be the case (Bayramicli, Yilmaz, & Numanoglu 1998a) and an increased contraction of the necrotic right hemiflap is the most likely explanation for the observed differences in flap survival between the two series. Of note also is the higher variability around the mean seen in the Finseth and Cutting series compared to the present one.

Other reported series providing data on the survival of rat epigastric island flaps where both the medial and lateral branches of the SIEA have been retained, are given in Table 1.3. My results are in broad agreement with these findings.

Table 2.2 - Published series of rat epigastric island flap survival.

Reference	Skin paddle dimensions	Flap survival
Sasaki <i>et al.</i> 1980	8x8 cm	76.7 %
Roberts <i>et al.</i> 1995	anatomical landmarks, 7 cm width	81.2 %
Bayramicli <i>et al.</i> 1998	anatomical landmarks used	75.4 %

The extent to which our flaps underwent contraction (final area reduced to on average, 78.8 % of starting area at one week) was however, significantly less than that reported by Bayramicli (flap area reduced to 54.12 % of starting area) (Bayramicli, Yilmaz, & Numanoglu 1998a). The reasons for this discordance are not apparent.

The striking difference between the angiographic appearance of the native abdominal rat skin and the that of the flap at one week, is in keeping with Taylor's angiosome model of the vascular territories of the body, each being separated by a series of choke vessels from its neighbour (Taylor & Palmer 1987). The presence of these choke vessels along the ventral mid line of our flap is evidenced by the lack of contrast medium crossing the midline and gaining access to the right hemiflap in group 1. When contrast was seen to have passed in to the right side, it was most frequent proximally, probably reflecting a higher luminal pressure generated by the syringe at more proximal branches of the main artery and overcoming the resistance of the choke vessels.

The opening of choke vessels to permit the passage of blood from one angiosome to another under the influence of an ischaemic insult formed part of Taylor's original model and has subsequently been confirmed by others (Cederna *et al.* 1997; Taylor & Palmer 1987). This phenomenon is well demonstrated in the present series.

Fewer reports however, objectively describe the angiographic changes that accompany these events, and none have done so for a rat model as far as I am aware.

Cederna *et al.* found an increase in overall vessel numbers in delayed rabbit epigastric island flaps, but noted this did not reach statistical significance until day 21 following the delay procedure (Cederna *et al.* 1997). Our results in the rat show a significant difference by day seven. Comparisons are difficult since the flap design and choice of animal model are different however, one explanation for these differences may be the more severely ischaemic conditions present in our model (designed to produce tissue necrosis), a condition absent from the rabbit model.

In considering these results however, I recognise the limitations inherent within the angiographic data. The increased numbers of vessels imaged in group 2 flaps cannot be considered a definitive reflection of angiogenesis since the same number of vessels may have been present in group 1 flaps where, in the 'resting' state their small calibre placed them beyond the imaging resolution of the angiographic technique. The differences in angiographic appearances may therefore be accounted for to some degree by changes in flow characteristics rather than new vessel formation. The area where the angiographic appearances are most likely to represent true angiogenesis is within the zone of demarcation where high densities of fine vessels are consistently observed in group 2 angiograms, appearances which are not seen elsewhere within the body of the flap. Thus although angiography is a useful guide to the gross vascular changes occurring within the flaps, definitive evidence of angiogenesis requires a more sensitive method of data collection.

2.6 Conclusions

1. The rat epigastric island flap, as first described by Finseth and Cutting and later modified by Padubidri and Brown, provides a reliable and reproducible model of tissue ischaemia.
2. The techniques employed to raise the experimental flap, monitor the animal in the postoperative period and collect relevant data, proved successful.
3. Analysis of microangiograms performed on day 0 confirmed the existence of a vascular watershed in the mid line. This watershed was not apparent on day 7. This change in the dynamics of blood flow was accompanied by a significant increase in the number of vessel branch points identified angiographically on both sides of the flap. This finding is consistent with, but not conclusive evidence of, angiogenesis occurring within the flap.

Chapter 3 – The assessment of average microvascular density in rat epigastric skin

3.1 Introduction

3.1.1 Overview

To detect evidence of induced angiogenesis in my experimental flaps I needed a reliable method to accurately quantify changes in their vascularity. The development of a quantitative technique to achieve this was therefore, central to my experimental work. Although some published work exists describing techniques that have been employed in other models of ischaemia, reports using the epigastric island flap model have tended to comment on an observed change in vascularity without objective measurement (Ishiguro *et al.* 1994; Padubidri A & Browne E 1996). Similarly, no published data was available regarding the native vascularity of abdominal rat skin.

One suitable method to achieve an objective analysis of vascularity is the assessment of microvascular density (MVD) (Gowdak *et al.* 2000; Walter *et al.* 1996).

Microvessel quantification in this way was first described by researchers interested in tumour angiogenesis and has led to the development of internationally recognised guidelines for its use in the histological assessment of solid tumours (Vermeulen *et al.* 2002; Weidner *et al.* 1991).

To obtain a measure of microvascular density, counts of microvessels are performed on paraffin embedded tissue or frozen sections and the results expressed either per unit area or by the use of a grading system (Pazouki *et al.* 1997; Saclarides *et al.* 1994). Microvascular density may be reported either in terms of the highest value for the sample examined, highest microvascular density (h-MVD), most commonly used in the evaluation of malignant tumours, or as an average figure more representative of the section as a whole, average microvascular density (a-MVD) (Bosari *et al.* 1992; Chandrachud *et al.* 1997). In the case of the former, the tissue section to be analysed is first examined under low magnification (x40) to identify areas of maximal vascularity, often referred to as vascular hotspots, and vessel counts are then performed in these areas using a higher magnification (e.g. x200). The highest of these counts provides the final result (Weidner *et al.* 1991). In the assessment of a-MVD, the slide may first be orientated under low magnification before converting to high power and performing a series of microvessel counts at random positions throughout the specimen. The number of such counts performed varies between authors but typically 8 – 10 counts will be undertaken and the final result expressed as the sample mean (Chandrachud *et al.* 1997).

The practicalities of this technique most frequently involve the manual counting of blood vessels under a microscope. An eyepiece graticule is commonly used to place a grid over the section under review, allowing the counts to be performed in a systematic manner across the area of interest (Obermair *et al.* 1997). An alternative technique is to use an eyepiece containing a series of points instead of a grid, and count the number of stained vessels that coincide with these points (Chandrachud *et*

al. 1997). This is known as Chalkley point overlap morphometric analysis, and provides a relative area estimate rather than a true vessel count (Vermeulen *et al.* 2002). Vascular area measured in this way has been shown to correlate well with microvascular density expressed in terms of vessel numbers (Pendleton *et al.* 1998).

In an attempt to automate the counting process, computer programs have been developed to perform the same task using digitally captured images (Rendell *et al.* 1998). This technique is best suited to the analysis of intersection points, as is the case when performing Chalkley counts, or in analysing images of intact microvascular networks, as can be provided by fluorescent labelled 'whole mount' tissue preparations (Fox *et al.* 1995). In the latter case, the number of intersections that occur between the vascular network and a superimposed grid are counted (Rieder, Roman, & Greene 1997). This technique also correlates well with manual counting (Rieder, O'Drobinak, & Greene 1995).

To be successful however, any technique designed to quantify the vascularity of a tissue sample is heavily reliant on the ability of the process to accurately identify the vessels to be counted. The use of an appropriate histological stain is helpful in this respect. Furthermore, Schor *et al.* studied the relationship between methodology and outcome in the assessment of microvascular density for a series of solid tumours and in normal breast tissue. They identified the chosen staining protocol as the single factor most likely to introduce variability in the results (Schor *et al.* 1998).

Immunohistochemical staining is the most commonly used technique for the identification of blood vessels prior to counting (Weidner 1995). In this technique, specific antibodies are raised against known vessel-wall antigens. The resulting antibody-antigen complexes are then identified by an appropriate detection system allowing a positive result to be recorded. Such detection systems may incorporate a fluorescent marker for example, or induce a local colour change. Heyderman describes the technique in more detail as:

An immunological method which may be used for the demonstration of various substances in tissue sections and utilises labelled or unlabelled antibodies and the very stable enzyme, horseradish peroxidase. The most widely used substrate, diaminobenzidine, polymerises in the presence of peroxidase and hydrogen peroxide to form an insoluble brown polymer which is deposited at the site of antigen-antibody reaction.

Heyderman (1979)

There are several methods by which this technology may be employed however, only the indirect method is of relevance here (Heyderman 1979). This technique involves two antibodies, the primary antibody directed against the target antigen and a secondary antibody, conjugated with horseradish peroxidase, directed against the primary antibody. The peroxidase acts on 3,3'-diaminobenzidine (DAB) substrate to produce the typical brown colour indicating a positive result when viewed on light microscopy (Fig 3.1).

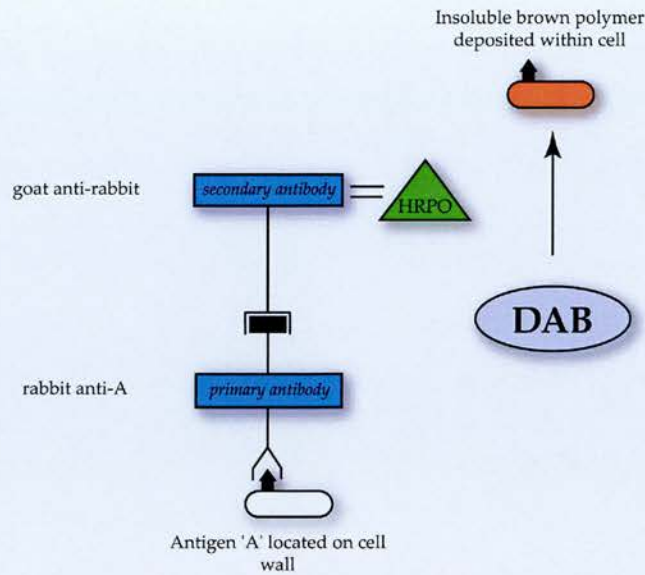


Figure 3.1 – Schematic diagram illustrating the principles of the immunoperoxidase stain technique where rabbit and goat antibodies are given as examples (HRPO: horse radish peroxidase, DAB: 3,3'-diaminobenzidine).

Many antigens may be targeted in this way, with vast arrays of complementary primary antibodies now available commercially (www.dako.co.uk). Target antigens useful in the identification of small blood vessels are usually located on the wall of endothelial cells, for example CD31, CD34, $\alpha_v\beta_3$ integrin, and von Willebrand factor have all been used successfully whilst many others are also recognised (Khan *et al.* 2002; Lindmark *et al.* 1996; Pazouki *et al.* 1997).

A further important factor in the planning of any immunohistochemical protocol is a consideration of the effects fixation may have on the antigen-antibody reaction (Montero 2003). In our experiments the tissue will be fixed using standard solutions of 10% formaldehyde in phosphate buffered saline. The binding of formaldehyde to the tissues, in addition to preservation, may also result in a degree of 'masking' of

antigen localisation sites, most likely as a result of cross-linking between elements of the antigen macromolecule (Ezaki 1996).

Formalin fixed tissue is therefore, often pre-treated before proceeding to immunohistochemical staining in an attempt to reverse this process (Cattoretti *et al.* 1993). Enzymatic or non-enzymatic techniques may be used. Both techniques aim to destroy the acquired cross-linkages so exposing the antigen binding site and permitting antibody localisation (Ezaki 1996). The use of proteolytic enzymes such as trypsin and protease constitute the former whereas in the non-enzymatic technique, referred to as antigen retrieval, heat may be used to achieve a similar effect, as first reported by Shi *et al.* in 1991 (Shi, Key, & Kalra 1991).

Ezaki *et al.*, in their review of these techniques, make a number of important points (Ezaki 1996). Firstly, the time with which the tissue is exposed to the fixative should be kept to a minimum to increase the likelihood of successful unmasking, secondly, increasing attempts at antigen retrieval by increasing the temperature and/or prolonging the time of heat exposure, are likely to result in a variable degree of unwanted, non-specific background staining. In addition, false positives may be observed making the interpretation of results difficult. In summary he concluded 'each antigen requires a "tailor-made" tissue preparation for optimal preservation of its antigenicity and precise localization.' My experiences in the use of this technique with my own tissue preparations concur entirely.

A further important consideration in the proposed use of immunohistochemical staining techniques for these experiments was that our specimens would be of animal and not human origin. Although not their intended use, anti-human antibodies have been successfully used for the identification of vascular endothelium in animal tissue (Ray *et al.* 2000). Two important factors however, must be considered in this situation. Firstly, the primary antibody is raised against a human antigen, and although a degree of cross-reactivity with the animal counterpart is common, this will vary dependant both on the target antigen chosen and the animal species in question. Species specific antibodies, which are increasingly now available, are therefore recommended (Azimzadeh *et al.* 1998). At the time this work was conducted however, no species specific endothelial stain for rats was available to us.

Secondly, from our own experience of the technique, a degree of cross-reactivity may be encountered between the primary and / or the secondary antibody and other, non specific antigens in the field resulting in a misleading level of background staining making interpretation difficult. This problem may be eased to a degree by the use of an affinity isolated secondary antibody, designed to be more specific to the binding site of the primary antibody and therefore reducing the incidence of background staining (Martinez-Hernandez *et al.* 1975).

If appropriate consideration be given to these technical aspects then the staining of animal preparations using antibodies raised against human antigens is likely to be successful and of significant value in the completion of microvascular counts for research purposes (Chichester *et al.* 2001).

In relation to our experimental model of tissue ischaemia, an important feature in the planning of a suitable technique for the assessment of MVD was that it should be comparable between animals. In this respect the counts should be performed in specific and well-defined locations. Similarly, we wished to analyse the vascularity of the flap in a generalised manner, to reduce the influence of any localised changes, and therefore we chose to measure average-MVD as offering the most useful assessment in this respect.

3.1.2 Aims

The aim of this study was to evaluate the use of immunohistochemical staining in formalin fixed, paraffin embedded rat tissue, as an aid to the assessment of microvascular density and apply this technology to an analysis of rat skin in the epigastric island flap model of tissue ischaemia.

3.2 Materials and Methods

Immunohistochemical preparations were performed with the help and advice of Ms P. Marr, Department of Anatomical Pathology, The St George Hospital, Sydney, Australia, to whom I am very grateful.

3.2.1 Antibodies

All antibodies were from Dako (Botany, NSW, Australia). The following panel of primary antibodies were evaluated, chosen on the basis of their successful use in microvascular analysis in humans: monoclonal mouse anti-human von Willebrand

factor (product code: M 0616), polyclonal rabbit anti-human von Willebrand factor (product code: A 0082) and monoclonal mouse anti-human CD31 (product ref: M 0823) (Lindmark *et al.* 1996). Biotinylated rabbit anti-mouse (product code: E 0354) and goat anti-rabbit (product code: E 0432) secondary antibodies were used accordingly.

3.2.2 Reagents

Bacterial protease type XXIV, 3,3'-diaminobenzidine (DAB) and TRIS buffer was from Sigma-Aldrich (Castle Hill, NSW, Australia). Streptavidin peroxidase label (product ref: 493000) was from Immunon (NSW). Xylene and ethanol were from Ajax Cehmicals, NSW, Australia. Imidazol was from Fluka (Australia). Skimmed milk powder was bought locally. Bovine serum albumin was from Life Technologies (New York, USA). Slides and cover slips were from Menzel Glaser (Germany). Harris' haematoxylin, deionised water, slide racks, glasswear, humidifier box and an oven were all available in the imunohistochemical laboratory at The St George Hospital. The microscope and eyepiece screen used was by Olympus.

3.2.3 Solutions

TRIS buffered saline: Dissolve 6 g TRIS (Sigma-Aldrich) in 1000 ml
normal saline and adjust to pH 7.6 using 10M HCl.
Final solution 0.06 mg/ml.

Endogenous peroxidase block: Add 20 ml hydrogen peroxide (30% w/v) to 180ml methanol (100%). Store at 4°C for a maximum of 2 days. Final solution 3% w/v hydrogen peroxide.

Non-specific block: Measure 200 ml TRIS buffer (pH 7.6) into a flask and add 6 g skimmed milk powder. Stir until dissolved. The solution will last for 5 days. Final solution 30 mg/ml (3% w/v).

Antibody diluent: Add 0.5 g bovine serum albumin to 50 ml TRIS buffer (pH 7.6). Final solution 0.1 mg/ml.

Protease Solution: Measure 50 ml TRIS buffer (pH 7.6) into a flask and add 25 mg protease and mix well. This solution should be used at 37°C and may be warmed in a microwave. Final solution 0.5 mg/ml.

Diaminobenzadine reagent: Measure 50ml TRIS buffer (pH 7.6, room temperature) into a flask and add 0.03 g of 3,3'-diaminobenzadine. To this add 1ml of a 0.1M solution of imidazole and 15µl 30% hydrogen peroxide solution. Stir gently.

3.2.4 Tissues

Formalin fixed, paraffin embedded tissue sections from rats in experimental groups 1 and 2 (8 animals in each group) were used in these experiments (see Chapter 2).

Details of the methodology used in the preparation of the histological blocks are given in Appendix 3. Sections were inked to allow orientation such that black ink denoted the medial extent of the section whilst blue ink was lateral. Full width sections of each flap were taken at two positions and divided as shown in Fig. 3.2, providing four blocks for analysis from each flap. Serial sections (5 μm , microtome) were cut from the blocks and lifted onto standard glass microscopy slides.

Optimization of the process was performed on tissue from a single group 1 flap.

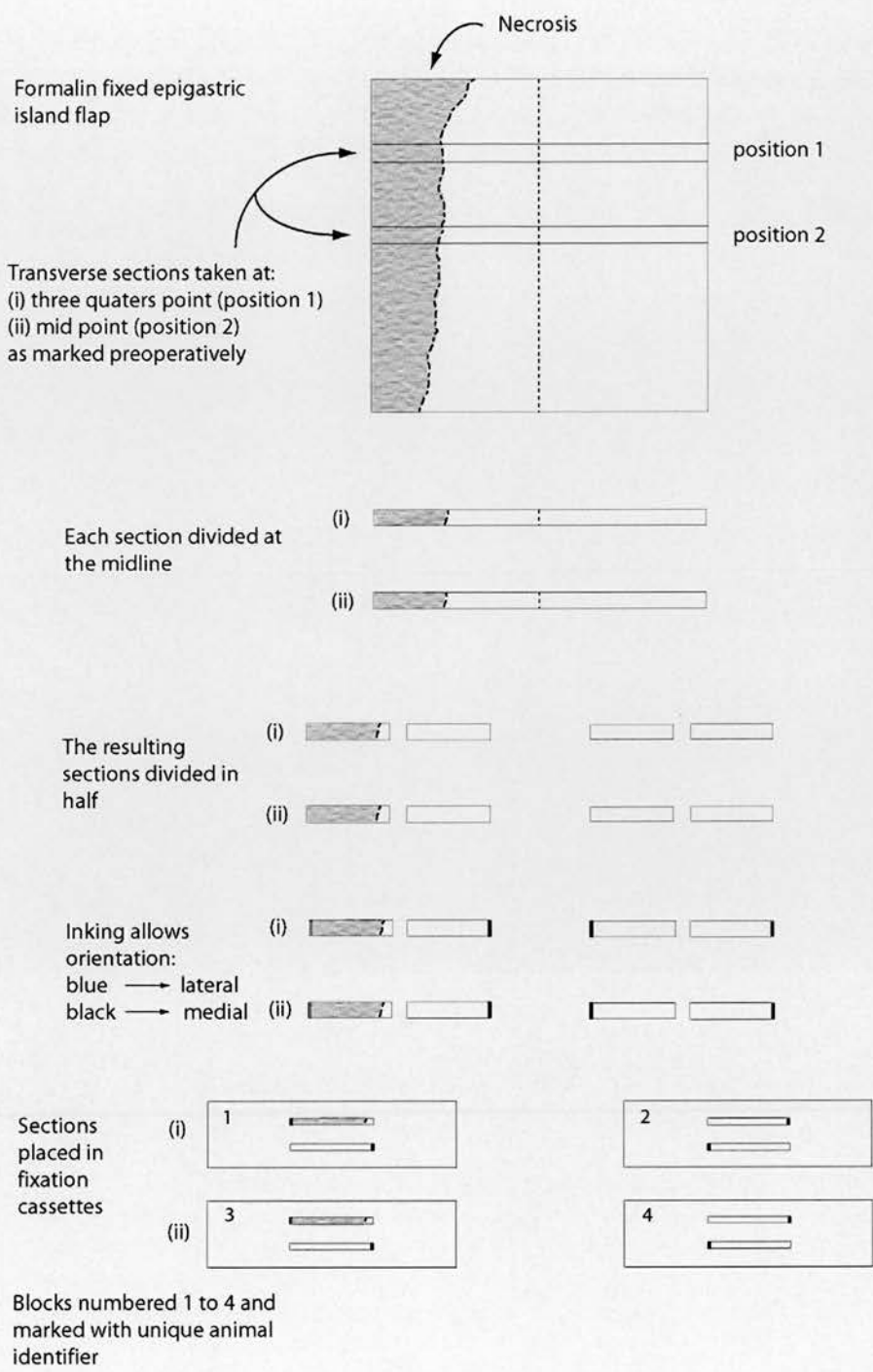


Figure 3.2 – Schematic diagram illustrating the technique devised for the preparation of histological sections from predetermined positions in rat epigastric island flaps. Transverse sections were taken at points three quarters of the way and half way along the flap (position 1 and position 2 respectively).

3.2.5 Immunohistochemistry

3.2.5.1 Pretreatment

Before immunostaining, the paraffin sections were dewaxed in xylene and processed to ethanol. Two techniques of antigen retrieval were evaluated. After washing in phosphate-buffered saline for 5 minutes, sections were submitted to either (i) microwave (750W) heating in 0.21% citric acid monohydrate for 10 minutes or (ii) treatment with 0.5 mg/ml protease type XXIV solution for 5 minutes at 37°C.

3.2.5.2 Immunostaining

Tissue sections were stained with each of the three trial antibodies (monoclonal mouse anti-human CD31, monoclonal mouse anti-human von Willebrand factor and polyclonal rabbit anti-human von Willebrand factor) in the following manner, based on standard procedure in our laboratory.

To block endogenous peroxidase activity, slides were treated with methanolic hydrogen peroxide (3% w/w hydrogen peroxide) for 10 minutes. Slides were rinsed in water and washed with PBS before transferring to non-specific binding block (3% skimmed milk in TRIS buffer) for a further 5 minutes. Following a further water rinse, tissue sections were localised with a PAP pen and an even coat of primary antibody applied at the highest concentration recommended by the manufacturer. Two primary antibody incubation times were evaluated. Slides were incubated at room temperature for either (i) 60 minutes or (ii) overnight. All specimens were given two further washes in PBS, 5 minutes each, incubated with the appropriate

secondary antibody for 30 minutes at the manufacturers recommended dilution and washed again in two changes of PBS. Staining was visualised using a streptavidin peroxidase label at a dilution of 1/150 applied evenly for 30 minutes followed by 2 washes with PBS and application of DAB reagent for 5 minutes. The slides were washed thoroughly in water and counterstained with Harris' Haematoxylin for 10 seconds, before dehydrating with absolute alcohol, clearing with xylene and transferring to an automated cover slip machine.

3.2.6 Quantification of a-MVD

Vessels were quantified by manual counting under light microscopy. A computer-assisted technique was evaluated but found to be unhelpful. An eyepiece incorporating a grid was fitted to the microscope to define the area to be counted. A countable vessel was defined as a cluster of positively staining endothelial cells of any size, with or without an identifiable lumen, clearly separated from neighbouring vessels defined similarly. Two regions of each flap were assessed, (i) the area adjacent to the zone of demarcation separating viable and non-viable tissue and (ii) the whole breadth of the flap. Each region was further subdivided into a superficial and deep plane separated by the *panniculus carnosus* muscle layer. Counts were performed for each of these regions and at both levels in the flap relative to the *panniculus*.

The assessment of a-MVD was performed as follows: Specimens were orientated under low magnification (x40). The *panniculus carnosus* was identified running through the specimen. The zone of demarcation was identified if present in the

specimen under review and the first series of counts performed at this point.

Magnification was increased to x400 and four sequential counts performed in the area bounded by the full grid square (0.0625 mm² at x400 magnification). For the first count the grid was positioned to abut the zone of demarcation. The subsequent three counts were performed in adjacent positions to count 1 moving towards the viable flap such that on completion, four consecutive grid areas had been assessed within the zone of demarcation. Results were recorded.

A series of 8 non-consecutive counts were then performed, using the same technique, at random positions across the full width of each section (also at x400 magnification). Both counts were performed at two levels within each specimen, namely above and below the *panniculus carnosus* muscle layer.

3.3 Statistics

Results for a-MVD are expressed as mean values with 95% confidence intervals for each region counted namely, position 1 and position 2 of both the axial pattern and random extension of each flap above and below the *panniculus carnosus*. Results for the zone of demarcation in group 2 flaps (one week post elevation) are presented similarly. Comparisons were performed using the Mann-Whitney U test and $p < 0.05$ was taken to represent statistical significance.

3.4 Results

3.4.1 Optimization of endothelial staining technique

Protease digestion appeared to be superior to antigen retrieval by heating, as a pre-treatment. Polyclonal rabbit anti-human von Willebrand factor was the best performing primary antibody with a higher density of staining observed than either of the monoclonal antibodies tested. There appeared to be no advantage to incubation with the primary antibody over night when compared to the standard incubation time of 60 minutes.

Our most successful protocol for the staining of formalin fixed, rat endothelium in epigastric island flaps can therefore be summarised as: protease pre-treatment, endogenous peroxidase block, non-specific binding block, polyclonal rabbit anti-human von Willebrand factor primary antibody (1 hr incubation), affinity isolated biotin labelled goat anti-rabbit secondary antibody (30 minute incubation), standard streptavidin detection system (Fig. 3.3).

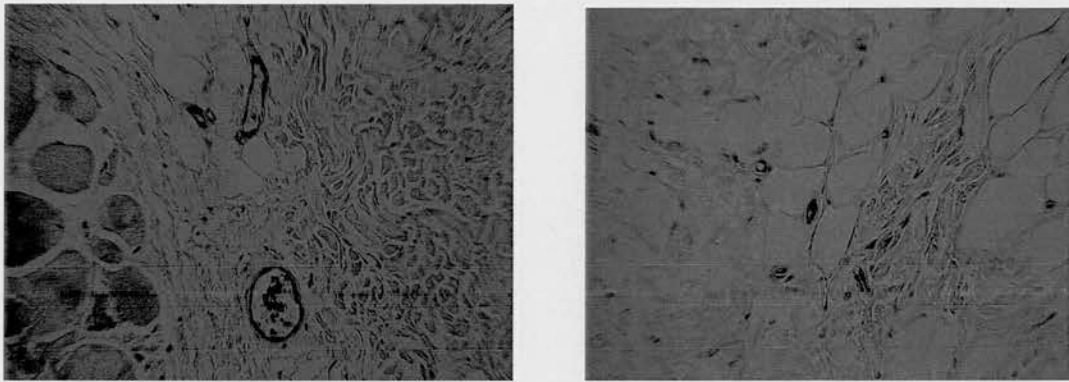


Figure 3.3 – Immunoperoxidase preparations of sections taken from rat epigastric island flaps (Polyclonal rabbit anti-vWF primary antibody, biotinylated goat anti-rabbit secondary antibody). Endothelium appears brown indicating positive staining.

3.4.2 Measurement of a-MVD

Tissue sections from all group 1 and group 2 animals were prepared for analysis using the above protocol. An independent assessor, not involved in the preparation of the tissue, performed all vessel counts. I am grateful for the help of Dr J. Clark, Surgical Resident, The St George Hospital, NSW Australia in this respect. All counts were performed blinded to the details of the specimen. All results are expressed as vessels per unit area of 0.0625 mm^2 .

3.4.2.1 Within-group analysis

Average MVD for group 1 animals for each of the locations examined is given in Table 3.1. In all positions a-MVD was significantly higher deep to the *panniculus* when compared to the superficial plane ($p < 0.01$). As expected however, within each tissue plane vascularity was found to be uniform, with no significant difference in a-MVD between positions 1 and 2 or in the axial pattern flap compared to the random pattern extension ($p > 0.15$). This being the case, the results for a-MVD in a given plane were pooled to provide an average measurement for the flaps taken as a whole. In this manner a-MVD for normal rat epigastric skin was found to be 8.6 C.I. (7.9, 9.3) vessels per unit area superficial to the *panniculus carnosus* whilst in the deep plane it was 12.7 C.I. (11.6, 13.8) ($p < 0.01$).

Table 3.1 – Average MVD for group 1 flaps (normal rat epigastric skin). Results are per unit area of 0.0625 mm² with 95% confidence intervals given in brackets. The observed differences in mean values for level ‘b’ (below the *panniculus*) compared to level ‘a’ (above the *panniculus*) were statistically significant at all locations examined (p<0.01).

		<i>Average MVD (95% C.I.)</i>	
		<i>random pattern flap</i>	<i>axial pattern flap</i>
Position 1 (n=8)	level a	9.2 (7.8, 10.6)	8.9 (7.9, 10.0)
	level b	12.6 (10.5, 14.7)	12.9 (10.9, 14.9)
Position 2 (n=8)	level a	8.0 (6.5, 9.5)	8.3 (6.9, 9.7)
	level b	12.4 (10.0, 14.7)	12.8 (10.6, 14.9)

Position 1 represents sections taken at a position three quarters of the way along the flap from caudal to cranial, marked at the time of elevation. Similarly, position 2 represents sections taken in the plane passing through the mid point of the flap (Fig. 3.2).

Level ‘a’ is above, and level ‘b’ below, the *panniculus carnosus* muscle.

Analysis of the data from group 2 animals (flap harvest at 1 week) found a similar pattern (Table 3.2). Average MVD deep to the *panniculus* was higher than in the superficial plane in all locations examined. In all but one location this difference reached statistical significance ($p < 0.04$). The exception was recorded for the axial pattern flap at position 1 where $p = 0.13$. Nevertheless, a-MVD at this location remained consistent with the general pattern of increased vascularity deep to the *panniculus* and may have reached statistical significance with further sampling. As for group 1 flaps, analysing the data for each region as a whole (excluding the zone of demarcation which represented a special and specific case), the vascularity within each layer was found to be uniform with no significant difference in a-MVD between positions 1 and 2 or in the axial pattern flap compared to the random pattern extension ($p > 0.24$). The data was therefore, pooled as before to give an a-MVD for the whole flap of 10.0 C.I. (8.8, 11.1) vessels per unit area above the *panniculus* and 14.9 C.I. (13.2, 16.5) below it ($p < 0.01$).

The highest a-MVD of all was recorded in the zone of demarcation in the plane deep to the *panniculus* (22.2 C.I. (17.0, 27.5)) where it was significantly higher than at any other location examined ($p < 0.01$).

Table 3.2 - Average MVD for group 2 flaps (rat epigastric island flap at one week). Results are per unit grid area of 0.0625 mm² with 95% confidence intervals given in brackets. Highest values for a-MVD were recorded deep to the *panniculus carnosus* within the zone of demarcation where they were significantly higher than at any other location examined (p<0.01).

		<i>Average MVD (95% C.I.)</i>		
		<i>Zone of demarcation</i>	<i>random pattern flap</i>	<i>axial pattern flap</i>
Position 1 (n=8)	level a	9.1 (6.5, 11.7)	9.8 (7.2, 12.4)	10.7 (7.8, 13.6)
	level b	23.4 (15.1, 31.8)	16.0 (11.9, 20.2)	13.7 (10.6, 16.8)
Position 2 (n=8)	level a	10.5 (7.7, 13.3)	10.1 (8.6, 11.6)	9.3 (7.6, 10.9)
	level b	22.3 (17.3, 27.3)	14.7 (11.4, 18.1)	15.3 (12.7, 17.9)

Position 1 & 2 and Levels 'a' & 'b' are as for Table 3.1

3.4.2.2 Between-group analysis

Comparisons were made between the results for group 1 and group 2 flaps. Average MVD was increased at all locations both above and below the *panniculus* in group 2 flaps when compared to the corresponding positions in group 1 flaps. This increase, with one exception, did not reach statistical significance ($p>0.06$). The exception was for the random pattern flap at position 2 above the *panniculus* where a-MVD had risen from 8.0 to 10.1 vessels per unit area in group 2 flaps ($p=0.02$). We did not consider this to be an important finding.

The data was further analysed by comparing the random and axial portions of each flap taken as a whole i.e. combining the results for position 1 and position 2 for each hemiflap. The change in a-MVD for the axial pattern flap was not significant on either side of the *panniculus carnosus* ($p>0.24$) however, for the random pattern flap the increase in a-MVD deep to the *panniculus* was significant ($p=0.04$) and tended to significance in the superficial layer ($p=0.06$).

The most impressive rise in a-MVD was however, within the zone of demarcation deep to the *panniculus* (Table 3.2). Since there is no zone of demarcation in group 1 flaps, comparisons were made instead with the results for the random pattern flap in these animals. The difference in a-MVD above the *panniculus* did not reach statistical significance ($p>0.2$) however, the observed rise in a-MVD below the *panniculus* was highly significant ($p<0.01$) (Figure 3.4).

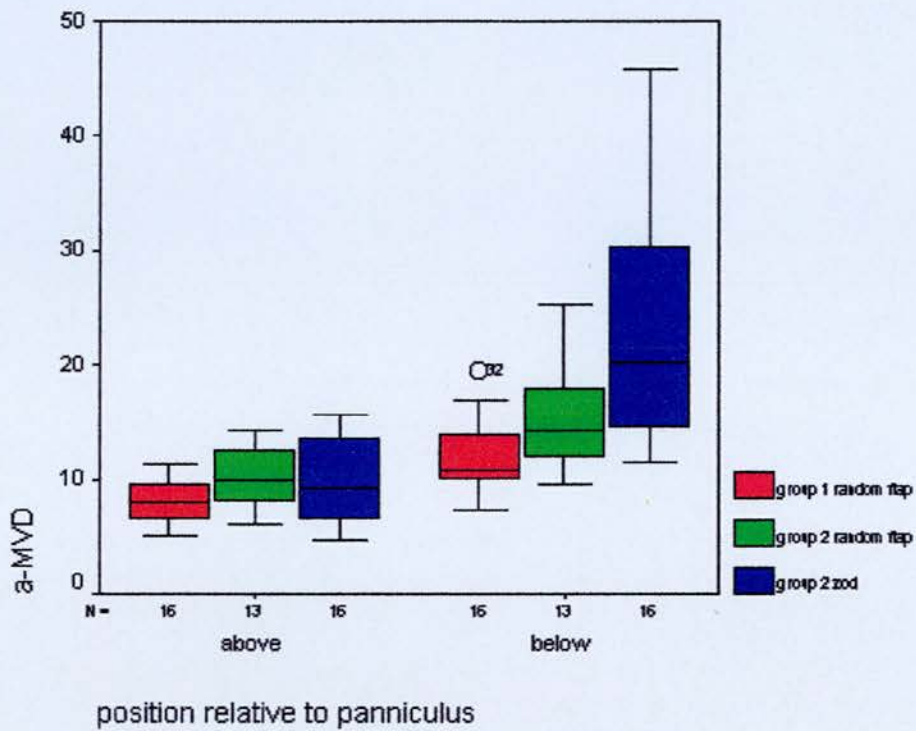


Figure 3.4 – Boxplot of a-MVD above and below the *panniculus carnosus* for the random pattern flaps in both groups and the zone of demarcation in group 2 flaps. Although no statistically significant change in vascularity is seen for the measurements taken above the *panniculus carnosus*, a-MVD below it is increased in both the random flap taken as a whole and at the zone of demarcation (zod) with the increase being most marked in the latter. In both these locations the observed increase in a-MVD was statistically significant ($p=0.04$ and $p<0.01$ respectively).

Evidence of physiological angiogenesis within the experimental flaps at one week was therefore most pronounced in the region deep to the *panniculus carnosus* within the zone of demarcation and more apparent in the random compared to the axial pattern flaps.

3.5 Discussion

Assessments of tissue vascularity may be made on standard Haematoxylin & Eosin stained slides, with some authors reporting their preference for this technique over the use of immunohistochemical stains (Rendell *et al.* 1998). In our experience however, the use of immunostaining was of great help in the identification of small blood vessels within our tissue blocks and as such, significantly improved our ability to count them accurately and reliably. Published protocols for use in the quantification of vascularity in human tumours, where the use of immunostains is routine, supports this view (Weidner 1995). In our work, an additional consideration was that the assessments were to be performed by a non pathologist, with limited experience in this field, to whom the benefits of accurate staining proved a great advantage.

In order to achieve consistent results however, time must be spent on the optimisation of any given protocol, both in terms of the reagents used and also the manoeuvres employed to enhance antigen staining whilst keeping confusing background stain to a minimum. Although the technology has been designed principally for use in human tissue, a degree of experimentation with standard protocols will give very satisfactory results for animal preparations. More recently,

primary antibodies raised against specific animal antigens have become increasingly available for research purposes and these are likely to further improve the quality of results in the future (www.serotec.com).

The vascularity of abdominal rat skin in its normal state has not been previously reported and represents a useful baseline with which to make comparisons. Average MVD was found to be consistently higher in the plane deep to the *panniculus carnosus* muscle relative to the superficial layer. Within each layer however, a-MVD was uniform across the flap as might be expected for normal rat abdominal skin. We were therefore encouraged that our technique for the collection of a-MVD data was effective and capable of providing a useful assessment of flap vascularity.

At one week post elevation, the pattern was similar in that a-MVD remained highest deep to the *panniculus carnosus* at all locations examined. Our results were similar in this respect to those reported by Taub *et al.* (Taub PJ *et al.* 1998), who also commented on the increased vascularity deep to the *panniculus* compared to the superficial plane. In addition however, we found increases in a-MVD in the random compared to the axial pattern flap and a marked increase in a-MVD deep to the *panniculus* in the location immediately adjacent to the necrotic front, in the zone of demarcation.

In attempting to explain these results it is helpful to consider the stimulus to angiogenesis acting on the flap as being made up of two components, namely a low local oxygen tension on the one hand and the requirements of tissue healing at the

interface between the flap and the abdominal wall on the other. Both of these conditions are known to act as independent promoters of angiogenesis via the activity of VEGF (Brown *et al.* 1992; Detmar *et al.* 1997).

Within the zone of demarcation, both of these stimuli will be active. On moving away from the ischaemic front however, the hypoxic drive will diminish, leaving the response to healing as the major promoter of angiogenesis. One might expect therefore, a-MVD to increase in all positions deep to the *panniculus* with further increases where tissue hypoxia is most marked, namely the random pattern flap and in particular within the zone of demarcation where oxygen tension is at the lowest level consistent with tissue viability.

The changes in a-MVD observed were, in general, in keeping with this view. The greatest increase was recorded at the zone of demarcation deep to the *panniculus* where it was significantly higher than at any other location. Smaller but definite increases were also noted for the random pattern flap on both sides of the *panniculus* although this fell just short of statistical significance in the superficial plane. The axial pattern flap showed no significant change in a-MVD in either plane where I would have expected to see a rise in the deep plane. Similarly the superficial plane within the zone of demarcation showed no significant increase in a-MVD where one may have been expected.

The pattern of changes in a-MVD observed was therefore, in general, consistent with expectation. The inconsistencies highlighted are most readily explained by a lack of

sensitivity of the test and increased sampling in the zone of demarcation superficial to the *panniculus* and in both planes of the axial pattern flap may have detected more subtle changes in a-MVD.

Where experimental flaps raised in rats have been used to investigate therapeutic angiogenesis to date, assessments of vascularity have tended to be subjective and made by examining the plane deep to the *panniculus carnosus* (Kryger *et al.* 2000; Taub PJ *et al.* 1998; Zhang *et al.* 2003). My results demonstrate that physiological angiogenesis is most apparent in this plane and that significant increases in microvessel numbers are to be expected within the zone of demarcation. When considering techniques most suited for the detection of stimulated angiogenesis, these results support the use of objective vessel counts and suggest they are best performed in the more stable vascular bed superficial to the *panniculus carnosus* where induced changes in vascularity will be more easily detected against the background of physiological angiogenesis.

3.6 Conclusions

1. Immunohistochemical staining of rat endothelium in formalin fixed tissue is effective and helpful in the assessment of microvascular density in epigastric island flaps.
2. Average microvascular density in the rat epigastric island flap at the time of elevation is 8.6 vessels per unit area of 0.0625 mm^2 in the superficial plane and 12.7 in the deep plane defined by the *panniculus carnosus*. Within each plane the vascularity is uniform across the flap.

3. Flaps analysed at one week demonstrate a significant increase in a-MVD in the plane deep to the *panniculus* in the random pattern flap compared to normal rat skin ($p=0.04$).
4. Highest a-MVD was recorded deep to the *panniculus* within the zone of demarcation where it was significantly higher than at any other position within the flap ($p<0.01$).
5. The optimal plane for the detection of induced angiogenesis in the rat epigastric island flap model is likely to be superficial to the *panniculus carnosus* in the random pattern flap where it is more easily distinguished from physiological angiogenesis.

Chapter 4 –Manufacture of pEF-BOS-VEGFB expression vector

4.1 Introduction

4.1.1 Objectives

One of our primary aims in this project was to evaluate the use of raw plasmid DNA, encoding the gene for VEGFB, as an agent for therapeutic angiogenesis. Amrad Corporation Ltd (Burnley, Victoria, Australia) could supply a suitable plasmid construct based on the mammalian expression plasmid pEF-BOS. Two plasmids were made available, one containing the VEGFB insert, and one without for use as a control. To proceed, we first required purified DNA stocks of both of these plasmids.

4.1.2 pEF-BOS mammalian expression plasmid

Plasmids that have been optimised for protein production are referred to as *expression vectors*. The pEF-BOS plasmid is one such expression vector designed for use in mammalian cells (Fig 4.2) (Mizushima & Nagata 1990). Uetsuki *et al.* in their work on the genetic structure of the polypeptide chain known as elongation factor 1 α (EF 1 α), reported the discovery of a powerful promoter sequence isolated from the human gene for EF- 1 α in fibroblast cells (Uetsuki *et al.* 1989). Subsequent experiments confirmed its great efficiency at inducing transcription *in vitro*. The

construction of an expression plasmid, pEF-BOS, based on this promoter soon followed (Mizushima & Nagata 1990). When tested against accepted standards, Mizushima *et al.* showed the superiority of the new construct in achieving protein production in a series of animal and human cell lines (Mizushima & Nagata 1990). Similar success with this plasmid has been reported by others (Kinoshita *et al.* 1999).

pEFBOS S FLAG/MYC Vectors For Secreted Expression

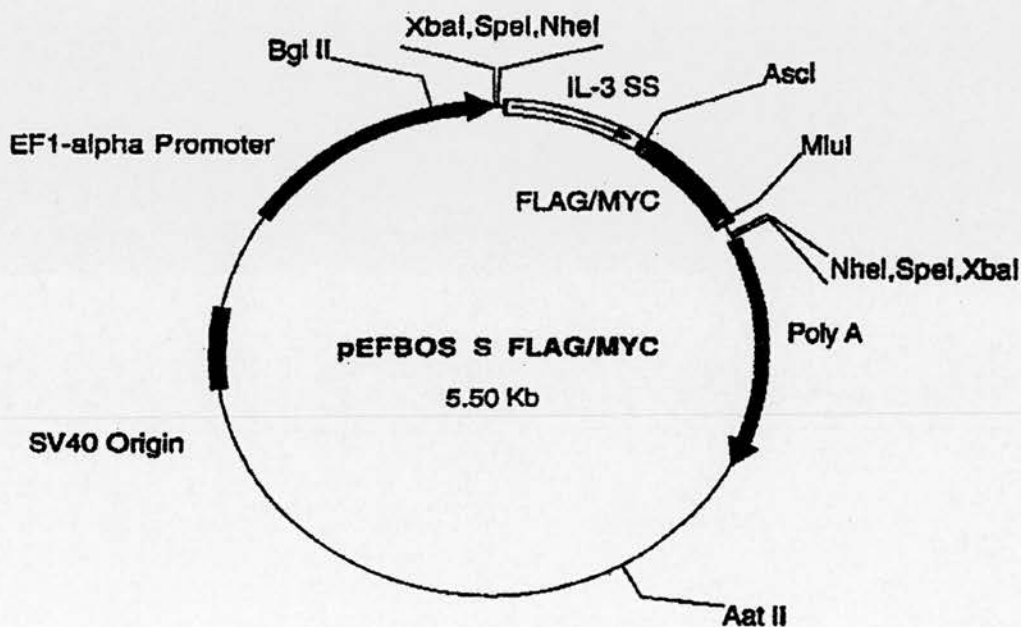


Figure 4.1 – Diagram of the mammalian expression plasmid pEF-BOS-FLAG indicating the position of a number of important restriction sites.

4.1.3 Purification of plasmid DNA

To be useful in the context of gene therapy, plasmid DNA must be pure. Extraction from the host bacterial cell population must therefore be achieved with as little contamination from other cellular components as possible. One highly successful method of achieving this was set out by Birnboim and Doly and relies on the existence of a narrow range of pH (12.0 – 12.5) within which linear DNA will undergo irreversible denaturation but in which covalently closed circular DNA remains intact (Birnboim & Doly 1979).

The published technique may be summarised as follows. The bacterial cell walls are first lysed with sodium hydroxide and sodium dodecyl sulphate (SDS) in a closely controlled manner. Chromosomal and plasmid DNA as well as proteins are denatured by the alkaline conditions. The lysate is neutralised with acidic sodium acetate. The high salt concentration causes SDS to precipitate, and the denatured proteins, chromosomal DNA and cellular debris become trapped in salt-detergent complexes. Plasmid DNA however, being smaller and covalently closed, renatures correctly and remains in solution. The precipitated debris can then be removed by centrifugation and the plasmid concentrated by ethanol precipitation (Birnboim 1983).

These principles have been incorporated into a number of commercially available kits for the extraction and purification of plasmid DNA which are highly efficient and offer excellent levels of plasmid yield and purity (Qiagen Pty Ltd 1997).

4.1.4 DNA sequencing

The ability to sequence DNA molecules is fundamental to much of the work in genetic engineering. Knowledge of the base sequence of a DNA region is essential if cutting and joining procedures are to be conducted accurately. In addition, sequencing allows confirmation that an intended genetic manipulation has indeed taken place, and at the location expected.

Franca *et al.* have recently reviewed the various methods by which DNA molecules may be sequenced (Franca, Carrilho, & Kist 2002). Of particular relevance to this work is the chain-terminator method of Sanger which provides the foundation for automated sequencing (Sanger, Nicklen, & Coulson 1977). The principles of this technique will be considered briefly here.

The enzyme DNA polymerase, under normal circumstances will synthesize complementary copies of a single-stranded DNA template from 2' deoxynucleoside triphosphate substrates. If present however, it will also incorporate the analagous 2',3' dideoxynucleotide into the growing chain. This is significant since the latter lacks a 3'- hydroxyl group on the pentose ring and as such will not allow further chain elongation. The growing chain is therefore terminated at this point. If a primer sequence is used to start a DNA polymerase reaction at a particular point in four separate incubation mixes, each of which contain the full complement of normal deoxynucleotide bases (radiolabelled), but in addition contain in turn, a single chain terminating dideoxynucleoside base (A, G, C or T), then a population of partially synthesised radioactive DNA molecules will result. These molecules will all have a

common 5'- end (the primer sequence) but vary in length to a base specific 3'- end.

Denaturing the DNA and performing electrophoresis of the four samples side by side allows the base sequence to be read directly from an autoradiograph (Fig. 4.3)

(Sanger, Nicklen, & Coulson 1977).

The replacement of radiolabels on the normal nucleotides with base specific fluorescent tags on the chain terminating ones has allowed automation of the system by the detection of each DNA band by its fluorescent colour as it electrophoreses past a detector (Prober *et al.* 1987).

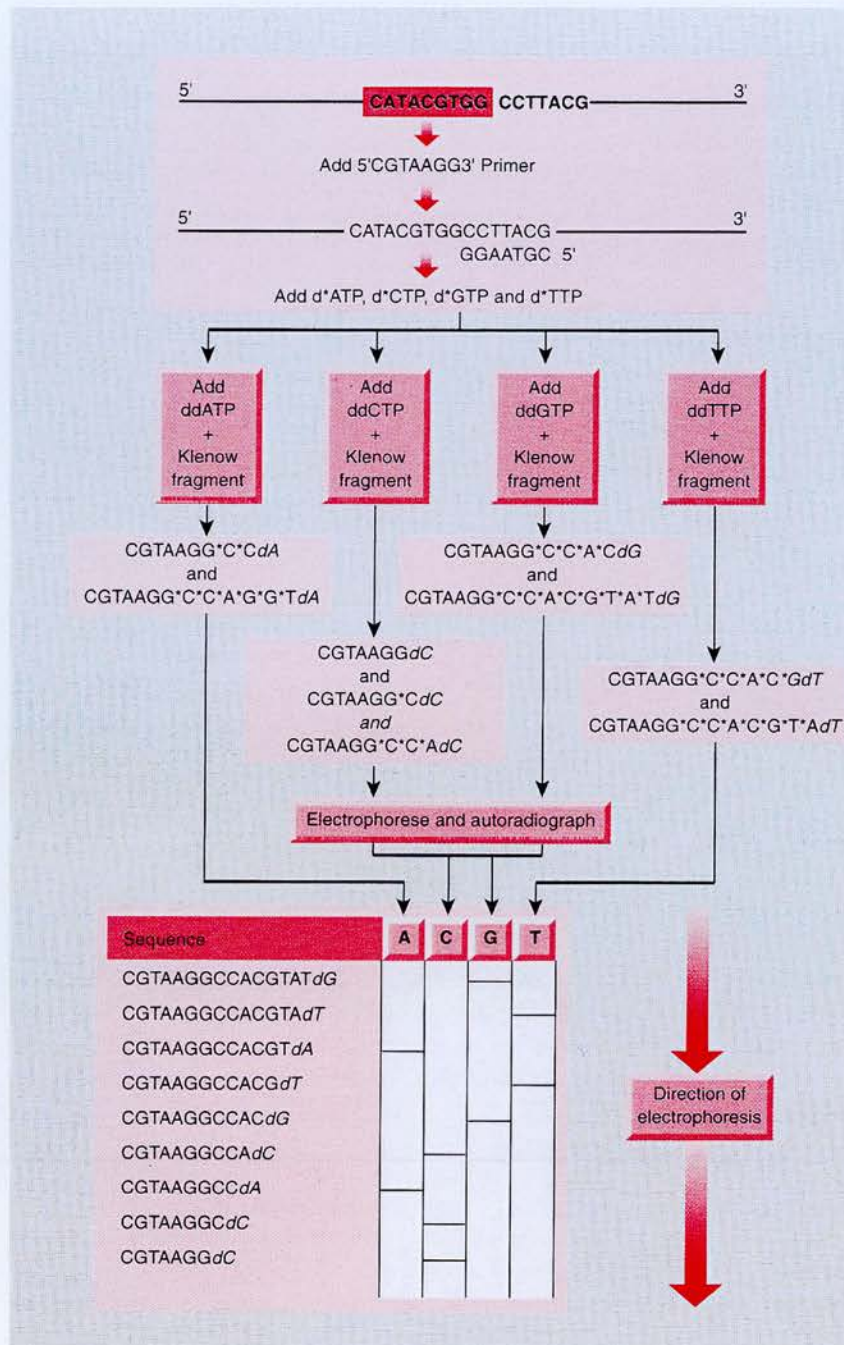


Figure 4.2 – DNA sequencing with dideoxynucleoside triphosphates as chain terminators after the method of Sanger. Asterisks indicate the presence of the radioactive label ‘³²P’ and the prefix ‘dd’ indicates the presence of a dideoxynucleoside. A, adenine; C, cytosine; G, guanine; T, thymidine (Taken from Old & Primrose, Principles of Gene Manipulation, 1994).

4.1.5 Aims

The aim of this work was to produce purified stocks of both pEF-BOS, and pEF-BOS-VEGFB plasmids and to confirm the integrity of the VEGF-B insert in the latter by sequencing the coding region.

4.2 Materials and Methods

4.2.1 Acknowledgments

Bacteriological culture work was performed with the aid of Dr S Tait, Department of Molecular Biology, Centre for Thrombosis and Vascular Research, University of New South Wales to whom I am very grateful. Amrad Corporation Operations (Burnley, Vic, Australia) provided a map of the correct base sequence for the VEGF-B gene to act as a reference (Fig. 4.4).

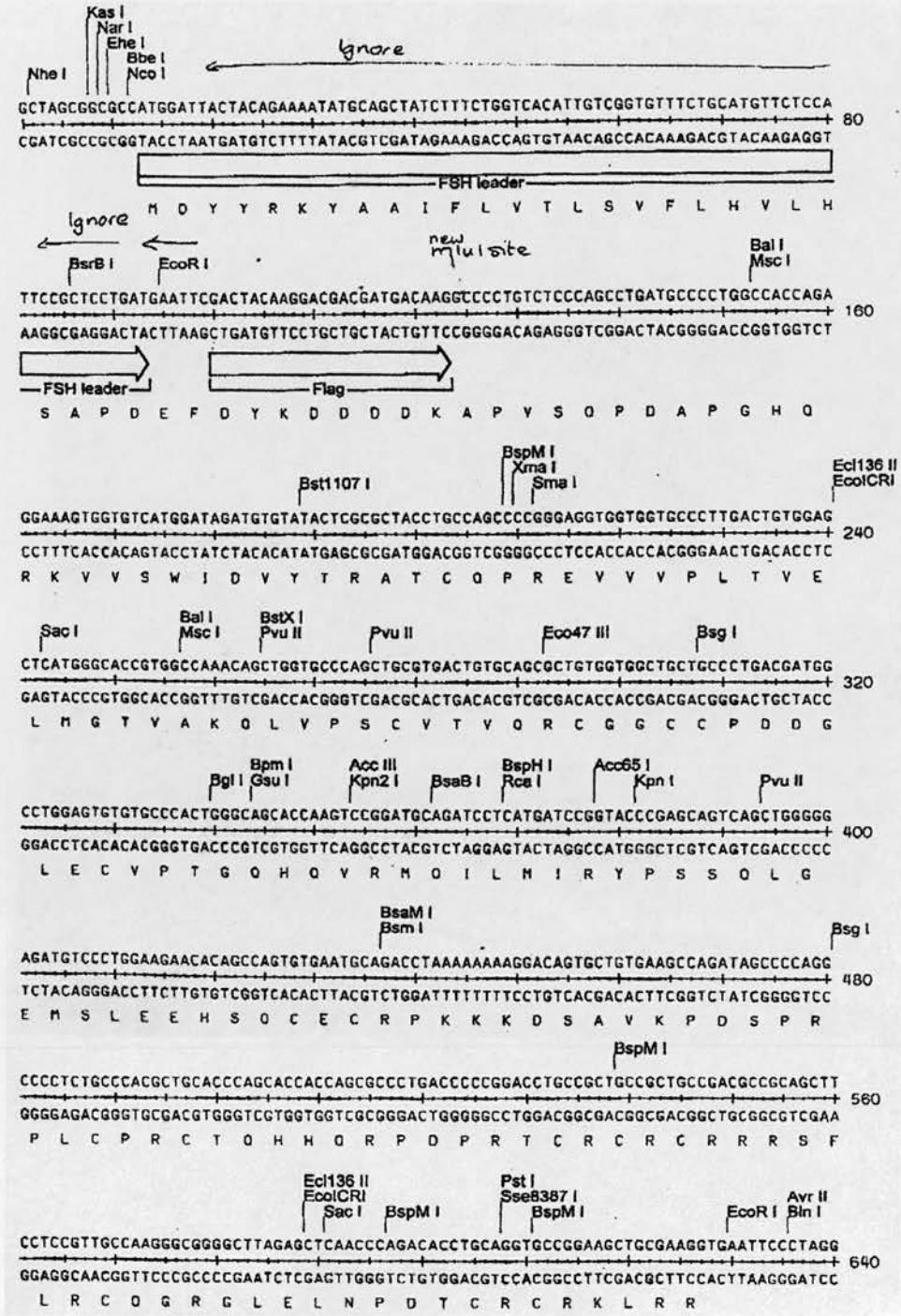


Figure 4.3 – Base and amino acid sequence for VEGFB provided by AMRAD Corporation seen here preceded by the Flag tag detection sequence (A, adenine; C, cytosine; G, guanine; T, thymidine)

4.2.2 Bacteriological reagents and equipment

Tryptone and yeast extract were from Difco (Becton Dickinson, MD, USA). Sodium chloride and ampicillin were from Sigma-Aldrich (St. Louis, USA). Distilled water was available in the laboratory. Isopropanol was from Lab Scan (Bangkok, Thailand) and ethanol from Ajax Chemicals (NSW, Australia). *Escherichia coli* transfected with the expression plasmids pEF-BOS and pEF-BOS-VEGFB were supplied by Amrad operations, as two separate glycerol stocks. The shaking device used was the B. Braun certomat and the centrifuge was the Beckman J2-21 model. Glassware and reaction tubes were from stock held in the haematology research laboratory at the Prince of Wales Hospital. Plasmid purification kit (QIAfilter Plasmid Maxi Kit, product no. 12262) was from Qiagen (Clifton Hill, Vic, Australia). The DNA spectrophotometer used was the Genequant from Pharmacia (Australia office). All DNA sequencing was performed using the multichannel sequencer at the University of New South Wales (Randwick, Australia).

4.2.3 Bacteriological solutions and media

Selective LB medium: Add 1 g tryptone, 1 g sodium chloride and 0.5 g yeast extract to 100 ml distilled water in a suitable flask and stir to dissolve. Sterilise by autoclaving for 15 minutes. Add 10 mg ampicillin and store at 4°C.

Dr S Tait supplied selective LB agar culture plates (ampicillin 100 µg/ml).

4.2.4 Plasmid preparation

Both experimental plasmids (pEF-BOS and pEF-BOS-VEGFB) were prepared in the same manner from glycerol stocks of *E. coli* transformants as described below.

4.2.4.1 Preparation of bacterial colonies

Selective LB agar culture plates were streaked with bacteria from each glycerol stock (pEF-BOS-VEGFB and pEF-BOS). The plates were incubated at 37°C for 16 hrs to provide single colonies, sealed with parafilm, labelled and stored in a cold room at 4°C.

4.2.4.2 Inoculation of minibroth

Two minibroths were started, one for each transformant. A single bacterial colony was selected from each culture plate and used to inoculate 3 ml selective LB medium in a 15 ml sterile plastic reaction tube. A heat sterilised wire loop was used to transfer each colony to the culture medium in the standard way. The tops of the tubes were covered but air was allowed to circulate. Each minibroth was incubated for 8 hrs at 37°C with vigorous shaking (B. Braun certomat equipment set at 225 rev/min).

4.2.4.3 Dilution of starter culture

A 500 µl aliquot from each minibroth was used to inoculate 100 ml of selective LB medium in a 1000 ml sterile glass container. A cotton wool bung was placed in the

mouth of each flask and the culture returned to the shaker and incubated at 37°C for 16 hrs at 225 rev/min as before.

4.2.4.4 Bacterial cell harvest

Each of the 100 ml cultures was divided into 25 ml balanced aliquots and centrifuged at 5000 rpm at 4°C for 10 min (Beckman J2-21 equipment). The supernatant was discarded to leave well-circumscribed bacterial pellets adherent to the base of each centrifuge tube.

4.2.4.5 Extraction of purified plasmid DNA

Plasmid DNA was extracted from the prepared bacterial pellets using the QIAfilter maxi kit (Qiagen Pty Ltd) according to manufacturer's instructions. The technique incorporates Qiagen modifications of the alkaline lysis technique of Birnboim and Doly (1979) outlined above. In summary, an anion-exchange resin, maintained at appropriate low-salt and pH conditions, is used to bind plasmid DNA released from host bacteria during alkaline lysis, avoiding the need for centrifugation. Impurities are then removed by a medium-salt wash while the plasmid DNA is tightly bound to the resin. Plasmid DNA is then eluted in a high-salt buffer before being concentrated and desalted by isopropanol precipitation. The manufacturer's expected yield is quoted as 500 µg plasmid DNA per QIAfilter cartridge used. Four cartridges were used to prepare purified plasmid DNA for each of the experimental plasmids pEF-BOS and pEF-BOS VEGFB. Following the final stage of air-drying each plasmid DNA pellet was dissolved in 1ml 10mM TRIS buffer (pH 8.0) and stored at -20°C.

4.2.4.6 Assessment of yield

Final plasmid yield was assessed for each of the eight DNA solutions prepared. The GeneQuant (Pharmacia) DNA spectrophotometer was used in accordance with the manufacturer's instructions. The DNA samples were analysed at 1:100 dilution in deionised water (5 µl in 495 µl). The same deionised water provided the reference.

4.2.5 Sequencing of purified pEF-BOS VEGFB

To verify the integrity of the VEGFB insert, the pEF-BOS-VEGFB plasmid was subjected to sequencing in this region. Since all stock preparations of the pEF-BOS-VEGFB plasmid had their origin in a single bacterial colony, confirmation of the correct sequence in one preparation only was necessary.

Sequencing was carried out at the automated DNA analysis facility housed at University of New South Wales, Sydney, Australia. The preparation of appropriate DNA samples was performed with the help and assistance of Dr Melissa Holmes, Senior Research Scientist, Centre for Thrombosis and Vascular Research, University of New South Wales.

The primer used corresponds to a sequence within the EF-1 alpha promoter, just upstream of an IL-3 signal sequence and Flag tag (used for detection of expressed protein) and the 5'- end of VEGFB. Sequencing used Perkin Elmer BigDye chemistry with 3.2 pmole primer and 500ng plasmid and standard cycle-sequencing conditions ie. 94°C for 1 min x1 cycle, then 94°C for 10 sec, 50°C for 10 sec and 60°C for 4 min for 25 cycles. The samples were then precipitated with 1/10 vol 3M

sodium acetate + 2.5 vol ethanol, rinsed with 70% ethanol, the pellet dried and sent for sequencing.

The first primer chosen resulted in inconsistencies in the sequence towards the 3'-end of the VEGFB coding region and so a second primer located further downstream, within the 5'- end of the VEGFB insert, was used to review this area.

4.3 Results

The plasmid DNA yield and purity I achieved for each of the final DNA solutions was in keeping with my expectations based on the kit manufacturers published information (Table 4.1). Preparation number 3 of the VEGFB series was the poorest quality sample. No difficulties were encountered during the preparation of this material and no specific reasons for the poor result were identified.

DNA from pEF-BOS-VEGFB sample 2 was taken as representative of the pEF-BOS-VEGFB plasmid preparations as a whole and subjected to sequencing. Sequencing data for this plasmid are given in Figures 4.5 and 4.6. Comparison of this sequence with that for the Flag tagged VEGFB gene sequence supplied by Amrad, showed complete agreement between the two bar a single base discrepancy at position 394 in our plasmid where a thymidine was recorded in place of the expected guanine base. This was felt most likely to have resulted from a sequencing error rather than a true base discrepancy

Table 4.1 – Plasmid yield and purity.

Plasmid	Sample	Yield	Purity
pEF-BOS	1	540µg	84%
	2	420µg	88%
	3	500µg	88%
	4	480µg	84%
pEF-BOS-VEGFB	1	490µg	82%
	2	490µg	86%
	3	700µg	75%
	4	440µg	84%

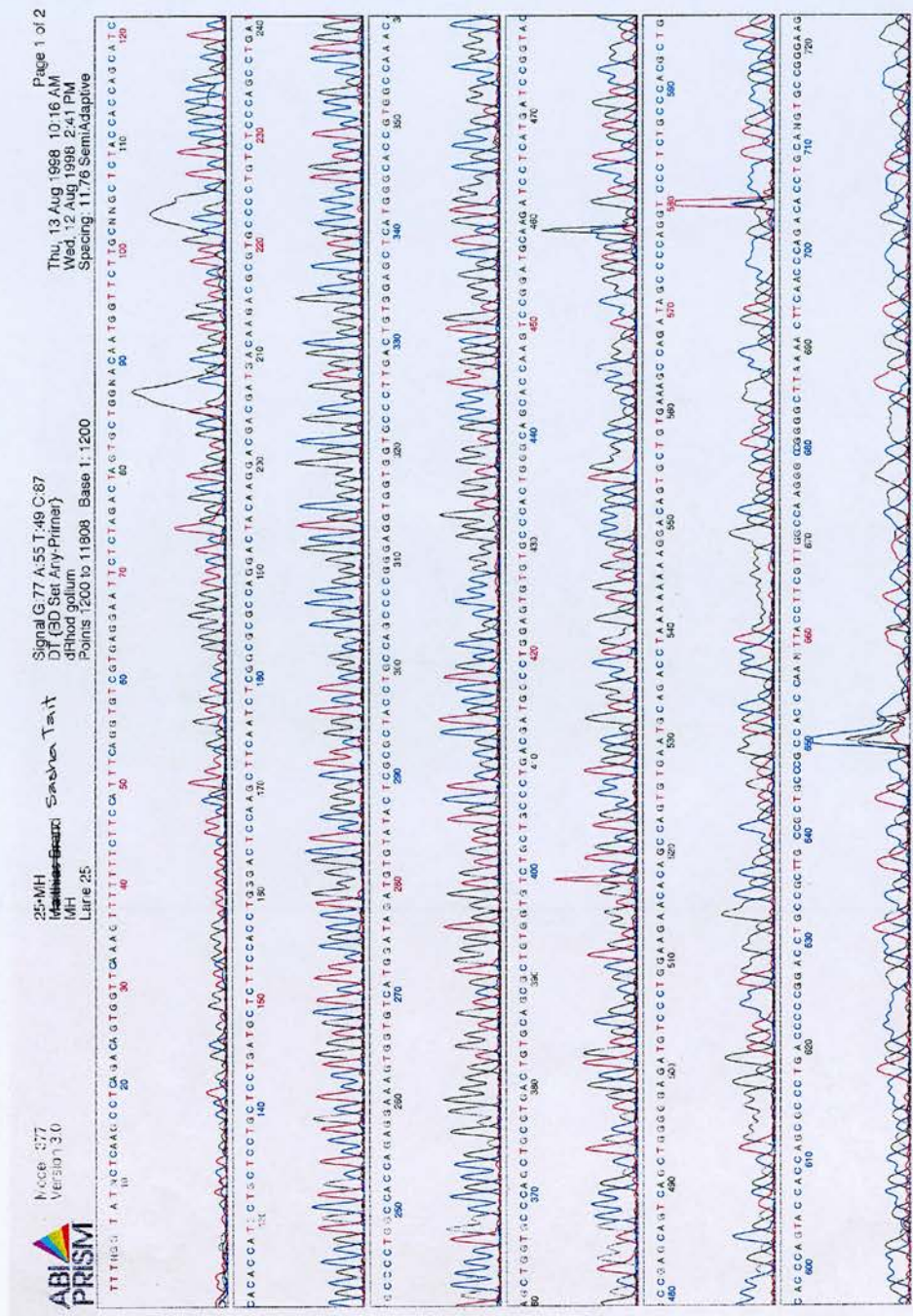


Figure 4.4a – First analysis sequencing data for purified pEF-BOS-VEGFB. Computer generated fluorescent traces using standard genetic code (A, adenine; C, cytosine; G, guanine; T, thymidine).

Untitled Sequence # 1 -> 3-phase Translation PRIMER -> GCCAGCTTG

DNA sequence 884 b.p. TTTGGTATCTCA ... GGCCCTTGCTTC linear

```

1/1                               31/11
TTT GGT ATC TCA AGC CTC AGA CAG TGG TTC AAA GTT TTT TTC TTC CAT TTC AGG TGT CGT
F G I S S L R Q W F K V F F F H F R C R
L V S Q A S D S G S K F F S S I S G V V
W Y L K P Q T V V Q S F F L P F Q V S *
61/21                               91/31
GAG GAA TTC TCT AGA CTA GTG CTG GAC AAT GGT TCT TGC GCT CTA CCA CCA GCA TCC ACA
E E F S R L V L D N G S C A L P P A S T
R N S L D * C W T M V L A L Y H Q H P H
G I L * T S A G Q W F L R S T T S I H T
121/41                               151/51
CCA TGC TGC TCC TGC TCC TGA TGC TCT TCC ACC TGG GAC TCC AAG CTT CAA TCT CGG CGC
P C C S C S * C S S T W D S K L Q S R R
H A A P A P D A L P P G T P S F N L G A
M L L L L L M L F H L G L Q A S I S A R
181/61                               211/71
GCC AGG ACT ACA AGG ACG ACG ATG ACA AGA CGC GTG CCC CTG TCT CCC AGC CTG ATG CCC
A R T T R T T M T R R V P L S P S L M R
P G L Q G R R * Q D A C P C L P A * C P
Q D Y K D D D D K T R A P V S Q P D A P
241/81                               271/91
CTG GCC ACC AGA GGA AAG TGG TGT CAT GGA TAG ATG TGT ATA CTC GCG CTA CCT GCC AGC
L A T K G K W C H G * M C I L A L P A S
W P P E E S G V M D R C V Y S R Y L P A
G H Q R K V V S W I D V Y T R A T C Q P
301/101                               331/111
CCC GGG AGG TGG TGG TGC CCT TGA CTG TGG AGC TCA TGG GCA CCG TGG CCA AAC AGC TGG
P G R W W C P * L W S S W A P W P N S W
P G G G G A L D C G A H G H R G Q T A G
R E V V V P L T V E L M G T V A K Q L V
361/121                               391/131
TGC CCA GCT GCG TGA CTG TGC AGC GCT GTG GTG TCT GCT GCC CTG ACG ATG GCC TGG AGT
C P A A * L C S A V V S A A L T M A W S
A Q L R D C A A L W C L L P * R W P G V
P S C V T V Q R C G (V) C C P D D G L E C
421/141                               451/151
GTG TGC CCA CTG GGC AGC ACC AAG TCC GGA TGC AAG ATC CTC ATG ATC CCG TAC CCG AGC
V C P L G S T K S G C K I L M I R Y P S
C A H W A A P S P D A R S S * S G T R A
V P T G Q H Q V R M Q D P H D P V P E Q
481/161                               511/171
AGT CAG CTG GGG GAG ATG TCC CTG GAA GAA CAC AGC CAG TGT GAA TGC AGA CCT AAA AAA
S Q L G E M S L E E H S Q C E C R P K K
V S W G R C P W K N T A S V N A D L K K
S A G G D V P G R T Q P V * M Q T * K K
541/181                               571/191
AAG GAC AGT GCT GTG AAA GCC AGA TAG CCC CAG GTC CCT CTG CCC ACG CTG CAC CCA GTA
K D S A V K A R * P Q V P L P T L H P V
R T V L * K P D S P R (S) L C P R C T Q (V)
G Q C C E S Q I A P G P S A H A A P S T
601/201                               631/211
CCA CCA GCG CCC TGA CCC CCG GAC CTG CCG CTT GCC GCT GCC CGC CAC CCA ATT ACC TTC
P P A P * P P D L P L A A A R H P I T F
H Q R P D P R T C R L P L P A T Q L P S
T S A L T P G P A A C R C (P) (P) (P) N Y L R
661/221                               691/231
GTT GGC CAA GGG CCG GGG CTT AAA ACT TCA ACC CAG ACA CCT GCA GTG CCG GGA AGC TTC
V G Q G P G L K T S T Q T P A V P G S F
L A K G R G L K L Q P R H L Q C R E A S
W P R A G A * N F N P D T C S A G K L Q
721/241                               751/251
AAA GGT GAC GCG TGC TTA CCA CTA GTC TAA ATG AAG GGT CCC CAC TGS GGA ACC TTG AAA
K G D A C L P L V * M K G P H W G T L K
K V T R A Y H * S K * R V P T G E P * N
R * R V L T T S L N E G S P L G N L E I
    
```

Figure 4.4b – Computer generated base and amino acid sequences from the first pEF-BOS-VEGFB analysis. The Flag tag sequence seen highlighted in yellow precedes the VEGFB coding sequence (standard abbreviations).

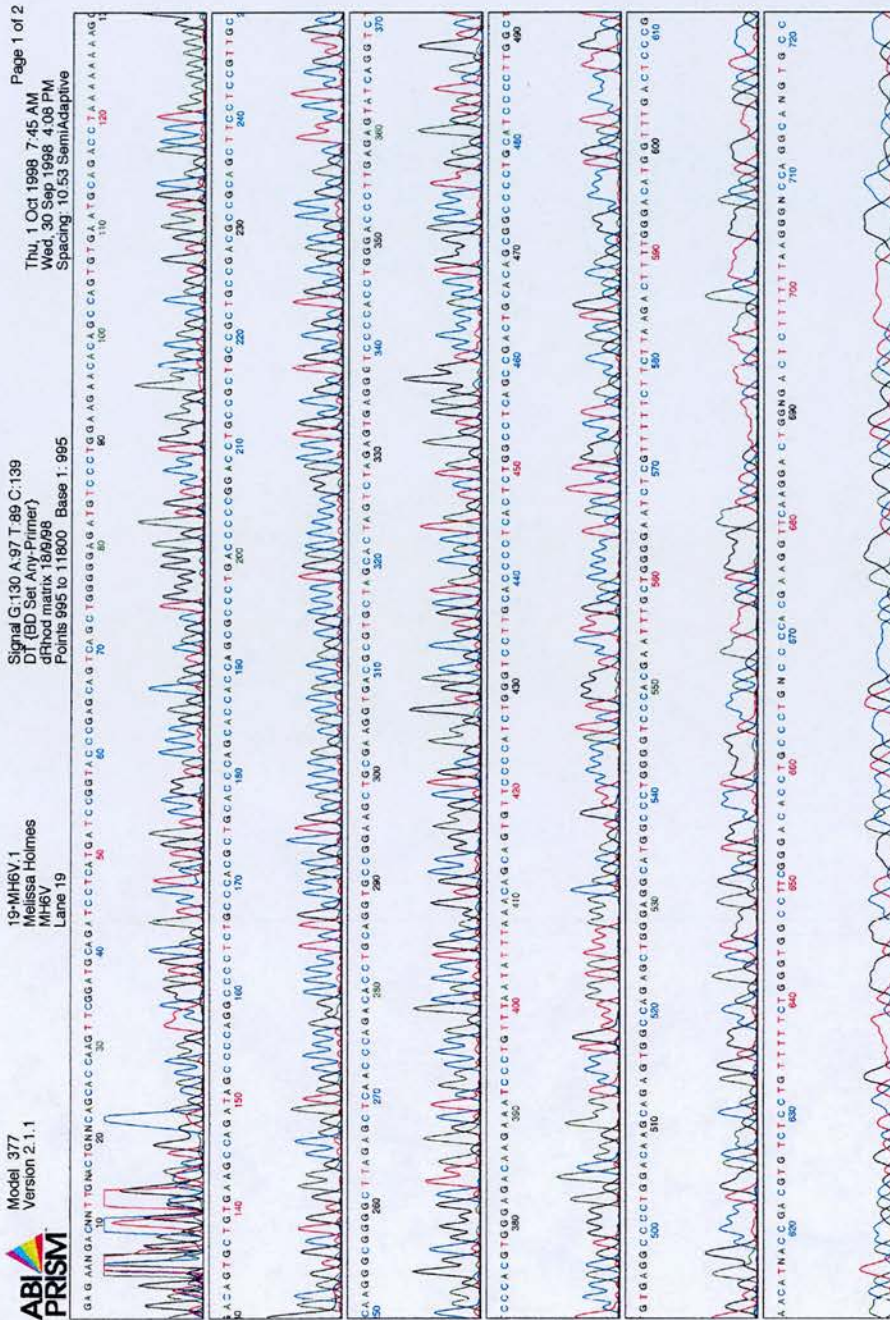


Figure 4.5a - Second analysis sequencing data for purified pEF-BOS-VEGF. Computer generated fluoroscopic traces using standard genetic code as before.

Untitled Sequence # 1 -> 3-phase Translation

DNA sequence 897 b.p. GAGAAGACTTGA ... AATTAAGGGTGTG linear

342 sequence II start

1/1 31/11
 → GAG AAG ACT TGA CTG CAG CAC CAA GTT CGG ATG CAG ATC CTC ATG ATC CGG TAC CCG AGC
 E K T * L Q H Q V R M Q I L M I R Y P S
 R R L D C S T K F G C R S S * S G T R A
 E D L T A A P S S D A D P H D P V P E Q
 61/21 91/31
 AGT CAG CTG GGG GAG ATG TCC CTG GAA GAA CAC AGC CAG TGT GAA TGC AGA CCT AAA AAA
 S Q L G E M S L E E H S Q C E C R P K K
 V S W G R C P W K N T A S V N A D L K K
 S A G G D V P G R T Q P V * M Q T * K K
 121/41 151/51
 AAG GAC AGT GCT GTG AAG CCA GAT AGC CCC AGG CCC CTC TGC CCA CGC TGC ACC CAG CAC
 K D S A V K P D S P R P L C P R C T Q H
 R T V L * S Q I A P G P S A H A A P S T
 G Q C C E A R * P Q A P L P T L H P A P
 181/61 211/71
 CAC CAG CGC CCT GAC CCC CGG ACC TGC CGC TGC CGC TGC CGA CGC CGC AGC TTC CTC CGT
 H Q R P D P R T C R C R C R R R S F L R
 T S A L T P G P A A A A A D A A S S V
 P A P * P P D L P L P L P T P Q L P P L
 241/81 271/91
 TGC CAA GGG CGG GGC TTA GAG CTC AAC CCA GAC ACC TGC AGG TGC CGG AAG CTG CGA AGG
 C Q G R G L E L N P D T C R C R K L R R
 A K G G A * S S S T Q T P A G A G S C E G
 P R A G L R A Q P R H L Q V P E A A K V
 301/101 331/111
 TGA CGC GTG CTA GCA CTA GTC TAG AGT GAG GGT CCC CAC CTG GGA CCC TTG AGA GTA TCA
 * R V L A L V * S E G P H L G P L R V S
 D A C * H * S R V R V P T W D P * E Y Q
 T R A S T S L E * G S P P G T L E S I R
 361/121 391/131
 GGT CTC CCA CGT GGG AGA CAA GAA ATC CCT GTT TAA TAT TTA AAC AGC AGT GIT CCC CAT
 G L P R G R Q E I P V * Y L N S S V P H
 V S H V G D K K S L F N I * T A V F P I
 S P T W E T R N P C L I F K Q Q C S P S
 421/141 451/151
 CTG GGT CCT TGC ACC CCT CAC TCT GGC CTC AGC CGA CTG CAC AGC GGC CCC TGC ATC CCC
 L G P C T P H S G L S R L H S G P C I P
 W V L A P L T L A S A D C T A A P A S P
 G S L H P S L W P Q P T A Q R P L H P L
 481/161 511/171
 TTG GCT GTG AGG CCC CTG GAC AAG CAG AGT GGC CAG AGC TGG GAG GCA TGG CCC TGG GGT
 L A V R P L D K Q S G Q S W E A W P W G
 W L * G P W T S R V A R A G R H G P G V
 G C E A P G Q A E W P E L G G M A L G S
 541/181 571/191
 CCC ACG AAT TTG CTG GGG AAT CTC GIT TTT CTT AAG ACT TTT GGG ACA TGG TTT GAC
 P T N L L G N L V F L L K T F G T W F D
 P R I C W G I S F F F L R L L G H G L T
 H E F A G E S R F S S * D F W D M V * L
 601/201 631/211
 TCC CGA ACA TAC CGA CGT GTC TCC TGT TTT TCT GGG TGG CCT TCG GGA CAC CTG CCC TGC
 S R T Y R R V S C F S G W P S G H L P C
 P E H T D V S P V F L G G L R D T C P A
 P N I P T C L L F F W V A F G T P A L P
 661/221 691/231
 CCC ACG AAG GTT CAA GGA CTG GGA CTC TTT TTT AAG GGC CAG GCA GTG CCT GGG ACA TTT
 P T K V Q G L G L F F K G Q A V P G T F
 P R R F K D W D S F L R A R Q C L G H F
 H E G S R T G T L F * G P G S A W D I L
 721/241 751/251
 TGC CTT TGC TTG GAA CGG GGA CTG GGG GAA TTT GGG GAA AGG GAA CCC ACG GGA AGG AAA
 C L C L E R G L G E F G E R E P T G R K
 A F A W N G D W G N L G K G N P R E G N
 P L L G T G T G G I W G K G T H G K E I

EJND

Figure 4.5b – Computer generated base and amino acid sequences from the second pEF-BOS-VEGFB analysis. Position 342 on the VEGFB reference map is indicated together with the end of the VEGFB coding sequence (standard abbreviations).

4.4 Discussion

The manufacturers expected yield for the plasmid extraction and purification technique employed was 500µg per preparation and I was pleased to get close to this figure for the majority of my stock solutions. The pEF-BOS-VEGFB sample 3 showed slightly different characteristics in comparison to the other samples. The yield appeared much higher but with a higher level of impurities than were present in the other preparations. Presumably this had resulted from technical errors at the time of DNA extraction leading to the persistence of some chromosomal DNA fragments in the final solution.

Although no literature is available on the specific influence of purity on the success of gene transfer experiments clearly the aim is to use solutions of the highest purity possible. In this respect I considered the plasmid DNA stocks in general to be of an acceptable quality for use in subsequent gene transfer experiments with the exception of sample 3 in the pEF-BOS-VEGFB group. The presence of significant quantities of impurities in this sample I believed would make interpretation of results difficult and I therefore decided to exclude this material from further experimental work.

The sequencing data was reassuring and confirmed the accurate inclusion of the full VEGFB-Flag sequence in sample 2 of my pEF-BOS-VEGFB plasmid preparations and that the orientation was correct. This sample was taken as representative of all four preparations which we therefore considered as similarly containing an accurate copy the VEGFB coding sequence also in the correct orientation.

4.5 Conclusions

1. Purified stocks of the two experimental plasmids pEF-BOS and pEF-BOS-VEGFB were produced according to expectations and in general an acceptable level of quality was achieved.
2. An accurate copy of the coding sequence for the VEGFB gene was confirmed as present within the purified pEF-BOS-VEGFB expression plasmids.

Chapter 5 – Transfection experiments with bovine aortic endothelial cells *in vitro*

5.1 Introduction

One of the primary aims of this project was to evaluate the pEF-BOS-VEGFB plasmid as an agent for therapeutic angiogenesis *in vivo*. To approach this problem effectively it was first necessary to investigate the properties of the plasmid *in vitro*. Two features were of particular importance. Firstly, I wanted to identify the optimum conditions for transferring plasmid DNA into endothelial cells and secondly, to confirm that once this had taken place, the plasmid was indeed capable of causing the production of VEGFB within the transfected cell.

Primarily cultured bovine aortic endothelial cells (BAECs) were readily available locally and although rat endothelial cells were on offer in the DMSZ (German Collection of Microorganisms and Cell Cultures) catalogue, their acquisition did not appear to offer significant advantage.

To identify successful gene transfer amongst the BAEC population post transfection, a detection system was also necessary. Since no monoclonal antibody was available to detect the VEGFB protein directly, the FLAG detection system had been incorporated into the design of the pEF-BOS-VEGFB expression plasmid. In this system, the coding sequence for the marker octapeptide FLAG, is cloned into the

plasmid in a position adjacent to the main protein sequence. The fusion protein that results now contains a FLAG epitope that is amenable to detection by immunostaining with commercially available anti-FLAG antibodies. The addition of this small polypeptide is considered to have no effect on the activity of the native protein (www.sigmaaldrich.com).

The use of immunostaining techniques for the detection of successful transfectants is however, a labour intensive process and necessitates fixing of the cells so preventing sequential data collection without the setting up of multiple cultures. In the initial work we therefore elected to use a reporter plasmid. These plasmids confer on their host cell a more easily measurable phenotype and simplify the detection process considerably (Naylor 1999). One such system codes for a green fluorescent protein (GFP) first isolated from the jellyfish *Aequorea Victoria* (Prasher *et al.* 1992). When viewed in the presence of long-wave ultraviolet light (e.g. from an appropriate microscope), the green fluorescence of GFP is easily detected and signals both successful gene transfer and, of particular importance to our work, successful protein production in the host cell. The non invasive nature of this detection system also makes possible the sequential monitoring of gene transfer and expression in a living cell population (Tsien 1998).

To achieve expression however, a plasmid must first reach the interior of a cell, and more specifically in the case of a mammalian cell, the nucleus (Goebel & Schiess 1975). In certain circumstances, DNA will pass spontaneously through a cell wall however, the mechanisms by which this occurs are poorly understood and in general,

appear highly inefficient (Danko & Wolff 1994). For this reason, a variety of techniques have been developed to facilitate the process (Schenborn 2000). One highly effective method, termed lipofection, was described by Felgner *et al.* and employs cationic liposomes as carriers for the raw DNA (Felgner *et al.* 1987a). When in solution, complexes form between liposomes and DNA and these complexes, having a net positive charge, allow associations to occur with the negatively charged cell surface. Interaction between the liposomes and cell wall membrane ultimately lead to the transfer of DNA into the cytoplasm, most likely by endocytosis (Zuhorn & Hoekstra 2002). This technique is now a well established method for gene transfer and more specifically, has been shown to be a highly effective method for the transfection of endothelial cells (Teifel *et al.* 1997).

The success of this technique has led to the development of commercially available liposomal transfection agents such as DOTAP (N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate) (<http://biochem.boehringer-mannheim.com>). This agent has proved helpful in achieving gene transfer to a wide variety of cell types, including endothelial cells, when tested *in vitro*. There is limited published work concerning its use *in vivo* however, some reports of similar success do exist (McLachlan *et al.* 1995; Uchida *et al.* 2002).

Optimal conditions for liposome-mediated transfection are known to vary between recipient cell types, dependant principally on the nature and concentration of the DNA construct being used and on the incubation time (Benhamou PY *et al.* 1997). Optimisation is therefore necessary. My priority in this respect was to achieve

maximum transfection efficiency whilst minimising incubation time (in view of the planned *in vivo* experiments where incubation time would equate to ischaemic time within the experimental flap). I therefore designed a series of experiments based on the GFP reporter plasmid and the transfection agent DOTAP to assess transfection efficiency for a variety of conditions in primary BAEC cultures before switching to the pEF-BOS-VEGFB study plasmid.

5.2 Materials and Methods

5.2.1 Acknowledgements

Primary cultured bovine aortic endothelial cells (BAECs) were provided at passage six by Dr L Juan, Cardiovascular Research Unit, University of New South Wales. I am grateful for the instruction given to me in the techniques fundamental to cell culture by Dr C Hicks, Department of Haematological Research, University of New South Wales.

5.2.2 Cell culture reagents and equipment

All reagents were tissue culture grade. DMEM MultiCel powder (product ref: 50-126-PA) and L-glutamine were from Trace Biosciences Pty Ltd (Noble Park, Victoria, Australia). Sodium bicarbonate and penicillin G/streptomycin were from Sigma-Aldrich (Castle Hill, NSW, Australia). Trypsin (0.25% in 1mM EDTA), phosphate buffer and fetal calf serum were from Gibco, Life Technologies (New York, USA). Sterile water was from Baxter Healthcare (Deerfield, IL, USA). Ultra filters (0.22 μ m, product ref: 4622) were from Gelman Sciences (Cheltenham,

Victoria, Australia). Falcon sterile pipettes and 75 cm³ cell culture flasks were from Becton Dickinson (Franklin Lakes, NJ, USA). Eppendorf tubes were from Eppendorf (Hamburg, Germany). Cell cytometer with Neubauer improved rulings was from Laboroptik (Friedrichsdorf, Germany). Eight-well chamber slides (product ref: 177445) were from Lab-Tek (Nalge Nunc International, Naperville, USA). A Westinghouse Pty (NSW, Australia) biological safety cabinet and Forma Scientific (Marietta, Ohio, USA) incubator were used for all cell culture work.

5.2.3 Plasmids and transfection reagents

pEGFP-N2 was from Clontech (Mountain View, California, USA) supplied at a stock concentration of 1 µg/µl DNA. pEF-BOS-VEGFB plasmid had previously been prepared from *E. coli* glycerol stock and was available at a concentration of 490 µg/ml (DNA ref #2). The transfection agent N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP) also at a stock concentration of 1 µg/µl was from Sigma-Aldrich. Hepes buffer (1M stock) was from Fluka (St. Gallen, Switzerland).

5.2.4 Immunohistochemical reagents and antibodies

General reagents and solutions used were as listed in Chapter 3. The M2 monoclonal mouse anti-FLAG primary antibody was from Sigma (product ref: F3165) whilst the biotinylated rabbit anti-mouse secondary antibody was from Dako (product ref: E 0354).

5.2.5 Preparation of cell culture medium and reagents

DMEM with 10% foetal calf serum: Add one preparation of DMEM powder to 900 ml sterile water and stir until dissolved. Add 2.4 g sodium bicarbonate and 1 ml penicillin/streptomycin solution (working concentration 5 u/ml penicillin, 5 µg/ml streptomycin) and adjust to pH 7.4 with 1M hydrochloric acid added dropwise. Transfer to biological safety cabinet and add 100 ml heat inactivated (56 °C, 30 min) foetal calf serum. Ultrafilter into sterile bottle and store at 4 °C. Replenish by adding 1:100 dilution L-glutamine (200 mM) every 14 days.

Phosphate buffered saline: Dissolve 5 tablets phosphate buffer in 500 ml sterile water. Ultrafilter into sterile bottle and store at 4 °C.

Hepes buffer: Use at 20 mM, pH 7.4. From 1M stock solution perform 1:50 dilution in sterile water and adjust pH.

5.2.6 Subculturing of BAEC's (1:3 split)

All reagents should be warmed to 37 °C in a waterbath. Remove medium from cell cultures and discard. Wash with one change of 5 ml PBS before adding 3 ml trypsin solution. Incubate at room temperature for 5 min with agitation until cells separate, neutralise trypsin with 3 ml media, transfer to 15 ml plastic reaction tube and centrifuge at 1000 rpm (Beckman J2-21) for 5 min. Discard supernatant and resuspend cell pellet in 3 ml medium, mixing gently to break up all cell clumps. A cell count may be performed at this stage (see below). Pipette 15 ml fresh medium

each into three sterile 75 cm³ culture flasks and transfer one third of the final cell suspension into each. Incubate at 37 °C, 5 % CO₂ with the culture flask lid loosened to allow gas exchange.

5.2.7 Estimation of cell concentration

A standard haemocytometer with Neubauer rulings was used in accordance with the manufacturers instructions. Since large culture flasks were being used, a preliminary dilution of 1:100 was carried out in fresh medium. Four grid counts were performed under x40 magnification and cell concentration calculated using the formula:

$$\begin{aligned}\text{Cell concentration} &= \text{mean cell count} \times \text{dilution factor} \times 10 \text{ cells/mm}^3 \\ &= \text{mean cell count} \times 100 \times 10 \times 1000 \text{ cells/ml} \\ &= \text{mean cell count} \times 10^6 \text{ cells/ml}\end{aligned}$$

5.2.8 Transfection of BAECs

All cell cultures were prepared in the same manner. Each well of an eight-well chamber slide was inoculated with 1×10^4 BAECs in 0.2 ml medium the day prior to transfection and returned to the incubator. The aim was to achieve a cell confluence of 60% – 80% at the time of DNA transfer.

The transfection procedure was conducted in accordance with the manufacturers instructions in all experiments using DOTAP i.e. DNA, at a concentration of 0.1 µg/ml in hepes buffer, was first combined with DOTAP in a sterile reaction tube and

incubated for 15 mins at room temperature before gently mixing with an appropriate volume of culture medium to achieve the desired final DNA concentration. 200 μ l of the resulting mixture was then transferred to the cell cultures in each well for the planned incubation time. On completion the transfection mixture was removed and replaced with fresh culture medium. The slide chamber lids were gently replaced and the cultures incubated at 37 °C and 5 % ambient CO₂ for the duration of the experiments.

5.2.9 pEGFP-N2 plasmid

5.2.9.1 Experiment 1

The influence of DOTAP concentration on transfection efficiency was investigated for two concentrations of DNA (2.5 μ g/ml and 5 μ g/ml), two incubation times (1 hr and 3 hrs) and at two DNA:DOTAP ratios. The manufacturer's recommended transfection conditions (2.5 μ g/ml DNA, DOTAP:DNA 6:1, incubation time 3 hrs) were incorporated as base line.

5.2.9.2 Experiment 2

In the light of the results from experiment 1, the influence of DNA concentration and incubation time on transfection efficiency was further investigated with a fixed DOTAP:DNA ratio of 6:1. DNA concentrations of 5 μ g/ml, 10 μ g/ml and 20 μ g/ml were each investigated with incubation times of 1 hr, 3 hrs and 12 hrs. Control groups comprised one series of wells incubated with DOTAP only (60 μ l/ml in

DMEM representing the median DOTAP concentration used) as above and 2 wells that underwent changes of medium only during the experimental period.

5.2.10 pEF-BOS-VEGFB plasmid

In three separate chamber slides, two wells each were inoculated with 4×10^4 , 2×10^4 and 1×10^4 BAECs respectively. A transfection mixture of 10 $\mu\text{g/ml}$ plasmid DNA containing DOTAP at a ratio of 6:1 was incubated with the BAEC cultures for 3 hrs. To monitor the number of successful transfections, one slide per day was fixed (10 % buffered formalin applied for 20 mins followed by two exchanges of PBS) for a total of three days. Slides were then subjected to immunostaining using the anti-FLAG primary antibody as described below. Prior to fixation, all wells were examined daily.

5.2.11 Immunohistochemistry

The outer casing of the chamber slides were removed according to the manufacturers instructions and the cells washed with PBS. Anti-FLAG monoclonal antibody was diluted 1/100 in TRIS buffered saline as per the manufacturer and applied as an even coat to each slide, ensuring all cells were covered, and incubated at room temperature for 60 minutes. Two washes in PBS were performed before applying an even coat of biotinylated rabbit anti-mouse secondary antibody at a dilution of 1/200 in TRIS buffered saline. Incubation was for 30 mins at room temperature. Staining was visualised using a streptavidin peroxidase label at a dilution of 1/150 applied evenly

for 30 minutes followed by 2 washes with PBS and application of DAB reagent for 5 minutes. Slides were then washed in water, dried and coverslips applied.

5.3 Results

5.3.1 pEGFP-N2 plasmid

Examination of the BAEC culture wells was undertaken at 1, 24, 48 and 72 hrs post transfection using a Leica inverted fluorescence microscope (Wild Leitz).

5.3.1.1 General observations

The following trends were noted when transfected cells were compared to media-only controls:

1. All cultures, including DOTAP-only controls, displayed a change in cellular morphology appearing more rounded from 1 hr and persisting to 72 hrs
2. There appeared to be a reduction in cytoplasmic volume
3. Evidence of cytotoxicity was apparent in all experimental wells at 1 hr including the DOTAP-only control. This proved difficult to quantify accurately however, the most affected were wells where DNA and DOTAP concentration were highest and where incubation time was longest.

Approximately one quarter to one half of cells were lost at the time of transfection under these conditions however, some recovery was apparent by 24 hrs. The most significant cell loss however, occurred when transfection was conducted in the absence of medium. In this group over half of the cells were lost at 24 hrs and all had died by 48 hrs.

5.3.1.2 *Transfection efficiency*

Cells expressing the EGFP-N2 gene were readily identified by the green fluorescence of their cytoplasm when viewed in the presence of ultraviolet light (Figure 5.1). No fluorescence was seen in the control wells (medium plus DOTAP, medium only) at any time.

The mean number of successfully transfected cells per well ($n=2$) for each set of transfection conditions is shown graphically for experiments 1 and 2 in Figures 5.2 and 5.3 respectively. Experiment 2 proved more successful than experiment 1 possibly reflecting the use of a new supply of DOTAP for this work. The most successful transfer of pEGFP-N2 into the BAECs appeared to occur at a DNA concentration of 10 $\mu\text{g/ml}$, with a DOTAP:DNA ratio of 6:1 and an incubation time of 3 hrs.

In general, the highest numbers of GFP-producing cells were seen on the second post transfection day for the time period studied. To investigate the relationship between each variable and transfection success, this set of observations was subjected to further analysis.



Figure 5.1 –BAEC cultures following transfection with pEGFP-N2. (A) low and (B) high magnification. The detection of the characteristic green fluorescence of GFP confirms both successful gene transfer and active protein synthesis (Inverted fluorescence microscope with ultra-violet illumination).

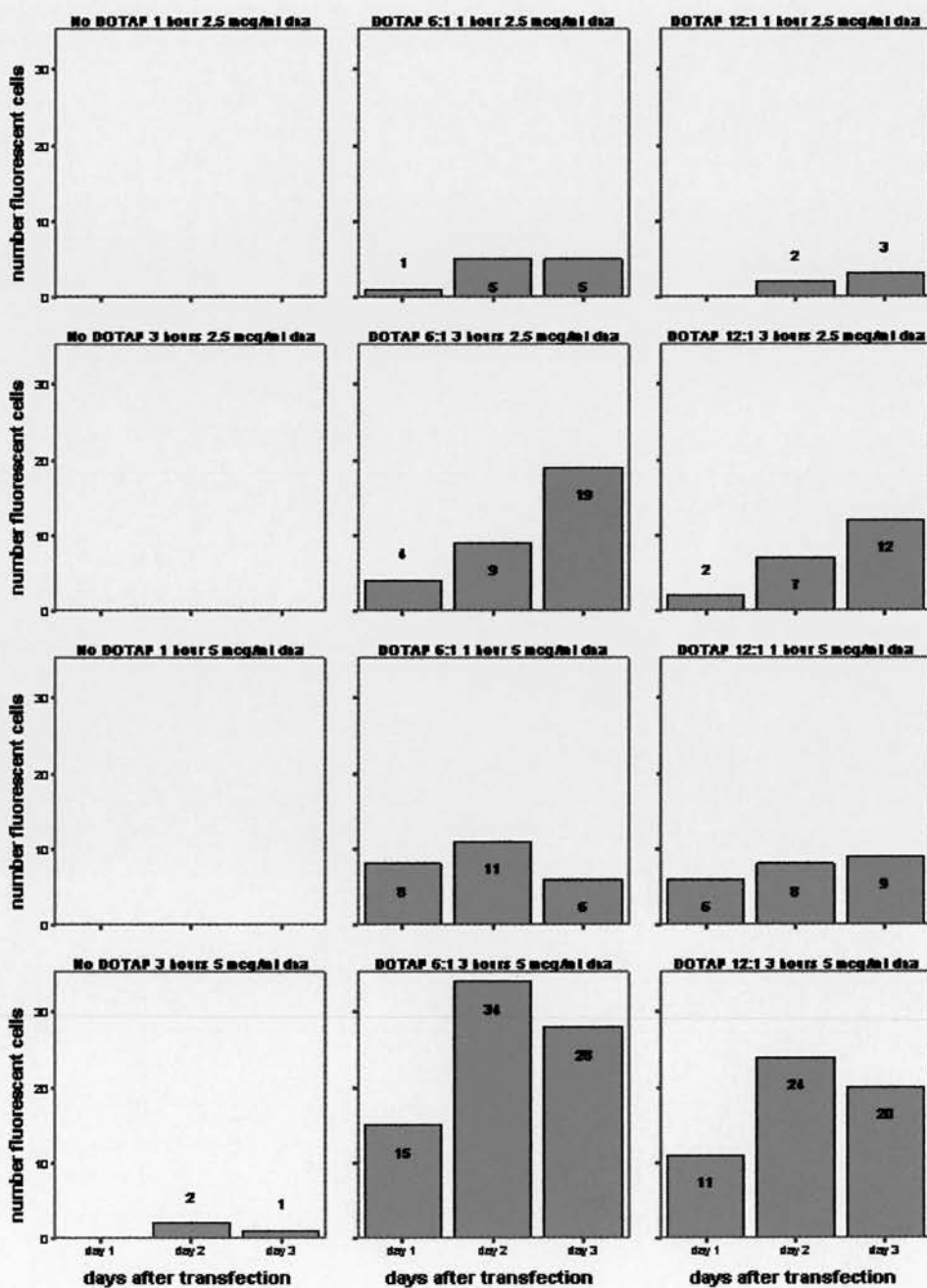


Figure 5.2 – Bar charts showing the effect of increasing DNA concentration, DOTAP:DNA ratio and incubation time on transfection efficiency.

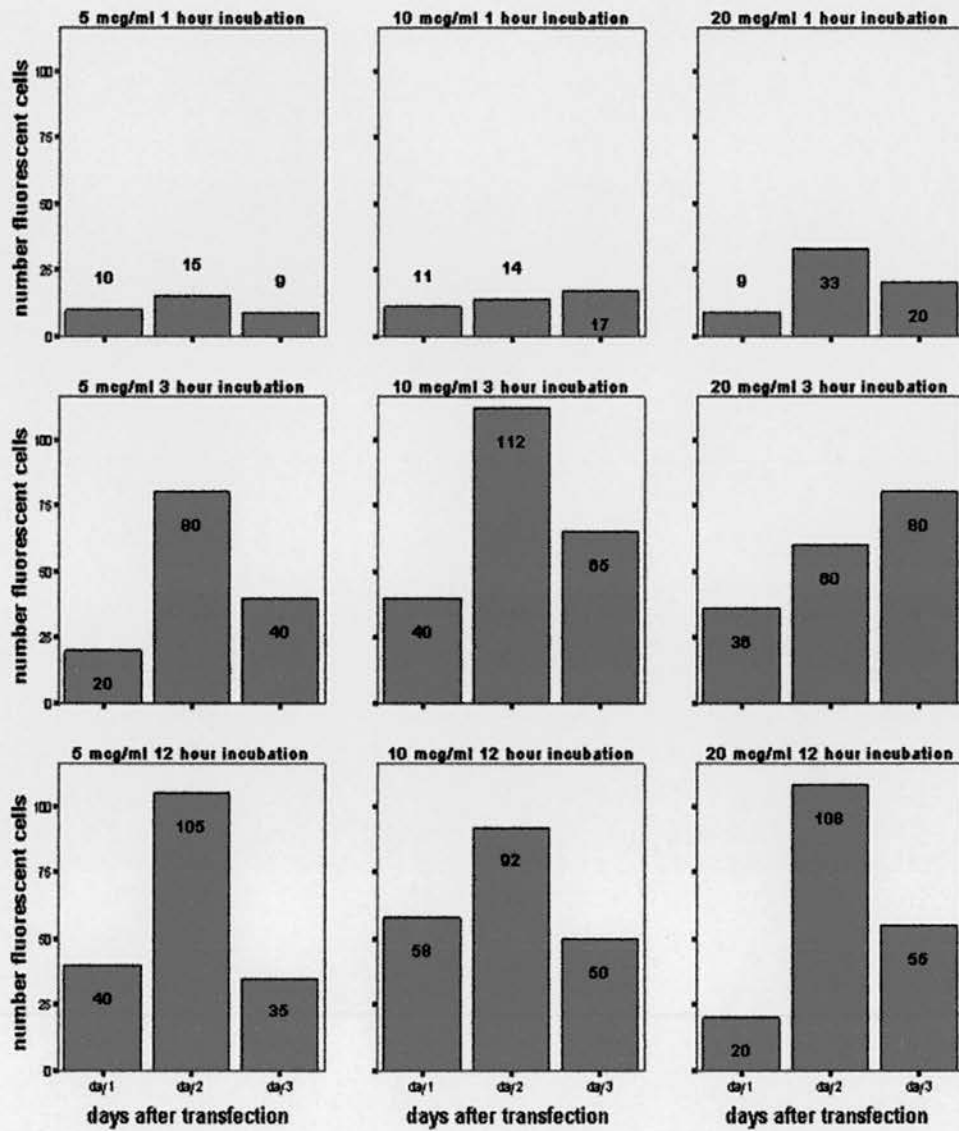


Figure 5.3 – Bar charts showing the effect of further increases in DNA concentration and incubation time on transfection efficiency using a DOTAP:DNA ratio of 6:1 in all cases.

5.3.1.3 DOTAP to DNA ratio

In all cases, the presence of DOTAP significantly improved transfection efficiency. There appeared however, no advantage in increasing DOTAP:DNA ratio above 6:1 (Figure 5.4).

5.3.1.4 Duration of incubation

Increased transfection efficiency was noted with increased incubation time up to the maximum of 12 hrs for DNA concentrations of 5 and 20 $\mu\text{g/ml}$. This did not appear to hold for the 10 $\mu\text{g/ml}$ concentration due to a very high reading for the 3 hour incubation samples (Figure 5.5).

5.3.1.5 DNA concentration

No consistent relationship between DNA concentration and transfection efficiency for a given incubation time was identified.

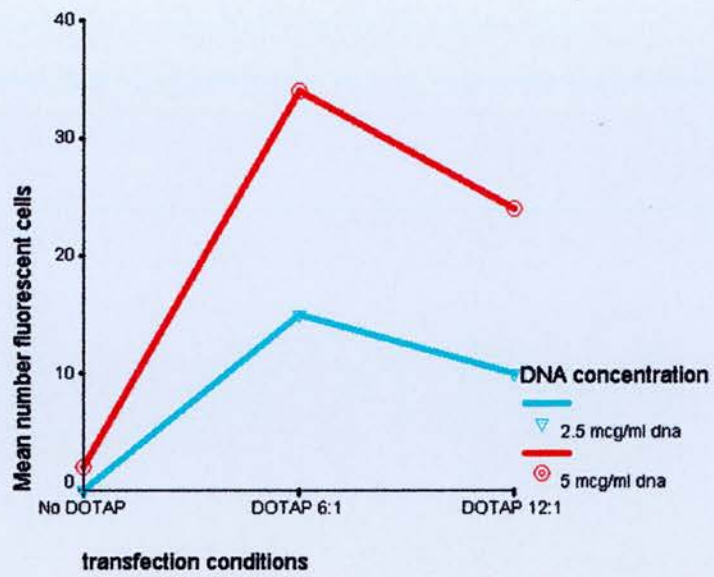


Figure 5.4 – Line chart showing the effect of increasing DOTAP:DNA concentration on transfection efficiency for two starting concentrations of DNA.

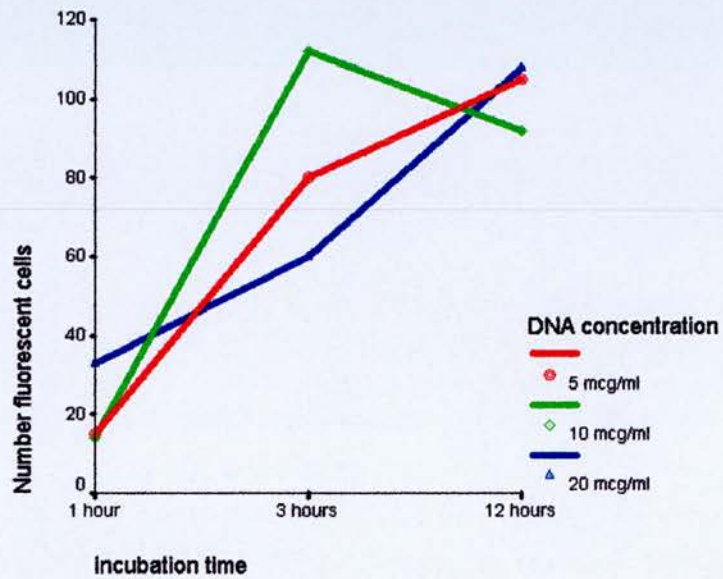


Figure 5.5 – Line chart showing the effect of increased incubation time on transfection efficiency for three starting concentrations of DNA.

5.3.2 pEF-BOS-VEGFB plasmid

Assessment of transfection efficiency was more difficult in this group reflecting a less sensitive detection system. Cells staining positive for the FLAG antigen proved more difficult to identify, and therefore quantify, with certainty than was the case for GFP transfectants. The pEF-BOS-VEGFP plasmid appeared however, to behave similarly to pEGFP-N2 in that the first signs of FLAG expression were noted at 24 hrs with a significant increase in the numbers of positive cells seen by 48 hrs followed by a decline to 72 hrs post transfection (Figure 5.6). The numbers of successful transfectants identified in general, appeared similar to the pEGFP-N2 plasmid.

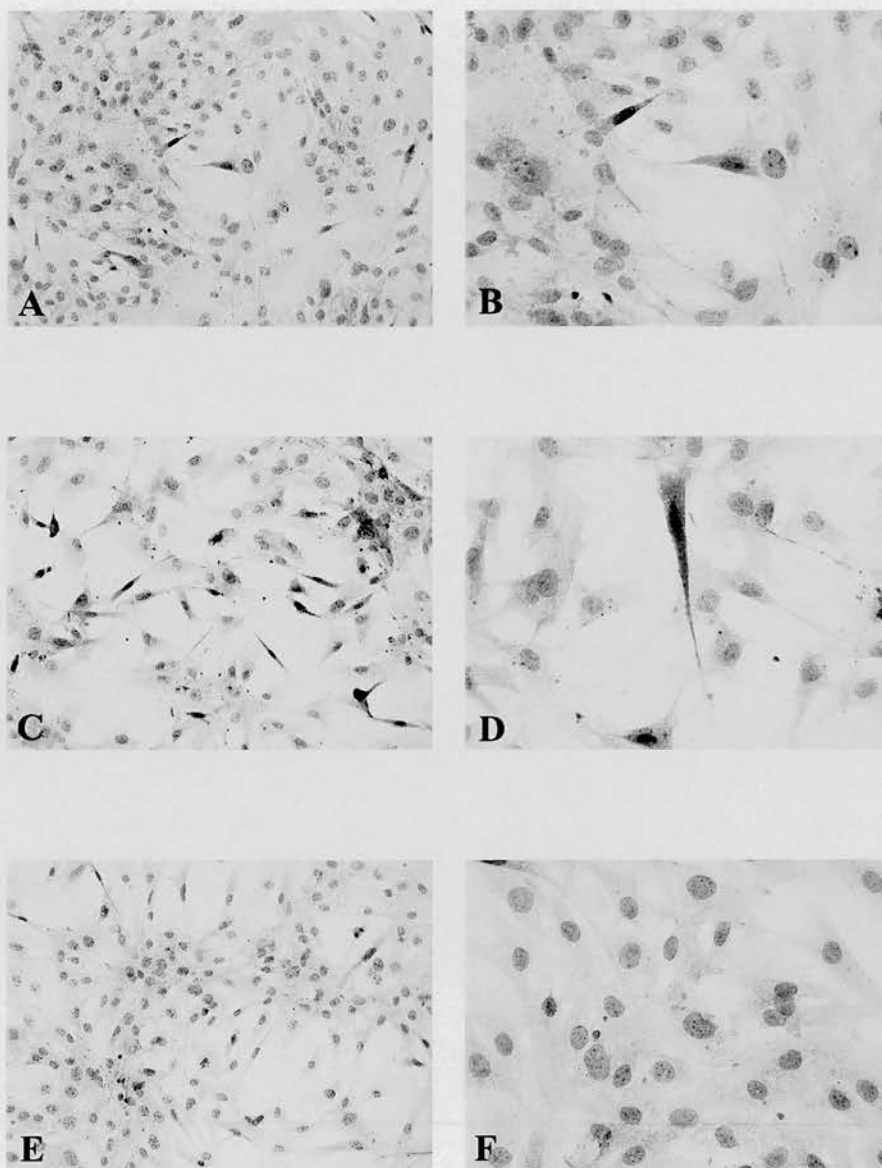


Figure 5.6 – Whole BAEC cell preparations stained for FLAG. (A) & (B) seen at 24 hrs and (C) & (D) at 48 hrs post transfection with 10 µg/ml pEF-BOS-VEGFB DNA, DOTAP:DNA 6:1 with an incubation time of 3hrs. (E) & (F) represent a control population of BAECs also stained for FLAG. Cells containing brown pigment are positive for the FLAG antigen and are most obvious in the 48 hr post transfection specimens.

5.4 Discussion

Since first described, cationic liposomes have been employed to achieve successful gene transfer to a wide variety of cells *in vitro* with, more recently, some authors reporting encouraging results using this technology *in vivo* (Audouy & Hoekstra 2001). In this series of *in vitro* experiments I was similarly successful in achieving GFP gene transfer to, and subsequent expression in, cultured BAECs. Also recognised is the variability that exists in the susceptibility of any given cell to this form of manipulation and like others, I found significant variation in the extent to which gene transfer occurred dependant on conditions at the time of transfection (Smyth 2003). Maximal transfection efficiency was achieved at a DNA concentration of 10 $\mu\text{g/ml}$ which is high relative to the manufacturers recommendation of 1-2.5 $\mu\text{g/ml}$ however, is consistent with previously reported protocols (Benhamou PY *et al.* 1997). The apparent cytotoxic effect of the transfection mixture at higher DNA concentrations is perhaps one reason why further increases in DNA concentration do not translate into improved transfection efficiency. Other conditions proved optimal as suggested by the manufacturer.

In general, maximal gene expression was observed at 48 hrs post transfection, a finding consistent with previous reports (Misteli & Spector 1997).

The pEF-BOS-VEGFB plasmid appeared equally as effective as pEGFP-N2 in achieving successful transfection of BAECs in culture and this was encouraging.

In these experiments I have identified the optimum conditions for gene transfer to BAECs in culture using the transfection agent DOTAP. I have also demonstrated the ability of the pEF-BOS-VEGFB plasmid to transfer the VEGFB gene to mammalian endothelial cells *in vitro* and to achieve expression of that gene as evidenced by positive staining using the FLAG detection system. These results will prove useful in attempting to achieve similar gene transfers *in vivo*.

5.5 Conclusions

1. The transfection agent DOTAP substantially improved transfection of BAECs with pEFGP-N2 compared to DNA alone.
2. Optimal transfection was achieved with a DNA concentration of 10 µg/ml, DOTAP:DNA ratio of 6:1 and an incubation time of 3 hrs.
3. pEF-BOS-VEGFB proved successful in achieving transfer and expression of the VEGFB gene to mammalian endothelial cells in culture.

Chapter 6 – Transfection experiments with pEF-BOS and pEGFP-N2 in vivo

6.1 Introduction

6.1.1 Overview

Strategies for the transfer of genetic material to the vascular tree *in vivo* have been the subject of much research (George & Baker 2002). Success depends on many factors however, two of the most important considerations are the choice of vector to facilitate the transfer and the proposed method of administration (Nishikawa & Hashida 2002).

In the search for a suitable vector, raw plasmid DNA in particular, has been a major focus of attention. The low toxicity of DNA in solution makes for easy handling and with few reported side effects, it contrasts markedly with the problems that must be faced if for example, viral vectors are to be used (Young & Dean 2002).

Unfortunately however, transfection efficiency with raw plasmid DNA has proved consistently low. Reissen *et al.* reported this to be in the order of 0.5% following application of raw plasmid DNA to rabbit carotid arteries using an angioplasty catheter (Reissen *et al.* 1993). As a result, many of the techniques known to improve transfection efficiency *in vitro*, including the use of cationic liposomes, have been applied, with varying degrees of success, *in vivo* (Smyth 2002). One additional strategy has been to use very high concentrations of DNA, far in excess of the

cytotoxic limit for cells in culture (Tsurumi *et al.* 1996). Using these methods techniques for gene transfer using raw plasmid DNA have advanced however, low overall efficiency remains a problem (Young & Dean 2002).

In the context of therapeutic angiogenesis however, this low efficiency is, to some extent, balanced by the biological activity of VEGF which is known to remain active even at picomolar concentrations (Ferrara N *et al.* 1991). This was demonstrated by Takeshita *et al.* in the rabbit hind limb model of ischaemia. In these experiments VEGFA was transferred to both iliac arteries in each rabbit, using the same technique as Reissen. Notwithstanding the low transfection efficiency, a significantly greater angiogenic response was detected in animals treated in this way when compared to controls, leading the authors to conclude the production of VEGFA was at least within the range of biological activity (Takeshita *et al.* 1996). There is some evidence to suggest that the uptake of raw DNA by endothelial cells is enhanced in the presence of ischaemia, making for a greater transfection efficiency in this model. The exact mechanism by which this occurs however, remains a point of speculation (Takeshita, Isshiki, & Sato 1996).

Equally important in the success of any given technique for gene transfer to the vasculature is the route of administration. These may be considered in two groups, intra- and extra-arterial. In the case of the later, the most popular method is that of Wolff *et al.* and involves the direct intra-muscular injection of plasmid DNA (Wolff *et al.* 1990). High levels of reporter gene expression have been achieved with this technique although there appears considerable variability in results dependant not

only on the plasmid construct but also the target muscle, species and even age of the experimental animal (Danko *et al.* 1997). Nevertheless, expression of VEGFA transferred to muscle in this way has resulted in an angiogenic response in animal models when compared to controls (Tsurumi *et al.* 1996). Successful gene expression has also been reported following subcutaneous administration of plasmid DNA although the exact fate of the exogenous DNA in this technique is not clear (Bohm *et al.* 1998).

Intra-arterial administration of plasmid DNA has proved similarly effective in animal models and more recently has formed the basis of a number of clinical trials (Amant, Berthou, & Walsh 1999). Where the size of the target vessel permits, Riessens method is effective (Riessen *et al.* 1993). In this technique plasmid DNA is applied to the surface of a balloon angioplasty catheter by means of a thin hydrogel coating. Inflation of the balloon within the vessel lumen results in site-specific gene transfer to the arterial wall (Takeshita *et al.* 1996). An alternative method described is the direct intra-arterial injection of plasmid DNA which is also effective, although non specific, with most successful transfections appearing to take place within the supplied muscle groups (Zhang *et al.* 2001).

Our aim was to use this technology to influence the survival of ischaemic epigastric flaps in rats and in this respect, there was little published work to guide us in the planning of our experiments. The small size of the SIEA precluded the use of a catheter-based technique for plasmid delivery. Intra-arterial administration however was feasible. The ability to access the vascular network within the ischaemic half of

the flap, via the divided contralateral SIEA, coupled with an ability to control the circulation through the flap by manipulating the pedicle on the ipsilateral side, served as additional attractions of this method. In this manner we could effect a uniform distribution of plasmid DNA throughout the vascular system in the ischaemic flap and provide for the target incubation period of 3 hrs.

6.1.2 Aims

Having shown that pEF-BOS-VEGFB was effective in transfecting bovine endothelial cells *in vitro* causing them to produce VEGFB, in these experiments I planned to investigate the possibility that this plasmid could be used to stimulate angiogenesis *in vivo*. In addition, transfection efficiency would be evaluated using pEGFP-N2.

6.2 Materials and Methods

6.2.1 Animals

Fourteen male Sprague-Dawley rats weighing between 300 g and 400 g were used in these experiments. The supply and care of the animals was as described in Chapter 2. Full ethical approval for this work was sought, and granted, from the Animal Care and Ethics Committee of the University of New South Wales.

6.2.2 Plasmids

pEF-BOS-VEGFB was from stock (preparation numbers 2 (490 µg/ml DNA) and 4 (440 µg/ml DNA) were used in this work {Chapter 4}) while pEGFP-N2 was from Clontech (product ref: U57608).

6.2.3 Experimental groups

Three experimental groups comprised four rats each and a fourth group, 2 rats.

Transfection conditions were based on the most successful combinations identified *in vitro*. In addition, a bolus dose of 100 µg, without the transfection agent, was also investigated. DOTAP, when used, was always at the preferred ratio of 6:1, DOTAP:DNA.

Group 1 (n=4)

The random pattern flap was infused with a transfection mixture comprising 10 µg/ml pEF-BOS-VEGFB with 60 µg/ml DOTAP in hepes buffer via the right SIEA. Flap circulation was arrested for 3 hrs to allow incubation of the mixture with the vessel wall.

Group 2 (n=4)

The random flap was infused with a transfection mixture comprising 20 µg/ml pEF-BOS-VEGFB and 120 µg/ml DOTAP in hepes buffer. Incubation was for 3 hrs.

Group 3 (n=4)

The random flap was infused with 100 µg raw plasmid DNA (pEF-BOS-VEGFB) in hepes buffer, no transfection agent was used in this group. Incubation was also for 3 hrs.

Group 4 (n=2)

Both right and left SIEAs were infused with pEGFP-N2 at a concentration of 10 µg/ml DNA and DOTAP at 60 µg/ml in hepes buffer. Incubation was for 3 hrs.

6.2.4 Transfection mixtures

From my angiogram work I knew that the volume of the arterial system of the flap was approximately 200 µl and this would be the minimum volume of each transfection mixture necessary per animal. Where practicable I prepared 400 µl to allow for losses. Preparation of these mixtures was conducted in the Haematology Research Laboratory, Prince of Wales Hospital, Sydney before transfer on ice, to the operating facility at the Prince Henry Hospital.

6.2.4.1 Transfection mixture 1 (10 µg/ml DNA + DOTAP 60 µg/ml):

36 µl stock 4 DNA was pipetted into a sterile eppendorf reaction tube and reduced to a working concentration of 0.1 µg/ml by the addition of 124 µl hepes buffer, pH 7.4. In a separate reaction tube, 96 µl DOTAP was diluted to a working concentration of 0.3 µg/µl by adding 224 µl hepes buffer. The two solutions were combined and

incubated at room temperature with gentle mixing for 15 mins. Hepes buffer was added to a final volume of 1600 μl again with gentle mixing. 400 μl of the final solution was placed into each of 4 sterile eppendorf tubes for transport.

6.2.4.2 Transfection mixture 2 (20 $\mu\text{g}/\text{ml}$ DNA + DOTAP 120 $\mu\text{g}/\text{ml}$):

Prepared in exactly the same manner using 72 μl stock 4 DNA made up to 320 μl with hepes buffer and added to 640 μl of 0.3 $\mu\text{g}/\text{ml}$ DOTAP. Incubation was for 15 mins and preparations for transport as above.

6.2.4.3 Transfection mixture 3 (100 μg DNA stat):

204 μl of stock 2 DNA would give the required dose. Administration of a volume to this level of accuracy was not practicable therefore 210 μl was placed in each of four sterile eppendorf tubes with the intention of using the full volume.

6.2.4.4 Transfection mixture 4 (10 $\mu\text{g}/\text{ml}$ pEGFP-N2 + DOTAP 60 $\mu\text{g}/\text{ml}$):

This plasmid is supplied at a stock concentration of 1 $\mu\text{g}/\mu\text{l}$. 8 μl stock DNA was diluted to a final volume of 80 μl in hepes buffer and gently mixed with 160 μl DOTAP at 0.3 $\mu\text{g}/\text{ml}$, incubated for 15 mins and made up to a final volume of 800 μl with hepes buffer. The final volume was divided between 4 sterile eppendorf tubes in preparation for transport.

6.2.5 Surgical procedure

General anaesthesia was induced and maintained and the surgical field prepared and draped as previously described (Chapter 2).

Groups 1 to 3 (n=4)

8 x 8cm epigastric island flaps were marked and raised preserving both left and right superficial inferior epigastric pedicles in the first instance. On the left side the femoral artery proximal to the origin of the left SIEA was dissected and controlled with a rubber vascular sloop initially left slack. The right SIEA was then dissected under the operating microscope and cannulated using a 24 gauge intravenous catheter secured using a single 7/0 prolene ligature with the main vessel cauterised proximally. A 0.5ml saline flush was performed to ensure freedom of flow before tightening the sloop on the left SIEA, thus arresting the circulation in the whole flap. 400 µl of the appropriate transfection mixture was then introduced into the right hemiflap via the right SIEA. The cannula was then removed and the right pedicle divided completely. The flap was then stitched in position using 5/0 nylon suture leaving the left groin open. Circulation to the flap was restored after a 3 hr incubation period by removing the sloop from the left femoral artery. The left groin wound was then similarly closed.

Group 4 (n=2)

Since this group would provide information on transfection efficiency only, no flap was necessary. Both groins were opened and the femoral and superficial inferior

epigastric arteries dissected. Proximal control of the femoral artery was achieved with a rubber vascular sloop before introducing a 24 gauge intravenous cannula in a retrograde manner distal to the origin of the SIEA. In this way it was possible to infuse the SIEA system while avoiding injury to the artery itself. 200 μ l of pEGFP-N2 transfection mixture was introduced into both right and left SIEAs using this technique. The distal femoral artery was ligated on each side following withdrawal of the cannula and the transfection mixture incubated in contact with the vessel wall for 3 hrs. The vascular sloops were then removed restoring circulation to the SIEA's and the groin wounds closed as before.

Recovery and postoperative management and observation of these animals were as previously described however, group 4 rats did not require a protective collar.

6.2.6 Data collection

Animals in groups 1 – 3 underwent data collection as previously described. They were killed on the seventh postoperative day and templates drawn for the assessment of flap survival, microangiography performed and tissue blocks prepared for immunohistochemical staining prior to the quantification of a-MVD. Group 4 animals were killed on day 2 and both right and left SIEAs harvested over a length of approximately 1 cm. Under the operating microscope each vessel was dilated and opened along its length before placing luminal side uppermost on a microscope slide, fixing and securing with a glass coverslip.

6.3 Statistics

Results are expressed as means \pm standard deviation and confidence intervals are quoted at the 95% level. Comparisons between groups were made using the Mann Whitney U test where $p < 0.05$ was taken to represent statistical significance.

6.4 Results

6.4.1 General observations

All surgical procedures were uncomplicated and each animal remained well during the period of observation. Similarly, all flaps were in good condition at the time of harvest. Two flaps from group 3 were noted to be mildly oedematous on the first postoperative day however, this resolved in both cases by day 2. Evidence of demarcation was first noted on day 3 for the majority of flaps in all groups (range 2 to 4).

6.4.2 pEF-BOS-VEGFB

6.4.2.1 Flap survival

The mean flap survival was 81.5 ± 4.8 % in the pEF-BOS-VEGFB 10 $\mu\text{g/ml}$ plus DOTAP group, 83.3 ± 2.5 % in the 20 $\mu\text{g/ml}$ plus DOTAP group and 81.8 ± 2.9 % in the 100 μg stat group (Tables 6.1 to 6.3). There was no significant difference in flap survival between any of the groups studied when compared to my control group of standard flaps ($p > 0.67$). The data is represented graphically in Fig. 6.1.

Table 6.1 – Flap survival at one week for rats receiving 10 µg/ml pEF-BOS-VEGFB with DOTAP transfection agent into the right SIEA. W1 refers to the weight of a template drawn from the whole flap while W2 is from the viable area only.

<i>Animal reference</i>	<i>Animal weight (g)</i>	<i>W1 (g)</i>	<i>W2 (g)</i>	<i>Flap survival (%)</i>
PVg1	380	1.27	0.99	78
PVg2	360	1.28	0.98	77
PVg3	380	1.31	1.10	84
PVg4	350	1.42	1.23	87
Mean				81.5
C.I.				73.9, 89.1

Table 6.2 – Flap survival at one week for rats receiving 20 µg/ml pEF-BOS-VEGFB with DOTAP transfection agent into the right SIEA.

<i>Animal reference</i>	<i>Animal weight (g)</i>	<i>W1 (g)</i>	<i>W2 (g)</i>	<i>Flap survival (%)</i>
PVg5	390	1.30	1.04	80
PVg6	350	1.21	1.00	83
PVg7	350	1.28	1.07	84
PVg8	350	1.40	1.20	86
Mean				83.3
C.I.				79.3, 87.2

Table 6.3 – Flap survival at one week for rats receiving 100 µg stat pEF-BOS-VEGFB (no transfection agent) into the right SIEA.

<i>Animal reference</i>	<i>Animal weight (g)</i>	<i>W1 (g)</i>	<i>W2 (g)</i>	<i>Flap survival (%)</i>
PVg9	340	1.57	1.35	86
PVg10	340	1.24	0.99	80
PVg11	330	1.35	1.09	81
PVg12	330	1.34	1.07	80
Mean				81.8
C.I.				77.2, 86.3

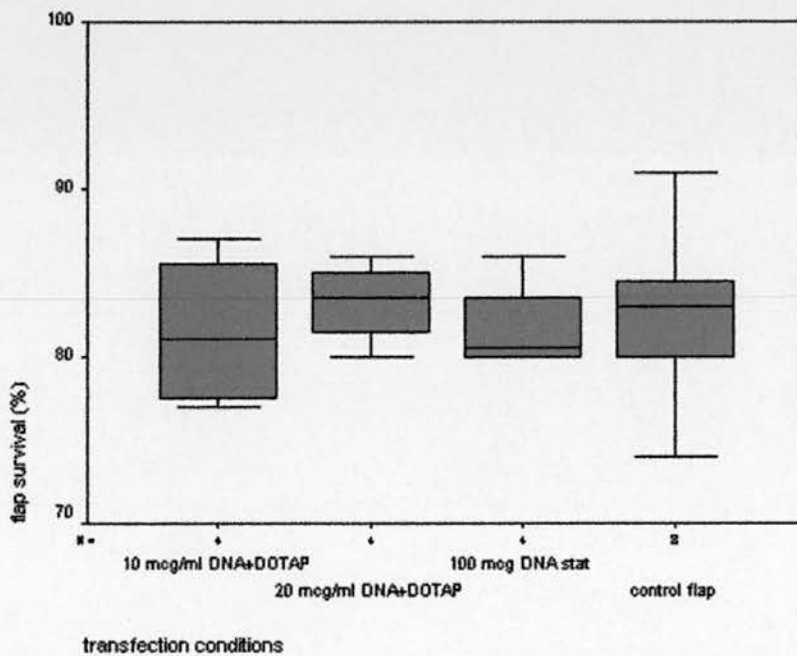


Figure 6.1 – Boxplot of percentage flap survival against transfection conditions. No difference between the groups is evident ($p>0.67$).

6.4.2.2 Microangiography

Mean HBPC/cm² for each group are shown in Table 6.4. No significant difference in mean HBPC was identified for any of the experimental groups when compared to the control group of standard flaps ($p>0.34$) (Figure 6.2). Examples of microangiograms obtained from each of the experimental groups are shown in Figure 6.3.

Table 6.4 – Mean HBPC/cm² recorded for the random and axial portions of each experimental group. 95% confidence intervals are given in brackets.

<i>Site</i>	<i>Group 1</i> <i>(10 µg pEF-BOS-VEGFB)</i> <i>n=4</i>	<i>Group 2</i> <i>(20 µg pEF-BOS-VEGFB)</i> <i>n=4</i>	<i>Group 3</i> <i>(100 µg pEF-BOS-VEGFB)</i> <i>n=3</i>	<i>Control</i> <i>n=8</i>
random pattern	17.5	17.5	15.3	16.1
flap	(14.2, 20.8)	(13.7, 21.3)	(9.1, 21.6)	(13.1, 19.2)
axial pattern	15.0	16.0	17.0	16.9
flap	(10.9, 19.1)	(13.1, 18.9)	(12.7, 21.3)	(13.8, 19.9)

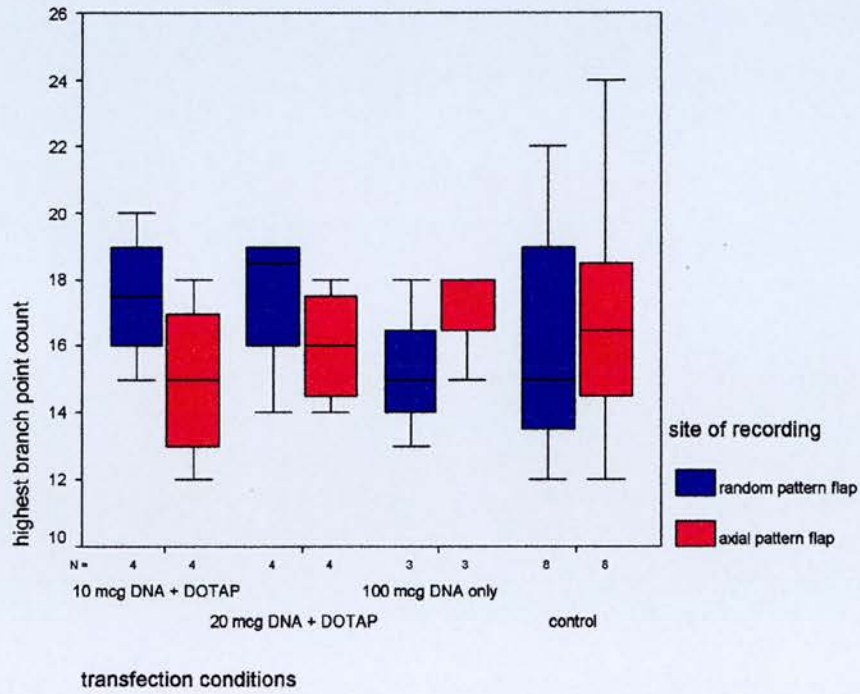


Figure 6.2 – Highest angiographic branch point count/cm² recorded for the axial and random pattern flaps for each transfection group and controls. Differences between experimental groups and controls did not reach statistical significance for either the random or axial pattern flaps (p>0.34)

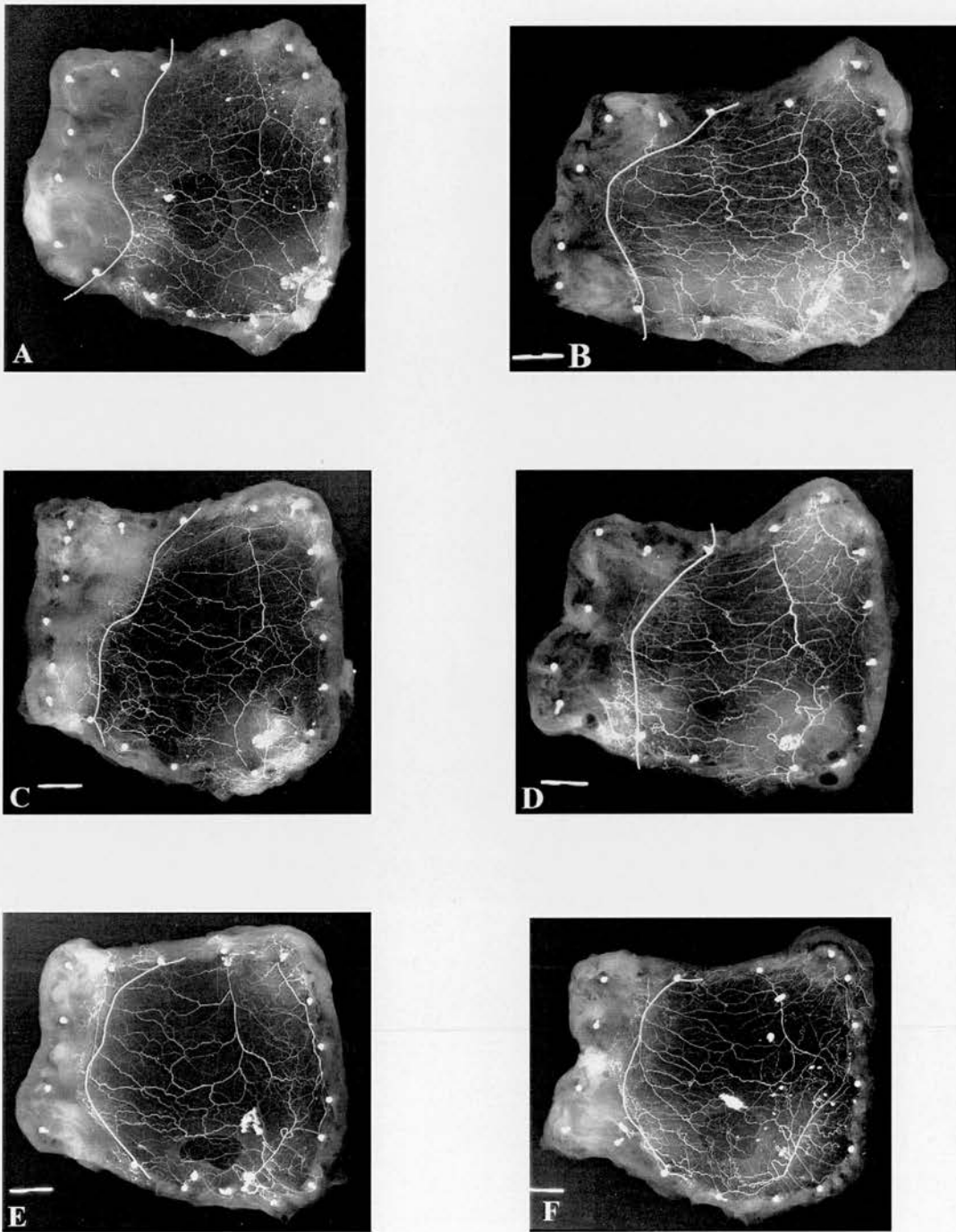


Figure 6.3 – Examples of microangiograms obtained from group 1 animals {(A) and (B)}, group 2 {(C) and (D)} and group 3 animals {(E) and (F)}. No statistically significant difference in mean HBPC/cm² was apparent when compared to controls (p>0.34).

6.4.2.3 Microvascular density

Histological examination of the flaps in each group revealed a pattern similar to that encountered in standard flaps, namely a-MVD was higher below compared to above the *panniculus carnosus* at all sites sampled and the highest a-MVD was observed deep to the *panniculus* within the zone of demarcation (Tables 6.5 to 6.7). When compared to the control group of standard flaps however, no statistically significant difference in a-MVD was noted in any of the positions examined ($p > 0.17$). Figure 6.4 shows typical distributions of a-MVD for each group taking position 2 of the random pattern flap as an example.

6.4.3 pEGFP-N2

There was no evidence of localising green fluorescence in any of the four femoral arteries examined suggesting that gene transfer had been unsuccessful (Figure 6.5).

Table 6.5 – Average MVD for rats receiving 10 µg/ml pEF-BOS-VEGFB into the right SIEA. Results are per unit area of 0.0625 mm², 95% confidence intervals given in brackets.

		<i>Average MVD (vessels per 0.0625 mm²) (95% C.I.)</i>		
		<i>Zone of demarcation</i>	<i>random pattern flap</i>	<i>axial pattern flap</i>
Position 1 (n=4)	level a	11.3 (4.8, 17.7)	9.8 (5.2, 14.3)	8.8 (6.0, 11.5)
	level b	28.0 (21.0, 35.0)	17.5 (11.3, 23.7)	16.8 (11.0, 22.5)
Position 2 (n=4)	level a	11.3 (5.1, 17.4)	8.5 (4.7, 12.3)	8.5 (4.3, 12.7)
	level b	22.8 (12.4, 33.1)	17.0 (12.3, 21.7)	18.0 (13.3, 22.7)

Position 1 represents sections taken at a position three quarters of the way along the flap from caudal to cranial, marked at the time of elevation. Similarly, position 2 represents sections from the mid point (see Fig. 3.2)

Level 'a' is above, and level 'b' below, the panniculus carnosus muscle.

Table 6.6 – Average MVD for rats receiving 20 µg/ml pEF-BOS-VEGFB into the right SIEA. Results are per unit area of 0.0625 mm², 95% confidence intervals given in brackets.

		<i>Average MVD (vessels per 0.0625 mm²) (95% C.I.)</i>		
		<i>Zone of demarcation</i>	<i>random pattern flap</i>	<i>axial pattern flap</i>
Position 1 (n=4)	level a	11.0 (5.1, 17.0)	9.5 (5.7, 13.3)	8.8 (5.2, 12.3)
	level b	19.5 (15.7, 23.3)	14.0 (11.1, 16.9)	15.0 (9.6, 20.4)
Position 2 (n=4)	level a	10.5 (1.7, 19.3)	9.3 (4.0, 14.5)	8.0 (3.9, 12.1)
	level b	21.5 (8.9, 34.1)	17.5 (12.9, 22.1)	15.3 (8.7, 21.8)

Position 1 & 2 and Levels 'a' & 'b' are as for Table 6.4

Table 6.7 – Average MVD for rats receiving 100 µg pEF-BOS-VEGFB stat into the right SIEA. Results are per unit area of 0.0625 mm² with 95% confidence intervals given in brackets.

		<i>Average MVD (vessels per 0.0625 mm²) (95% C.I.)</i>		
		<i>Zone of demarcation</i>	<i>random pattern flap</i>	<i>axial pattern flap</i>
Position 1 (n=4)	level a	10.3 (7.0, 13.5)	8.3 (5.5, 11.0)	9.3 (5.1, 13.4)
	level b	20.3 (12.0, 28.5)	16.0 (9.8, 22.2)	17.5 (11.3, 23.7)
Position 2 (n=4)	level a	12.3 (7.3, 17.2)	10.5 (3.4, 17.6)	8.5 (6.5, 10.6)
	level b	22.5 (12.4, 32.6)	16.5 (8.4, 24.6)	18.3 (14.7, 21.8)

Position 1 & 2 and Levels 'a' & 'b' are as for Table 6.4

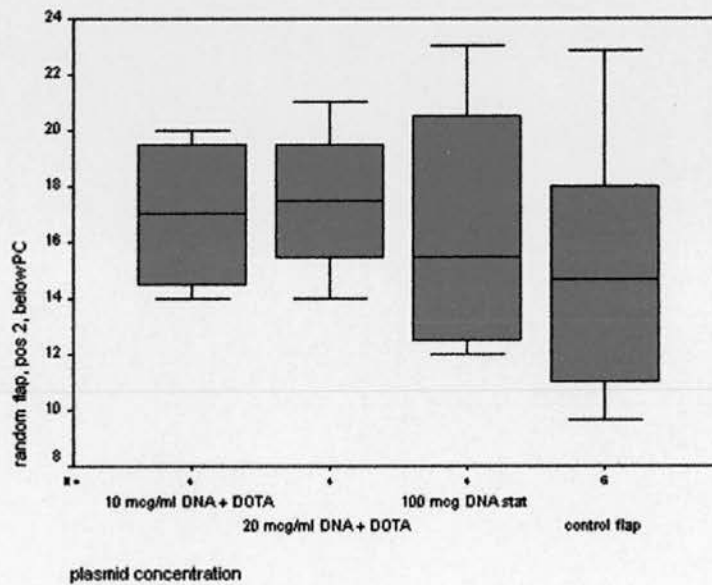
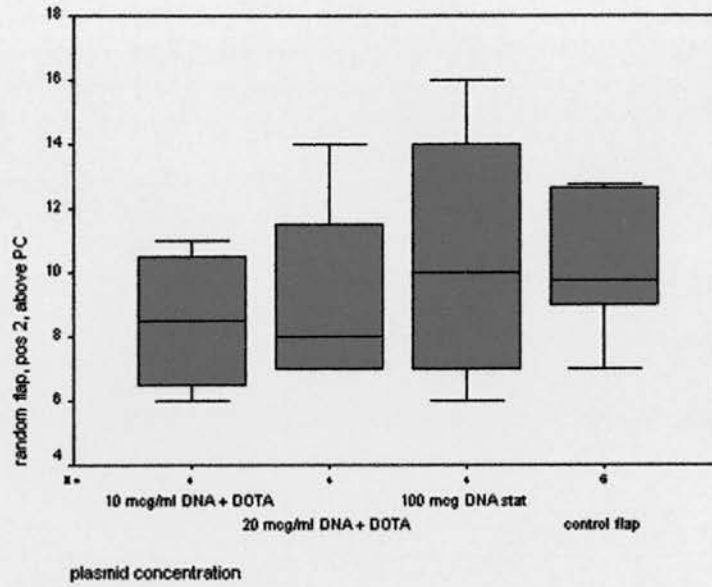


Figure 6.4 – Boxplots of a-MVD recorded for the random pattern flaps at position 2 (three quarters of the way along the flap) above (upper chart) and below (lower chart) the *panniculus carnosus* muscle layer. These results are typical of the data set as a whole. No significant difference in a-MVD was noted for any of the experimental flaps in any of the locations examined when compared to the control group

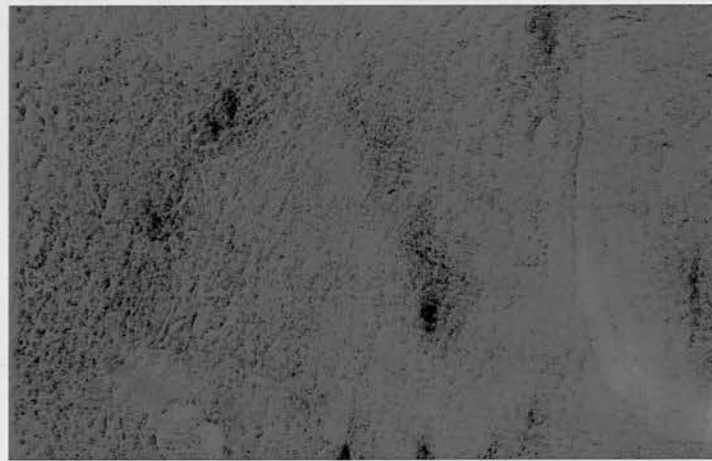
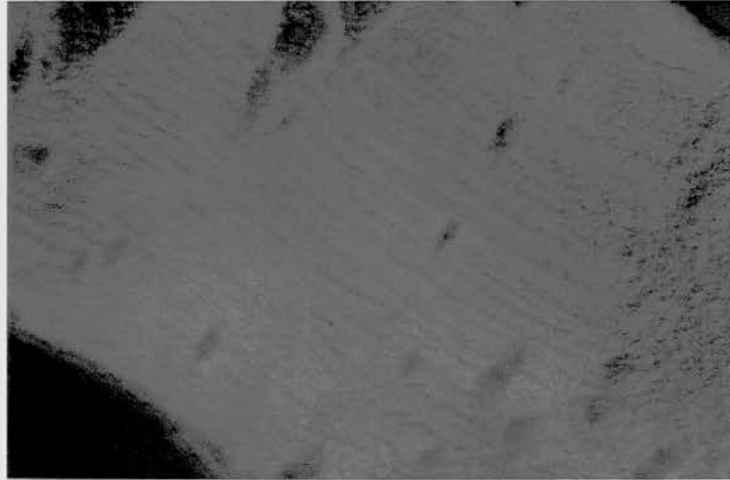


Figure 6.5 – Full thickness arterial wall specimens taken from the SIEA's of group 4 animals illuminated with ultraviolet light. No evidence of GFP expression was detected.

6.5 Discussion

In these experiments I failed to detect any evidence that pEF-BOS-VEGFB was capable of inducing angiogenesis in the rat epigastric island flap when administered intra-arterially in the manner described. In considering these results it is worth reviewing the requirements for success. Firstly a suitable expression vector for VEGF-B is required. I believe that pEF-BOS-VEGFB was such a vector. Having purified the plasmid from *E. coli* stock, preliminary work had confirmed its ability to induce expression of the VEGF-B gene in mammalian endothelial cells in culture. Although I did not test expression in rat endothelial cells specifically, the success of lipofection in achieving gene transfer in these cells has been previously reported by others (Laulajainen *et al.* 1993). It seems likely therefore that the rat endothelial cells would respond in a similar manner to BAECs.

One significant difference between the cell population *in vivo* compared to the *in vitro* setting does however exist. Endothelial cells *in vivo* are known to be among the longest lived in the body with only 0.01% in the process of cell division in adults at any given time (Hobson & Denekamp 1984). This is significant since transfection is known to occur most efficiently in the log growth phase in culture with much poorer results in the lag or stationary phase (Rippe, Brenner, & Leffert 1990). Nevertheless, skeletal muscle is also a predominantly stable cell population *in vivo* and yet very high transfection efficiencies have been reported in this tissue following trans-arterial infusion of plasmid DNA (Zhang *et al.* 2001). This suggests endothelial cells in particular are less receptive to gene transfer by these methods. The lack of any

detectable reporter gene expression at 2 days post exposure supports the view that transfection was significantly less efficient *in vivo* than was observed *in vitro*.

Although rat endothelial cells are likely to express the VEGFB gene in response to pEF-BOS-VEGFB in a similar manner to bovine endothelial cells the timing of expression may vary. I first detected the presence of GFP in the BAEC cultures at 24 hrs rising to a peak at 48 hrs. Significant delay in expression of the reporter gene *in vivo* would lead to a false negative result at the 48 hr assessment. It is certainly possible that a cell population of the stability of endothelium may require a longer time for gene expression to become apparent. I was unable to identify any specific *in vivo* work looking at this issue however, Takeshita *et al.* working with an arterial organ culture model, also reported good evidence of protein secretion by 24 hrs following gene transfer (Takeshita *et al.* 1994a).

Takeshita *et al.* have also published work to suggest that maximal endothelial cell proliferation in response to VEGF can be expected to occur between day 3 and 5 days following administration (Takeshita *et al.* 1995). Since flap demarcation is clinically apparent by day three post-elevation, the lack of a survival advantage would not necessarily translate to a failure of gene expression and in this model therefore, flap survival represents the least sensitive indicator of VEGFB production. This being the case however, and accepting a possible delay in the anticipated timing of VEGFB production, I would still have expected to detect its activity in the flap by the seventh postoperative day as evidenced angiographically by an increase in mean

HBPC/cm² and histologically by an increase in a-MVD. Neither proved to be the case.

The experience of Taub *et al.* in similar experiments with VEGF-A was significantly different. Reviewing their experimental protocol one crucial difference was the administration of their plasmid construct 4 days prior to rendering the experimental flaps ischaemic. This may have helped in achieving the significant survival benefit reported in their series however, interestingly they achieved these results with only a very short incubation time of 10 mins. Immunoperoxidase staining for VEGF appeared to confirm transgene expression in the periluminal region of vessels in transfected flaps however, they also reported no increase in absolute vessel numbers within their flap specimens (Taub PJ *et al.* 1998). By definition therefore, any observed improvement in flap survival cannot be attributed directly to induced angiogenesis.

My results are most easily explained by a failure of the transfection process to achieve the necessary efficiency such that any VEGF-B production failed to reach biologically active levels.

6.6 Conclusions

1. pEF-BOS-VEGFB was unsuccessful as an agent for therapeutic angiogenesis when administered intra-arterially to the rat epigastric island flap.
2. The most likely point of failure was at the level of gene transfer to the arterial wall.

Chapter 7 – Experiments with Chinese Hamster Ovary cells genetically modified to produce VEGFB

7.1 Introduction

An alternative strategy for the targeted transfer of genetic material *in vivo* is to make use of cultured cells as intermediaries (Plautz, Nabel, & Nabel 1991). In this way gene transfer is performed in two stages with the initial transfection performed *in vitro* with subsequent transplantation of the genetically altered cells undertaken as a secondary procedure. This allows for greater control over the transfection conditions and, by selecting only positive phenotypes for transplantation, has the potential to greatly improve the efficiency with which functional gene transfer may be achieved. This two-stage approach is often referred to as *ex vivo* gene therapy.

A significant problem however, in the development of this technology has been to address the immune mediated rejection of the transplanted cells (Smythe, Hodgetts, & Grounds 2000). Machens *et al.*, working with a similar epigastric island flap model in rats, performed their transfections in autologous cells to overcome the problem (Machens *et al.* 1998). In this manner, fibroblasts carrying the gene for a platelet derived growth factor, were shown to promote angiogenesis without evidence of local inflammation. Their technique however requires the preliminary harvest and expansion of fibroblast cultures specific to their population of experimental animals. Another approach is to conceal the transplanted cells in such a way that allows them to remain undetected by the host immune system. Rinsch *et*

al. employed this technique to influence the survival of random pattern dorsal skin flaps in rats. In their technique microporous polymer membranes were used to encapsulate myoblasts transfected with the genes for basic fibroblast growth factor and vascular endothelial growth factor prior to injection under the distal ischaemic region of the flaps. Improved flap survival was observed in these animals when compared to controls (Rinsch *et al.* 2001b). Although potentially an attractive option, such encapsulation relies on sufficient experience with the technique to maintain cell viability during both the encapsulation and transplantation processes. The further development and simplification of this technique may ultimately however, provide a powerful tool for *ex vivo* gene transfer.

The cells made available to me for this work were Chinese Hamster Ovary (CHO) cells genetically modified to secrete VEGFB (Amrad Corporation, Burnley, Victoria, Australia). Although we anticipated that as foreign cells, they would be short-lived in our rat model of flap ischaemia, we also knew that even a brief exposure to VEGF can induce angiogenesis (Takeshita *et al.* 1994c). We reasoned therefore, that prolonged survival of the cells may not be a requirement for a measurable angiogenic response and may even be advantageous as a self limiting step in the system.

In addition to the gene for VEGFB, the genetically modified CHO cells also contained a gene coding for resistance to the cytotoxic antibiotic puromycin, allowing selection of the CHO-VEGFB phenotype in culture.

In considering my experimental approach I identified a number of important variables. Firstly I needed to know how many cells were likely to be required to deliver VEGFB to the tissues in a biologically active dose and secondly, I needed to identify the best way in which to deliver these cells to the experimental flaps *in vivo*.

There was no work to guide us on the bioactivity of VEGFB specifically however, Kryger *et al.* found a dose of 1 µg VEGFA delivered subdermally effective in improving the survival of their experimental skin flaps also in rats (Kryger *et al.* 2000). Amrad quoted their average yield of VEGFB protein from a CHO cell population of around 50×10^6 cells as approximately 12 µg VEGFB extracted from the supernatant daily (Drinkwater 1998). Thus a yield of 1 µg VEGFB per day would be expected from a cell population of approximately 4×10^6 cells. From my initial cell culture work using 75 cm² culture flasks I could expect a yield of approximately 8 – 10 x 10⁶ CHO-VEGFB cells per flask when harvested at a confluence of 80%. This number appeared to be a suitable starting point.

The effect that method of delivery may have on the ability of VEGF to influence flap survival was also investigated by Kryger and colleagues who reported success in a variety of methods tested including direct injection into the flap, implying strategies for delivery and site of administration may not be critical (Kryger *et al.* 2000). I decided therefore, to deliver CHO-VEGFB cells as direct injections to the deep surface of the experimental flaps following elevation, allowing careful placement of each instillation and so minimising injury to the native vasculature. I was also interested to know the significance, if any, that the site of injection had on

angiogenic activity within the flaps. This arose out of concerns that CHO-VEGFB cells injected into the ischaemic environment of the random pattern flap, would likely suffer a disturbance of their own metabolism resulting in an unknown effect on VEGFB protein production. In addition to injecting the random pattern flap I therefore proposed to examine the effects of CHO-VEGFB when injected into the axial pattern flap only where local oxygenation was presumed normal. To complete the series I decided to include a final group where the whole flap was injected with CHO-VEGFB cells at the same dose per unit area as planned for the first two groups.

Prior to any *in vivo* experiments it was necessary to confirm that the CHO-VEGFB cells were indeed producing VEGFB as expected. Since no commercially available VEGFB antibody existed at the time of this work, the VEGFB product had been artificially tagged with the FLAG antigen at the time of initial gene transfer. Immunohistochemical identification of this antigen is possible using the FLAG detection system as previously described in Chapter 5.

The aim of this work therefore was firstly, to confirm CHO-VEGFB cells as a source for VEGFB first *in vitro* and subsequently *in vivo* and secondly, to investigate their ability to act as agents for therapeutic angiogenesis in the rat epigastric island flap model of tissue ischaemia.

7.2 Materials and Methods

7.2.1 Acknowledgements

CHO cells stably transfected with VEGFB were supplied frozen, by Dr. C Drinkwater, Senior Molecular Biologist, Amrad Operations while Dr S Tait, Centre for Thrombosis and Vascular Research, The University of New South Wales, provided standard CHO cells, without the VEGFB insert. To distinguish these two cultures the former will be referred to as CHO-VEGFB and the latter, CHO-BLANK. Immunohistochemical preparations were performed with the help of Ms P Marr, Department of Anatomical Pathology, The St George Hospital, Kogarah, New South Wales, Australia.

7.2.2 Cell culture reagents and equipment

CHO cells, like BAECs, were cultured in DMEM with 10% foetal calf serum. The cell culture reagents and methods used for sub-culturing were, in general, the same for both cell lines and are listed in Chapter 5. There were however, some important differences and these are described below.

In addition to the materials listed previously the selective agent puromycin (product ref: P8833) was from Sigma-Aldrich, Castle Hill, NSW, Australia.

7.2.3 Preparation of cell culture medium and reagents

7.2.3.1 Puromycin aliquots:

Recommended working concentration is 25 µg/ml. Therefore require 375 µg puromycin per 75 cm² culture flask (15 ml medium). Stock puromycin prepared at 1.5 µg/µl and stored as 550 µl aliquots to provide sufficient agent for use in two culture flasks with 50 µl wastage. Prepare aliquots from one 25 mg ampoule of puromycin hydrochloride as follows: In the biological safety cabinet add 16.7 ml sterile water to a 50 ml sterile reaction tube. Take 1 ml of this volume to the puromycin ampoule and dissolve the powder by gentle pipetting before returning it to the 50 ml tube. Draw into a 50 ml syringe and ultrafilter (0.2 µm pore size) into a second sterile reaction tube. Pipette 550 µl aliquots into sterile 1 ml eppendorf tubes and seal. Store at -20°C and thaw immediately before use.

7.2.3.2 DMEM with 10% foetal calf serum:

Prepared as for BAEC culture however, the quantity of sodium bicarbonate used should be 3.7 g/l and the final solution adjusted to pH 7.2 with 1M hydrochloric acid.

7.2.4 CHO cell culture

7.2.4.1 Thawing from frozen:

Prepare a suitable culture flask containing fresh medium. Place cryotube containing frozen cells directly into a waterbath at 37 °C, use forceps. Once thawed, transfer the cell suspension dropwise to a sterile reaction tube containing 10 ml culture medium. Centrifuge at 1000 rpm (Beckman J2-21) for 5 mins. Discard supernatant and

suspend cell pellet in 4 ml fresh culture medium by gentle pipetting. Transfer to the culture flask and incubate at 37 °C, 5 % CO₂ with the lid loosened.

7.2.4.2 *Passaging:*

CHO cell cultures were split and sub-cultured in the same manner described for BAECs with two important differences. Selective pressure was applied to the CHO-VEGFB cultures by including puromycin (25 µg/ml) in the culture medium. 250 µl of stock puromycin (1.5 µg/µl) was therefore added to each 75 cm² culture flask containing 15 ml fresh medium.

In addition, CHO cells propagated much more vigorously in culture than was the case for BAECs, with the CHO-BLANK cells being especially prolific. Preliminary work identified the cell doubling times as 22 hrs for CHO-VEGFB whilst CHO-BLANK was significantly shorter at only 13 hrs. From this, an inoculation table was drawn up quantifying the number of cells needed to achieve the target cell population of 8 – 10 x 10⁶ cells per culture flask (approximately 70 – 90% confluent) on a given operating day (Table 7.1). Cell counts were performed using a standard haemocytometer as previously described. CHO-VEGFB cell aliquots used for *in vivo* experiments were transported to the operating theatre on ice in 2 ml fresh culture medium.

Table 7.1 – Inoculation schedule for CHO cells

<i>operating day</i>	<i>passage</i>	<i>inoculate per 75 cm² culture flask (cells)</i>	
		<i>CHO-VEGFB</i>	<i>CHO-BLANK</i>
Mon	Fri pm	1.5 x 10 ⁶	4 x 10 ⁵
Tue	Fri pm	6 x 10 ⁵	9 x 10 ⁴
Wed	Fri pm	4 x 10 ⁵	3 x 10 ⁴
Thu	Mon am	9 x 10 ⁵	2.5 x 10 ⁵
Fri	Mon am	4 x 10 ⁵	8 x 10 ⁴

7.2.5 Immunohistochemical testing

Reagents, primary and secondary antibodies for immunohistochemical FLAG stains were as previously described (Chapter 5). For *in vivo* stains, goat anti-rat immunoglobulin was from Serotec (Centennial Campus, Raleigh, NC, USA, product ref: STAR 71).

7.2.5.1 CHO-VEGFB cells *in vitro*

A single, 8-well chamber slide was used to prepare CHO-VEGFB and CHO-BLANK cultures prior to immunostaining. Since the culture area of each well was 1 cm² i.e. 1/75th of the standard culture bottle area, an appropriate inoculation for each well was calculated using the schedule in table 7.1 to allow expansion of the population for between two and three days. Each of 4 wells were therefore inoculated with 1 x 10⁴ CHO-VEGFB cells whilst the remaining 4 wells each received 3 x 10³ CHO-BLANK cells. The slide was fixed (10 % buffered formalin applied for 20 mins

followed by two exchanges of PBS) when cell confluence had reached 70 – 80% and transferred to the immunohistochemical laboratory for immediate FLAG staining. The technique used was identical to that described in Chapter 5.

7.2.5.2 CHO-VEGFB cells in vivo

To assess the potential of the FLAG detection system to monitor VEGFB production *in vivo*, 2 ml of an $8 - 10 \times 10^6$ CHO-VEGFB cell suspension was injected subcutaneously into the midline ventral skin of 2 male Sprague-Dawley rats under general anaesthetic. Biopsies of this area were performed immediately following injection and then daily for the following 4 days. Each biopsy was fixed in formalin, blocked and cut in preparation for immunostaining using the FLAG detection system. Animals were monitored daily for signs of distress and surgical wounds were checked for evidence of infection or dehiscence.

7.2.5.3 Immunohistochemical technique

Immunostaining was performed in essentially the same manner as previously described for the localisation of von Willibrand factor on rat endothelium (Chapter 3). One significant problem however, arose from the fact that the primary anti-FLAG antibody is supplied as mouse anti-FLAG making it necessary to use an anti-mouse secondary antibody. This unfortunately led to a significant problem with cross reactivity, with native rat IgG staining positive in addition to the target mouse IgG. In an attempt to address this problem we used goat anti-rat immunoglobulin as a blocking agent. Briefly, sections were dewaxed and taken to ethanol and

endogenous peroxidase block applied. A protease digest was performed (5 min, 37 °C) and non-specific binding blocked with casein. Goat anti-rat immunoglobulin was applied at this stage (diluted 1/50 in TRIS buffered saline) followed by mouse anti-FLAG primary antibody (1/100 dilution). Incubation was for 10 mins and 60 mins at room temperature respectively. Biotinylated rabbit anti-mouse secondary antibody and streptavidin detection system were used as already described.

7.2.6 CHO-VEGFB cells as agents for therapeutic angiogenesis

7.2.6.1 Animals

Sixteen male Sprague-Dawley rats weighing between 350 – 450g were used in this work. All animals were supplied and cared for at the animal house facility, Prince Henry Hospital, NSW, Australia. All cell cultures were grown at the Haematology Research Unit, Prince of Wales Hospital, Randwick, harvested on the morning of surgery when cell yield was estimated, and transported to the operating theatre on ice in sealed sterile reaction tubes.

7.2.6.2 Experimental groups

Four experimental groups comprised four rats each. Cells were delivered to the experimental animals as multiple instillations using a 22G hypodermic needle mounted on a 5 ml syringe, attempting an even distribution to the area under test. Injections to the flap were all performed from the deep surface avoiding damage to the vascular network as far as was possible (Figure 7.1).

Groups 1 – 3 received $2 - 3 \times 10^6$ cells/cm² to either the random pattern flap (group 1), the axial pattern flap (group 2) or the whole experimental flap (group 3). Group 4 animals received twice this dose to the axial pattern flap only.

7.2.6.3 Surgical procedure

All experimental epigastric island flaps were 8 x 8 cm, based on the left superficial inferior epigastric vascular pedicle and raised under general anaesthesia as previously described (Chapter 2). CHO-VEGFB cells suspended in 2 – 4 ml culture medium were then injected into the test area of the flap as described above before returning the flap to its bed and closing the skin with 4/0 prolene. The postoperative care of the animals was provided as before.

7.2.6.4 Data collection

All animals underwent data retrieval in a similar manner to that described in Chapter 2. Daily flap observations were performed until the seventh postoperative day when the animals were killed by CO₂ overdose and microangiography performed through the left SIEA. Measurement of flap survival was performed, as before, by planimetry using a single, hand drawn template.

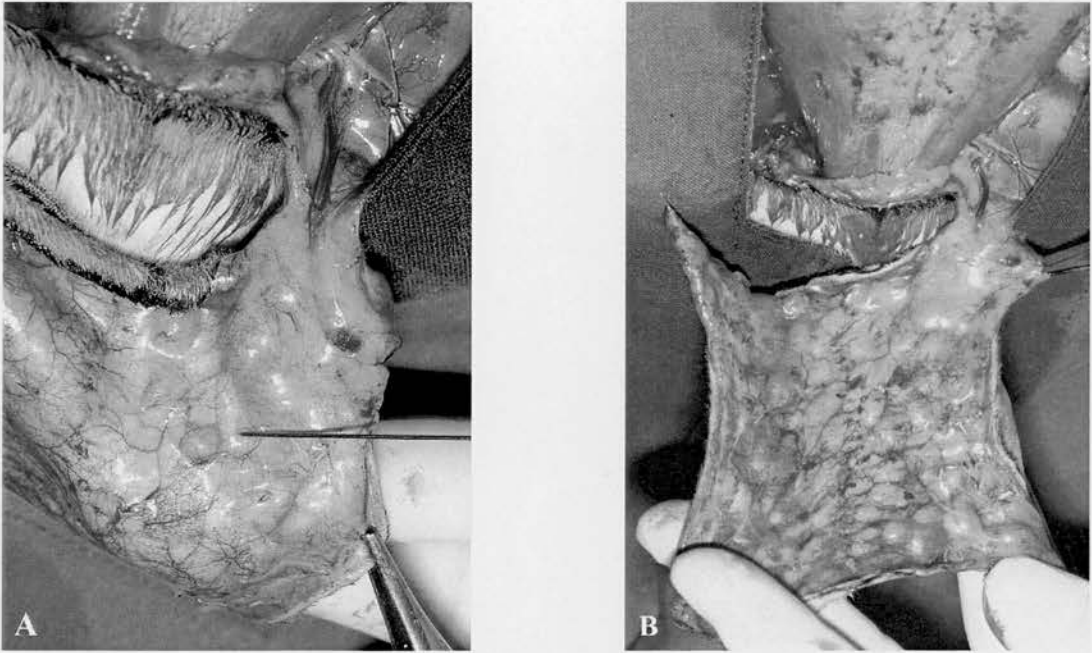


Figure 7.1 – Technique for injection of CHO-VEGFB cells suspended in culture medium into the rat epigastric island flap. **(A)** The flap has been raised on the left superficial inferior epigastric neurovascular bundle and reflected over the animals tail. Instillations begin in the vicinity of the pedicle with care taken to avoid injury to the vascular network, and proceed outwards. **(B)** In this animal the whole flap has been treated.

7.3 Statistics

The results from these experiments were compared with those obtained from unmodified flaps presented in Chapter 2 and any differences subjected to statistical analysis. Results for area flap survival were analysed using a one-way analysis of variance (ANOVA) incorporating a post-hoc test while the Mann-Whitney U test

was used to analyse angiographic data. Confidence intervals are reported at the 95% level and $p < 0.05$ was taken to represent statistical significance.

7.4 Results

7.4.1 Immunohistochemistry

Immunoperoxidase staining for the FLAG antigen *in vitro* was positive for CHO-VEGFB cells confirming VEGFB production, whilst CHO-BLANK cells stained negative (Figure 7.2). Optimisation of the FLAG stain *in vivo* proved more difficult. Serial biopsies identified the presence of CHO cells within rat abdominal skin for up to 3 days however the FLAG stain proved unreliable for the detection of VEGFB production (Figure 7.3).

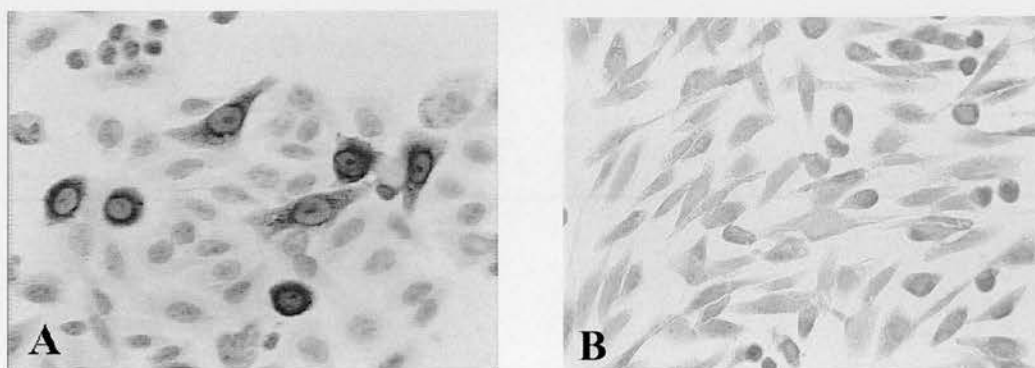


Figure 7.2 – CHO cell monolayers immunostained for the FLAG antigen. In (A) a number of CHO - VEGFB cells have stained positive indicating the presence of the FLAG-VEGFB fusion protein whilst in (B) CHO cells without the FLAG-VEGFB insert (CHO-BLANK) stain negative.

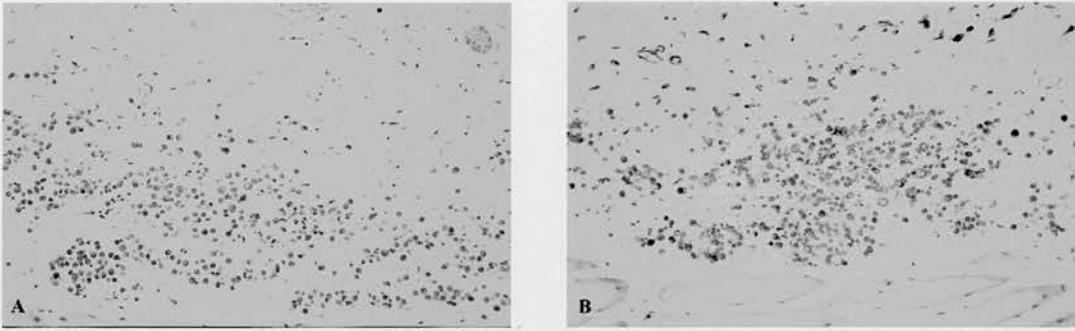


Figure 7.3 – Flap section immediately following CHO-VEGFB transfer (A) H & E (B) immunostain for the FLAG antigen. CHO cells are clearly seen in both sections and although some cells in (B) appear to stain positive for the FLAG antigen, the presence of background stain makes interpretation unreliable.

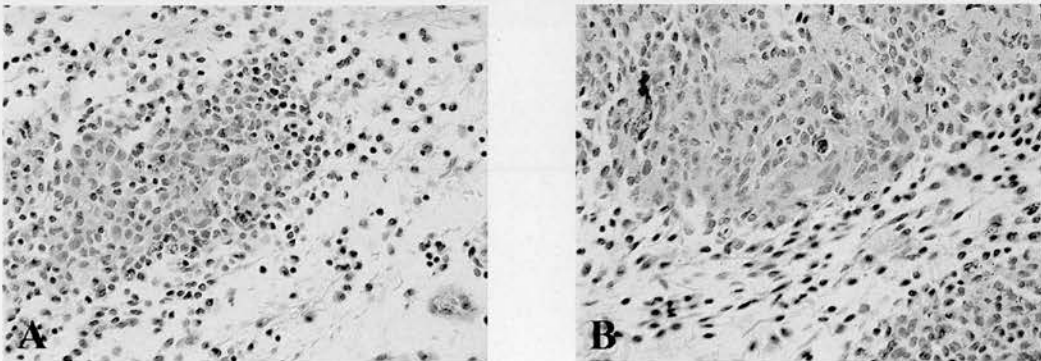


Figure 7.4 – Flap sections stained for the FLAG antigen at (A) 24 hrs and (B) 48 hrs post CHO-VEGFB injection. Although CHO cells are clearly seen (containing the large nuclei towards the centre of each image) all stain negative for FLAG.

7.4.2 Flap survival

There were no intra-operative complications and all animals recovered from anaesthesia without event. Mean operating time for completion of the surgery was 40 mins. Mild localised oedema was noted in two flaps receiving CHO-VEGFB into their random halves, one each in the axially injected and whole injected groups and two in the higher dose axially injected group. In all cases bar one, this had settled by day two. The animal with prolonged oedema had received the higher concentration of CHO-VEGFB cells to the axial flap only and flap oedema persisted until day 3. All animals remained alive and well during the study period and no adverse reactions were noted at the injection sites.

Mean flap survival for each experimental group is given in table 7.2. One group 5 flap showed complete survival (Figure 7.5). This was the first occasion for a flap to show complete survival in any of the experimental work performed thus far. A statistically significant difference was noted for percentage flap survival between the five experimental groups, $F(4, 19) = 5.87, p < 0.01$. Post-hoc testing indicated that the flaps receiving double quantities of CHO-VEGFB showed, on average, a 10.3% higher mean flap survival than the control group ($p=0.04$). There was no significant difference between any of the remaining groups when compared to controls ($p>0.98$). The results are displayed graphically in Figure 7.6.

Table 7.2 - Mean flap survival expressed as a percentage of final flap area for each experimental group. Standard flaps were used as controls.

<i>Injection group</i>	<i>n</i>	<i>mean CHO-VEGFB dose ($\times 10^5$ cells/cm²)</i>	<i>mean flap survival (%)</i>	<i>SD</i>
Random flap	4	3.1	79.3	3.6
Axial flap 1	4	3.7	86.0	6.4
Whole flap	4	2.5	84.3	6.0
Axial flap 2	4	5.9	95.3	3.8
Control flaps (no injection)	8	0	85.0	4.3

The control group was taken from the series presented in Chapter 2.

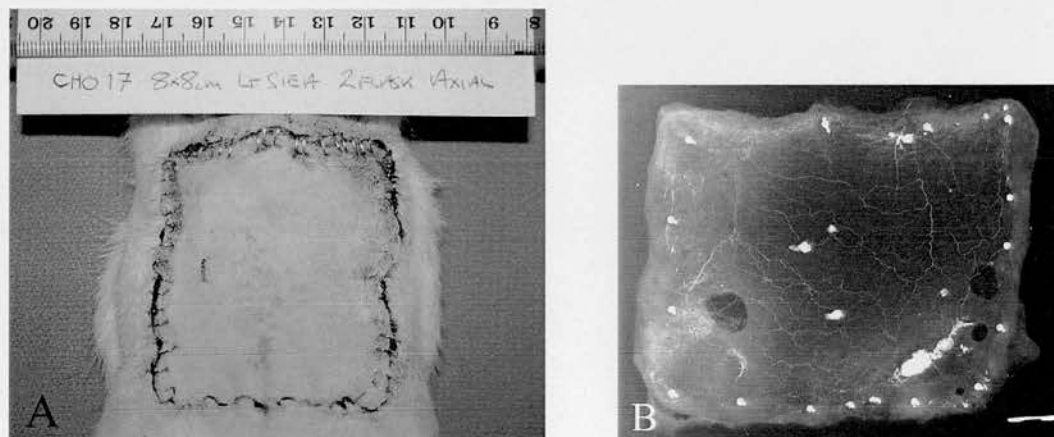


Figure 7.5 – (A) Complete survival of one of the experimental flaps receiving the higher dose of CHO-VEGFB cells to the axial portion of the flap. (B) Angiographic appearance of the same flap.

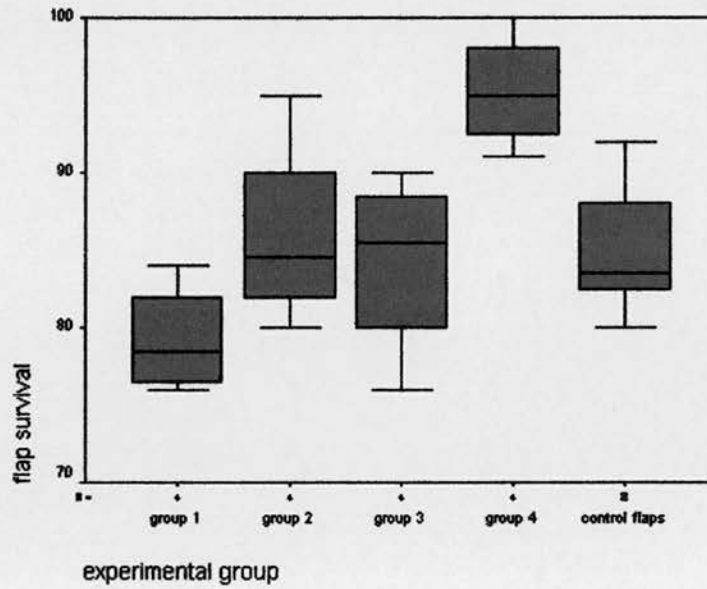


Figure 7.6 – Boxplot of flap survival against experimental group. The flaps receiving the higher CHO-VEGFB dose to the axial portion (group 4) showed on average a 10.3% increase in survival at seven days ($p=0.04$).

7.4.3 Microangiography

All angiograms demonstrated an increase in vascular markings at the zone of demarcation between viable and non-viable tissue within the random pattern flap as seen previously (Chapter 2). Mean HBPC/cm² for each experimental group is given in Table 7.3. Mean HBPC/cm² for the ^{random}~~axial~~ pattern region of group 4 angiograms (20.8± 2.2) was higher than at any other position investigated within any of the experimental groups and when compared to the control group, the difference just reached statistical significance for this location (p=0.05). Differences in mean HBPC/cm² recorded from all other sites and in all experimental groups when compared to the control group were not statistically significant (p>0.23). The results are displayed graphically in Figure 7.7. Examples of angiograms from each of the groups are given in Figure 7.8

Table 7.3 – Mean HBPC/cm² recorded for the random and axial portions of each flap in each experimental group. 95% confidence intervals are given in brackets.

Site	Group 1 (CHO-VEGFB random flap) n=4	Group 2 (CHO-VEGFB axial flap) n=4	Group 3 (CHO-VEGFB whole flap) n=4	Group 4 (CHO-VEGFB high dose axial flap) n=4	Control n=8
random pattern flap	18.3 (12.8, 23.7)	15.8 (11.0, 20.5)	17.8 (12.0, 23.5)	20.8* (17.2, 24.3)	16.1* (13.1, 19.2)
axial pattern flap	16.8 (14.8, 18.8)	14.3 (9.7, 18.8)	16.8 (14.8, 18.8)	17.5 (15.5, 19.6)	16.9 (13.8, 19.9)

* p=0.05

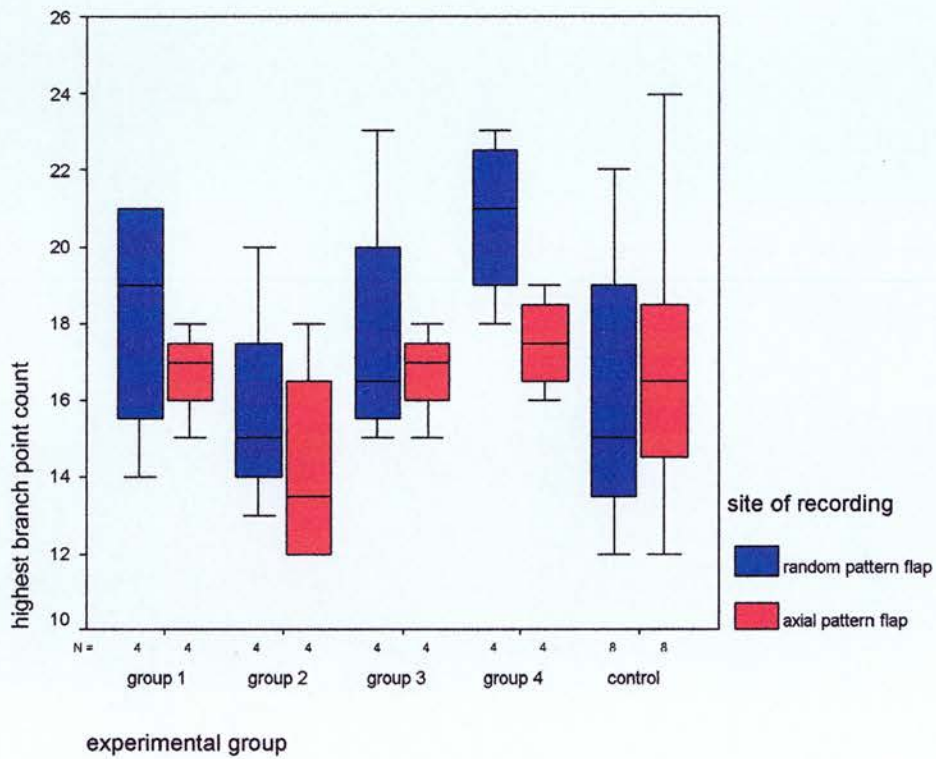


Figure 7.7 – Boxplot of HBPC/cm² recorded from the angiograms of each experimental group and a control group of standard flaps. Mean HBPC/cm² recorded from the random pattern area of group 4 flaps was significantly higher than that found in the control group (p=0.05).

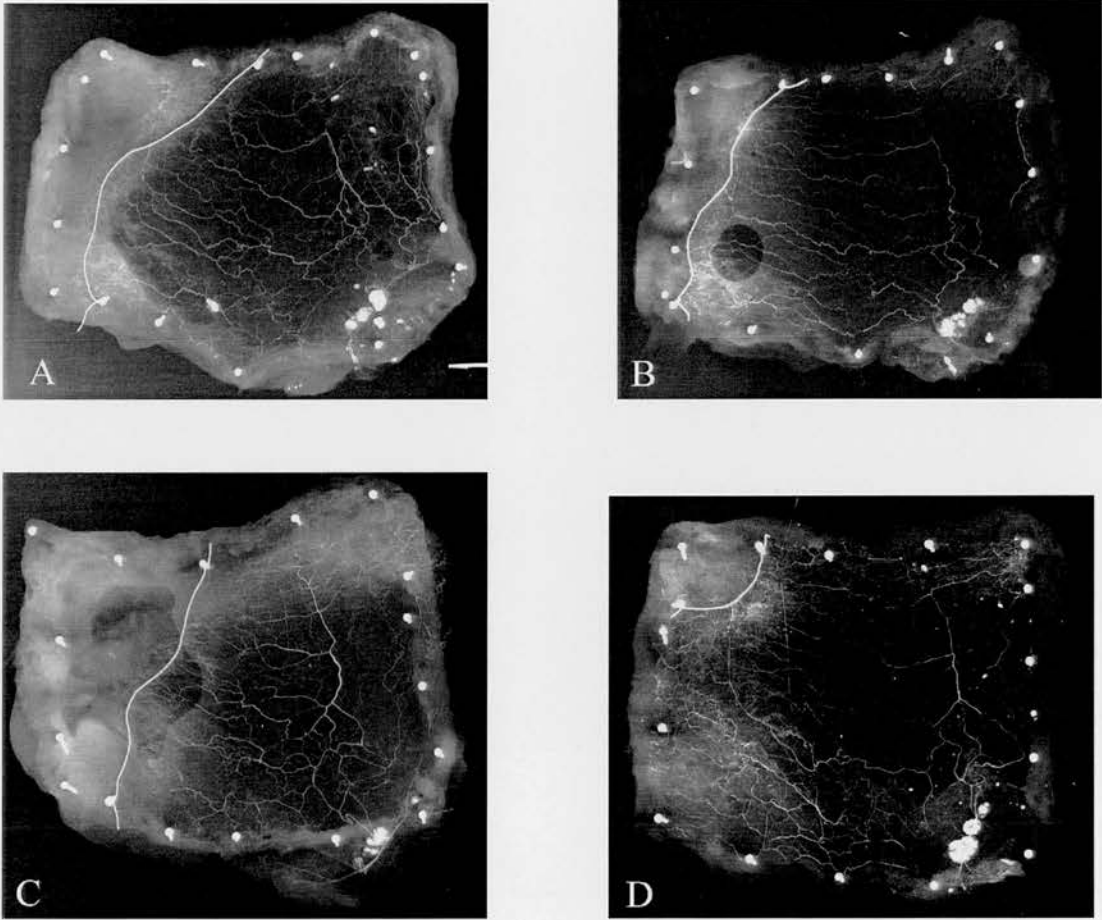


Figure 7.8 – Example angiograms from each experimental group. (A) Group 1 (CHO-VEGFB random flap), (B) group 2 (CHO-VEGFB axial flap), (C) group 3 (CHO-VEGFB whole flap) and (D) group 4 flaps (CHO-VEGFB high dose axial flap).

7.5 Discussion

These results were encouraging. CHO-VEGFB stained positive for FLAG in vitro confirming the presence of the VEGFB-FLAG fusion protein. Interestingly however, positive staining was not uniform within the CHO population. The use of puromycin as a selective agent would prevent the survival of non-VEGFB containing CHO cells since both genes, for VEGFB and puromycin resistance, were transferred as a single coding sequence. The heterogeneity of FLAG staining may therefore either indicate a true variability in the expression of the FLAG-VEGFB sequence or reflect a limitation in our ability to detect it using the FLAG system. Although no data is available for VEGFB specifically, the half-lives of VEGF species in general are known to be short (estimated by Ferrara *et al.* to be less than 6 mins) making any variations in the rate of synthesis more apparent (Takeshita *et al.* 1994c).

The technique used to introduce the cells into the flap appeared effective with no adverse events or reactions and their survival for three days was in keeping with our expectations.

The apparent increased incidence of temporary flap oedema in the CHO injected flaps compared with controls may have occurred as a consequence of the activity of inflammatory mediators released in response to the presence of CHO cells themselves, particularly since it was observed in all injected groups. It is worth noting however, that temporary oedema is considered by some a constant feature of VEGFA induced angiogenesis and although the situation for VEGFB is less clear,

recent evidence suggests that increased vascular permeability accompanies angiogenesis regardless of the stimulus (Bates *et al.* 2002;Dvorak *et al.* 1995a).

The improvement in mean flap survival noted for flaps receiving the higher dose of CHO-VEGFB cells was especially encouraging although the numbers of animals in each group were small. To achieve the complete survival of one of the experimental flaps was something I had not encountered in any of my previous experiments and gave us cause for optimism in the planning of further work. The lack of any significant improvement in flap survival in any of the groups receiving the lower dose of CHO-VEGFB cells confirmed the watershed for activity to be in excess of 3×10^5 cells/cm².

Interpretation of the angiographic data however, proved more difficult. Although the increase in mean HBPC/cm² for group 4 flaps was consistent with the observed increased area survival in this group we found it surprising that this increase was not apparent at the site of CHO cell injection but rather on the contralateral side. Two important physiological properties of VEGF and its receptors may help explain this finding. Firstly the bioactivity of VEGF is potentiated by an upregulation of its KDR receptors in the presence of local ischaemia (Brogi *et al.* 1996). Such ischaemic conditions are expected within the random but not the axial pattern flap. Secondly, VEGF is known to act on endothelial cells as a freely diffusible protein (Ferrara 1999).

We postulated therefore, that VEGFB entering the blood stream within the axial pattern flap would exert maximal effect on the ischaemic tissues of the random flap. The effect of local ischaemia on the ability of VEGF to induce angiogenesis was studied by Pu *et al.* (Pu *et al.* 1993a). Working with a rabbit model of hindlimb ischaemia and delivering VEGF protein intramuscularly to both ischaemic and non ischaemic muscles they found evidence of induced angiogenesis only in the former when compared to controls. The bioactivity of exogenously administered VEGF would appear therefore, to be significantly influenced by the prevailing levels of tissue oxygenation. Our findings were consistent with this view.

My experience with the FLAG detection system for the identification of VEGFB-FLAG fusion protein *in vivo* was, on the whole, disappointing. Cross reactivity and background staining limited our ability to achieve clean stains when applied to fixed tissue. Despite the various manoeuvres employed to improve the results, they remained unreliable. As a result I could not reliably detect the presence of VEGFB in my rat flap sections and was therefore unable to assess the duration of VEGFB production by CHO-VEGFB cells following transfer using this technique.

The higher dose of CHO-VEGFB proved successful in improving mean flap survival in our experimental model. Performed principally as a pilot however, there are obvious weaknesses in the design of this study; the groups were small, there was no randomisation and the controls used were from a previous series. To subject the technique to a more critical analysis I proposed a randomised controlled trial using

the higher dose of CHO-VEGFB cells and investigating all three sites of injection i.e. axial pattern flap, random pattern flap and the whole flap.

7.6 Conclusions

1. Production of VEGFB by CHO-VEGFB was heterogeneous *in vitro*.
2. CHO-VEGFB cells survived within rat tissue for a maximum of 3 days
3. Given at a dose of approximately $5 - 6 \times 10^5$ cells/cm² and administered to the axial portion of the flap, CHO-VEGFB cells appeared to improve the survival of experimental skin flaps compared to controls.
4. Increased area flap survival was associated with an increase in mean angiographic HBPC/cm² in the random portion of flaps injected with the higher dose of CHO-VEGFB cells.
5. A randomised controlled trial would provide more substantive evidence of the activity of CHO-VEGFB cells as agents for therapeutic angiogenesis.

Chapter 8 – Randomised controlled trial to evaluate CHO-VEGFB cells as agents for therapeutic angiogenesis

8.1 Introduction

8.1.1 Overview

To test the hypothesis that CHO-VEGFB cells could influence the survival of ischaemic rat epigastric island flaps by the induction of angiogenesis I proposed to conduct a randomised controlled trial. I decided to examine the effect of CHO-VEGFB cells when administered subcutaneously to the same three regions of the flap as the pilot study, i.e. random flap only, axial flap only and to the whole flap, using the higher dose of approximately 6×10^5 cells/cm². Control animals would receive the same numbers of CHO-BLANK cells and flap survival, a-MVD and angiographic data would be collected as previously described.

8.1.2 Power calculation

The pilot study data indicated that the injection of CHO-VEGFB cells into the axial region of the epigastric island flap at a dose of 5.9×10^5 cells/cm², on average, resulted in a 10.3% improvement in flap survival ($p=0.04$) (Chapter 7). To determine an appropriate sample size for the proposed randomised trial, a power calculation was performed using 10% as the difference between the experimental and control group means that the trial was designed to detect.

The equation can be written:

$$(u_1 - u_2)^2 = f(\alpha, P)\sigma^2 (1/n_1 + 1/n_2)$$

where $(u_1 - u_2)$ is the difference between the means of two experimental groups u_1 and u_2 , $f(\alpha, P)$ is a constant for a given power (P) and significance level (α), σ^2 the variance of the measurements and n_1 and n_2 the two respective sample sizes (Bland 2000a). If each group contains equal numbers i.e. $n_1 = n_2$ then:

$$n = 2f(\alpha, P)\sigma^2 / (u_1 - u_2)^2$$

I chose a high value for $P = 0.95$ making it likely that my trial would detect this difference should it exist, giving the value of $f(\alpha, P)$ at the 0.05 significance level as 13.0 taken from a published table (Bland 2000a). The standard deviation recorded for the control flaps was 5.3. Therefore,

$$n = 2 \times 13.0 \times 5.3^2 / 10^2$$

$$n = 7.3$$

To allow for potential losses I decided to design the trial around a group size of 10 rats in each of the 6 groups making a total of 60 rats.

8.1.3 Randomisation

Attempts were made to standardise the rats to be used in the trial in that they were all male rats taken from the same breeding colony and only those weighing 350 – 450 g were selected. To avoid any bias that may have resulted from an improvement in technique over the course of the trial, the operative schedule was randomised using a table of random numbers (Bland 2000b). Details of this schedule are given in Appendix 4.

8.2 Materials and methods

8.2.1 Animals

Sixty male Sprague-Dawley rats were used weighing between 350-450 g. All animals were supplied and cared for as before in the animal house, prince Henry Hospital, NSW, Australia.

8.2.2 Preparation of CHO cell cultures

All CHO cells were cultured in the Department of Haematology Research, Prince of Wales Hospital, Randwick, Sydney, Australia as set out in Chapter 7 following the same subculturing schedule as used in the pilot study.

The cell culturing process was labour-intensive and on a scale that inevitably led to some logistical problems in terms of accessing sufficient incubator space. As a result, some departures from the planned schedule did occur, most frequently involving the whole-flap injection group where insufficient capacity was available to

culture the required cells (four culture bottles required per animal). In this situation a swap was done within the schedule to make the most use of the available capacity. Cell counts were performed on a representative sample of flasks from each cell culture batch where again, the large turnover made counts on every flask unrealistic. The target cell population was $8 - 10 \times 10^6$ cells per culture flask at a confluence of 70 – 90%. Although in general the cell yield was very reliable, there were occasions where cell counts outside the target range were used, usually higher, to keep up with the operating schedule.

Cells were transported to the operating theatre on ice within sterile 15 ml reaction tubes containing either 2 ml fresh culture medium (random and axial flap injection groups) or 4ml (whole flap injection groups).

8.2.3 Surgical procedure

All experimental epigastric island flaps were 8 x 8 cm, based on the left superficial inferior epigastric vascular pedicle and raised under general anaesthesia as previously described. CHO-VEGFB or CHO-BLANK were delivered to the deep surface of the flap in accordance with the operating schedule using the same technique described for the pilot study (Chapter 7). All instruments, sutures, syringes and pipettes were used sterile. Skin closure was with 4/0 prolene to the inferior border of the flap and staples elsewhere. The animals were recovered and monitored in the postoperative period as previously described (Chapter 2).

8.2.4 Data Collection

8.2.4.1 General observations

All animals were examined daily for signs of flap oedema and for evidence of demarcation between viable and non-viable tissue. All were killed by CO₂ overdose on the seventh postoperative day, the flaps photographed and further data collected as follows:

8.2.4.2 Flap survival

Measured by planimetry using three consecutive hand drawn templates as previously described (Chapter 2). A mean value for percentage flap survival based on the final flap area was thus calculated for each flap and used in all subsequent analyses.

8.2.4.3 Angiography

Performed as described in Chapter 2 i.e. 0.2 ml barium contrast medium infused into the flap following cannulation of the feeding artery (left SIEA). Imaging was performed using mammography film with the previously noted equipment and settings. An assessment of highest branch point density was performed for each angiogram as before by a single examiner (CR) blinded to the nature of cells injected.

8.2.4.4 Average microvascular density

Assessment of a-MVD followed the protocol described in Chapter 3. Each flap was fixed in 10% buffered formalin, sectioned, blocked, cut and stained for von Willebrand factor and all sections were examined by a single independent observer (Dr. J Clark) blinded to the experimental group. In view of the large numbers of sections to be analysed, five representative flaps only from each group were subject to further analysis in this way. All results are expressed as previously described namely, vessels counted per grid area of 0.0625 mm².

8.3 Statistics

For parametric data, sample mean and standard deviation are quoted with confidence intervals presented at the 95% level. Non-parametric data are summarised by the median and inter-quartile range. Differences in results were subjected to statistical analysis using a one-way analysis of variance (ANOVA) or the Mann – Whitney U test as appropriate. $P \leq 0.05$ was taken to represent statistical significance.

8.4 Results

8.4.1 General observations

8.4.1.1 Overview

There were no intra- or immediate postoperative complications. Mean operating time for the completion of each procedure was 38 mins (range 30 – 50 mins).

There was no statistically significant difference in the time taken for flap demarcation to become clinically apparent between any of the groups and the incidence of temporary flap oedema was low (Table 8.1). In all cases the oedema involved the whole flap, was noted on the first postoperative day and lasted only 24 hrs.

Table 8.1 – Incidence of temporary flap oedema and time to demarcation.

<i>injection group</i>	<i>n</i>	<i>number of flaps with temporary oedema</i>	<i>mean time to demarcation (days)*</i>
CHO-VEGFB random flap	10	2	2.9
CHO-BLANK random flap	10	0	2.8
CHO-VEGFB axial flap	10	1	3.1
CHO-BLANK axial flap	10	0	3.1
CHO-VEGFB whole flap	10	1	2.9
CHO-BLANK whole flap	10	1	2.9
Population	60	5 (8%)	3.0

* $F(5, 54) = 0.45$, $p = 0.81$.

8.4.1.2 Complications

Postoperative complications are detailed in Table 8.2. Two rats required replacement of their protective collars and a further two, repair of minor wound dehiscences.

Both procedures were performed under general anaesthetic.

Table 8.2 – Postoperative complications.

<i>injection group</i>	<i>n</i>	<i>wound dehiscence</i>	<i>infection</i>	<i>seroma</i>	<i>autocannibalism</i>
CHO-VEGFB random flap	10				1
CHO-BLANK random flap	10	1		4	
CHO-VEGFB axial flap	10			1	
CHO-BLANK axial flap	10	1		3	
CHO-VEGFB whole flap	10		1	3	1
CHO-BLANK whole flap	10		1	3	
Total	60	2 (3%)	2 (3%)	14 (23%)	2 (3%)

The incidence of seroma (23%) was higher than previously experienced. Typically, a small volume of fluid was encountered beneath the flap at the time of excision and did not interfere with data collection. In 4 rats however, large seromas started to collect between days 4 and 5 and by the time of excision (day 7), the flaps were thickened and inflamed with exudates present on the deep surface. Specimens were

submitted for microbiological analysis, which revealed a heavy bacterial growth in two of the cases (enterobacter species) but no growth in the other two.

In two animals, flaps were damaged by autocannibalism despite the presence of a protective head collar. Both animals were killed before completion of the trial period.

8.4.2 Exclusions

A total of six animals were excluded from the trial. The condition of the four flaps associated with large fluid collections made them unsuitable for data collection (CHO-VEGFB axial: 1 flap; CHO-VEGFB whole: 1 flap; CHO-BLANK whole: 2 flaps) and they were excluded. Both autocannibalised flaps were also excluded. Flap survival, microvascular density and angiographic data were therefore available for analysis in 54 of the 60 flaps.

8.4.3 Flap survival

Mean CHO-cell dose administered to each flap was similar for each group ($p > 0.06$). There was no statistically significant difference in percentage flap survival between any of the experimental groups when compared to their respective controls ($p > 0.15$) (Table 8.3)

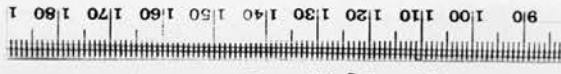
Table 8.3 - Median flap survival expressed as a percentage of final flap area and mean cell infiltrate for each group.

<i>Injection group</i>	<i>n</i>	<i>mean CHO cell dose (x10⁵ cells/cm²)</i>	<i>median flap survival (%)</i>	<i>IQR</i>
CHO-VEGFB random flap	9	5.5	80*	21.0
CHO-BLANK random flap	10	7.1	78*	12.3
CHO-VEGFB axial flap	9	6.3	79 [§]	26.7
CHO-BLANK axial flap	10	5.4	81 [§]	9.0
CHO-VEGFB whole flap	8	6.3	79 [†]	14.0
CHO-BLANK whole flap	8	6.6	82 [†]	5.8

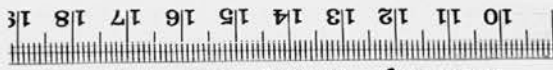
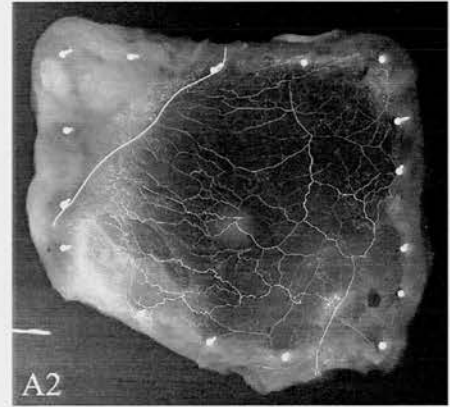
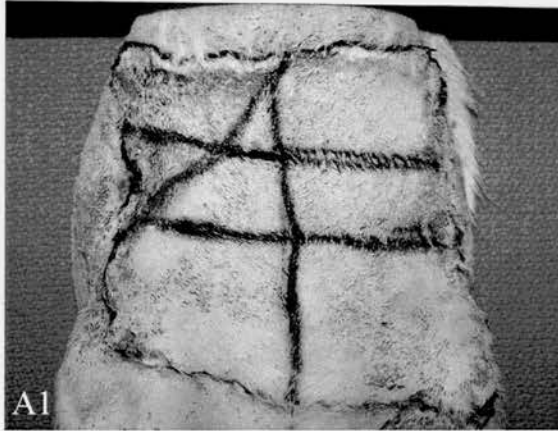
*p = 0.71; [§]p = 0.35; [†]p = 0.15.

8.4.4 Microangiography

Gross angiographic appearances were as previously noted i.e. increased vascular markings were observed in the zone of demarcation between the viable and non-viable tissue when compared to other locations in the flap and this finding was consistent across all experimental groups (Figures 8.1 to 8.3). In one angiogram, no vascular markings were seen within the random pattern region of the flap as a result of greater than expected flap necrosis suggestive of a pedicle injury at the time of elevation. In all other flaps, angiographic data was available for collection from both regions i.e. random and axial pattern.



VFC - 9 CHO-VEGFβ RANDOM



VFC - 43 CHO-BLANK RANDOM

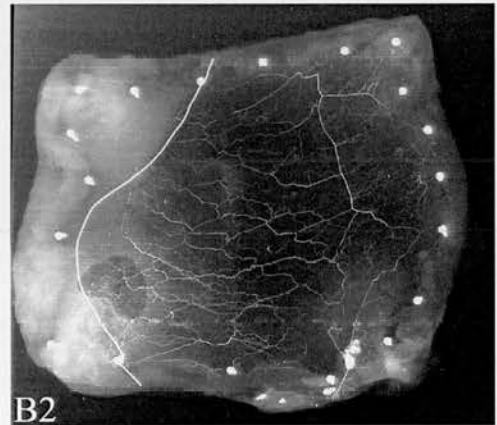
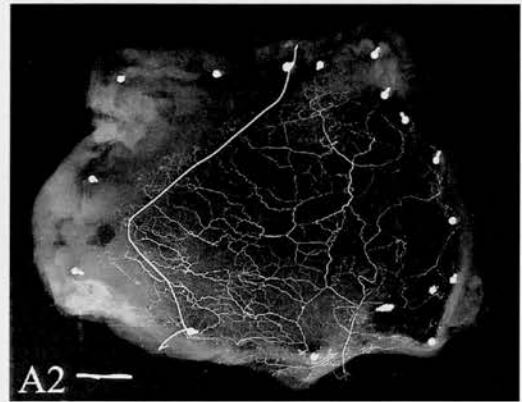
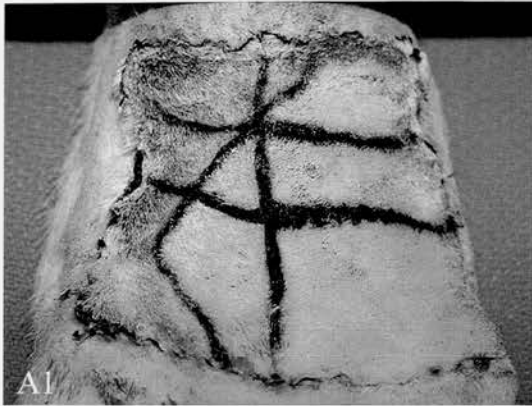


Figure 8.1 – Examples of epigastric island flaps and microangiograms at one-week post CHO injection: (A) CHO-VEGFβ and (B) CHO-BLANK injected into the *random* portion of the flap. A vascular ‘blush’ at the zone of demarcation was evident in the majority of flaps across all experimental groups.

9 10 11 12 13 14 15 16 17 18 19
VFC 30 CHO-VEGFB AXIAL



9 10 11 12 13 14 15 16 17 18 19
VFC 28 CHO-BLANK AXIAL

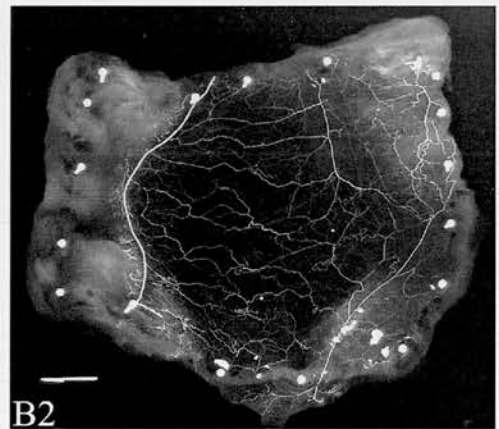
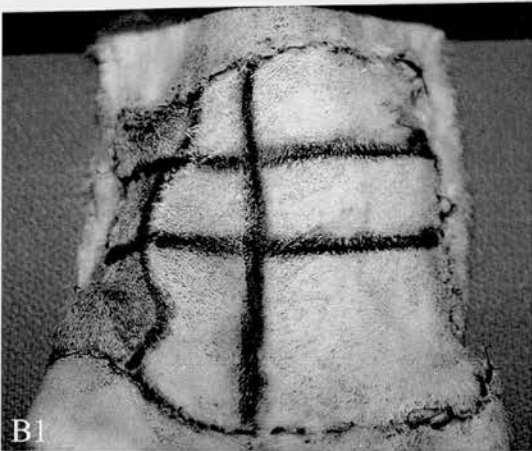
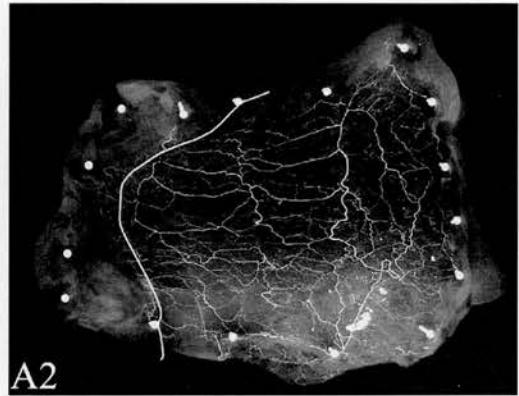
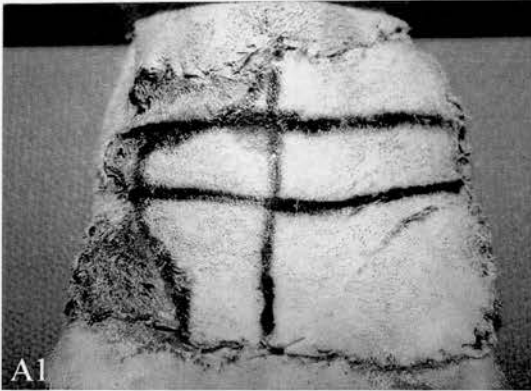


Figure 8.2 – Examples of epigastric island flaps and microangiograms at one-week post CHO injection: (A) CHO-VEGFB and (B) CHO-BLANK injected into the *axial* portion of the flap.

9 10 11 12 13 14 15 16 17 18 19

VFC 7 CHO-VEGFB WHOLE



90 100 110 120 130 140 150 160 170 180

VFC - 8 CHO-BLANK WHOLE FLAP

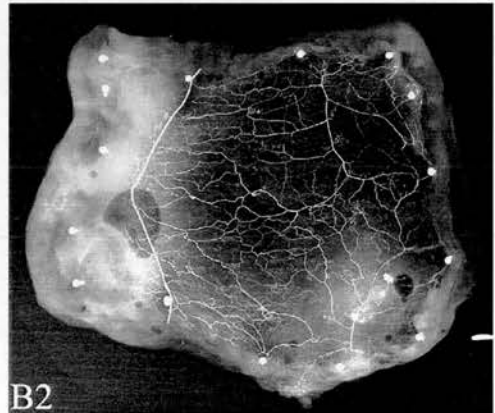
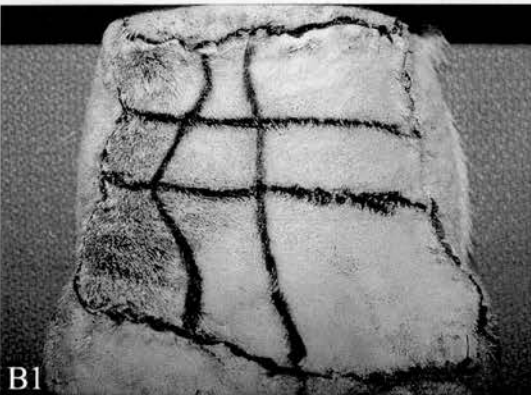


Figure 8.3 – Examples of epigastric island flaps and microangiograms at one-week post CHO injection: (A) CHO-VEGFB and (B) CHO-BLANK injected into *both* the random and axial portions of the flap (whole flap injected).

Mean values for HBPC/cm² for each flap region in each experimental group are presented in Table 8.4 and the population data is displayed graphically in Figure 8.4.

Differences in mean HBPC/cm² for each group were subjected to statistical analysis. Whole flaps injected with CHO-BLANK cells were found to have a significantly higher mean HBPC/cm² when compared to whole flaps receiving CHO-VEGFB cells ($p = 0.02$). This held for both the random and axial flap regions. This result was unexpected and must be regarded as spurious. It may have arisen as a consequence of the high exclusion rate, and therefore data loss, from this arm of the trial (four animals excluded). Any variation in the technical quality of the remaining angiograms would therefore be more likely to result in a skewing of the data. Comparisons between the other experimental groups found no statistically significant differences in mean HBPC/cm² between CHO-VEGFB and CHO-BLANK injected flaps in either of the regions examined ($p > 0.06$).

Table 8.4 – Mean HBPC/cm² recorded for the random and axial portions of each flap in each experimental group. 95% confidence intervals are given in brackets.

Site	CHO-VEGFB random flap n=9	CHO-BLANK random flap n=10	CHO-VEGFB axial flap n=9	CHO-BLANK axial flap n=10	CHO-VEGFB whole flap n=8	CHO-BLANK whole flap n=8
random pattern flap	16.9 (14.1, 19.7)	15.0 (12.8, 17.2)	17.3 (13.3, 21.3)	17.3 (15.5, 19.1)	17.6* (15.2, 20.1)	21.4* (19.5, 23.2)
axial pattern flap	16.1 (13.4, 18.8)	16.7 (14.9, 18.6)	18.4 (15.2, 21.7)	16.0 (14.3, 17.7)	17.8[§] (15.7, 19.8)	20.5[§] (18.7, 22.3)

* denotes p = 0.02; § denotes p = 0.02

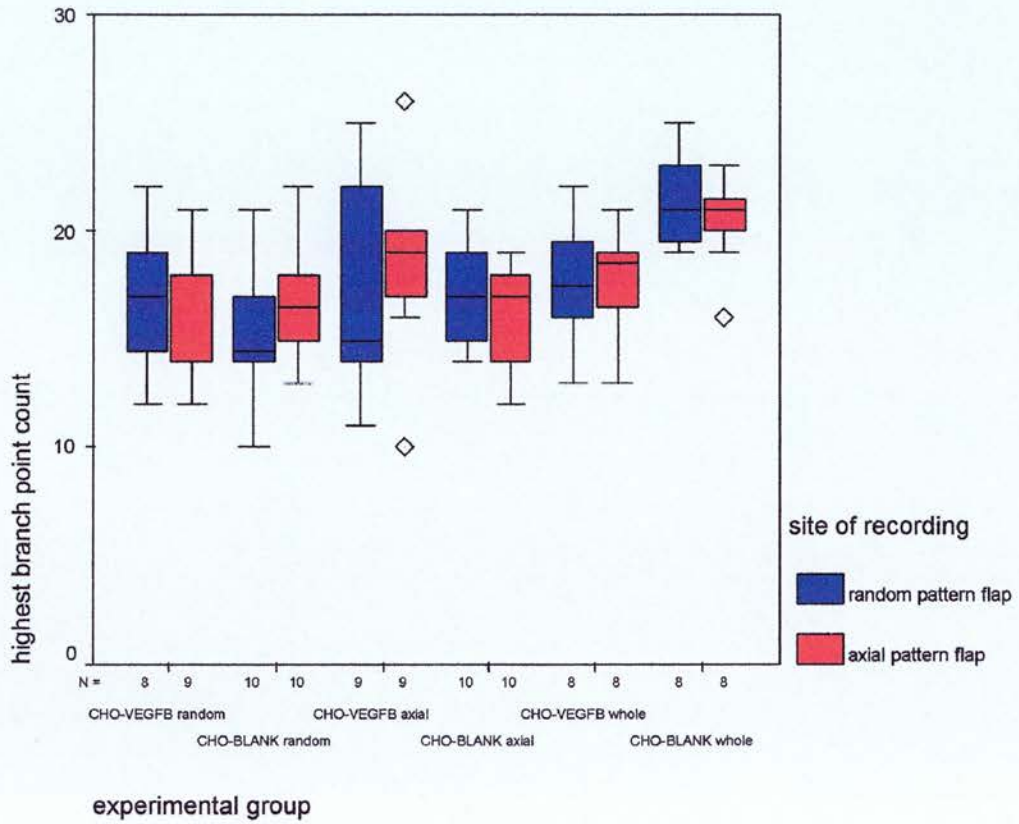


Figure 8.4 – Boxplot of HBPC/cm² for each experimental group. Diamonds represent outliers. In the ‘whole flap’ injected group, the higher readings in the CHO-BLANK compared to the CHO-VEGFB flaps was unexpected and likely to be spurious.

8.4.5 Average microvascular density

Average MVD for each of the groups followed the same pattern as seen previously i.e. a-MVD was higher below the panniculus carnosus than above it (Tables 8.5 to 8.7). In the majority of cases, there was no statistically significant difference in a-MVD at any location tested when compared with controls. In four locations however, a significant difference was identified ($p < 0.05$) (Tables 8.5 and 8.7). In three of these cases the CHO-BLANK injected flaps recorded the greater a-MVD. It is unlikely therefore that the CHO-VEGFB cells were the cause of the higher a-MVD in the final case and this result must again be regarded as spurious.

Table 8.5 – Average MVD for rats receiving CHO cells into the *random* pattern flap only. Results are expressed as vessel counts per grid area of 0.0625 mm² with 95% confidence intervals given in brackets. Position 1 represents sections taken at a position three quarters of the way along the flap from caudal to cranial, marked at the time of elevation. Similarly, position 2 represents sections from the mid point (see Fig. 3.2) Level ‘a’ is above, and level ‘b’ below, the *panniculus carnosus* muscle.

		average MVD (vessels per 0.0625 mm ²) (95% C.I.)					
		zone of demarcation		random pattern flap		axial pattern flap	
		CHO-VEGFB	CHO-BLANK	CHO-VEGFB	CHO-BLANK	CHO-VEGFB	CHO-BLANK
Position 1	level a	16.0 (3.7, 28.3)	12.5 (5.4, 19.6)	11.6 (6.2, 17.1)	13.0 (5.1, 20.9)	9.8 (5.7, 13.9)	7.8 (4.0, 11.5)
	level b	23.8 (14.9, 32.7)	31.8 (15.8, 47.7)	22.4 (12.1, 32.7)	22.0 (9.9, 34.1)	17.4 (9.3, 25.5)	19.5 (15.3, 23.7)
Position 2	level a	14.0 (10.8, 17.2)*	9.2 (3.7, 14.8)*	11.6 (3.7, 19.5)	10.5 (5.6, 15.5)	8.2 (4.8, 11.7)	11.8 (6.0, 17.5)
	level b	19.8 (13.2, 26.5)	26.0 (-11.2, 63.2)	21.4 (8.2, 34.6)	22.8 (12.2, 33.3)	16.4 (9.1, 23.7)*	24.0 (14.1, 33.9)*

* denotes p < 0.05

Table 8.6 – Average MVD for rats receiving CHO cells into the *axial* pattern flap only. Results are expressed as vessel counts per grid area of 0.0625 mm² with 95% confidence intervals given in brackets. Position 1 & 2 and Levels ‘a’ & ‘b’ are as for Table 8.5.

		<i>average MVD (vessels per 0.0625 mm²) (95% C.I.)</i>					
		<i>zone of demarcation</i>			<i>axial pattern flap</i>		
		<i>CHO-VEGFB</i>	<i>CHO-BLANK</i>	<i>CHO-VEGFB</i>	<i>CHO-BLANK</i>	<i>CHO-VEGFB</i>	<i>CHO-BLANK</i>
Position 1	level a	9.6 (4.0, 15.2)	11.2 (6.9, 15.5)	9.8 (5.2, 14.4)	11.6 (5.4, 17.9)	10.0 (4.5, 15.5)	11.8 (6.2, 17.5)
	level b	26.4 (3.6, 49.2)	25.0 (16.9, 33.1)	19.0 (6.8, 31.2)	23.0 (13.2, 32.8)	18.8 (14.0, 23.6)	23.8 (13.0, 34.6)
Position 2	level a	10.8 (2.6, 19.0)	12.4 (7.3, 17.5)	9.6 (4.7, 14.5)	10.2 (5.3, 15.1)	9.2 (5.8, 12.7)	9.8 (5.6, 14.1)
	level b	29.2 (14.5, 43.9)	26.2 (16.1, 36.3)	22.2 (14.4, 30.0)	26.8 (16.4, 37.2)	19.8 (8.6, 31.0)	22.2 (16.2, 28.2)

$p \geq 0.21$ for differences in a-MVD between experimental groups and controls at any given position

Table 8.7 – Average MVD for rats receiving CHO cells into the *whole* flap. Results are expressed as vessel counts per grid area of 0.0625 mm² with 95% confidence intervals given in brackets. Position 1 & 2 and Levels ‘a’ & ‘b’ are as for Table 8.5.

		<i>average MVD (vessels per 0.0625 mm²) (95% C.I.)</i>					
		<i>zone of demarcation</i>		<i>random pattern flap</i>		<i>axial pattern flap</i>	
		<i>CHO-VEGFB</i>	<i>CHO-BLANK</i>	<i>CHO-VEGFB</i>	<i>CHO-BLANK</i>	<i>CHO-VEGFB</i>	<i>CHO-BLANK</i>
Position 1	level a	7.8 (3.4, 12.2)	10.0 (4.4, 15.6)	7.4 (5.5, 9.3)	10.0 (5.6, 14.4)	9.2 (4.1, 14.4)	10.0 (5.3, 14.7)
	level b	20.2 (15.3, 25.1)*	27.6 (23.6, 31.6)*	16.0 (9.8, 22.2)*	23.0 (19.2, 26.8)*	19.8 (13.3, 26.3)	18.2 (12.4, 24.1)
Position 2	level a	9.0 (6.4, 11.6)	10.0 (6.4, 13.6)	8.6 (7.2, 10.0)	9.4 (5.8, 13.0)	8.4 (3.7, 13.1)	9.8 (5.9, 13.7)
	level b	21.6 (13.2, 30.0)	31.0 (17.6, 44.4)	23.0 (11.9, 34.1)	21.8 (13.6, 30.0)	19.6 (13.2, 26.0)	21.0 (14.4, 27.6)

* denotes $p < 0.05$

8.5 Discussion

In contrast to the positive findings of pilot study, these results were disappointing. The survival advantage apparent for the axially injected flaps initially was not reproduced in this trial. Similarly, there was no consistent evidence of induced angiogenesis in either the histological or angiographic data. Some of the flaps underwent more extensive necrosis than is usual, presumably as a result of vascular injury sustained during the injection process. This resulted in some skewing of the data however, even allowing for this, there was no significant difference in flap survival between any of the CHO-VEGFB treated groups and their respective controls.

The discordance in results for average flap survival between the pilot study and the randomised controlled trial is difficult to explain. The CHO-VEGFB cells used in the RCT were cultured from the same stock as those used in the pilot study, administered in the same way and in comparable numbers. One possible explanation was that expression of the VEGFB gene by the CHO-VEGFB cell population had declined by the time of the second study compared to the first possibly as a consequence of their increased age. The most likely explanation I believe however, for the initial false positive was that it occurred by chance. The probability for this being the case is 1 in 25 ($p = 0.04$).

The angiographic data suggestive of VEGFB activity in the pilot study was also not reproduced in this trial. I found no evidence of increased HBPC/cm² in any of the CHO-VEGFB injected flaps in either the random or axial regions when compared to

controls. The lack of reproducibility again suggests that the result of the pilot study had been spurious in this respect and ultimately misleading.

The histological assessment of flap vascularity was in broad agreement with the angiographic findings i.e. there was no convincing evidence of stimulated angiogenesis over and above that which was apparent in the control flaps and which represented the activity of native angiogenic pathways. The significant differences that were noted followed no pattern and were most frequently associated with a higher vascularity in the control flaps. They were therefore considered as erroneous.

The single animal that recorded a flap survival of 100% in the pilot study had a significant impact both on the average flap survival for the CHO-VEGFB double dose axially injected group but also on our belief that the technique had been successful. This result shows that an island flap raised as I have described, can survive completely purely by chance and in the absence of any effective therapeutic intervention.

In reviewing these data however, I believe the experimental approach taken was appropriate and maintain that the early successes were deserving of further evaluation. I also believe the most significant factor in the failure of the CHO-VEGFB cells to induce angiogenesis in our rat model was the low VEGFB yield as demonstrated by our FLAG stain work in Chapter 7. This required the transplantation of large quantities of cells that we knew to be immunogenic in their host limiting still further the likely VEGFB yield *in vivo*.

8.6 Conclusions

1. CHO-VEGFB cells do not act as agents for therapeutic angiogenesis in the rat epigastric island flap model of tissue ischaemia.
2. The most significant factors in the failure of the technique are likely to be the generally low yield of VEGFB by the genetically modified CHO cells and the activities of the host immune system in creating a hostile environment for the transplanted cells, limiting VEGFB production still further.
3. The false positive results seen in the pilot study are difficult to explain however, are most likely to have arisen by chance reflecting the small number of animals in the test group.

Chapter 9 – Concluding remarks and future work

The aim of this project was to evaluate two separate agents, both incorporating the gene for VEGF-B, for their ability to induce therapeutic angiogenesis in experimental skin flaps raised in rats. In the course of this evaluation I found no evidence to suggest that either agent had been successful in achieving this goal by the methods examined. The most likely explanation for this failure, I believe, was an inability to deliver sufficiently high quantities of cytokine to the tissues of the experimental flaps such that the threshold for biological activity was never reached. These results were disappointing and contrast with the success reported by a number of other groups using similar strategies and based on a variety of angiogenic agents including VEGF-A (Ishiguro *et al.* 1994; Machens *et al.* 2002; Padubidri & Browne 1996; Takeshita *et al.* 1994c; Taub *et al.* 1998).

Comparisons of technique and outcome are however, hampered by the diversity of agents and experimental approaches that have been adopted by each individual research group. Within the plastic surgery literature the two most commonly used angiogenic agents have been VEGF-A and bFGF however, dosages, delivery strategies, animal model used and data collection techniques vary widely between authors (Lubiatowski *et al.* 2002; Rinsch *et al.* 2001b; Tucci *et al.* 2001; Zhang *et al.* 2003). In accounting for these different approaches, one contributory factor is likely to be the relative difficulty in accessing the raw materials i.e. pure cytokine preparations and cDNA constructs, used in the more successful experiments. In

addition, the fact that to date, no vector has proved vastly superior to any other has provided a stimulus for the development and use of more novel agents and delivery strategies in the hope that existing results may be further improved.

This was the case in my project where I was offered the opportunity to be amongst the first to study VEGF-B as an agent for therapeutic angiogenesis. At the time this project was undertaken, details on the biology of VEGF-B were only just emerging and there were certainly no reports of its use for therapeutic angiogenesis. The activity of VEGF-B in this respect was therefore completely unknown. Published data was however, encouraging and suggested that VEGF-B, like VEGF-A, was a powerful mitogen for endothelial cells implicated in the control of both normal and pathological angiogenesis (Olofsson *et al.* 1996). More recent work has since confirmed its status as a potent stimulator of angiogenesis in a variety of circumstances (Olofsson *et al.* 1999).

An obvious starting point for the investigation of VEGF-B as an agent for therapeutic angiogenesis was to evaluate the purified VEGF-B protein itself in a similar manner to that performed by Padubidri and Browne for VEGF-A (Padubidri A & Browne E 1996). We sourced our raw materials from the biotechnology company Amrad and although they were prepared to offer us VEGF-B both as a plasmid construct and contained within a culture of stably transfected CHO cells, we were not given access to the purified VEGF-B protein. This preliminary work was therefore not possible. The successes reported by Isner and others in stimulating angiogenesis with plasmids

however, encouraged us to proceed in our attempts to apply this technology to plastic surgery (Isner *et al.* 1996; Takeshita *et al.* 1996).

As more companies have become interested in this area of research the availability of raw materials has similarly increased. Kryger *et al.* for example sourced the recombinant human VEGF-A necessary for their work, as a purified protein preparation directly from the biotechnology company Genentech* (Kryger *et al.* 2000). Plasmid constructs containing the cDNA for angiogenic cytokines produced by the company may be obtained similarly. As the technology advances and as the number of interested companies increases, both purified agents and plasmid constructs are set to become increasingly easy to obtain. For now however, it remains the case that little of what has been published has undergone the test of independent reproducibility.

Not all experiments however, have met with success. Hayward *et al.* were amongst the first to evaluate an angiogenic growth factor for its ability to influence the survival of ischaemic skin flaps in rats. In a large study comprising 100 experimental flaps they reported no evidence of induced angiogenesis in flaps injected subdermally with a relatively large dose (100µg) of basic fibroblast growth factor (bFGF) when compared to control flaps receiving saline vehicle only. In the discussion of their findings they comment on the large body of evidence confirming

* Genentech will provide raw materials as part of their 'material transfer' service following consideration and acceptance of a formal application.

bFGF as a potent stimulator of new vessel growth in a variety of experimental settings and raise the possibility that, as yet undefined, co-factors may be required for the induction of angiogenesis in more complex models of tissue ischaemia such as the rat epigastric island flap (Hayward *et al.* 1991).

This theme has been developed more recently by Brindle *et al.* who make the point that although relief of ischaemia is directly related to improved blood flow it does not necessarily follow from an increased number of vascular channels observed histologically since newly formed capillaries are known to be fragile and are often not patent (Brindle, McCarthy, & Bell 1999). In this state they are therefore unlikely to make a significant impact on blood flow. Furthermore, there is some experimental evidence to suggest that new vessels produced by the action of VEGF collapse and regress once this stimulus to growth is removed (Benjamin *et al.* 1999). An important distinction may therefore need to be made between the ability of an agent, or agents, to induce angiogenesis *per se* as against their potential to enhance the vascularity of ischaemic tissue by the local production of functional blood vessels.

One agent known to promote the integrity and stability of newly formed blood vessels is angiopoietin-1 and early experience in using this agent in combination with VEGF appear to support its role as a 'survival factor' for the newly produced capillaries (Suri *et al.* 1996). Asahara *et al.* (1998) using a corneal model of angiogenesis reported a significant increase in the number of patent vessels using the two agents together when compared with VEGF alone. Similarly Chae *et al.* (2000) compared the efficacy of single versus combined agent delivery in the rabbit model

of hind limb ischaemia and recorded angiographic evidence of increased collateral vessel formation in the animals receiving angiopoietin-1 and VEGF when compared to those receiving VEGF alone.

Although these results are encouraging and will certainly help direct future research it is still too early to know the full impact they and others like them, may have on the field as a whole. Undoubtedly as more experimental results become available, other equally successful agents and combinations of agents will be identified. In the interpretation of such results, one important aim will be the identification of critical steps and agents in the process of therapeutic angiogenesis, which are fundamental to success. This in turn will allow optimisation and simplification of the technique as unnecessary steps are discarded.

Although such dual agent use may ultimately prove superior, the majority of published reports to date have concentrated on single agent techniques for therapeutic angiogenesis. Like us, other groups have explored a potential role for VEGF-B in this respect. Although at the time of writing it remains the case that purified VEGF-B protein has yet to be shown to induce angiogenesis *in vivo* in the same way that has been shown for VEGF-A (Takeshita *et al.* 1994b; Takeshita *et al.* 1994c), I am aware of two reports where expression vectors encoding the gene for VEGF-B have been used for this purpose, with mixed results. Silvestre *et al.* (2003) working with a mouse model of hindlimb ischaemia, successfully induced angiogenesis, as assessed angiographically, using a plasmid encoding for VEGF-B administered as a single intramuscular injection.

Rissanen *et al.* (2003) however, compared four species of VEGF (A, B, C and D) for their ability to induce angiogenesis in rabbit hindlimb skeletal muscle following adenovirus-assisted gene transfer. They recorded the strongest angiogenic response following administration of the VEGF-D containing virus and found no evidence of angiogenesis at all amongst animals receiving VEGF-B virus. It is possible therefore, that the presence of local ischaemia is an important co-factor in the induction of angiogenesis by VEGF-B in a way that is not shared by all VEGF species (Elcin, Dixit, & Gitnick 2001). Silvestre's detection of VEGF-B induced angiogenesis in mice however, supports my view that insufficient delivery of cytokine to the tissues of the flap was the point of failure in my experiments rather than the cytokine itself being ineffective in stimulating angiogenesis.

Perhaps the most significant addition to the literature in the field of therapeutic angiogenesis however, has been the recent publication of the results of the VIVA (Vascular endothelial growth factor in ischaemia) trial (Henry *et al.* 2003). This work constitutes the first randomised, double-blind, placebo controlled trial using recombinant human VEGF-A for therapeutic angiogenesis in patients with myocardial ischaemia. Previous clinical trials had yielded some promising results however, were often small in size and lacking in a control arm (Hendel *et al.* 2000; Henry *et al.* 2001; Schumacher *et al.* 1998). The VIVA trial was therefore constructed to provide a rigorous examination of the efficacy of this technique using the best available evidence as a starting point.

The study population comprised 178 patients with stable exertional angina, unsuitable for standard revascularisation. Patients were randomised to two treatment groups (low and high dose rhVEGF administered as an intracoronary infusion over 20 minutes and supplemented by three intravenous infusions on days 3, 6 and 9) or a control group receiving placebo. The authors found no significant difference between any of the trial groups in terms of myocardial perfusion or exercise tolerance over the study period of 60 days. There was therefore no evidence that VEGF-A had acted therapeutically to improve myocardial vascularity in this study. These results again fell short of expectations and the authors cite suboptimal dosaging and route of administration as potential explanations for their findings. The systemic administration of rhVEGF did however, appear safe with no evidence that pathological angiogenic states (e.g. tumour growth, atherosclerosis, retinopathy) were induced or accelerated in the treatment groups compared to controls, albeit over a relatively short follow-up period of four months. The authors did point out the need for longer follow-up data before a definitive assessment on safety can be made.

The potential for serious side effects however, cannot be ignored and Epstein emphasises the importance of vigilance and long term data collection as dose escalating trials are contemplated (Epstein *et al.* 2001). Bliznakov similarly draws attention to the potential hazards of providing an angiogenic stimulus to untargeted tissue where significant pathologies (e.g. rheumatoid arthritis, retinopathy, psoriasis) may be initiated or aggravated and in addition points to the extensive literature linking angiogenesis with neoplasia (Bliznakov 2002). These are genuine concerns

that must be acknowledged in the selection criteria and design of future clinical trials.

There are, as yet, no reports of this technology being applied to plastic surgery within the setting of a clinical trial.

In summary, although therapeutic angiogenesis holds much promise for the future management of ischaemic conditions, including those relevant to plastic surgery, results to date have lacked consistency making a reliable assessment of the technology at this time difficult. It is anticipated however, that a better understanding of the detailed biology of angiogenesis combined with the application of increasingly sophisticated therapeutic techniques will lead to improvements in both the consistency and reproducibility of results as the technology advances.

In completing this work I would like to make a number of recommendations for future research based on my results and experience with this project. The CHO-VEGFB cells were ultimately found to be inefficient in their ability to produce active VEGF-B cytokine. To continue this work requires an improved cell culture capable of producing significantly higher quantities of VEGF-B and preferably of Sprague-Dawley rat origin. There is little published work to guide the choice of such a cell however, Machens *et al.* report the successful use of fibroblasts for this purpose while Rinsch and colleagues preferred myoblasts (Machens *et al.* 2002; Rinsch *et al.* 2001a). Sprague-Dawley fibroblasts can be bought directly from DSMZ and would seem a reasonable starting point (www.dsmz.de). Transfection of these cells could

be performed using pEF-BOS-VEGFB and DOTAP transfection agent in the manner I described for BAEC's with clonal selection achieved using puromycin. The immunohistochemical staining protocol that we developed for the identification of FLAG tagged proteins would provide an assessment of the efficiency of VEGF-B expression within the transfected cell cultures prior to any *in vivo* work.

To offer some protection against the host immune response Rinsch *et al.* encapsulated their genetically modified cells in microporous polymer membrane microspheres prior to implantation and this technique may have merit as a way to extend the life of transplanted cells. The efficacy of the genetically modified fibroblasts so produced as agents for therapeutic angiogenesis could then be evaluated using the rat epigastric island flap model in a manner similar to that I described for CHO-VEGFB cells.

Although the rat epigastric island flap model is appropriate for the testing of genetically modified cells in this way, it may be less suited for use in gene transfer experiments where the evidence suggests that skeletal muscle is a more receptive target for transfection. Several authors have demonstrated reporter gene expression in skeletal muscle following direct plasmid injection or intravascular infusion which contrasts with the relative difficulty reported in achieving gene expression by vascular endothelium and this was certainly my experience (Budker *et al.* 1998; Teifel *et al.* 1997; Wolff *et al.* 1990). The use of an animal model based on skeletal muscle may therefore offer the greatest chance of success. Kryger *et al.* (1999) describe a rat gracilis muscle flap model that has potential in this respect.

In the first instance I would plan to use the reporter plasmid pEGFP-N2 initially to confirm successful gene transfer and expression and then to optimise the transfection conditions. The construction of a hybrid plasmid comprising the pEF-BOS backbone and containing the GFP reporter gene would permit evaluation of the pEF-BOS expression vector *in vivo* using the gracilis flap model. Fluorescence of muscle biopsies taken sequentially beginning at 48 hrs would confirm successful gene transfer and expression as well as providing data on the timing of gene expression. Further optimisation of the system would allow identification of the most successful conditions for gene transfer prior to investigating the angiogenic potential of the pEF-BOS-VEGFB plasmid in this system. Microangiographic and histological data would be collected from both the injected and contralateral uninjected gracilis muscles at one week. Data analysis would be similar to that described for the epigastric island flap (i.e. highest angiographic branch point counts and average microvascular density on histology). Evidence of enhanced vascularity in the injected muscle compared to controls would confirm the pEF-BOS-VEGFB plasmid as capable of inducing angiogenesis *in vivo*. On the basis of these results experiments could then be designed to assess the rate at which new vessels are formed by performing data collection at serial time intervals following gene transfer.

With respect to the use of therapeutic angiogenesis to influence the survival of ischaemic flaps, I now believe that administration of the angiogenic agent synchronous with flap elevation is unlikely to provide sufficient time for the development of functional blood vessels within the flap, capable of preventing ischaemic necrosis. I would favour instead the preoperative administration of these

agents. My *in vitro* transfection data using pEGFP-N2 and BAECs found maximal gene expression 48 hrs post transfection. There is some evidence to suggest this may occur slightly later *in vivo* at around day 7 (Takeshita, Isshiki, & Sato 1996).

Takeshita *et al.* (1995) studied the timecourse of increased cellular proliferation in collateral arteries exposed to VEGF-A and found it to be maximal on day 5. The optimal interval between administration of the angiogenic agent and flap elevation is therefore likely to be 5 days for genetically modified cells where cytokine production is continuous and between 7 and 12 days for plasmid based techniques allowing a lag phase for gene expression to take place.

Although much has still to be learned before the considerable power that is angiogenesis can be truly controlled for the benefit of patients, I remain optimistic that with an increased understanding of the mechanisms involved, therapeutic angiogenesis will one day become a reality.

Bibliography

Akita, S., Rashid, M. A., Ishihara, H., Daian, T., Dazai, S., & Fujii, T. 2002, "Cytokine-dependent gp130 receptor subunit regulates rat modified axial-pattern epigastric flap", *Journal of Investigative Surgery.*, vol. 15, no. 3, pp. 137-151.

Akyurek, M., Kayikcioglu, A., Mavili, M. E., & Safak, T. 1999, "Evaluation of skin flap survival in rats.[comment]", *Plastic & Reconstructive Surgery.*, vol. 103, no. 1, pp. 337-338.

Amant, C., Berthou, L., & Walsh, K. 1999, "Angiogenesis and gene therapy in man: dream or reality?. [Review] [15 refs]", *Drugs.*, vol. 58, p. Spec-6.

Arranz Lopez, J. L., Suarez, N. C., Barthe, G. P., & Rojo Ortega, J. M. 1995, "Evaluation of angiogenesis in delayed skin flaps using a monoclonal antibody for the vascular endothelium", *British Journal of Plastic Surgery.*, vol. 48, no. 7, pp. 479-486.

Asahara, T., Chen, D., Takahashi, T., Fujikawa, K., Kearney, M., Magner, M., Yancopoulos, G. D., & Isner, J. M. 1998, "Tie2 receptor ligands, angiopoietin-1 and angiopoietin-2, modulate VEGF-induced postnatal neovascularization.[see comment]", *Circulation Research.*, vol. 83, no. 3, pp. 233-240.

Audouy, S. & Hoekstra, D. 2001, "Cationic lipid-mediated transfection in vitro and in vivo (review). [Review] [155 refs]", *Molecular Membrane Biology.*, vol. 18, no. 2, pp. 129-143.

Azimzadeh, A., Romain, N., Vermot-Desroches, C., Ravanat, C., Chenard, M. P., Wijdenes, J., Herve, P., Jaeck, D., & Wolf, P. 1998, "Antibodies to human adhesion molecules and von Willebrand factor: in vitro cross-species reactivity in the xenotransplantation setting", *Xenotransplantation.*, vol. 5, no. 4, pp. 284-291.

Balbas, P., Soberon, X., Bolivar, F., & Rodriguez, R. L. 1988, "The plasmid, pBR322. [Review] [180 refs]", *Biotechnology.*, vol. 10, pp. 5-41.

Bates, D. O., Hillman, N. J., Williams, B., Neal, C. R., & Pocock, T. M. 2002, "Regulation of microvascular permeability by vascular endothelial growth factors. [Review] [125 refs]", *Journal of Anatomy.*, vol. 200, no. 6, pp. 581-597.

Bayati, S., Russell, R. C., & Roth, A. C. 1998, "Stimulation of angiogenesis to improve the viability of prefabricated flaps", *Plastic & Reconstructive Surgery.*, vol. 101, no. 5, pp. 1290-1295.

Bayramicli, M., Yilmaz, B., & Numanoglu, A. 1998a, "Contraction of experimental skin flaps", *Annals of Plastic Surgery.*, vol. 41, no. 2, pp. 185-190.

Bayramicli, M., Yilmaz, B., & Numanoglu, A. 1998b, "Modified protective rat vest", *Plastic & Reconstructive Surgery.*, vol. 101, no. 3, pp. 859-860.

- Bendig, M. M. 1988, "The production of foreign proteins in mammalian cells. [Review] [103 refs]", *Genetic Engineering*, no. 7, pp. 91-127.
- Benhamou PY, Moriscott C, Prevost P, Rolland E, Halimi S, & Chroboczek J 1997, "Standardization of Procedure for efficient ex-vivo gene transfer into porcine pancreatic islets with cationic liposomes.", *Transplantation*, vol. 63, no. 12, pp. 1798-1803.
- Benjamin, L. E., Golijanin, D., Itin, A., Pode, D., & Keshet, E. 1999, "Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal.[see comment]", *Journal of Clinical Investigation*, vol. 103, no. 2, pp. 159-165.
- Birnboim, H. C. 1983, "A rapid alkaline extraction method for the isolation of plasmid DNA", *Methods in Enzymology*, vol. 100, pp. 243-255.
- Birnboim, H. C. & Doly, J. 1979, "A rapid alkaline extraction procedure for screening recombinant plasmid DNA", *Nucleic Acids Research*, vol. 7, no. 6, pp. 1513-1523.
- Bland, M. 2000a, "Determination of sample size," in *An introduction to medical statistics*, 3rd edn, Oxford University Press, Oxford, pp. 335-347.
- Bland, M. 2000b, "The design of experiments.," in *An introduction to medical statistics*, 3rd edn, Oxford University Press, Oxford, pp. 5-25.
- Bliznakov, E. G. 2002, "Therapeutic angiogenesis: hope or hype.[comment]", *Circulation*, vol. 106, no. 25, p. e220-e221.
- Bohm, W., Mertens, T., Schirmbeck, R., & Reimann, J. 1998, "Routes of plasmid DNA vaccination that prime murine humoral and cellular immune responses", *Vaccine*, vol. 16, no. 9-10, pp. 949-954.
- Bosari, S., Lee, A. K., DeLellis, R. A., Wiley, B. D., Heatley, G. J., & Silverman, M. L. 1992, "Microvessel quantitation and prognosis in invasive breast carcinoma", *Human Pathology*, vol. 23, no. 7, pp. 755-761.
- Braithwaite, F. 1951, "Some observations on the vascular channels in tubed pedicles. II", *British Journal of Plastic Surgery*, vol. 4, no. 1, pp. 28-37.
- Brindle, N. P., McCarthy, M. J., & Bell, P. R. 1999, "Angiogenic revascularisation in ischaemic disease. Molecular techniques hold promise, though they are still some way off.[comment]", *BMJ*, vol. 318, no. 7197, pp. 1500-1501.
- Brock, T. & Madigan MT 1994, "Cell Biology," in *Biology of microorganisms*, 6th Edition edn, Prentice Hall International, pp. 81-83.
- Broggi, E., Schatteman, G., Wu, T., Kim, E. A., Varticovski, L., Keyt, B., & Isner, J. M. 1996, "Hypoxia-induced paracrine regulation of vascular endothelial growth factor receptor expression", *J.Clin.Invest*, vol. 97, no. 2, pp. 469-476.

- Brown, L. F., Yeo, K. T., Berse, B., Yeo, T. K., Senger, D. R., Dvorak, H. F., & van de, W. L. 1992, "Expression of vascular permeability factor (vascular endothelial growth factor) by epidermal keratinocytes during wound healing", *Journal of Experimental Medicine*, vol. 176, no. 5, pp. 1375-1379.
- Budker, V., Zhang, G., Danko, I., Williams, P., & Wolff, J. 1998, "The efficient expression of intravascularly delivered DNA in rat muscle", *Gene Therapy*, vol. 5, no. 2, pp. 272-276.
- Callegari, P. R., Taylor, G. I., Caddy, C. M., & Minabe, T. 1992, "An anatomic review of the delay phenomenon: I. Experimental studies", *Plastic & Reconstructive Surgery*, vol. 89, no. 3, pp. 397-407.
- Cattoretti, G., Pileri, S., Parravicini, C., Becker, M. H., Poggi, S., Bifulco, C., Key, G., D'Amato, L., Sabattini, E., & Feudale, E. 1993, "Antigen unmasking on formalin-fixed, paraffin-embedded tissue sections.[comment]", *Journal of Pathology*, vol. 171, no. 2, pp. 83-98.
- Cederna, P. S., Chang, P., Pittet-Cuenod, B. M., Razaboni, R. M., & Cram, A. E. 1997, "The effect of the delay phenomenon on the vascularity of rabbit abdominal cutaneous island flaps", *Plastic & Reconstructive Surgery*, vol. 99, no. 1, pp. 183-193.
- Chae, J. K., Kim, I., Lim, S. T., Chung, M. J., Kim, W. H., Kim, H. G., Ko, J. K., & Koh, G. Y. 2000, "Coadministration of angiopoietin-1 and vascular endothelial growth factor enhances collateral vascularization", *Arteriosclerosis, Thrombosis & Vascular Biology*, vol. 20, no. 12, pp. 2573-2578.
- Chandrachud, L. M., Pendleton, N., Chisholm, D. M., Horan, M. A., & Schor, A. M. 1997, "Relationship between vascularity, age and survival in non-small-cell lung cancer", *British Journal of Cancer*, vol. 76, no. 10, pp. 1367-1375.
- Chang, A. C. & Cohen, S. N. 1974, "Genome construction between bacterial species in vitro: replication and expression of Staphylococcus plasmid genes in Escherichia coli", *Proceedings of the National Academy of Sciences of the United States of America*, vol. 71, no. 4, pp. 1030-1034.
- Cheng, S. H., Chan, P. K., & Wu, R. S. 2001, "The use of microangiography in detecting aberrant vasculature in zebrafish embryos exposed to cadmium", *Aquatic Toxicology*, vol. 52, no. 1, pp. 61-71.
- Chichester, P., Schroder, A., Horan, P., & Levin, R. M. 2001, "Vascular response of the rabbit bladder to chronic partial outlet obstruction", *Molecular & Cellular Biochemistry*, vol. 226, no. 1-2, pp. 1-8.
- Chow, S. P., Chen, D. Z., & Gu, Y. D. 1993, "The significance of venous drainage in free flap transfer", *Plastic & Reconstructive Surgery*, vol. 91, no. 4, pp. 713-715.

Chu, G., Hayakawa, H., & Berg, P. 1987, "Electroporation for the efficient transfection of mammalian cells with DNA", *Nucleic Acids Research.*, vol. 15, no. 3, pp. 1311-1326.

Connolly, D. T., Heuvelman, D. M., Nelson, R., Olander, J. V., Eppley, B. L., Delfino, J. J., Siegel, N. R., Leimgruber, R. M., & Feder, J. 1989, "Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis", *Journal of Clinical Investigation.*, vol. 84, no. 5, pp. 1470-1478.

Danko, I., Williams, P., Herweijer, H., Zhang, G., Latendresse, J. S., Bock, I., & Wolff, J. A. 1997, "High expression of naked plasmid DNA in muscles of young rodents", *Human Molecular Genetics.*, vol. 6, no. 9, pp. 1435-1443.

Danko, I. & Wolff, J. A. 1994, "Direct gene transfer into muscle. [Review] [15 refs]", *Vaccine.*, vol. 12, no. 16, pp. 1499-1502.

Denhardt, D. T., Edwards, D. R., Kowalski, J., Parfett, C. L., & Waterhouse, P. 1988, "Specialized plasmid vectors for cloning cDNA. [Review] [48 refs]", *Biotechnology.*, vol. 10, pp. 237-251.

Detmar, M., Brown, L. F., Berse, B., Jackman, R. W., Elicker, B. M., Dvorak, H. F., & Claffey, K. P. 1997, "Hypoxia regulates the expression of vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) and its receptors in human skin", *Journal of Investigative Dermatology*, vol. 108, no. 3, pp. 263-268.

Dhar, S. C. & Taylor, G. I. 1999, "The delay phenomenon: the story unfolds", *Plastic & Reconstructive Surgery.*, vol. 104, no. 7, pp. 2079-2091.

Dower, W. J., Miller, J. F., & Ragsdale, C. W. 1988, "High efficiency transformation of *E. coli* by high voltage electroporation", *Nucleic Acids Research.*, vol. 16, no. 13, pp. 6127-6145.

Drinkwater, C. VEGFB production by CHO cultures. Raine, C. 15-7-0098.
Ref Type: Personal Communication

Dunn, R. M. & Mancoll, J. 1992, "Flap models in the rat: a review and reappraisal.[comment]. [Review] [58 refs]", *Plastic & Reconstructive Surgery.*, vol. 90, no. 2, pp. 319-328.

Duprez, K., Bour, C., Merle, M., & Duprez, A. 1991, "Human nerve xenografting in nude mouse: experimental study of graft revascularization", *Microsurgery.*, vol. 12, no. 1, pp. 1-8.

Dvorak, H. F., Brown, L. F., Detmar, M., & Dvorak, A. M. 1995b, "Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. [Review] [121 refs]", *American Journal of Pathology.*, vol. 146, no. 5, pp. 1029-1039.

Dvorak, H. F., Brown, L. F., Detmar, M., & Dvorak, A. M. 1995a, "Vascular permeability factor/vascular endothelial growth factor, microvascular

- hyperpermeability, and angiogenesis. [Review] [121 refs]", *American Journal of Pathology.*, vol. 146, no. 5, pp. 1029-1039.
- Earle, A. S., Fratianne, R. B., & Nunez, F. D. 1974, "The relationship of hematocrit levels to skin flap survival in the dog", *Plastic & Reconstructive Surgery.*, vol. 54, no. 3, pp. 341-344.
- Ehrlich, S. D. 1977, "Replication and expression of plasmids from *Staphylococcus aureus* in *Bacillus subtilis*", *Proceedings of the National Academy of Sciences of the United States of America.*, vol. 74, no. 4, pp. 1680-1682.
- Elcin, Y. M., Dixit, V., & Gitnick, G. 2001, "Extensive in vivo angiogenesis following controlled release of human vascular endothelial cell growth factor: implications for tissue engineering and wound healing", *Artificial Organs.*, vol. 25, no. 7, pp. 558-565.
- Eliceiri, B. P., Paul, R., Schwartzberg, P. L., Hood, J. D., Leng, J., & Cheresh, D. A. 1999, "Selective requirement for Src kinases during VEGF-induced angiogenesis and vascular permeability", *Molecular Cell.*, vol. 4, no. 6, pp. 915-924.
- Enholm, B., Paavonen, K., Ristimaki, A., Kumar, V., Gunji, Y., Klefstrom, J., Kivinen, L., Laiho, M., Olofsson, B., Joukov, V., Eriksson, U., & Alitalo, K. 1997, "Comparison of VEGF, VEGF-B, VEGF-C and Ang-1 mRNA regulation by serum, growth factors, oncoproteins and hypoxia", *Oncogene*, vol. 14, no. 20, pp. 2475-2483.
- Epstein, S. E., Kornowski, R., Fuchs, S., & Dvorak, H. F. 2001, "Angiogenesis therapy: amidst the hype, the neglected potential for serious side effects", *Circulation.*, vol. 104, no. 1, pp. 115-119.
- Erol, O. O. 1976, "The transformation of a free skin graft into a vascularized pedicled flap", *Plastic & Reconstructive Surgery.*, vol. 58, no. 4, pp. 470-477.
- Ezaki, T. 1996, "Antigen retrieval: its significance and drawbacks in immunohistochemistry. [Review] [53 refs] [Japanese]", *Anatomical Science International.*, vol. 71, no. 6, pp. 615-628.
- Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J. P., Ringold, G. M., & Danielsen, M. 1987a, "Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure", *Proceedings of the National Academy of Sciences of the United States of America.*, vol. 84, no. 21, pp. 7413-7417.
- Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J. P., Ringold, G. M., & Danielsen, M. 1987b, "Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure", *Proceedings of the National Academy of Sciences of the United States of America.*, vol. 84, no. 21, pp. 7413-7417.

- Ferrara N, Houck KA, Jakeman LB, & Winer J 1991, "The vascular endothelial growth factor family of polypeptides.", *Journal of Cellular Biochemistry*, vol. 47, no. 3, pp. 211-218.
- Ferrara, N. 1999, "Molecular and biological properties of vascular endothelial growth factor. [Review] [230 refs]", *Journal of Molecular Medicine.*, vol. 77, no. 7, pp. 527-543.
- Ferrara, N. 2001, "Role of vascular endothelial growth factor in regulation of physiological angiogenesis. [Review] [115 refs]", *American Journal of Physiology - Cell Physiology.*, vol. 280, no. 6, p. C1358-C1366.
- Ferrara, N. & Davis-Smyth, T. 1997, "The biology of vascular endothelial growth factor. [Review] [250 refs]", *Endocrine Reviews.*, vol. 18, no. 1, pp. 4-25.
- Ferrara, N. & Henzel, W. J. 1989, "Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells", *Biochemical & Biophysical Research Communications.*, vol. 161, no. 2, pp. 851-858.
- Finseth F & Cutting C 1978, "An experimental neurovascular island skin flap for the study of the delay phenomenon.", *Plastic and Reconstructive Surgery*, vol. 61, no. 3, pp. 412-420.
- Finseth, F. 1979, "Clinical salvage of three failing skin flaps by treatment with a vasodilator drug", *Plastic & Reconstructive Surgery.*, vol. 63, no. 3, pp. 304-308.
- Folkman, J. & Shing, Y. 1992, "Angiogenesis. [Review] [113 refs]", *Journal of Biological Chemistry.*, vol. 267, no. 16, pp. 10931-10934.
- Fox, S. B., Leek, R. D., Weekes, M. P., Whitehouse, R. M., Gatter, K. C., & Harris, A. L. 1995, "Quantitation and prognostic value of breast cancer angiogenesis: comparison of microvessel density, Chalkley count, and computer image analysis", *Journal of Pathology.*, vol. 177, no. 3, pp. 275-283.
- Fraley, R. T., Rogers, S. G., Horsch, R. B., Sanders, P. R., Flick, J. S., Adams, S. P., Bittner, M. L., Brand, L. A., Fink, C. L., Fry, J. S., Galluppi, G. R., Goldberg, S. B., Hoffmann, N. L., & Woo, S. C. 1983, "Expression of bacterial genes in plant cells", *Proceedings of the National Academy of Sciences of the United States of America.*, vol. 80, no. 15, pp. 4803-4807.
- Franca, L. T., Carrilho, E., & Kist, T. B. 2002, "A review of DNA sequencing techniques. [Review] [194 refs]", *Quarterly Reviews of Biophysics.*, vol. 35, no. 2, pp. 169-200.
- Friebs, K. & Reardon, K. F. 1993, "Parameters influencing the productivity of recombinant E. coli cultivations. [Review] [164 refs]", *Advances in Biochemical Engineering-Biotechnology.*, vol. 48, pp. 53-77.

- George, S. J. & Baker, A. H. 2002, "Gene transfer to the vasculature: historical perspective and implication for future research objectives. [Review] [104 refs]", *Molecular Biotechnology*, vol. 22, no. 2, pp. 153-164.
- Goebel, W. & Schiess, W. 1975, "The fate of a bacterial plasmid in mammalian cells", *Molecular & General Genetics*, vol. 138, no. 3, pp. 213-223.
- Goshen, J., Wexler, M. R., & Peled, I. J. 1985, "The use of two alpha blocking agents, phenoxybenzamine and phentolamine, in ointment and injection form to improve skin flap survival in rats", *Annals of Plastic Surgery*, vol. 15, no. 5, pp. 431-435.
- Gowdak, L. H., Poliakova, L., Li, Z., Grove, R., Lakatta, E. G., & Talan, M. 2000, "Induction of angiogenesis by cationic lipid-mediated VEGF165 gene transfer in the rabbit ischemic hindlimb model", *Journal of Vascular Surgery*, vol. 32, no. 2, pp. 343-352.
- Grossman, J. A., McGonagle, B. A., Dowden, R. V., & Dinner, M. I. 1983, "The effect of hyaluronidase and dimethyl sulfoxide (DMSO) on experimental skin flap survival", *Annals of Plastic Surgery*, vol. 11, no. 3, pp. 223-226.
- Guba, A. M., Jr. 1979, "Study of the delay phenomenon in axial pattern flaps in pigs", *Plastic & Reconstructive Surgery*, vol. 63, no. 4, pp. 550-554.
- Hanahan, D. & Folkman, J. 1996, "Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis.", *Cell*, vol. 86, pp. 353-364.
- Hanahan, D. 1983, "Studies on transformation of Escherichia coli with plasmids", *Journal of Molecular Biology*, vol. 166, no. 4, pp. 557-580.
- Harley, C. B. & Reynolds, R. P. 1987, "Analysis of E. coli promoter sequences", *Nucleic Acids Research*, vol. 15, no. 5, pp. 2343-2361.
- Hartl, D. L. 1994, "The genetics of bacteria and viruses," in *Genetics*, 3rd edn, Jones and Bartlett, London, pp. 290-291.
- Hayward, P. G., Alison, W. E., Jr., Carp, S. S., Hui, P. S., & Robson, M. C. 1991, "Local infiltration of an angiogenic growth factor does not stimulate the delay phenomenon", *British Journal of Plastic Surgery*, vol. 44, no. 7, pp. 526-529.
- Hendel, R. C., Henry, T. D., Rocha-Singh, K., Isner, J. M., Kereiakes, D. J., Giordano, F. J., Simons, M., & Bonow, R. O. 2000, "Effect of intracoronary recombinant human vascular endothelial growth factor on myocardial perfusion: evidence for a dose-dependent effect.[see comment]", *Circulation*, vol. 101, no. 2, pp. 118-121.
- Henry DT. Can we really grow new blood vessels? *The Lancet* 351[June 20], 1826-1827. 1998.
- Ref Type: Abstract

- Henry, T. D. 1999, "Therapeutic angiogenesis.[comment]. [Review] [19 refs]", *BMJ.*, vol. 318, no. 7197, pp. 1536-1539.
- Henry, T. D., Annex, B. H., McKendall, G. R., Azrin, M. A., Lopez, J. J., Giordano, F. J., Shah, P. K., Willerson, J. T., Benza, R. L., Berman, D. S., Gibson, C. M., Bajamonde, A., Rundle, A. C., Fine, J., McCluskey, E. R., & Investigators, V. I. V. A. 2003, "The VIVA trial: Vascular endothelial growth factor in Ischemia for Vascular Angiogenesis", *Circulation.*, vol. 107, no. 10, pp. 1359-1365.
- Henry, T. D., Rocha-Singh, K., Isner, J. M., Kereiakes, D. J., Giordano, F. J., Simons, M., Losordo, D. W., Hendel, R. C., Bonow, R. O., Eppler, S. M., Zioncheck, T. F., Holmgren, E. B., & McCluskey, E. R. 2001, "Intracoronary administration of recombinant human vascular endothelial growth factor to patients with coronary artery disease", *American Heart Journal.*, vol. 142, no. 5, pp. 872-880.
- Heyderman, E. 1979, "Immunoperoxidase technique in histopathology: applications, methods, and controls. [Review] [25 refs]", *Journal of Clinical Pathology.*, vol. 32, no. 10, pp. 971-978.
- Hinnen, A., Hicks, J. B., & Fink, G. R. 1978, "Transformation of yeast", *Proceedings of the National Academy of Sciences of the United States of America.*, vol. 75, no. 4, pp. 1929-1933.
- Hobson, B. & Denekamp, J. 1984, "Endothelial proliferation in tumours and normal tissues: continuous labelling studies", *British Journal of Cancer.*, vol. 49, no. 4, pp. 405-413.
- Im, M. J., Kim, Y. S., Edwards, R. J., Hoopes, J. E., & Fenselau, A. 1992, "The effect of bovine basic fibroblast growth factor on skin flap survival in rats", *Annals of Plastic Surgery.*, vol. 28, no. 3, pp. 242-245.
- Iruela-Arispe, M. L. & Dvorak, H. F. 1997, "Angiogenesis: a dynamic balance of stimulators and inhibitors. [Review] [60 refs]", *Thrombosis & Haemostasis.*, vol. 78, no. 1, pp. 672-677.
- Ishiguro, N., Yabe, Y., Shimizu, T., Iwata, H., & Miura, T. 1994, "Basic fibroblast growth factor has a beneficial effect on the viability of random skin flaps in rats", *Annals of Plastic Surgery.*, vol. 32, no. 4, pp. 356-360.
- Isner, J. M., Walsh, K., Symes, J., Pieczek, A., Takeshita, S., Lowry, J., Rosenfield, K., Weir, L., Brogi, E., & Jurayj, D. 1996, "Arterial gene transfer for therapeutic angiogenesis in patients with peripheral artery disease", *Human Gene Therapy.*, vol. 7, no. 8, pp. 959-988.
- Itakura, K., Hirose, T., Crea, R., Riggs, A. D., Heyneker, H. L., Bolivar, F., & Boyer, H. W. 1977, "Expression in *Escherichia coli* of a chemically synthesized gene for the hormone somatostatin", *Science.*, vol. 198, no. 4321, pp. 1056-1063.
- Jensen, P. R. & Hammer, K. 1998, "Artificial promoters for metabolic optimization. [Review] [17 refs]", *Biotechnology & Bioengineering.*, vol. 58, no. 2-3, pp. 191-195.

- Jonsson, K., Hunt, T. K., Brennan, S. S., & Mathes, S. J. 1988, "Tissue oxygen measurements in delayed skin flaps: a reconsideration of the mechanisms of the delay phenomenon", *Plastic & Reconstructive Surgery.*, vol. 82, no. 2, pp. 328-336.
- Kerrigan, C. L. 1983, "Skin flap failure: pathophysiology", *Plastic & Reconstructive Surgery.*, vol. 72, no. 6, pp. 766-777.
- Kerrigan, C. L. & Daniel, R. K. 1982, "Pharmacologic treatment of the failing skin flap", *Plastic & Reconstructive Surgery.*, vol. 70, no. 5, pp. 541-549.
- Khan, A. W., Dhillon, A. P., Hutchins, R., Abraham, A., Shah, S. R., Snooks, S., & Davidson, B. R. 2002, "Prognostic significance of intratumoural microvessel density (IMD) in resected pancreatic and ampullary cancers to standard histopathological variables and survival", *European Journal of Surgical Oncology.*, vol. 28, no. 6, pp. 637-644.
- Khouri, R. K., Brown, D. M., Leal-Khouri, S. M., Tark, K. C., & Shaw, W. W. 1991, "The effect of basic fibroblast growth factor on the neovascularisation process: skin flap survival and staged flap transfers", *British Journal of Plastic Surgery.*, vol. 44, no. 8, pp. 585-588.
- Kinoshita, T., Takahashi, Y., Sakashita, T., Inoue, H., Tanabe, T., & Yoshimoto, T. 1999, "Growth stimulation and induction of epidermal growth factor receptor by overexpression of cyclooxygenases 1 and 2 in human colon carcinoma cells", *Biochimica et Biophysica Acta.*, vol. 1438, no. 1, pp. 120-130.
- Klagsbrun, M. & D'Amore, P. A. 1996, "Vascular endothelial growth factor and its receptors. [Review] [136 refs]", *Cytokine & Growth Factor Reviews.*, vol. 7, no. 3, pp. 259-270.
- Kobayashi, M., Kurusu, Y., & Yukawa, H. 1991, "High-expression of a target gene and high-stability of the plasmid. [Review] [116 refs]", *Applied Biochemistry & Biotechnology.*, vol. 27, no. 2, pp. 145-162.
- Komorowska-Timek, E., Newlin, L., Zhang, F., Dogan, T., Lineaweaver, W. C., & Buncke, H. J. 1999, "Shortening of rat teeth prevents autocannibalization of surgical flaps", *Journal of Reconstructive Microsurgery.*, vol. 15, no. 4, pp. 303-306.
- Kryger, Z., Zhang, F., Dogan, T., Cheng, C., Lineaweaver, W. C., & Buncke, H. J. 2000, "The effects of VEGF on survival of a random flap in the rat: examination of various routes of administration", *British Journal of Plastic Surgery.*, vol. 53, no. 3, pp. 234-239.
- Ku, D. D., Zaleski, J. K., Liu, S., & Brock, T. A. 1993, "Vascular endothelial growth factor induces EDRF-dependent relaxation in coronary arteries", *American Journal of Physiology.*, vol. 265, no. 2:Pt 2, p. t-92.
- Kuwano, M., Fukushi, J., Okamoto, M., Nishie, A., Goto, H., Ishibashi, T., & Ono, M. 2001, "Angiogenesis factors. [Review] [57 refs]", *Internal Medicine.*, vol. 40, no. 7, pp. 565-572.

- Laulajainen, T., Julkunen, I., Haltia, A., Knuutila, S., Miettinen, A., & Holthofer, H. 1993, "Establishment and characterization of a rat glomerular endothelial cell line", *Laboratory Investigation.*, vol. 69, no. 2, pp. 183-192.
- Lee, K. Y., Peters, M. C., & Mooney, D. J. 2003, "Comparison of vascular endothelial growth factor and basic fibroblast growth factor on angiogenesis in SCID mice", *Journal of Controlled Release.*87(1-3):49-56.
- Leung, D. W., Cachianes, G., Kuang, W. J., Goeddel, D. V., & Ferrara, N. 1989, "Vascular endothelial growth factor is a secreted angiogenic mitogen", *Science.*, vol. 246, no. 4935, pp. 1306-1309.
- Lindmark, G., Gerdin, B., Sundberg, C., Pahlman, L., Bergstrom, R., & Glimelius, B. 1996, "Prognostic significance of the microvascular count in colorectal cancer.[comment]", *Journal of Clinical Oncology.*, vol. 14, no. 2, pp. 461-466.
- Lobb, R. R., Key, M. E., Alderman, E. M., & Fett, J. W. 1985, "Partial purification and characterization of a vascular permeability factor secreted by a human colon adenocarcinoma cell line", *International Journal of Cancer.*, vol. 36, no. 4, pp. 473-478.
- Lubiatowski, P., Goldman, C. K., Gurunluoglu, R., Carnevale, K., & Siemionow, M. 2002, "Enhancement of epigastric skin flap survival by adenovirus-mediated VEGF gene therapy", *Plastic & Reconstructive Surgery.*, vol. 109, no. 6, pp. 1986-1993.
- Machens, H. G., Morgan, J. R., Berthiaume, F., Stefanovich, P., Reimer, R., & Berger, A. C. 1998, "Genetically modified fibroblasts induce angiogenesis in the rat epigastric island flap", *Langenbecks Archives of Surgery.*, vol. 383, no. 5, pp. 345-350.
- Machens, H. G., Morgan, J. R., Berthiaume, F., Stefanovich, P., Siemers, F., Krapohl, B., Berger, A., & Mailander, P. 2002, "Platelet-derived growth factor-AA-mediated functional angiogenesis in the rat epigastric island flap after genetic modification of fibroblasts is ischemia dependent", *Surgery.*, vol. 131, no. 4, pp. 393-400.
- Martinez-Hernandez, A., Merrill, D. A., Naughton, M. A., & Geczy, C. 1975, "Letter: Acrylamide affinity chromatography for immunohistochemistry. Purification of specific antibodies", *Journal of Histochemistry & Cytochemistry.*, vol. 23, no. 2, pp. 146-148.
- McGregor, A. D. 1992, "The angiosome--an in vivo study by fluorescein angiography.[see comment]", *British Journal of Plastic Surgery.*, vol. 45, no. 3, pp. 219-221.
- McLachlan, G., Davidson, D. J., Stevenson, B. J., Dickinson, P., Davidson-Smith, H., Dorin, J. R., & Porteous, D. J. 1995, "Evaluation in vitro and in vivo of cationic liposome-expression construct complexes for cystic fibrosis gene therapy", *Gene Therapy.*, vol. 2, no. 9, pp. 614-622.

- Misteli, T. & Spector, D. L. 1997, "Applications of the green fluorescent protein in cell biology and biotechnology. [Review] [45 refs]", *Nature Biotechnology*, vol. 15, no. 10, pp. 961-964.
- Mizushima, S. & Nagata, S. 1990, "pEF-BOS, a powerful mammalian expression vector", *Nucleic Acids Research*, vol. 18, no. 17, p. 5322.
- Montero, C. 2003, "The antigen-antibody reaction in immunohistochemistry. [Review] [31 refs]", *Journal of Histochemistry & Cytochemistry*, vol. 51, no. 1, pp. 1-4.
- Morbidelli, L., Chang, C. H., Douglas, J. G., Granger, H. J., Ledda, F., & Ziche, M. 1996, "Nitric oxide mediates mitogenic effect of VEGF on coronary venular endothelium", *American Journal of Physiology*, vol. 270, no. 1:Pt 2, p. t-5.
- Morrissey, W. M., Jr. & Hallock, G. G. 2000, "The increase in TRAM flap survival after delay does not diminish long term", *Annals of Plastic Surgery*, vol. 44, no. 5, pp. 486-490.
- Myers, M. B. & Cherry, G. 1969, "Mechanism of the delay phenomenon", *Plastic & Reconstructive Surgery*, vol. 44, no. 1, pp. 52-57.
- Naylor, L. H. 1999, "Reporter gene technology: the future looks bright. [Review] [120 refs]", *Biochemical Pharmacology*, vol. 58, no. 5, pp. 749-757.
- Nichter, L. S., Sobieski, M. W., Morgan, R. F., Rodeheaver, G., & Edlich, R. F. 1984, "Quantitation of skin-flap survival: a computer-based method", *Plastic & Reconstructive Surgery*, vol. 73, no. 4, pp. 684-686.
- Nishikawa, H., Manek, S., & Green, C. J. 1991, "The oblique rat groin flap", *British Journal of Plastic Surgery*, vol. 44, no. 4, pp. 295-298.
- Nishikawa, M. & Hashida, M. 2002, "Nonviral approaches satisfying various requirements for effective in vivo gene therapy. [Review] [125 refs]", *Biological & Pharmaceutical Bulletin*, vol. 25, no. 3, pp. 275-283.
- Norrby, K. 1996, "Vascular endothelial growth factor and de novo mammalian angiogenesis", *Microvascular Research*, vol. 51, no. 2, pp. 153-163.
- Obermair, A., Bancher-Todesca, D., Bilgi, S., Kaider, A., Kohlberger, P., Mullauer-Ertl, S., Leodolter, S., & Gitsch, G. 1997, "Correlation of vascular endothelial growth factor expression and microvessel density in cervical intraepithelial neoplasia", *Journal of the National Cancer Institute*, vol. 89, no. 16, pp. 1212-1217.
- Okamoto, Y., Nakajima, T., & Yoneda, K. 1993, "Augmentation of skin flap survival by selective intraarterial infusion of prostaglandin E1: experimental and clinical studies", *Annals of Plastic Surgery*, vol. 30, no. 2, pp. 154-158.

- Old, R. W. & Primrose, S. B. 1995a, "Gene manipulation: an all-embracing technique," in *Principles of Gene Manipulation: An Introduction to Genetic Engineering*, 5th edn, Blackwell Science, pp. 1-5.
- Old, R. W. & Primrose, S. B. 1995b, "The impact of recombinant DNA technology: the generation of novelty," in *Principles of Gene Manipulation: An Introduction to Genetic Engineering*, 5th edn, Blackwell Science, pp. 369-407.
- Olofsson, B., Jeltsch, M., Eriksson, U., & Alitalo, K. 1999, "Current biology of VEGF-B and VEGF-C. [Review] [86 refs]", *Current Opinion in Biotechnology.*, vol. 10, no. 6, pp. 528-535.
- Olofsson, B., Pajusola, K., Kaipainen, A., von Euler, G., Joukov, V., Saksela, O., Orpana, A., Pettersson, R. F., Alitalo, K., & Eriksson, U. 1996, "Vascular endothelial growth factor B, a novel growth factor for endothelial cells", *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 6, pp. 2576-2581.
- Padubidri A & Browne E. Effect of vascular endothelial growth factor(VEGF) on survival of random extension of axial pattern skin flaps in the rat. *Annals of Plastic Surgery* 37[6], 604-611. 1996.
Ref Type: Abstract
- Padubidri, A. N. & Browne E Jr 1997, "Modification in flap design of the epigastric artery flap in rats--a new experimental flap model", *Annals of Plastic Surgery.*, vol. 39, no. 5, pp. 500-504.
- Padubidri, A. N., Browne, E., Banbury, J., & Seimionow, M. 1997, "Arteriovenous cross-flow flap in rats: a novel skin flap", *Plastic & Reconstructive Surgery.*, vol. 100, no. 5, pp. 1218-1226.
- Pagano, R. E. & Weinstein, J. N. 1978, "Interactions of liposomes with mammalian cells. [Review] [184 refs]", *Annual Review of Biophysics & Bioengineering.*, vol. 7, pp. 435-468.
- Pal, S., Khazanchi, R. K., & Moudgil, K. 1991, "An experimental study on the effect of nifedipine on ischaemic skin flap survival in rats", *British Journal of Plastic Surgery.*, vol. 44, no. 4, pp. 299-301.
- Pang, C. Y., Forrest, C. R., & Morris, S. F. 1989, "Pharmacological augmentation of skin flap viability: a hypothesis to mimic the surgical delay phenomenon or a wishful thought. [Review] [140 refs]", *Annals of Plastic Surgery.*, vol. 22, no. 4, pp. 293-306.
- Pang, C. Y., Forrest, C. R., Neligan, P. C., & Lindsay, W. K. 1986, "Augmentation of blood flow in delayed random skin flaps in the pig: effect of length of delay period and angiogenesis", *Plastic & Reconstructive Surgery.*, vol. 78, no. 1, pp. 68-74.
- Park, J. E., Keller, G. A., & Ferrara, N. 1993, "The vascular endothelial growth factor (VEGF) isoforms: differential deposition into the subepithelial extracellular

matrix and bioactivity of extracellular matrix-bound VEGF", *Molecular Biology of the Cell.*, vol. 4, no. 12, pp. 1317-1326.

Pazouki, S., Chisholm, D. M., Adi, M. M., Carmichael, G., Farquharson, M., Ogden, G. R., Schor, S. L., & Schor, A. M. 1997, "The association between tumour progression and vascularity in the oral mucosa", *Journal of Pathology.*, vol. 183, no. 1, pp. 39-43.

Pendleton, N., Pazouki, S., Heerkens, E., Smither, R. L., Chisholm, D. M., Moore, J. V., Howell, A., Horan, M. A., & Schor, A. M. 1998, "Relationships between different measurements of vascularity and clinico-pathological parameters in breast cancer", *Anticancer Research.*, vol. 18, no. 6B, pp. 4565-4568.

Petry, J. J. & Wortham, K. A. 1984, "The anatomy of the epigastric flap in the experimental rat", *Plastic & Reconstructive Surgery.*, vol. 74, no. 3, pp. 410-413.

Plate, K. H. & Warnke, P. C. 1997, "Vascular endothelial growth factor. [Review] [75 refs]", *Journal of Neuro-Oncology.*, vol. 35, no. 3, pp. 365-372.

Plautz, G., Nabel, E. G., & Nabel, G. J. 1991, "Introduction of vascular smooth muscle cells expressing recombinant genes in vivo", *Circulation.*, vol. 83, no. 2, pp. 578-583.

Poliakova, L., Kovesdi, I., Wang, X., Capogrossi, M. C., & Talan, M. 1999, "Vascular permeability effect of adenovirus-mediated vascular endothelial growth factor gene transfer to the rabbit and rat skeletal muscle", *Journal of Thoracic & Cardiovascular Surgery.*, vol. 118, no. 2, pp. 339-347.

Prasher, D. C., Eckenrode, V. K., Ward, W. W., Prendergast, F. G., & Cormier, M. J. 1992, "Primary structure of the *Aequorea victoria* green-fluorescent protein", *Gene.*, vol. 111, no. 2, pp. 229-233.

Prober, J. M., Trainor, G. L., Dam, R. J., Hobbs, F. W., Robertson, C. W., Zagursky, R. J., Cocuzza, A. J., Jensen, M. A., & Baumeister, K. 1987, "A system for rapid DNA sequencing with fluorescent chain-terminating dideoxynucleotides", *Science.*, vol. 238, no. 4825, pp. 336-341.

Pu, L. Q., Sniderman, A. D., Arekat, Z., Graham, A. M., Brassard, R., & Symes, J. F. 1993a, "Angiogenic growth factor and revascularization of the ischemic limb: evaluation in a rabbit model", *Journal of Surgical Research.*, vol. 54, no. 6, pp. 575-583.

Pu, L. Q., Sniderman, A. D., Brassard, R., Lachapelle, K. J., Graham, A. M., Lisbona, R., & Symes, J. F. 1993b, "Enhanced revascularization of the ischemic limb by angiogenic therapy", *Circulation.*, vol. 88, no. 1, pp. 208-215.

Qiagen Pty Ltd. Qiagen plasmid purification handbook. 1997. Clifton Hill, Victoria, Australia, Qiagen.

Ref Type: Pamphlet

Quinodoz, P., Quinodoz, M., Nussbaum, J. L., Montandon, D., & Pittet, B. 2002, "Barium sulphate and soft-tissue radiology: allying the old and the new for the investigation of animal cutaneous microcirculation", *British Journal of Plastic Surgery.*, vol. 55, no. 8, pp. 664-667.

Ray, P. S., Estrada-Hernandez, T., Sasaki, H., Zhu, L., & Maulik, N. 2000, "Early effects of hypoxia/reoxygenation on VEGF, ang-1, ang-2 and their receptors in the rat myocardium: implications for myocardial angiogenesis", *Molecular & Cellular Biochemistry.*, vol. 213, no. 1-2, pp. 145-153.

Rees, M. J. & Taylor, G. I. 1986, "A simplified lead oxide cadaver injection technique", *Plastic & Reconstructive Surgery.*, vol. 77, no. 1, pp. 141-145.

Rendell, M. S., Finnegan, M. F., Healy, J. C., Lind, A., Milliken, B. K., Finney, D. E., & Bonner, R. F. 1998, "The relationship of laser-Doppler skin blood flow measurements to the cutaneous microvascular anatomy", *Microvascular Research.*, vol. 55, no. 1, pp. 3-13.

Rieder, M. J., O'Drobinak, D. M., & Greene, A. S. 1995, "A computerized method for determination of microvascular density", *Microvascular Research.*, vol. 49, no. 2, pp. 180-189.

Rieder, M. J., Roman, R. J., & Greene, A. S. 1997, "Reversal of microvascular rarefaction and reduced renal mass hypertension", *Hypertension.*, vol. 30, no. 1:Pt 1, pp. 1-7.

Riessen, R., Rahimizadeh, H., Blessing, E., Takeshita, S., Barry, J. J., & Isner, J. M. 1993, "Arterial gene transfer using pure DNA applied directly to a hydrogel-coated angioplasty balloon", *Human Gene Therapy.*, vol. 4, no. 6, pp. 749-758.

Rinsch, C., Quinodoz, P., Pittet, B., Alizadeh, N., Baetens, D., Montandon, D., Aebischer, P., & Pepper, M. S. 2001a, "Delivery of FGF-2 but not VEGF by encapsulated genetically engineered myoblasts improves survival and vascularization in a model of acute skin flap ischemia", *Gene Therapy.*, vol. 8, no. 7, pp. 523-533.

Rinsch, C., Quinodoz, P., Pittet, B., Alizadeh, N., Baetens, D., Montandon, D., Aebischer, P., & Pepper, M. S. 2001b, "Delivery of FGF-2 but not VEGF by encapsulated genetically engineered myoblasts improves survival and vascularization in a model of acute skin flap ischemia", *Gene Therapy.*, vol. 8, no. 7, pp. 523-533.

Rippe, R. A., Brenner, D. A., & Leffert, H. L. 1990, "DNA-mediated gene transfer into adult rat hepatocytes in primary culture", *Molecular & Cellular Biology.*, vol. 10, no. 2, pp. 689-695.

Risau, W. 1997, "Mechanisms of angiogenesis. [Review] [53 refs]", *Nature.*, vol. 386, no. 6626, pp. 671-674.

Rissanen, T. T., Markkanen, J. E., Gruchala, M., Heikura, T., Puranen, A., Kettunen, M. I., Kholova, I., Kauppinen, R. A., Achen, M. G., Stacker, S. A., Alitalo, K., & Yla-Herttuala, S. 2003, "VEGF-D is the strongest angiogenic and lymphangiogenic

- effector among VEGFs delivered into skeletal muscle via adenoviruses", *Circulation Research*, vol. 92, no. 10, pp. 1098-1106.
- Roberts, A. P., Cohen, J. I., & Cook, T. A. 1996, "The rat ventral island flap: a comparison of the effects of reduction in arterial inflow and venous outflow.[comment]", *Plastic & Reconstructive Surgery*, vol. 97, no. 3, pp. 610-615.
- Roberts, W. G. & Palade, G. E. 1997, "Neovasculature induced by vascular endothelial growth factor is fenestrated", *Cancer Research*, vol. 57, no. 4, pp. 765-772.
- Rosengart, T. K., Lee, L. Y., Patel, S. R., Kligfield, P. D., Okin, P. M., Hackett, N. R., Isom, O. W., & Crystal, R. G. 1999, "Six-month assessment of a phase I trial of angiogenic gene therapy for the treatment of coronary artery disease using direct intramyocardial administration of an adenovirus vector expressing the VEGF121 cDNA", *Annals of Surgery*, vol. 230, no. 4, pp. 466-470.
- Ross, M. & Reith, E. 1985, "The cardiovascular system," in *Histology: a text and atlas*, 1st edition edn, Harper, Row & Lippincott, p. 287.
- Saclarides, T. J., Speziale, N. J., Drab, E., Szeluga, D. J., & Rubin, D. B. 1994, "Tumor angiogenesis and rectal carcinoma", *Diseases of the Colon & Rectum*, vol. 37, no. 9, pp. 921-926.
- Safi, J., Jr., Gloe, T. R., Riccioni, T., Kovesdi, I., & Capogrossi, M. C. 1997, "Gene therapy with angiogenic factors: a new potential approach to the treatment of ischemic diseases. [Review] [128 refs]", *Journal of Molecular & Cellular Cardiology*, vol. 29, no. 9, pp. 2311-2325.
- Sanger, F., Nicklen, S., & Coulson, A. R. 1977, "DNA sequencing with chain-terminating inhibitors", *Proceedings of the National Academy of Sciences of the United States of America*, vol. 74, no. 12, pp. 5463-5467.
- Schaffner, W. 1980, "Direct transfer of cloned genes from bacteria to mammalian cells", *Proceedings of the National Academy of Sciences of the United States of America*, vol. 77, no. 4, pp. 2163-2167.
- Schenborn, E. T. 2000, "Transfection technologies. [Review] [47 refs]", *Methods in Molecular Biology*, vol. 130, pp. 91-102.
- Schor, A. M., Pendleton, N., Pazouki, S., Smither, R. L., Morris, J., Lessan, K., Heerkens, E., Chandrachud, L. M., Carmichael, G., Adi, M., Chisholm, D. M., & Stevenson, H. 1998, "Assessment of vascularity in histological sections: effects of methodology and value as an index of angiogenesis in breast tumours", *Histochemical Journal*, vol. 30, no. 12, pp. 849-856.
- Schumacher, B., Pecher, P., von Specht, B. U., & Stegmann, T. 1998, "Induction of neoangiogenesis in ischemic myocardium by human growth factors: first clinical results of a new treatment of coronary heart disease.[see comment]", *Circulation*, vol. 97, no. 7, pp. 645-650.

- Senger, D. R., Galli, S. J., Dvorak, A. M., Perruzzi, C. A., Harvey, V. S., & Dvorak, H. F. 1983, "Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid", *Science.*, vol. 219, no. 4587, pp. 983-985.
- Sherratt, D. J. 1979, "Plasmid vectors for genetic manipulation in vitro. [Review] [23 refs]", *Biochemical Society Symposia.*, vol. 44, pp. 29-36.
- Shi, S. R., Key, M. E., & Kalra, K. L. 1991, "Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections", *Journal of Histochemistry & Cytochemistry.*, vol. 39, no. 6, pp. 741-748.
- Shweiki, D., Itin, A., Neufeld, G., Gitay-Goren, H., & Keshet, E. 1993, "Patterns of expression of vascular endothelial growth factor (VEGF) and VEGF receptors in mice suggest a role in hormonally regulated angiogenesis", *Journal of Clinical Investigation.*, vol. 91, no. 5, pp. 2235-2243.
- Silvestre, J. S., Tamarat, R., Ebrahimian, T. G., Le Roux, A., Clergue, M., Emmanuel, F., Duriez, M., Schwartz, B., Branellec, D., & Levy, B. I. 2003, "Vascular endothelial growth factor-B promotes in vivo angiogenesis.[see comment]", *Circulation Research.*, vol. 93, no. 2, pp. 114-123.
- Singer, A. J. & Clark, R. A. 1999, "Cutaneous wound healing. [Review] [86 refs]", *New England Journal of Medicine.*, vol. 341, no. 10, pp. 738-746.
- Smyth, T. N. 2002, "Liposomal delivery of nucleic acids in vivo. [Review] [34 refs]", *DNA & Cell Biology.*, vol. 21, no. 12, pp. 857-867.
- Smyth, T. N. 2003, "Myths concerning the use of cationic liposomes in vivo", *Expert Opinion on Biological Therapy.* 3(1):57-69.
- Smythe, G. M., Hodgetts, S. I., & Grounds, M. D. 2000, "Immunobiology and the future of myoblast transfer therapy. [Review] [120 refs]", *Molecular Therapy: the Journal of the American Society of Gene Therapy.*, vol. 1, no. 4, pp. 304-313.
- Sowter, H. M., Corps, A. N., Evans, A. L., Clark, D. E., Charnock-Jones, D. S., & Smith, S. K. 1997, "Expression and localization of the vascular endothelial growth factor family in ovarian epithelial tumors", *Laboratory Investigation.*, vol. 77, no. 6, pp. 607-614.
- Suri, C., Jones, P. F., Patan, S., Bartunkova, S., Maisonpierre, P. C., Davis, S., Sato, T. N., & Yancopoulos, G. D. 1996, "Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis.[see comment]", *Cell.*, vol. 87, no. 7, pp. 1171-1180.
- Takayanagi, S. & Ogawa, Y. 1980, "Effects of Pentoxifylline on flap survival", *Plastic & Reconstructive Surgery.*, vol. 65, no. 6, pp. 763-767.
- Takeshita, S., Isshiki, T., Mori, H., Tanaka, E., Tanaka, A., Umetani, K., Eto, K., Miyazawa, Y., Ochiai, M., & Sato, T. 1997, "Microangiographic assessment of

collateral vessel formation following direct gene transfer of vascular endothelial growth factor in rats", *Cardiovascular Research*, vol. 35, no. 3, pp. 547-552.

Takeshita, S., Isshiki, T., & Sato, T. 1996, "Increased expression of direct gene transfer into skeletal muscles observed after acute ischemic injury in rats", *Laboratory Investigation*, vol. 74, no. 6, pp. 1061-1065.

Takeshita, S., Losordo, D. W., Kearney, M., Rossow, S. T., & Isner, J. M. 1994a, "Time course of recombinant protein secretion after liposome-mediated gene transfer in a rabbit arterial organ culture model", *Laboratory Investigation*, vol. 71, no. 3, pp. 387-391.

Takeshita, S., Pu, L. Q., Stein, L. A., Sniderman, A. D., Bunting, S., Ferrara, N., Isner, J. M., & Symes, J. F. 1994b, "Intramuscular administration of vascular endothelial growth factor induces dose-dependent collateral artery augmentation in a rabbit model of chronic limb ischemia", *Circulation*, vol. 90, no. 5:Pt 2, p. t-34.

Takeshita, S., Rossow, S. T., Kearney, M., Zheng, L. P., Bauters, C., Bunting, S., Ferrara, N., Symes, J. F., & Isner, J. M. 1995, "Time course of increased cellular proliferation in collateral arteries after administration of vascular endothelial growth factor in a rabbit model of lower limb vascular insufficiency", *American Journal of Pathology*, vol. 147, no. 6, pp. 1649-1660.

Takeshita, S., Weir, L., Chen, D., Zheng, L. P., Riessen, R., Bauters, C., Symes, J. F., Ferrara, N., & Isner, J. M. 1996, "Therapeutic angiogenesis following arterial gene transfer of vascular endothelial growth factor in a rabbit model of hindlimb ischemia", *Biochemical & Biophysical Research Communications*, vol. 227, no. 2, pp. 628-635.

Takeshita, S., Zheng, L. P., Brogi, E., Kearney, M., Pu, L. Q., Bunting, S., Ferrara, N., Symes, J. F., & Isner, J. M. 1994c, "Therapeutic angiogenesis. A single intraarterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hind limb model", *Journal of Clinical Investigation*, vol. 93, no. 2, pp. 662-670.

Taub PJ, Marmur JD, Zhang WX, Senderoff D, Nhat PD, Phelps R, Urken ML, Silver L, & Weinberg H 1998, "Locally administered vascular endothelial growth factor cDNA increases survival of ischaemic experimental skin flaps.", *Plastic & Reconstructive Surgery*, vol. 102, no. 6, pp. 2033-2039.

Taylor, G. I. & Palmer, J. H. 1987, "The vascular territories (angiosomes) of the body: experimental study and clinical applications", *British Journal of Plastic Surgery*, vol. 40, no. 2, pp. 113-141.

Teifel, M., Heine, L. T., Milbredt, S., & Friedl, P. 1997, "Optimization of transfection of human endothelial cells", *Endothelium*, vol. 5, no. 1, pp. 21-35.

Togari, A., Dickens, G., Kuzuya, H., & Guroff, G. 1985, "The effect of fibroblast growth factor on PC12 cells", *Journal of Neuroscience*, vol. 5, no. 2, pp. 307-316.

Tsien, R. Y. 1998, "The green fluorescent protein. [Review] [114 refs]", *Annual Review of Biochemistry.*, vol. 67, pp. 509-544.

Tsurumi, Y., Takeshita, S., Chen, D., Kearney, M., Rossow, S. T., Passeri, J., Horowitz, J. R., Symes, J. F., & Isner, J. M. 1996, "Direct intramuscular gene transfer of naked DNA encoding vascular endothelial growth factor augments collateral development and tissue perfusion.[comment]", *Circulation.*, vol. 94, no. 12, pp. 3281-3290.

Tucci, M. G., Scalise, A., Lucarini, G., Pierangeli, M., Pugnali, A., Bertani, C., Orlando, F., Aldini, N. N., Santoli, M., Campodonico, A., Gavaudan, F., Ricotti, G., Bertani, A., & Biagini, G. 2001, "rhVEGF and experimental rat skin flaps: systemic or local administration and morphological characteristics", *International Journal of Artificial Organs.*, vol. 24, no. 10, pp. 743-751.

Uchida, E., Mizuguchi, H., Ishii-Watabe, A., & Hayakawa, T. 2002, "Comparison of the efficiency and safety of non-viral vector-mediated gene transfer into a wide range of human cells", *Biological & Pharmaceutical Bulletin.*, vol. 25, no. 7, pp. 891-897.

Uetsuki, T., Naito, A., Nagata, S., & Kaziro, Y. 1989, "Isolation and characterization of the human chromosomal gene for polypeptide chain elongation factor-1 alpha", *Journal of Biological Chemistry.*, vol. 264, no. 10, pp. 5791-5798.

Uhl, E., Barker, J. H., Bondar, I., Galla, T. J., Lehr, H. A., & Messmer, K. 1994, "Improvement of skin flap perfusion by subdermal injection of recombinant human basic fibroblast growth factor", *Annals of Plastic Surgery.*, vol. 32, no. 4, pp. 361-365.

Vermeulen, P. B., Gasparini, G., Fox, S. B., Colpaert, C., Marson, L. P., Gion, M., Belien, J. A., de Waal, R. M., Van Marck, E., Magnani, E., Weidner, N., Harris, A. L., & Dirix, L. Y. 2002, "Second international consensus on the methodology and criteria of evaluation of angiogenesis quantification in solid human tumours. [Review] [143 refs]", *European Journal of Cancer.*, vol. 38, no. 12, pp. 1564-1579.

Walter, D. H., Hink, U., Asahara, T., Van Belle, E., Horowitz, J., Tsurumi, Y., Vandlen, R., Heinsohn, H., Keyt, B., Ferrara, N., Symes, J. F., & Isner, J. M. 1996, "The in vivo bioactivity of vascular endothelial growth factor/vascular permeability factor is independent of N-linked glycosylation", *Laboratory Investigation.*, vol. 74, no. 2, pp. 546-556.

Wang HJ, Chen TM, Chow LS, Cheng TY, & Chen JL 1997, "Recipient bed vascularity and survival of ischaemic flaps.", *British Journal of Plastic Surgery.*, vol. 50, pp. 266-271.

Webster, K. A. 2003, "Therapeutic angiogenesis: a complex problem requiring a sophisticated approach. [Review] [104 refs]", *Cardiovascular Toxicology.*, vol. 3, no. 3, pp. 283-298.

- Weidner, N. 1995, "Current pathologic methods for measuring intratumoral microvessel density within breast carcinoma and other solid tumors. [Review] [66 refs]", *Breast Cancer Research & Treatment.*, vol. 36, no. 2, pp. 169-180.
- Weidner, N., Semple, J. P., Welch, W. R., & Folkman, J. 1991, "Tumor angiogenesis and metastasis--correlation in invasive breast carcinoma", *New England Journal of Medicine.*, vol. 324, no. 1, pp. 1-8.
- Wolff, J. A., Malone, R. W., Williams, P., Chong, W., Acsadi, G., Jani, A., & Felgner, P. L. 1990, "Direct gene transfer into mouse muscle in vivo", *Science.*, vol. 247, no. 4949:Pt 1, p. t-8.
- Young, J. L. & Dean, D. A. 2002, "Nonviral gene transfer strategies for the vasculature. [Review] [83 refs]", *Microcirculation.*, vol. 9, no. 1, pp. 35-49.
- Zhang, F., Oswald, T., Lin, S., Cai, Z., Lei, M., Jones, M., Angel, M. F., & Lineaweaver, W. C. 2003, "Vascular endothelial growth factor (VEGF) expression and the effect of exogenous VEGF on survival of a random flap in the rat", *British Journal of Plastic Surgery.*, vol. 56, no. 7, pp. 653-659.
- Zhang, G., Budker, V., Williams, P., Subbotin, V., & Wolff, J. A. 2001, "Efficient expression of naked dna delivered intraarterially to limb muscles of nonhuman primates", *Human Gene Therapy.*, vol. 12, no. 4, pp. 427-438.
- Zimmermann, U. & Vienken, J. 1982, "Electric field-induced cell-to-cell fusion. [Review] [90 refs]", *Journal of Membrane Biology.*, vol. 67, no. 3, pp. 165-182.
- Zuhorn, I. S. & Hoekstra, D. 2002, "On the mechanism of cationic amphiphile-mediated transfection. To fuse or not to fuse: is that the question?. [Review] [105 refs]", *Journal of Membrane Biology.*, vol. 189, no. 3, pp. 167-179.

Appendices

Appendix 1 – Weight-area standard for exposed radiographic film

To calculate percentage flap survival from templates constructed from exposed radiographic film requires that it be of constant weight: area ratio. To investigate this, ten samples of exposed radiographic film were selected at random and cut to an area measured at 8 x 8 cm. Each template obtained in this way was weighed and a weight: area ratio calculated. The results compared and a mean value with 95% confidence intervals calculated.

Results

The results are given in Table A1.1. Mean weight:area ratio was found to be $2.54 \times 10^{-2} \text{ g/cm}^2$ (95% C.I. 2.52×10^{-2} , 2.55×10^{-2}). The narrow overall range of the results and confidence intervals suggest uniformity. We concluded that radiographic film was a good choice of material for our purposes.

Table A1.1 – Weight : area ratios calculated for exposed radiographic film samples

Template	Weight (g)	Weight : area ratio
1	1.66	0.0259
2	1.61	0.0252
3	1.63	0.0255
4	1.63	0.0255
5	1.63	0.0255
6	1.62	0.0253
7	1.62	0.0253
8	1.62	0.0253
9	1.60	0.0250
10	1.60	0.0250

Appendix 2– Estimate of right hemiflap survival area

Finseth & Cutting, in their original description of the rat epigastric island flap, chose to express their data as an area survival for the right hemiflap only. To make the two series more comparable requires the conversion of my data to this format. The calculation may be performed in the following manner:

Mean starting template weight (8 x 8 cm)	=	1.63 g
Mean right hemiflap template weight (8 x 4 cm)	=	0.815 g
Allowing for flap contracture to 78.8 % of starting area at one week		
Mean expected right hemiflap template weight (1 wk)	=	0.788 x 0.815
	=	0.642 g

Right hemiflap survival (RHS) is given by:

$$\frac{\text{Expected hemiflap template weight} - \text{necrotic template weight}}{\text{Weight-area standard}}$$

Using the data in Table 2.1, necrotic template weight = $W1 - W2$

Therefore,

$$\text{RHS} = \frac{0.642 - (W1 - W2)}{0.0254}$$

Mean survival of the randomly supplied right hemiflap in my series of group 2 flaps was therefore, approximately $17 \pm 2 \text{ cm}^2$. The purpose of this calculation was to enable a board comparison with the results of Finseth and Cutting. The error incurred by the assumption that flap contraction is uniform, which it is not, means that this approximation is likely to be an overestimate of the true result.

Table A2.1 – Calculation of right hemiflap survival (RHS) for group 2 flaps from the data given in Chapter 2.

<i>Animal reference</i>	<i>W1 – W2 (g)</i>	<i>RHS (cm²)</i>
P1	0.20	17.4
P2	0.21	17.0
P3	0.10	21.3
P4	0.22	16.6
P5	0.13	20.2
P6	0.20	17.4
P7	0.21	17.0
P8	0.26	15.0
	Mean	17.7
	S.D.	2.0

Appendix 3 – Operating procedure used for the histological preparation of rat skin

PURPOSE

To prepare rat ventral skin flaps for histological staining and examination. The flaps will be pinned out to prevent shrinkage at the time of harvest and presented in formalin.

SUPPLIES

scalpel blade
cutting board
specimen cassettes with lids
coloured ink

histological stains
10% formaldehyde

SOURCE

All supplies and equipment are available at the Anatomical Pathology laboratory at The St. George Hospital

EQUIPMENT

paraffin embedding apparatus

PROCEDURE

This operating procedure has been designed for use in a pathology laboratory with appropriate ‘cut - up’ facilities.

1. Complete St George pathology department request form using the following identifiers:

Name: RESEARCH - POOLE

MRN: T 0008041

DOB: 05/06/98

Specimen: unique specimen identification number

2. Record unique specimen identification number on project record sheet
3. Remove flap from formalin and record total time in preservative on project record sheet
4. Place flap on cutting board, leave holding pins in-situ
5. Perform gross pathological examination of tissue recording flap dimensions and any striking features on project specimen sheet
6. Orientate specimen (generally the experimental half of the flap will be on the right *in-vivo*)
7. Section specimen as marked (horizontal sections will be taken at a point 75% of the way up from the base of the flap [strip 1] and at the mid-point [strip 2]) to produce strips approximately 2-3mm wide
8. Store residual flap in formalin
9. Orientate specimen strips on cutting board
10. Further divide each strip into four blocks by sectioning at the midline and half way to the edge of the flap on each side
11. For the **medial** specimens, mark the edges closest to the **midline** with **black ink**

12. For the **lateral** specimens, mark the edges closest to the **sides** of the flap with **blue ink**

13. Transfer marked specimens (2 specimens per cassette) to histology cassettes in the following manner maintaining orientation and with the specimens nearest the **midline lowermost** in the cassette:

75% strip[strip 1]: random pattern blocks(RHS) cassette 1

axial pattern blocks(LHS) cassette 2

midpoint strip[strip 2]: random pattern blocks(RHS) cassette 3

axial pattern blocks(LHS) cassette 4

14. Seal cassettes with metal lids provided

15. Transfer completed cassettes to formalin racks for paraffin embedding

16. Place completed pathology request form in file tray provided

17. Return project specimen sheet to file

Appendix 4 – Randomisation details for CHO-VEGFB trial

The proposed trial comprised six experimental groups of 10 rats each giving a total of 60 rats. To reduce bias, the experimental order was randomised using a table of random sampling numbers according to the technique of random permutation. Each individual rat was given a number form 1 – 60 as shown below:

Group 1 (CHO-VEGFB cells random flap)	1 - 10
Group 2 (CHO-BLANK cells random flap)	11 – 20
Group 3 (CHO-VEGFB cells axial flap)	21 – 30
Group 4 (CHO-BLANK cells axial flap)	31 – 40
Group 5 (CHO-VEGFB cells whole flap)	41 – 50
Group 6 (CHO-BLANK cells whole flap)	51 – 60

The final position of each individual in the overall experimental order was generated using the random number table and is given in Table A4.1 below (animal number is given in black, experimental position is in red):

Table A4.1 – Randomisation table for CHO-VEGFB trial. Animal number is given in black and trial number is in red.

1	14	11	11	21	58	31	50	41	35	51	18
2	9	12	16	22	30	32	34	42	2	52	24
3	24	13	3	23	47	33	20	43	37	53	49
4	13	14	48	24	52	34	10	44	7	54	54
5	21	15	29	25	41	35	18	45	5	55	29
6	49	16	4	26	15	36	42	46	39	56	30
7	1	17	26	27	31	37	25	47	36	57	28
8	45	18	51	28	57	38	43	48	46	58	21
9	19	19	32	29	55	39	22	49	53	59	40
10	17	20	6	30	56	40	59	50	38	60	58

The final operating schedule for the ventral flap CHO (VFC) trial is therefore given in Table A4.2

Table A4.2 – Final operating schedule for CHO-VEGFB randomised controlled trial.

<i>Animal reference</i>	<i>CHO delivery</i>	<i>Animal reference</i>	<i>CHO delivery</i>
VFC – 1	CHO-VEGFB random pattern flap	VFC – 6	CHO-BLANK random pattern flap
VFC – 2	CHO-VEGFB whole flap	VFC – 7	CHO-VEGFB whole flap
VFC – 3	CHO-BLANK random pattern flap	VFC – 8	CHO-BLANK whole flap
VFC – 4	CHO-BLANK random pattern flap	VFC – 9	CHO-VEGFB random pattern flap
VFC – 5	CHO-VEGFB whole flap	VFC – 10	CHO-BLANK axial pattern flap

Table A4.2(continued) – Final operating schedule for CHO-VEGFB randomised controlled trial.

<i>Animal reference</i>	<i>CHO delivery</i>	<i>Animal reference</i>	<i>CHO delivery</i>
VFC – 11	CHO-BLANK random pattern flap	VFC – 36	CHO-VEGFB whole flap
VFC – 12	CHO-BLANK whole flap	VFC – 37	CHO-VEGFB whole flap
VFC – 13	CHO-VEGFB random pattern flap	VFC – 38	CHO-VEGFB whole flap
VFC – 14	CHO-VEGFB random pattern flap	VFC – 39	CHO-VEGFB whole flap
VFC – 15	CHO-VEGFB axial pattern flap	VFC – 40	CHO-BLANK whole flap
VFC – 16	CHO-BLANK random pattern flap	VFC – 41	CHO-VEGFB axial pattern flap
VFC – 17	CHO-VEGFB random pattern flap	VFC – 42	CHO-BLANK axial pattern flap
VFC – 18	CHO-BLANK axial pattern flap	VFC – 43	CHO-BLANK axial pattern flap
VFC – 19	CHO-VEGFB random pattern flap	VFC – 44	CHO-BLANK whole flap
VFC – 20	CHO-BLANK axial pattern flap	VFC – 45	CHO-VEGFB random pattern flap
VFC – 21	CHO-VEGFB random pattern flap	VFC – 46	CHO-VEGFB whole flap
VFC – 22	CHO-BLANK axial pattern flap	VFC – 47	CHO-VEGFB axial pattern flap
VFC – 23	CHO-BLANK whole flap	VFC – 48	CHO-BLANK random pattern flap
VFC – 24	CHO-BLANK random pattern flap	VFC – 49	CHO-VEGFB random pattern flap
VFC – 25	CHO-BLANK axial pattern flap	VFC – 50	CHO-BLANK axial pattern flap
VFC – 26	CHO-BLANK random pattern flap	VFC – 51	CHO-BLANK random pattern flap
VFC – 27	CHO-BLANK whole flap	VFC – 52	CHO-VEGFB axial pattern flap
VFC – 28	CHO-BLANK whole flap	VFC – 53	CHO-VEGFB whole flap
VFC – 29	CHO-BLANK random pattern flap	VFC – 54	CHO-BLANK whole flap
VFC – 30	CHO-VEGFB axial pattern flap	VFC – 55	CHO-VEGFB axial pattern flap
VFC – 31	CHO-VEGFB axial pattern flap	VFC – 56	CHO-VEGFB axial pattern flap
VFC – 32	CHO-BLANK random pattern flap	VFC – 57	CHO-VEGFB axial pattern flap
VFC – 33	CHO-BLANK whole flap	VFC – 58	CHO-VEGFB axial pattern flap
VFC – 34	CHO-BLANK axial pattern flap	VFC – 59	CHO-BLANK axial pattern flap
VFC – 35	CHO-VEGFB whole flap	VFC – 60	CHO-BLANK whole flap