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**Therapeutic Applications of Endothelial
Progenitor Cells for Diabetic Ulcer Healing**

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**Thesis for the degree of PhD.
of the National University of Ireland, Galway**

Submitted October 2016



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This candidate confirms that the work submitted is his own and that appropriate credit has been made to the work of others

This thesis is dedicated to my parents, Con and Phil

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ABBREVIATIONS

AAV	Adeno-associated virus
Ac-LDL	acetylated low density lipoprotein
Ad	adenovirus
AKT	protein kinase B
ApoE ^{-/-}	apolipoprotein deficient
bFGF	basic Fibroblast Growth Factor
BH ₄	tetrahydrobiopterin
Bps	Base Pairs
CAC	Circulating angiogenic cell
CAD	Coronary artery disease
CAR	Coxsackievirus and adenovirus receptor
CD**	Cluster of differentiation**
cDNA	complementary DNA
CFU-Hill	Colony forming unit-Hill
CMV	Cytomegalovirus
cGMP	Cyclic guanosine-3',5'-monophosphate
CXCR4	CXC chemokine receptor 4 (Fusin)
DiI	1,1 dioctadecyl-6,6-dil(4-sulfophenyl)-3,3,3indocarbocyanine
DNA	Deoxy-ribonucleic acid
dsDNA	Double stranded DNA
EBM	Endothelial basal medium
ECFC	Endothelial colony forming cell
EGFP	Enhanced green fluorescent protein
EGM	Endothelial growth medium
EDRF	Endothelium derived relaxing factor
eNOS	Endothelial nitric oxide synthase (NOS3)
EPC	Endothelial progenitor cell
Epo	Erythropoietin
FAD	Flavin adenine dinucleotide
FGF-2	Fibroblast growth factor-2
FIV	Feline immunodeficiency virus
Flk-1	Foetal liver kinase

FMN	Flavin mononucleotide
FSS	Fluid shear stress
G-CSF	Granulocyte-colony stimulating factor
GFP	Green fluorescent protein
GM-CSF	Granulocyte/macrophage-colony stimulating factor
Gro- α	Growth regulated oncogene alpha
HDL	High density lipoprotein
HIV-1	Human immunodeficiency virus 1
HPRT	Hypoxanthine phosphoribosyltransferase
ICAM	Intercellular adhesion molecule
IGF	Insulin-like growth factor
IL-3	Interleukin 3
IL-8	Interleukin 8
iNOS	Inducible nitric oxide synthase (NOS1)
ITR	Inverted terminal repeat
Kb	Kilobases
Kg	Kilogram
KDR	Kinase domain receptor
L	Litre
LFA-1	Leukocyte/lymphocyte function antigen-1
MCP-1	Monocyte chemoattractant protein-1
mKitL	Membrane bound kit ligand
ml	Millilitre
mm	Millimetre
mmol	milimole
MMP	Matrix metalloproteinase
MnSOD	Manganese superoxide dismutase
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NLS	Nuclear localisation signal
nM	Nanometers
nNOS	neuronal nitric oxide synthase (NOS2)
NO	Nitric oxide
NO ₂	Nitrite
NO ₃	Nitrate

NOS	Nitric oxide synthase
PlGF	Placenta growth factor
PAD	Peripheral arterial disease
PAR-1	Proteinase activated receptor-1
PBMN	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PFU	Plaque forming unit
PGE ₂	Prostaglandin E ₂
PGI ₂	Prostaglandin I ₂ (prostacyclin)
PMN	Polymorphonuclear
PPAR γ	Peroxisome proliferator-activated receptor gamma
OEC	Outgrowth endothelial cell
ORF	Open reading frame
oxLDL	oxidised low density lipoprotein
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RRE	<i>rev</i> response region
RT	Reverse transcription
SDF-1	Stromal cell derived factor-1
SIN	Self-inactivating
SIV	Simian immunodeficiency virus
sKitL	Soluble kit ligand
SMC	Smooth muscle cell
ssDNA	single stranded deoxy-ribonucleic acid
TGF- β	Transforming growth factor beta
TNF- α	Tumour necrosis factor alpha
UEA-1	Ulex europaeus agglutinin-1
uPA	urokinase plasminogen activator
VCAM	Vascular adhesion molecule
VE-Cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VEGF-R2	Vascular endothelial growth factor receptor-2
VSV-G	Vesicular stomatitis virus glucoproteins
vWF	von Willebrand factor

ABSTRACT

Diabetic ulcer healing is a major problem in health care. Impaired circulation has long been associated with diabetes resulting in an increased risk of foot ulceration and making an increase in blood flow an excellent candidate for a therapeutic intervention. Since being identified in 1997, much interest has surrounded endothelial progenitor cells (EPCs) as a means of facilitating neovascularisation. In this study we aimed to explore the use of EPCs to enhance blood vessel formation in the diabetic ulcer and to examine their effects on the healing of these wounds. Firstly, culture and characterisation of the cells had to be optimised. Subsequently a rabbit ear ulcer model was developed and used to explore the potential therapeutic effects of EPC transplantation in vivo. We isolated two types of cell from peripheral blood. The first type, being consistent with previously described outgrowth endothelial cells, displayed endothelial characteristics and showed good clonogenic potential. These cells correspond to endothelial colony forming cells (ECFC) as described in the review by Hirschi et al (Hirschi et al, 2008). However in spite of the fact that this cell type may represent true endothelial progenitor cells, the relative unreliability of culturing these populations from a given sample of peripheral blood made them unsuitable for subsequent therapeutic in vivo work. ECFCs directly incorporate into newly forming blood vessels. The second type also displayed endothelial characteristics, manifest by uptake of acetylated LDL and binding of isolectin, and was consistent with described early endothelial progenitors. It has recently become evident that these cells are monocytic in nature, do not directly differentiate to endothelial cells and support angiogenesis not by direct incorporation but by paracrine means. These cells have been designated circulating angiogenic cells by Hirschi et al. It was this population of cells, CACs, that was used for the subsequent in vivo experiments. Although ECFCs show greater clonogenic potential and a greater transduction efficiency they were a less

suitable cell choice for the work reported here compared to CACs as ECFCs are not as reliably isolated from relatively small volumes of blood. This likely represents relatively fewer numbers circulating in peripheral blood. Since autologous cells were used in this work, only small volumes of blood could be used from which to isolate cells and so CACs were the cell of choice for this work.

Gene therapy is also an area of research being applied to a wide range of topics. In this study we also looked at the use of genetically modified EPCs and their applications to wound healing. Specifically, the gene encoding endothelial nitric oxide synthase was used as the transgene as the angiogenic and vasodilatory properties of nitric oxide have been well established. We initially explored the use of a variety of vector systems for genetic modification of this cell type. After comparing different viral as well as non-viral vectors, adenovirus was selected as the vector of choice. In addition two different cell subpopulations could be identified based on relative transduction efficiencies.

A model of diabetic wound healing was initially established for the in vivo experiments. We chose the alloxan induced diabetic ear wound to avoid problems with wound contraction which can occur in other models using the dorsum of the animal. After establishing the model, we studied the effect of duration of diabetes on wound healing. Diabetes was induced 1 week or 4 weeks before creation of the wound and healing was measured after 1 week. A significant restriction of healing was noted in the group where diabetes was induced 4 weeks before wound induction with less restriction being observed where wounds were induced 1 week after the induction of diabetes mellitus. Next, transduced as well as non-transduced cells were delivered to the wound site by way of a collagen scaffold. In terms of the treatment of the wounds with transduced or non-transduced EPCs, no differences were seen in the healing parameters measured when examined at 1 week wound induction after either 2 or 5 weeks hyperglycaemia.

Since diabetic ulcer healing has been long identified as an issue of significant clinical concern the aim of this work was to explore strategies for promoting and accelerating healing. Angiogenesis is integral to wound healing and a growing body of work demonstrates the contribution of EPCs in a variety of clinical and pre-clinical settings through promotion of angiogenesis. NO has also been shown to have significant functions regarding angiogenesis and endothelial function. In addition, functional impairment of EPCs has been demonstrated in the diabetic setting and so the aim of this work was to explore whether functionality could be restored to diabetic CACs through gene modification to overexpress eNOS and the effect on diabetic ulcer healing of treatment with these modified cells. A secondary aim was to establish the most efficient vector for transduction of these cells.

1. Background and Introduction

1.1 Vasculogenesis

1.1.1 Angiogenesis and Vasculogenesis

It has long been known that patients with occlusive or ischemic vascular diseases spontaneously develop collateral vessels to bypass the occlusion or to restore perfusion to the ischemic area thus preserving tissue survival and organ function (Heil et al., 2006). Therapeutic approaches that stimulate angiogenesis would be a less invasive and potentially more desirable option in conditions such as myocardial infarction, peripheral artery disease and stroke than current treatments which include angioplasty, surgery, amputation and bypasses. The concept of therapeutic angiogenesis has been explored since the suggestion by Folkman et al in 1971 who proposed that angiogenic growth factors contribute to tumour formation via neovascularisation of the tumour site (Folkman, 1971). These factors may be introduced as a result of recombinant protein therapy or of gene therapy strategies. Endothelial cells are considered the critical mediators of angiogenesis and strategies to promote endothelial cell proliferation and migration represent an important aspect of therapeutic angiogenesis. The cytokine vascular endothelial growth factor (VEGF) is a potent stimulator of endothelial cells and stimulation of endothelial cells with VEGF leads to increased production of the cytokine from the cells themselves resulting in paracrine and autocrine effects which further stimulate endothelial cell proliferation and inhibit apoptosis.

Blood vessel formation may be categorised into distinct types:

Angiogenesis: In the past post-natal blood vessel formation was thought to occur exclusively through angiogenesis, a term used to describe sprouting of new capillaries from pre-existing vessels (Risau, 1997). This results in the formation of capillary networks composed of endothelial tubes which lack the stability of a vessel wall structure provided by smooth muscle cells or adventitia. Angiogenesis can be driven by hypoxia in the surrounding tissues and the resulting increased capillary density allows for increased blood perfusion which restores local oxygen levels and nutrient supply. Reduced oxygen tension in tissues leads to expression of the VEGF family of genes. A key mediator of this effect has been identified as the transcription factor hypoxia-inducible factor-1 (HIF-1) (Semenza, 2002). HIF-1 promotes transcription of VEGF-A leading to proliferation and increased permeability of endothelial cells. VEGF is also upregulated by other growth factors including TGF- β , FGF and PDGF (Pertovaara et al., 1994).

Arteriogenesis: Remodelling of pre-existing collateral anastomoses and growth of functional collateral arteries may be termed arteriogenesis (Heil et al., 2006). This can be triggered by the physical force of altered shear stress due to increased blood flow which can be caused by pressure difference between branches upstream and downstream of an occlusion. Also characteristic of arteriogenesis is the vascular wall remodelling process facilitated by cell proliferation and migration (Scholz et al., 2000, Cai et al., 2004). Arteries can regress when not constantly perfused, enlarge under sustained increased flow and increase in wall thickness under high blood pressures making physical forces due to increased pressure likely candidates to be triggers for arteriogenesis but work by Pipp et al suggests fluid shear stress to be the pivotal early trigger (Pipp et al., 2004). In the hindlimb ischemia model arteriogenesis has been observed in well oxygenated tissues with no increase in HIF-1 or VEGF expression (Deindl et al., 2001, Helisch et al., 2006).

Vasculogenesis: In the embryo blood vessel formation occurs through a mechanism known as vasculogenesis. This involves in situ differentiation of undifferentiated

precursor cells (angioblasts) into endothelial cells, which then begin to form a vascular plexus. Traditionally it had been thought that postnatal vessel formation occurred exclusively through angiogenesis and arteriogenesis but the recent isolation of endothelial progenitor cells – circulating bone marrow derived cells which incorporate into sites of neovascularisation and differentiate into endothelial cells in situ – suggests that vasculogenesis may represent a complementary mechanism of postnatal vessel formation. The discovery of these cells has led to much work concerning their potential for use in therapeutic neovascularisation. Full scale therapeutic endothelial cell transplantation would be difficult if even feasible. It is known that hematopoietic stem cells circulate in the blood in quantities sufficient for transplantation and based on an inference that endothelial progenitor cells also exist due to the common hematopoietic and endothelial precursor – the hemangioblast – CD34/Flk-1 positive cells were isolated from the leukocyte fraction of peripheral blood and found to differentiate into endothelial cells in vitro (Asahara et al., 1997). These cells expressed endothelial markers such as CD31, tie-2 and mRNA for eNOS. They also incorporated acetylated low density lipoprotein (ac-LDL) and bound Ulex-lectin (UEA-1) – both characteristic of endothelial cells – formed tube-like structures in vitro and produced nitric oxide in response to VEGF. The finding that there exist circulating endothelial lineage cells that can home to and incorporate into areas of neovascularisation is consistent with vasculogenesis or the development of de novo blood vessels in postnatal vessel formation. Further evidence that not only proliferation of differentiated endothelial cells from the established vasculature was involved in the growth of blood vessels was provided by findings that hematopoietic stem cells coated the lumen of Dacron grafts in dogs (Shi et al., 1998) and ventricular assist devices in humans (Peichev et al., 2000).

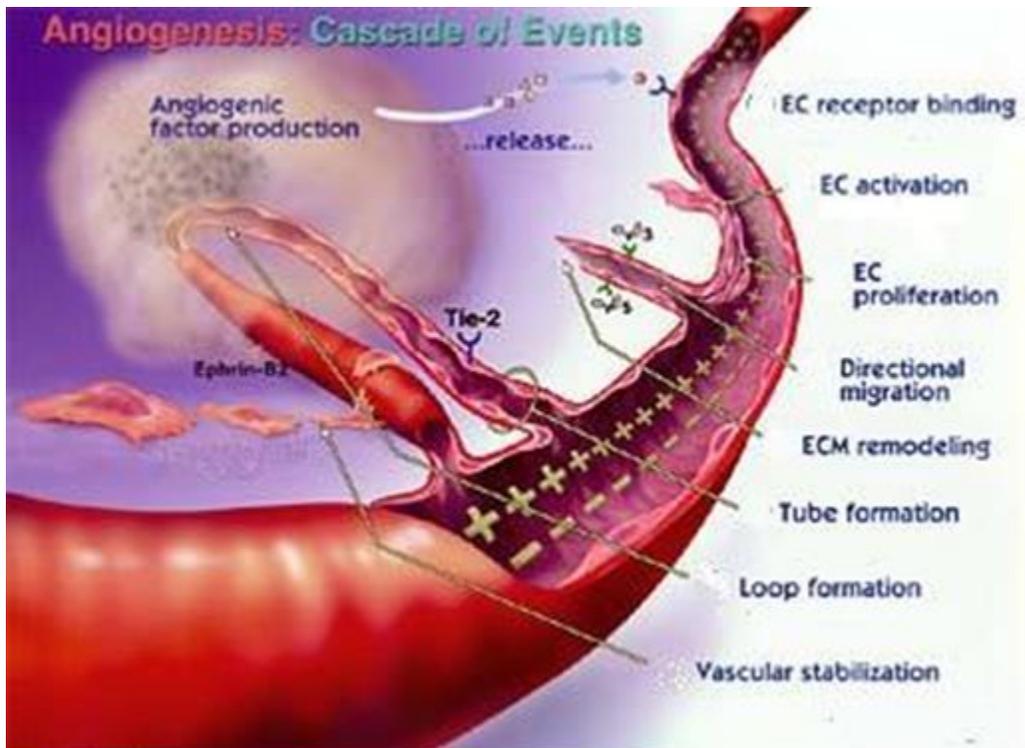


Figure 1.1: The cascade of events leading to formation of blood vessels in the angiogenic paradigm. Taken from <http://people.bath.ac.uk/pr1cemb/Tumour.htm>

1.2 Bone marrow stem cells

Bone marrow stem cells are known to be migratory but until recently factors influencing their movement and homing abilities were poorly understood. Despite this bone marrow cells have been in widespread clinical use, such as in transplantation to cancer and leukaemia patients after pharmaceutical stimulation and collection from normal donors (Bradstock, 2001).

Recent experiments using a murine syngeneic transplant model helped to give a picture of a specific process of migration based on a hierarchy of stem cell maturity within the bone marrow. Labelled marrow stem cells were injected intravenously which then rapidly localised to the marrow. It was found that the most immature cells localised to the endosteal region (closest to the inner surface of the bone cortex, and furthest from the central venous sinusoids). The more mature cells, however, migrated to the central area of the marrow (Nilsson et al., 2001). This implies that cells occupy specific niches within the bone marrow where stromal cells can provide appropriate survival, growth and maturation signals. In haematopoiesis signals passing between stem cells and stroma lead to exposure of stem cells to regulatory cytokines such as G-CSF, GM-CSF, interleukin-3 (IL-3) and stem cell factor (Gupta et al., 1998). G-CSF has also been shown to have a direct effect on numbers of circulating EPCs (Honold et al., 2006, Powell et al., 2005). Various integrins, such as VLA-4 and other adhesion receptors, such as CD44 have been shown to be essential for homing of stem cells to the bone marrow (Papayannopoulou et al., 1995) but these adhesion receptors are not specific which suggests the involvement of a unique adhesion protein or a chemoattractant which would encourage stem cells to selectively lodge in the marrow. One candidate for such a role is the chemokine stromal derived factor-1 (SDF-1) which is secreted by bone marrow stromal cells including endothelial cells, and which binds to a cellular receptor called CXCR4 (Mohle et al., 1998). This receptor is expressed by bone marrow stem cells and homing of human stem cells to murine bone marrow has been shown to be severely disrupted if it is switched off (Peled et al., 1999b).

It appears that a cascade of events involving these cytokines and receptors governs the eventual fate of the stem cell once it reaches the vasculature of the marrow. SDF-1, produced by endothelial cells, binds to CXCR4 on the stem cell surface which activates VLA-4 (Peled et al., 2000). This activated integrin binds to the receptor VCAM-1, which is expressed on the surface of the marrow endothelium, thus arresting the progress of the cell (Peled et al., 1999a). The cell then migrates through the endothelium into the bone-marrow microenvironment, where a gradient of SDF-1 guides its migration towards the endosteal region of the marrow cavity. From here regulatory cytokines influence cell development.

In states of stress stem cells may be recruited from the bone marrow into the blood. Clinically, the release of large numbers of cells into circulation has been achieved by administration of G-CSF (Sheridan et al., 1992). To facilitate detachment of the cells from the bone marrow stroma, neutrophil-derived proteases have been shown to cleave the adhesion of VLA-4 to VCAM-1, thus allowing the cells to be released from their niches in the marrow and into the circulation (Levesque et al., 2000). After the interaction between the stem cells and bone marrow stromal cells has been disrupted the stem cells can pass through the sinusoidal endothelium to enter the blood stream. These proteinases which include elastase, cathepsin G and matrix metalloproteinases (MMPs) and can also cleave the SDF-1/CXCR4 interaction (Levesque et al., 2003). Another important mediator of bone-marrow cell mobilisation is eNOS which appears to affect the levels of mobilisation-induced MMP-9. As a consequence of reduced levels of MMP-9 there is a reduction in the release of soluble Kit ligand (sKitL, another important agent of cell mobilisation) from membrane bound Kit ligand (mKitL) leading to an impaired mobilisation response.

EPCs have been shown to differentiate from bone-marrow derived multipotent adult progenitor cells (Reyes et al., 2002) and a number of studies have identified them as being of bone-marrow origin as discussed below.

1.2.1 Stem and Progenitor Cells

Progenitor cells are bone-marrow derived cells at a relatively primitive stage of development that possess an innate ability to proliferate, migrate and differentiate into various cell types.

Although vessel wall injury is recognised as a stimulus for EPC release, patients at high risk for such events may have reduced numbers of circulating progenitors. However, it is unclear whether this relates to an exhaustion of cells mobilised from the bone marrow to partake in vascular repair, or to impaired differentiation or function of these cells. Oxidative stress, nitric oxide, inflammation and thrombosis and their by-products are also likely to be mediators of the EPC response.

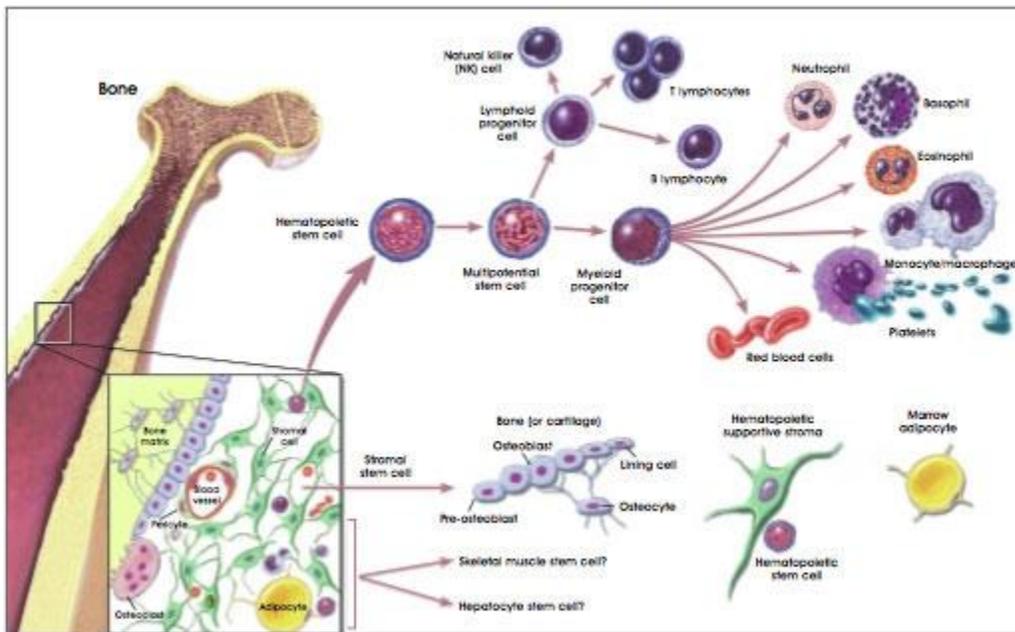


Figure 1.2: Schematic showing possible fates of bone marrow derived stem cells and lineages including progenitor cell stages of various cell types. Taken from <http://stemcells.nih.gov/info/scireport/chapter4.asp>

1.3 Endothelial Progenitor Cells (EPCs)

Since first being described by Asahara et al, much work has been done to define and characterise endothelial progenitor cells and to elucidate their potential therapeutic properties. However, to date there exists no single marker to definitively characterise these cells but a consensus is emerging regarding common properties to the populations isolated in various studies.

Although some differences exist in culture conditions and characterization criteria in studies on cells termed EPCs and even seemingly different cell populations are classified as EPCs, the common theme in these studies is the potential of a diverse range of cells to acquire or exhibit an endothelial-like phenotype in the presence of endothelial growth factors or to incorporate into sites of neovascularisation. The recent article by Yoder has resulted in a more stringent definition of what is termed a true endothelial progenitor cell. That group also describes a distinct population of cells which do not directly incorporate into newly forming blood vessels but support angiogenesis in a paracrine manner. They point out that these cells are CD14 positive and likely to be monocytic in origin. They are found in culture on fibronectin after 4-7 days. They are readily isolated and due to their angiogenic potential may be used to induce therapeutic angiogenesis. These cells have also been termed early EPCs and will be the main subject of the current thesis. They have been entitled circulating angiogenic cells (CACs) by Yoder et al (Yoder., 2007). Some authors would suggest that they are not true EPCs in that they do not have the capacity to differentiate into endothelial cells and do not incorporate into the blood vessel wall. They do however support angiogenesis in a paracrine manner. In addition this cell population is defective in disease states such as diabetes mellitus. In contrast, what may be referred to as true EPCs, termed endothelial colony forming cells (ECFCs) by Yoder, are a rare cell population which are inconsistently isolated by culture of mononuclear cells at about 20 days, directly incorporate into the blood vessel wall following in vivo transplantation and may also be defective in disease states. Yoder has described these cells as being positive for CD 34, KDR, and negative for CD 45. These cells have been termed late outgrowth endothelial cells by others. For the purposes of this

discussion EPC is used as a blanket term for cells with, or with the potential to develop, endothelial characteristics, isolated from peripheral blood unless otherwise specified. Notwithstanding the fact that the term has yet to be specifically defined, due in part to the lack of a specific marker or consensus on specific characteristics of these cells, this approach appears to be consistent with nomenclature over much of the literature (Yoder., 2012) although efforts are ongoing to define these cell populations (Masuda et al., 2011, Fadini et al., 2012).

1.3.1 EPC Origin

EPCs have been isolated from different sources but are commonly isolated from the peripheral blood mononuclear cell fraction although they have been derived from the more differentiated CD34 or CD14 positive population or from immature CD133+ hematopoietic progenitor cells (Peichev et al., 2000, Gulati et al., 2003a, Romagnani et al., 2005, Rookmaaker et al., 2003, Shi et al., 1998, Gehling et al., 2000, Harraz et al., 2001). By way of comparison, similar cell populations have been isolated separately from both peripheral blood and bone marrow (Sharpe et al., 2006).

It is believed that these cells share a common precursor with hematopoietic cells, termed the hemangioblast. Such a cell has been identified in the mouse and a possible human equivalent has also been described (Loges et al., 2004).

To date no single surface marker that can definitively identify a cell as an EPC has been reported.

Although EPCs may be isolated from different sources they all show expression of endothelial markers such as VEGFR-2/KDR, vWF and eNOS (Asahara et al., 1997, Shi et al., 1998) and many studies have attributed improvements in vessel formation and blood perfusion, with subsequent benefits in a range of models, to these cells. Cells grown from the MNC fraction of blood have been shown to develop endothelial characteristics after 1 week of culture with 50% of cells expressing VEGF-R2 and VE-Cadherin and 70% of cells expressing PECAM 1 while 50% and 70% of cells continued

to express monocyte and macrophage markers CD14 and CD45 respectively. Cultured cells also consistently express endothelial markers CD31, tie-2 and von Willebrand factor as well as exhibiting uptake of acetylated low density lipoprotein (acLDL) and binding UEA-1 lectin (Bellik et al., 2005, Burger et al., 2002). Expression of CD34, CD133 or VEGF-R2 are often taken as adequate markers of EPCs.

In addition to cells originating in the bone marrow there have also been reports of tissue resident endothelial progenitors which have been isolated from side populations resident in vessel walls (Sainz et al., 2006) and such cells have been shown to have clonogenic potential (Ingram et al., 2005) as well as exhibiting a hierarchy comparable to that established for cells of a hematopoietic lineage (Ingram et al., 2004).

1.3.2 Mobilization and Recruitment of EPCs

That EPCs originate in the bone marrow has been established by numerous studies (Lin et al., 2000, Gill et al., 2001, Takahashi et al., 1999, Shi et al., 1998). From here they are mobilized and home to sites of neovascularisation. Bone-marrow derived EPCs have been shown to account for up to 26% of endothelial cells after growth factor induced neovascularisation (Murayama et al., 2002). There is even evidence that they may participate in the formation of newly formed lymphatic vessels (Religa et al., 2005).

Mobilisation of EPCs from the bone marrow into circulation is a key event in the functioning of EPCs and can be induced by a number of agents including VEGF, stromal cell-derived factor-1 (SDF-1), angiopoietin-1, granulocyte/macrophage-colony stimulating factor (GM-CSF), granulocyte-colony stimulating factor (G-CSF), erythropoietin (Epo), angiopoietin-II oestrogen and statins as well as exercise (Honold et al., 2006, Gill and Brindle, 2005, George et al., 2005, Yamaguchi et al., 2003, Sandri et al., 2005, Iwakura et al., 2003, Kong et al., 2004a, Seiler et al., 2001) with many of these factors also promoting differentiation, recruitment, adhesion and capacity for neovascularisation (Iwaguro et al., 2002, Asahara et al., 1999a, Asahara et al., 1999b,

Asai et al., 2006a, Massberg et al., 2006, Kalka et al., 2000c, Kalka et al., 2000a). IGF-1 and bFGF also stimulate the differentiation of EPCs (de Boer et al., 2006). In contrast, the PPAR- α agonist rosiglitazone which inhibits vascular smooth muscle proliferation and migration has also been shown to inhibit EPC differentiation and survival which may provide a link between inflammation and cardiovascular disease (Verma et al., 2004). Similar to other bone marrow derived cells, CD34 positive cells have intracellular CXCR4 receptors which can be expressed on the cell surface under cytokine influence suggesting a mechanism by which cytokines may increase EPC proliferation (Peled et al., 2000). The reversal of the marrow/periphery SDF-1 gradient following its release by SDF-1 expressing tissues mobilised EPCs to those tissues. SDF-1 expression in target tissues and CXCR-4 expression on EPCs as well as expression of VEGF and other angiogenic cytokines is thought to be regulated by HIF-1 α . Increased proliferation and enhanced homing in CAD following G-CSF administration has also been linked to upregulated expression of CXCR4 (Powell et al., 2005). Caveolin has been shown to aid in both the mobilization and homing of EPCs (Sbaa et al., 2006) and is expressed by early EPCs suggesting possible paracrine or autocrine effects of these cells (Gulati et al., 2003a). Increases in EPC number and enhancement in terms of adhesion and incorporation into vascular structures both in vivo and in vitro were seen after administration or stimulation of endogenous production of these agents (Takahashi et al., 1999, Iwaguro et al., 2002). Indeed early EPCs secrete many pro-angiogenic cytokines such as VEGF, HGF, G-CSF and GM-CSF which gives further credence to their paracrine signalling role in vasculogenesis as orchestrators of blood vessel formation (Urbich et al., 2003, Rehman et al., 2003). Conversely, administration of agents such as COX inhibitors and myoseverin may result in a reduction in EPC proliferation (Colleselli et al., 2006, Park et al., 2006) and there is a possibility that G-CSF can reduce the migratory response to SDF-1 and the in vivo capacity of cells (Honold et al., 2006). In addition, increased levels of oxidized low density lipoprotein (oxLDL) and C-reactive protein which are risk factors cardiovascular disease are associated with decreased EPC number and function (Ma et al., 2006, Verma et al., 2004).

At the gene level defects in the cell cycle inhibitor genes p130^{Rb2} and p27^{kip1} have been associated with impaired mobilization of endothelial progenitors from the bone marrow (Vidal et al., 2005). Migration from the bone marrow is also influenced by proteinase activated receptor-1 (PAR-1), the main thrombin receptor on vascular cells and platelets. A number of ligand receptor interactions influence the homing of EPCs. Integrin $\alpha_4\beta_1$ (VLA-4) promotes progenitor homing to the VCAM and cellular fibronectin and smooth muscle cells may also play a role in EPC development and adhesion by secreting TGF β -1.

EPCs are also mobilised endogenously in response to disease states such as ischaemia or injury (Takahashi et al., 1999, Sandri et al., 2005) and have been shown to be more effective than mature endothelial cells in revascularisation in adult ischemic tissue (Kalka et al., 2000b, Kocher et al., 2001, Urbich et al., 2003). In the ischaemic setting intercellular adhesion molecule (ICAM-1) is upregulated and contributes to EPC recruitment (Yoon et al., 2006) as well as integrin-linked kinase which is produced in response to hypoxia (Lee et al., 2006). In the hypoxic milieu, expressing high levels of MnSOD is a mechanism by which EPCs are themselves protected from oxidative stress (He et al., 2004a). The degree of ischaemia may also affect the magnitude of the EPC response (Tepper et al., 2005). In addition, high HDL levels have been shown to increase EPC numbers in an eNOS dependent manner (Noor et al., 2006) and haemostatic factors such as those in the initial stages after vascular injury provoke a rise in EPC recruitment. Apoptotic bodies from damaged endothelial cells also appear to have a role in proliferation and differentiation of EPCs (Hristov et al., 2004).

1.3.3 Attraction of circulating cells in collateral vessel formation.

Fluid shear stress (FSS) has been shown to be an activator of endothelial cells. After an initial cell swelling (Ziegelhoeffer et al., 2003, Barakat, 1999) an upregulation of several genes encoding for chemoattractants or activating cytokines for adhesion molecules occurs (Lee et al., 2004), which is fundamental in the attraction of circulating cells. Of particular importance in the attraction of monocytes is monocyte chemoattractant protein

1 (MCP-1). A chemoattractant gradient is built up in endothelial cells by transfer of MCP-1 to the cell surface where it is immobilized by proteoglycans. Stimulated increase of this chemoattractant gradient has been shown to markedly enhance arteriogenesis (Ito et al., 1997, Pipp et al., 2004). Attraction, adhesion and invasion of leukocytes into the vessel is mediated by changes in expression and conformation of adhesion molecules which transform the quiescent endothelium to a surface that circulating cells adhere to. Focal adhesion clusters of attractant molecules are formed by the increased expression of selectins, intercellular adhesion molecules (ICAM 1 and 2) and vascular adhesion molecules (VCAM-1) (Scholz et al., 2000).

Since the mid-1970s when electron microscopic images of heart collaterals in dogs showed massive adhesion of monocytes at sites of activated endothelium (Schaper et al., 1976), much work has gone into elucidating the role of circulating monocytes in arteriogenesis and blood monocyte concentrations have been shown to have an important influence on the dynamics of arteriogenesis (Pipp et al., 2003, Heil et al., 2002).

The binding of circulating monocytes is facilitated by integrin receptors like Mac-1 and LFA-1 which are heterodimers expressed on monocytes and are the counterparts of ICAM 1, 2 and VCAM-1. Expression of these integrin receptors can be upregulated by growth factors such as VEGF, PlGF and TGF- β and by chemokines such as MCP-1 (Pipp et al., 2003, van Royen et al., 2002, Heil et al., 2000).

After adhesion, monocytes migrate into the deeper parts of the vessel wall and their accumulation is characterized by appearance of clusters rather than a uniform distribution. Monocytes, including those that have matured into macrophages, seem to have significant involvement in induction of vascular wall proliferation and remodelling as indicated by the monocytic expression of the proteases matrix-metalloproteinases and uPA (Kusch et al., 2002, Menshikov et al., 2002). Proliferation and migration of smooth muscle cells is initiated by elastin-derived fragments which may be formed by proteolytic cleavage of the elastic lamina (Mochizuki et al., 2002). As the monocytes migrate through the vessel wall proteolytic degradation of the extracellular structures may thus

generate the proliferation signal for smooth muscle cells (SMCs). In addition, release of growth factors such as fibroblast growth factor-2 (FGF-2) by macrophages has been shown to enhance SMC proliferation within the vessel wall (Arras et al., 1998). As early EPCs (CACs) appear to be derived from, or at least have features closely resembling, circulating monocytes, this homing mechanism is of interest to the field of EPC biology.

Although tissue resident progenitors have been suggested as an alternative source of monocytes (Khmelewski et al., 2004), the fast recovery of blood flow deficits seen in some in vivo models suggests that the time taken for recruitment and subsequent proliferation of these cells makes it less likely that these represent a substantial resource from which monocytes are recruited. Skeletal muscle resident cells represent only a rare population compared to the blood and bone marrow pathways acting together.

An additional pathway of formation has been proposed for de novo vessel formation. Here, monocytes/macrophages form tunnel-like structures in the extracellular compounds using the proteolytic activity of their matrix-metallo-proteinases and these tunnels are later colonized by circulating endothelial progenitors (Moldovan, 2002). Furthermore, it has been suggested that pericytes or SMC progenitors are attracted to such endothelial tubules and form the basis of a new smooth muscle layer.

1.3.4 EPC Homing

Ischemia or cytokines like VEGF lead to mobilisation from bone-marrow. EPCs are preferentially recruited to sites of neovascularisation and can improve the process by direct incorporation or through paracrine effects (Takahashi et al., 1999, Kalka et al., 2000b, Aicher et al., 2003a, Urbich and Dimmeler, 2004). Distinct cell populations may play different roles in this process. For instance the circulating angiogenic cell as defined by Yoder has a paracrine effect in enhancing angiogenesis but does not become directly incorporated into the blood vessel wall. In contrast ECFCs directly incorporate into the blood vessel wall and do not have paracrine mediated effects.

Homing of EPCs to sites of ischemia is comparable to that of other bone marrow derived cells and is dependent on interaction of chemokines and their receptors, intracellular and intercellular signalling, and expression and activity of adhesion molecules such as selectins and integrins and of proteases. In the ischemic environment tissue pH decreases and proteolytic enzymes are released from damaged lysosomes. Accumulation of reactive oxygen species (ROS) and oxidized metabolites results from reperfusion at infarct border zones and invading neutrophils and macrophages contribute further oxygen radicals. Thus, any pro-angiogenic cells working to re-establish perfusion in such an environment need to be able to withstand and tolerate oxidative stress. It has been shown by He et al that EPC viability and function are preserved after exposure to ROS-generating TNF- α and LY83583, a superoxide generator (He et al., 2004a). This effect was not seen in mature endothelial cells and they also showed a 3- to 4-fold higher expression of manganese superoxide dismutase (MnSOD) in EPCs compared with mature ECs and that adenoviral mediated over-expression of MnSOD enhances resistance of mature EPCs to oxidative stress.

EPC homing may have some aspects in common with homing of leukocytes in the inflammatory response. In vivo blocking experiments have shown that initial arrest of embryonic EPCs is mediated by E- and P- selectin (Vajkoczy et al., 2003) and human adult and murine EPCs express the leukocyte β 2 integrins which mediate adhesion to mature endothelial monolayers, in vitro transendothelial migration and homing to ischemic tissues in vivo (Chavakis et al., 2005). As with leukocytes, EPCs also require matrix degrading proteases for matrix invasion during homing (Luster et al., 2005, Urbich et al., 2005). The inflammatory chemokines IL-8/Gro- α and their receptors CXCR2 and CXCR1 have been presented as having an important role in EPC recruitment to ischemic tissues but inhibition of the interaction of these chemokines and their receptors only induced a partial reduction in neovascularisation suggesting a possible role for other chemokine factors (Kocher et al., 2006). Indeed, disruption of the SDF-1/CXCR4 interaction, which is upregulated with ischemia (Abbott et al., 2004), impeded migration and adhesion to endothelial monolayers of EPCs in vitro (Ceradini et al., 2004) and homing of EPCs to the ischemic limb in vivo (Walter et al., 2005). Other cytokines

may also have a role in EPC migration and recruitment and MCP-1 and VEGF can induce transendothelial migration of EPCs (Chavakis et al., 2005). Whether homing mechanisms are tissue and/or context specific or whether different subpopulations of EPCs capable of supporting neovascularisation use the same mechanisms is still unclear (Chavakis, 2006).

1.3.5 Early and Late EPCs

The definition of cells isolated from the peripheral blood that are capable of acquiring an endothelial phenotype does not stand as a complete description of all EPC populations. Many different culture methods and identification protocols have been used in a variety of studies which all use the term EPC and although many of these studies may indeed involve the same cell type, the term EPC may be used to broadly describe two distinct types, isolated in a similar manner and displaying many characteristics in common but which nonetheless exhibit marked differences. These different populations may be referred to as early and late EPCs (the latter also being referred to as outgrowth endothelial cells or OECs) (Timmermans et al., 2009) but Yoder has suggested terminology of circulating angiogenic cells (CACs) and endothelial colony forming cells (ECFCs), respectively which is the terminology which will be adhered to hereafter. However, in reviewing the literature there is no consensus as to a single definition of an EPC with variations between studies in both isolation procedure and characterisation of cells, Therefore, when discussing the published literature it appears necessary to adhere to the original author's nomenclature. Broadly speaking, the term EPC is overwhelmingly the most commonly used term throughout the literature and whether the cells under investigation are more likely CACs or ECFCs in a particular study may be suggested by the culture methods of the study. For an overview of the current literature however, it is very difficult in practical terms to avoid use of the blanket term EPC.

CACs and ECFCs

CACs are a more heterogeneous population consisting of relatively non-proliferative cells while the ECFC population displays more stem cell like characteristics in terms of their clonogenic potential with high proliferative rates giving rise to an expandable homogenous population with a characteristic endothelial cobblestone monolayer developing. The clonogenic potential of these cells sets them apart from mature endothelial cells which have a lower proliferative rate and shows that ECFCs are distinct from circulating mature endothelial cells which have merely sloughed off established vessels.

Early cells appear around 4-7 days after isolation with the ECFC colonies developing usually after 2-4 weeks. A comparison by Hur et al showed expression of endothelial markers VE-Cadherin, KDR, Flt-1, eNOS and vWF in both cell types although expression of KDR and VE-Cadherin was increased in OECs (Hur et al., 2004). The leukocyte marker CD45 decreased gradually after isolation and CD31, a marker shared by endothelial cells and monocytes, was found to be expressed by ECFCs but not by CACs. ECFCs were also found to produce more NO, incorporate into an endothelial monolayer and form capillary tubes more readily than CACs but vasculogenic potential in a hindlimb ischemia model was comparable between the two cell types (Hur, 2004). This was attributed to increased production of angiogenic cytokines by the CACs and this mode of action appears to be a major contributor to their angiogenic potential. CACs do incorporate into neovasculature but their contribution to vasculogenesis is likely to have more to do with paracrine effects (Ziegelhoeffer et al., 2004) as they have been shown to secrete various angiogenic cytokines (Hur et al., 2004, Rehman et al., 2003).

Accumulating evidence suggests that CACs are of monocytic origin (Rehman et al., 2003, Schmeisser et al., 2001). A study by Gulati et al cultured both CACs and ECFCs from a blood mononuclear cell starting population and was able to separate the two end populations based on expression of the monocyte marker CD14 (Gulati et al., 2003a). Those cells initially expressing CD14 gave rise to the early population while those not

expressing the marker could be expanded into an ECFC culture. This makes the reported absence of CD31 expression by early cells (Hur et al., 2004), as investigated using western blotting, all the more intriguing since this marker is known to be present on monocytes and indeed was detected on freshly isolated MNCs. However, other studies have detected this marker using flow cytometry (Burger et al., 2002, Kaushal et al., 2001 Asahara et al., 1997).

The non-proliferative nature of CACs calls into question their true stem or progenitor nature and the question of these cells simply being transdifferentiated monocytes has been raised. Transdifferentiation ability of CACs into cardiomyocytes has been demonstrated (Badorff et al., 2003), suggesting stem cell abilities but expression of the stem cell marker CD133 is minimal on these cells (Rehman et al., 2003).

Other possible stages in EPC lineage progression are intimated by the findings that myelomonocytic cells, hematopoietic stem cells and mesenchymal stem cells all have endothelial differentiation capacity. A side population of human stem cells has also been shown to regenerate endothelium in vivo. In addition, cardiac and skeletal muscle stem and progenitor populations have been described which can give rise to endothelial cells and contribute to neovasculature and endothelial progenitors have also been identified in adipose and splenic tissue (Doyle et al., 2006).

1.3.6 EPCs and Disease States.

EPCs are associated with a number of disease states either as predictors of such conditions or being themselves attenuated by such states and EPC dysfunction has been proposed as a contributing factor to the pathogenesis of many conditions. The number and migratory activity of EPCs inversely correlate with risk factors for CAD (Vasa et al., 2001b) and with C reactive protein, a risk factor for cardiovascular disease (Verma et al., 2004). In one study a strong correlation was seen between the number of circulating EPCs and combined Framingham risk factor score (Hill et al., 2003) and this correlation with cardiovascular end-points was observed after 30 days and up to 1 year (Lee et al.,

2005). The study by Hill *et al* suggested that sufficient levels of EPCs may halt the progression of cardiovascular disease. Hypertension has been found to be a strong predictor of impaired EPC number and function while other studies have also made the link between EPCs and cardiovascular disorders and outcomes (Vasa *et al.*, 2001b, Shantsila *et al.*, 2007, Werner *et al.*, 2005). However disagreement in the literature exists regarding hypertension with one study finding no significant difference in EPC numbers between essential hypertensive patients and controls (Delva *et al.*, 2007) while another found increased number of EPCs in hypertensive patients over controls, although hypertensive patients with more advanced vascular and cardiac disease had fewer EPCs than hypertensive patients with less advanced disease (Mandraffino *et al.*, 2014). They postulate that different EPC phenotypes may behave differently in different subtypes of hypertensive patients. Findings may also be complicated by co-morbidities or different concomitant treatments. In hypercholesterolaemic states EPC number and function have been shown to be reduced (Chen *et al.*, 2004, Imanishi *et al.*, 2004, Rossi *et al.*, 2010) and the ratio of CD31⁺/CD42⁻ cells to EPCs was found to be a marker of atherosclerosis (Pirro *et al.*, 2006). Correlations were also found in another study between EPC number and severity of atherosclerosis (Bogoslovsky *et al.*, 2010). High levels of circulating EPCs also correlate with a poor prognosis in non-small cell lung carcinoma with post-treatment EPC levels being lower in responders to treatment (Dome *et al.*, 2006) with mobilization of EPCs observed in association with pancreatic cancer growth (Li *et al.*, 2011).

Many disease states are associated with a reduction of EPC numbers or functional impairment. Peripheral arterial disease (PAD) may be associated with decreased expression of EPC specific molecules in bone marrow and blood, and transplantation of BM cells can go some way to correcting this deficiency (Yamamoto *et al.*, 2004). Cardiac transplant arteriopathy, pre-eclampsia, chronic renal failure and coronary artery disease are all associated with reduced EPC numbers and in some cases with impaired function (Simper *et al.*, 2003, Sugawara *et al.*, 2005, Choi *et al.*, 2004, Hristov *et al.*, 2006, Lorenzen *et al.*, 2010). Decreased levels of EPCs were also found in patients with heart failure (Chiung, 2012). Smoking had also been shown to affect EPC numbers but results

are somewhat contradictory. This may be in part due to EPC dysfunction with established cardiovascular disease compared to mobilisation of EPCs in other states. Lack of consensus on the cell type being investigated may also be a factor in the discrepancy with potentially different phenotype of cells used in different studies (Yue et al., 2010, Werner et al., 2005, Mobarrez et al., 2014, Kondo et al., 2004, Michaud et al., 2006, Mandraffino et al., 2010, Lamirault et al., 2013).

Other disease states may lead to a sudden rise in the number of EPCs released into the circulation. An acute ischemic event such as myocardial infarction leads to an elevated EPC count (Shintani et al., 2001) and a rapid but transient increase has been observed after burns or coronary artery bypass grafting (Gill et al., 2001). Raised EPC counts have also been observed associated with arthritis raising the possibility of EPC involvement in the pathogenesis of the disease (Kurosaka et al., 2005). Chronic idiopathic myelofibrosis is another disorder in which a raised EPC count has been observed (Leibundgut et al., 2006). In these conditions whether EPC numbers are involved in pathogenesis or are a compensatory response to pathogenesis is difficult to determine.

Changes in EPC number and function have also been observed in other settings which are not disease states such as in an elderly population (Jie et al., 2009, Xia et al., 2012). In an elderly population higher mortality was associated with lower baseline EPCs while higher levels of EPCs were associated with longevity (Mandraffino et al., 2012).

1.3.7 EPCs and Diabetes Mellitus

Some degree of EPC dysfunction has been demonstrated in a number of disease states and other settings such as from aged donors (or increased consumption of EPCs in aged atherosclerotic milieu) (Heiss et al., 2005, Shaffer et al., 2006, Zhu et al., 2006) but possibly the most studied of these settings is that of diabetes. Lower cell numbers have consistently been demonstrated in subjects with both type 1 and type 2 diabetes mellitus with one study finding fewer differentiated endothelial cells being cultured from type 1 than type 2 patients but both being lower than those isolated from non-diabetic controls

(Schatteman et al., 2000). EPCs isolated from diabetic patients have been shown to have decreased survival and proliferation rates and lower numbers have been isolated compared to non-diabetic controls (Awad et al., 2005, Tepper et al., 2002, Fadini et al., 2005). Functionally they have also been found to have compromised ability to differentiate or to adhere and incorporate into an endothelial monolayer (Tamarat et al., 2004, Fadini et al., 2006, Tepper et al., 2002). In addition, conditioned media from type 1 diabetic patients showed a reduced ability to promote tube formation by mature endothelial cells (Loomans et al., 2004). In vivo an augmented recovery was seen after transplantation of diabetic EPCs in response to hindlimb ischaemia (Awad et al., 2005, Tamarat et al., 2004) as well as reduced neovascularisation of healing wounds (Awad et al., 2005), although one study surprisingly found enhanced healing despite reduced neovascularisation (Stepanovic et al., 2003). Improvement of glycaemic control, however, has been shown to improve EPC numbers (Kusuyama et al., 2006). Reduction in EPCs has been found to worsen over time in diabetic patients and it has been postulated that these low levels may be due to an altered process of maturation and/or commitment rather than a failure of production and/or mobilization (Fadini, 2014). In addition, while EPC number was reduced in diabetic patients compared to controls, EPC number was found to be reduced further with the presence of diabetic retinopathy (Asnaghi et al., 2006) and treatment with insulin sensitizing drugs was found to restore EPC number in type 2 diabetes mellitus independent of glycaemic control (Liao et al., 2010, Pistrosch et al., 2005). All studies that we are aware of have focused on early EPCs (CACs) and there have been no publications to our knowledge on late outgrowth (ECFC) cells from patients with diabetes mellitus.

1.3.8 Therapeutic Effects of EPCs

Transplantation of EPCs has been examined in a wide variety of disease states using a variety of animal models to explore their potential for healing parameters such as angiogenesis, re-endothelialisation and reduction of neointimal formation. Although certain beneficial effects have been seen following the administration of total MNC populations, studies have raised concerns about the use of undifferentiated cell fractions.

Transplantation of a potentially mixed population of cell types has the potential to increase the risk of undesirable side effects. In a rat model of myocardial infarction comparing effects of G-CSF mobilised CD34⁺ cells and unfractionated MNCs increased haemorrhagic infarctions were seen in the MNC administered group with abundant infiltration of CD45⁺ inflammatory cells after 3 days. In addition, capillary density was greater, fibrotic area was reduced and functional parameters were better in the CD34⁺ administered group (Kawamoto et al., 2006). In another study myocardial calcification was observed in a rat myocardial infarction model after total MNC administration (Yoon et al., 2004). These studies highlight some of the benefits of using fractionated or differentiated cell populations.

EPCs have been found to have therapeutic benefit in many settings including; regeneration in vein graft atherosclerosis (Xu et al., 2003); neovascularisation in the mouse cornea; and after induction of hindlimb ischaemia with concomitant restoration of blood flow (Takahashi et al., 1999, Urbich et al., 2003, Asahara et al., 1999a, Awad et al., 2006, Kalka et al., 2000b). The improvement in the hindlimb ischaemia model has been observed with both early and late outgrowth cells, the effect of transplantation of both populations being better than that of either population alone (Yamaguchi et al., 2003, Yoon et al., 2005) and clinical trials have been undertaken with encouraging results (Burt et al., 2010).

The therapeutic effects of EPCs in cardiovascular disease have also been explored (Hung et al., 2009). Incorporation of EPCs has been noted at foci of neovascularisation at the border of myocardial infarction and conferring improvement in cardiac function with reduced cardiomyocyte apoptosis and cardiac remodelling (Asahara et al., 1999a, Hamada et al., 2006, Kocher et al., 2001). Transplantation of EPCs has also been shown to improve recovery after myocardial ischaemia (Kawamoto et al., 2003, Kawamoto et al., 2001). Increased survival areas have also been observed around ischaemic flaps with augmented neovascularisation after EPC transplantation, with the effect being enhanced further when the cells over-expressed VEGF (Yi et al., 2006). Furthermore, in patients with acute myocardial infarction treated with coronary stenting who underwent intracoronary infusion of EPCs, persistent improvement in left ventricular ejection

fraction and reduction in infarct size after 5 years was reported (Leistner et al., 2011). Beneficial neovascularisation effects have also been observed in a mouse model of focal cerebral ischaemia (Zhang et al., 2002) as well as in engraftment and repair of damaged lung tissue (Zhao et al., 2005) and alleviating progression of pulmonary hypertension (Takahashi et al., 2004) although their involvement in compensatory lung growth has been questioned (Voswinckel et al., 2003). EPCs have also been shown to improve endothelium-dependent vasorelaxation in apoE^{-/-} mice on a high cholesterol diet with impaired vasorelaxation (Wassmann et al., 2006).

There is accumulating evidence of EPC mediated improvements in re-endothelialisation and reduction in neointimal thickening after arterial injury as well as moderating lesion development and improving vasoreactivity (Werner et al., 2003, Werner et al., 2002, Fujiyama et al., 2003, Griese et al., 2003, Gulati et al., 2004, Gulati et al., 2003b, He et al., 2004b, Kong et al., 2004b, Kong et al., 2004a).

EPCs have also been used to colonise tissue engineered patches and bioprosthetic and Dacron grafts (Schmidt et al., 2005, Shi et al., 1998, Griese et al., 2003) with grafts seeded with EPCs staying patent for longer (Kaushal et al., 2001).

The effects of statins on EPCs have also been a topic of study. In the CAD setting it has been found that use of statins augmented EPC numbers and function as well as mobilization and differentiation with a concomitant increase in re-endothelialisation after arterial balloon-injury through stimulation of the Akt signalling pathway (Vasa et al., 2001a, Walter et al., 2002, Dimmeler et al., 2001). However other studies have cautioned that while statins may enhance EPC number and function after 1 month, numbers may decrease after 3 months and while low doses may be beneficial, at higher doses, despite an increased stability of eNOS mRNA, endothelial apoptosis was increased (Hristov et al., 2006, Urbich et al., 2002).

1.3.9 EPCs in Clinical Trials

Therapeutic use of EPCs has also been investigated in clinical trials. Infusion of unfractionated bone marrow progenitor cells were shown to improve left ventricular function and physical capacity in patients with diffuse coronary artery disease and angina pectoris (Tse et al., 2003, Hamano et al., 2001, Perin et al., 2003) and also has shown positive effects on left ventricular function after myocardial infarction (Strauer et al., 2002, Assmus et al., 2002, Fernandez-Aviles et al., 2004) with improvement sustained at 1 and 5 year follow-up (Schachinger et al., 2004, Leistner et al., 2011). Indeed, improvement in left ventricular function has been shown after myocardial infarction after administration of G-CSF due to mobilization of bone-marrow derived progenitor cells (Ohtsuka et al., 2004).

In chronic limb ischaemia direct administration of EPCs improved local neovascularisation with reduced limb amputation at 3 years and an increase in local endothelial markers was observed in this setting after intramuscular injection of autologous bone-marrow derived MNCs containing 1% CD34+ cells (Yamamoto et al., 2004, Lenk et al., 2005). Improvement in endothelial function and wall motion abnormalities has also been observed after administration of autologous EPCs in symptomatic coronary atherosclerosis (Erbs et al., 2005).

Trials are currently registered to investigate the therapeutic effects of EPCs in advanced liver cirrhosis, autologous EPC transplantation for chronic ischaemic stroke, prognostic value of EPCs in aneurysmal subarachnoid haemorrhage, effects in aortic aneurysm. Trials have also been registered to evaluate EPCs in idiopathic pulmonary arterial hypertension, to quantify EPCs as markers of tumour angiogenesis in breast cancer and to investigate mobilization of EPCs by atorvastatin in patients with stable coronary artery disease

(clinicaltrials.gov/ct2/results?term=Endothelial+progenitor+cells&Search=Search).

1.4 Wound Healing

1.4.1 Skin

Structure of skin

Epidermis: The epidermis – the outermost layer of the skin - is principally made up of keratinocytes forming a continuously self-replicating stratified squamous epithelium. The different strata are can be thought of as compartments through which the cells pass as they change and differentiate from the metabolically active lower layers to the more superficial strata that contain cells achieving terminal keratinisation constituting the cornified zone. The keratinocyte population undergoes continuous renewal and keratinisation is the end result of cells moving from the mitotic basal layer and transforming from polygonal living cells to non-viable flattened squames which replace cells shed at the surface.

Also found in the epidermis are melanocytes or pigment-forming cells, the immunocompetent antigen-presenting Langerhans cells and lymphocytes in addition to sensory nerve endings. The functioning together of different elements of the epidermis (the epidermal symbionts) is of importance in the cascading pathways of development, growth, inflammation, immunology and wound healing.

Dermis: Below the epidermis is the dermis which is vital for the survival of the epidermal layer with morphogenic signals being exchanged between the two. The dermal connective tissue has a matrix composed of an interwoven collagenous and elastic network in an amorphous ground substance which accommodates nerves, blood vessels, lymphatics, epidermal appendages and a changing population of cells. Its collagen fibres provide tensile strength and elastic recoil is provided by its elastic fibres. Collagen types I and III predominate in proportions of 80-85% and 15-20% respectively. Interwoven between collagen bundles can be found the fibrous network of elastic fibres. The ground substance consists mostly of proteoglycans and fibronectins which are involved with

maintaining the cellular environment of the dermis. It is also concerned with cell movement and attachment to substratum during development and wound healing. Also found in the cell population of the dermal layer are fibroblasts, macrophages, mast cells, eosinophils, neutrophils (heterophils seeming to be some way between these cell types can be identified in rabbit skin), T and B-lymphocytes, dermal Langerhans cells and monocytes.

Vascularisation of the skin

Due to the thermoregulatory function of the cutaneous circulation, blood flow in the skin may exceed nutritional requirements by 10 times and may amount to 5% of cardiac output. The blood carrying capacity can be rapidly altered by as much as 20 times in either direction in response to the need for heat loss or conservation. Some arterioles penetrate deeply to supply the adipose tissue, sweat glands and hair follicles while others pass superficially, giving off anastomotic collaterals to glands and hair follicles within the middle of the dermis. Arteriovenous anastomoses in the deeper layers of the dermis are surrounded by thick muscular coats and normal vascular tone is balanced between vasoconstrictor and vasodilator and chemical influences. The deeper dermal arterioles have elastic tissue in the wall and are surrounded by several layers of smooth muscle cells. Two layers of contractile pericytes are found around the postcapillary venules and these can produce gaps between the endothelial cells to allow extravasation of fluid. The vessel wall is strengthened and stabilised by tight junctions between smooth muscle cells, pericytes and endothelial cells. Endothelial cells in the dermal microvasculature are particularly rich in microfilaments, which serve cytoskeletal and possibly contractile functions. In addition to acting as a semi-permeable barrier lining the vessels, the endothelium is involved in many other processes such as inflammatory, immunological and reparative responses. It has contractile and migratory abilities and expresses a large number of antigens and these properties allow it to interact with mast cells and nerves and are important in lymphocyte adhesion and migration and in recruitment of inflammatory cells into the skin.

Fluid and macromolecules that have leaked in to the skin are collected in lymph vessels through gaps in the wall and are returned to circulation. These vessels also allow transport of lymphocytes, Langerhans cells and macrophages involved in immunological reactions.

1.4.2 Dermal Repair and Wound Healing

Dermal wound healing is perhaps the best studied type of healing due to its frequency and accessibility for experimentation. Although most healing proceeds in an uncomplicated fashion there are a large number of chronic wounds which may be broadly divided into venous ulcers, pressure sores and diabetic foot ulcers. These wounds represent a significant health problem.

In order to understand these ulcer states it is desirable to first examine the normal wound healing process.

After injury the repair response may be broadly divided into 3 overlapping phases: inflammation, proliferation and remodelling.

Inflammation

The early stages of acute inflammation begin with activation of platelets and mast cells and the removal of damaged tissue and the initiation of formation of granulation tissue. The initial response to disruption of blood vessels by injury is bleeding which allows for clot formation to stop haemorrhage. This is an essential part of the healing process. Exposed collagen and basement membrane proteins initiate the classic clotting cascade via clotting factors in the blood leading to formation of fibrin which binds platelets in its meshwork. Growth factors produced by the platelets such as platelet derived growth factor (PDGR) and transforming growth factor- β (TGF- β) lead to proliferation and

chemotaxis of inflammatory cells, facilitating infiltration of these cells to the wound site. After transient vasoconstriction to reduce initial blood loss vasodilation occurs, allowing increased blood flow to supply necessary cells and substrate. Increased vascular permeability aids migration from the vasculature and both these processes are mediated by histamine, prostaglandin E₂ (PGE₂), vascular endothelial growth factor (VEGF) and prostaglandin I₂ (prostacyclin; PGI₂).

Production of anaphylatoxins attracts neutrophils and monocytes to the wound site. Debris from the initial injury such as devitalized tissue, clot, bacteria extravasated serum proteins and foreign material attracts polymorphonuclear (PMN) leukocytes (primarily neutrophils) immediately and these cells increase to large numbers within 24 hours and are followed by macrophages in the next 2-3 days. The monocyte population mature into macrophages which remain for the entire inflammatory period and also act to release over 30 biologically active materials including growth factors which induce fibroblast proliferation, angiogenesis and extracellular matrix production which are essential for the initiation of granulation tissue formation during the proliferative stage. Cytokines released by macrophages also attract additional macrophages to the wound site giving an amplified healing effect.

Although both PMNs and macrophages are phagocytic, once the bacterial contamination is under control, neutrophil infiltration ceases. These multiple interacting pathways of inflammation continue as long as the inflammatory stimuli persist. Delay in dissipation of these stimuli leads to delayed wound healing but it has also been shown that an excess of PMN depressed the wound healing response, probably due to an excess of reactive oxygen species (ROS) and proteases.

Proliferation

During the proliferative stage granulation tissue is formed at the wound site. This nutritive substrate allows for migration of the regenerating epidermis and gradually gives way to scar tissue. It is mainly composed of macrophages, pluripotent pericytes, fibroblasts and endothelial cell-lined capillaries all contained in a matrix of fibronectin, collagen and proteoglycans. During this stage a mutually dependant unit made up of macrophages, fibroblasts and capillaries, known as a wound module, migrates into the wound bed. The activated macrophages release chemoattractant agents that attract pericytes, fibroblasts and endothelial cells to the site. They also release growth factors that stimulate these cells to proliferate, and lactate which initiates synthesis of collagen from fibroblasts (which appear by day 3), thus strengthening the developing tissue. Maturing fibroblasts secrete a matrix through which infiltrating cells can migrate and which provides mechanical support for forming capillaries. These capillaries provide nutrients and oxygen allowing further development of the granulation tissue. In addition endothelial cells have been found to release collagenase which degrades the collagen of the basement membrane allowing the cells to migrate into the perivascular spaces where they form buds that are added to by proliferation of cells within or near the parent vessel forming a capillary plexus. This plexus gives the wound its pink or purple-red appearance. Although not fully identified, proposed factors to mediate this response include a macrophage-derived growth factor known to stimulate proliferation of endothelial cells in vitro, low oxygen tension, lactic acid, biogenic amines and hepatocyte growth factor. Chemoattractant factors such as platelet-derived substances, heparin and fibronectin are also important due to their role in cell migration and endothelial cells can themselves synthesize fibronectin, thus partially providing a matrix in which to move themselves. Fibroblasts also may develop into myofibroblasts which are involved in the process of wound contraction. Forming complexes through intercellular contacts and links with their substrates the collective forces of contraction of the myofibroblasts serve to effect contraction of the wound margin.

Remodelling

In the last phase of the healing process the granulation tissue gradually gives way to scar tissue. This may occur over months or years as the fibronectin is removed and there follows an accumulation of type I collagen bundles. Actual accumulation of collagen reaches a maximum 2-3 weeks after wounding but supranormal rates of synthesis and degradation continue throughout the remodelling phase. These cross-link to improve the tensile strength of the wound site. Initially the bundles are laid down in an irregular pattern but over time (possibly due to mechanical forces) they take on a more ordered orientation which gives more strength and extensibility to the tissue. The orientation of the fibres is dictated by the stresses placed on the wound. Evidence suggests that certain growth factors stimulate scarring while others reduce it. Therefore, manipulation of the growth factor profile may lead to improvements in the healing response. Scars increase in strength for six months or longer and may eventually reach 70% of the strength of unwounded skin.

Epithelial regeneration

Within a few hours of injury re-epithelialisation begins with keratinocytes at the edge of the wound migrating across the wound site. A change in gene expression allows for this and the cells are thought to move by elongation of the superficial cells of the epidermis which then move over adjacent cells until they reach the wound bed where they stop and divide. This process is repeated by other cells until it is stopped by contact inhibition. Cells from the basal layer, migrating across the wound can complete coverage in 18-24 hours in a coapted surgical wound. Any crust or eschar over an open wound can delay re-epithelialization and the advancing epithelium must burrow underneath, a process facilitated by collagenase and other metalloproteinases. Keeping the wound area moist prevents crusts and eschars from forming and thus aids in healing.

After the first layer of cells has migrated across the wound surface, the basilar-apical order is restored by additional layers developing and keratin formation is resumed as the cells mature. This regenerates the stratum corneum and provides stable coverage.

Cut hair follicles may act as a source of epidermal stem cells which can form a stem cell keratin that allows the cells to remain sufficiently flexible to migrate over the wound bed. After maturation the epidermis produces more rigid keratins. Keratinocytes also produce fibronectin providing a substrate over which to migrate. TGF- β has also been shown to encourage epithelial cell migration and fibronectin deposition. The keratinocytes secrete factors which clear the way for their migration and once re-epithelialisation is complete they revert to their original phenotype.

Restoration of the epidermis induces apoptosis of inflammatory cells and maturation of mesenchymal cells. Delayed epithelialisation in younger patients with prolonged inflammation can lead to hypertrophic excess scarring. Reconstitution of the stratum corneum restores the normal barrier to evaporation allowing homeostasis to be achieved.

Wound Contraction

All healing wounds generate a strong contractile force. The driving force for this contraction comes from fibroblasts. These cells, like muscle cells, contain actin myofilaments and when these filaments increase in number the cells take on the appearance of myofibroblasts. Myofibroblasts are seen in increasing numbers in contracting wounds but their mechanism of action is unclear.

Wound contraction allows surrounding skin to be pulled over the wound, quickly reducing its size and resurfacing the wound with normal skin. Skin that is not strongly attached to the underlying muscle fascia is capable of a great degree of contraction and wounds in some animal models may heal almost entirely by contraction. Human skin

however tends not to be as loose as animal skin allowing for less contraction in most sites.

When wounds contract over a joint it may result in a scar contracture which limits the mobility of the joint.

Recent research using transgenic mouse studies have suggested that under certain experimental conditions up to 30% of local wound healing cells may be bone marrow derived prompting great interest in EPCs and their potential role in the healing process (Mustoe. Dermal ulcer healing: Advances in understanding. Institute Pasteur. Tissue repair and ulcer/wound healing: molecular mechanisms, therapeutic targets and future directions. Paris, March 17-18 2005).

1.4.3 Chronic Ulcer Healing

A chronic wound may be described as one that has failed to heal after 3 months. Delayed healing may be due to lack of attention to basic care such as cleansing, debridement, oedema control, avoidance and treatment of ischaemia and maintenance of a moist healing environment. There are however, differences between chronic and acute wound environments. Increased levels of proteases and collagenases and degradation of growth factors can be seen in fluid from chronic wounds and this excessive protease activity may be from overexpression of MMPs (Harding et al., 2002). In particular reduced PDGF, bFGF, epidermal growth factor and TGF- β have been seen in chronic ulcers (Higley et al., 1995). There is suggestion that such factors may be trapped in the extracellular matrix or simply degraded (Bucalo et al., 1993, Barrick et al., 1999). Chronic wounds generally occur more frequently in aged patients who may have an impaired response to the stress factors of local tissue hypoxia and increased levels of ROS generated by the inflammatory response. There may also be cell senescence and impaired responsiveness of cells such as fibroblasts to growth hormone (Hasan et al., 1997, Stanley et al., 1997).

In pressure sores and diabetic foot ulcers, repeated cycles of transient ischaemia and reperfusion are more damaging than an ischaemic insult itself due to reperfusion injury.

There is some evidence that prolonged stimulus can lead to senescence of cells at the wound periphery.

Epithelialization only occurs from the edges of the wound at a maximal rate of 1-2 mm/day. Adequate coverage of sizable wounds is difficult to achieve and lower leg ulcers rarely heal faster than 1cm/month. In practice, adequate coverage of sizeable wounds is rarely achieved.

Contraction in the skin of the lower leg is relatively low. For a wound on the perineum, contraction may account for 90% of healing but only for a maximum of 30-40% on the lower leg which is one reason that lower limb ulcers are so slow to heal.

According to the National Institute of Diabetes and Digestive and Kidney diseases diabetic lesions are responsible for more hospitalizations than any other diabetic complication and account for up to 25% of all diabetic admissions in the United States and Britain (Pecoraro et al., 1990, Reiber et al., 1992). As well as resulting in lengthy hospital stays and periods of disability the diabetic foot and its sequelae account for billions of dollars in direct medical expenditure in the United States (Frykberg et al., 2000). In diabetic patients 15% develop a foot ulcer and 12-24% of these require amputation. Diabetic peripheral neuropathy confers the greatest risk of foot ulceration and is present in 80% of patients with diabetic foot ulcers. Most complications resulting in amputation begin with the formation of ulcers and detection and adequate care of these ulcers may prevent up to 85% of these amputations (Armstrong and Lavery, 1998). Arterial insufficiency is a major factor in the failure of ulcers to heal. In addition to diabetic neuropathy, which can contribute to ulcer formation due partly to lack of protective sensation, diabetic patients are at four times greater risk of peripheral arterial occlusive disease than non-diabetics (Kannel and McGee, 1979). While risk factors for cardiovascular disease contribute to increased prevalence of vascular occlusive disease, in diabetic patients there is an inverse correlation between cardiovascular risk factors and EPC number and function (Vasa et al., 2001b, Hill et al., 2003). Moreover EPC dysfunction is associated with diabetes mellitus suggesting an additional cause of

inadequate perfusion in the diabetic limb (Awad et al., 2005, Tepper et al., 2002, Fadini et al., 2005, Tamarat et al., 2004).

Diabetic Ulcer Healing

The cellular response to injury and the process of wound healing involves a complex interaction between many cell types including keratinocytes, fibroblasts, endothelial cells, macrophages and platelets. In addition, many growth factors and cytokines are needed to co-ordinate the healing process. In the diabetic setting over 100 known physiologic factors contribute to wound healing deficiencies including decreased or impaired growth factor production (Galkowska et al., 2006, Goren et al., 2006, Falanga, 2005) as well as angiogenic response (Falanga, 2005, Galiano et al., 2004), macrophage function (Maruyama et al., 2007), collagen accumulation, epidermal barrier function keratinocyte and fibroblast migration and proliferation quantity of granulation tissue, number of epidermal nerves (Falanga, 2005, Gibran et al., 2002), bone healing and balance between the accumulation of ECM components and their remodelling by MMPs (Lobmann et al., 2002). It has been shown in a murine model of diabetes that eNOS phosphorylation in the bone-marrow is impaired, limiting EPC mobilization and that SDF-1 α expression is decreased in epithelial cells and myofibroblasts preventing EPC homing to wounds (Gallagher et al., 2007). Thus it can be seen that the delayed healing of diabetic ulcer is a complex process involving many factors.

1.4.4 EPCs and Wound Healing

The contribution of EPCs to the healing of wounds has been documented by a variety of studies. An acceleration of diabetic wound healing has been observed in genetically diabetic mice after treatment with VEGF with the effect being due to recruitment of cells from the bone marrow (Galiano et al., 2004). Enhanced healing in response to increased recruitment of EPCs may be mediated in part by dibutyryl cAMP (Asai et al., 2006a).

Impaired EPC response from the bone marrow has been linked to the delayed healing of ischaemic wounds (Bauer et al., 2006) and wound revascularisation is compromised and even inhibited by injection of dysfunctional diabetic EPCs (Awad et al., 2005). EPC recruitment to the wound site with a corresponding increase in vascularisation and granulation tissue formation was demonstrated after EPC transplantation (Suh et al., 2005, Asahara et al., 1999a) but whether this increase is due to direct cell incorporation or secretion of angiogenic cytokines is unclear from these studies. In diabetic mice, accelerated wound healing has been shown to be mediated by expression of manganese superoxide dismutase (Marrotte et al., 2010).

1.5 Nitric Oxide

1.5.1 Nitric Oxide Synthase

Nitric oxide is a free radical, produced by the enzyme nitric oxide synthase (NOS). This enzyme is a cytochrome p450-like reductase that catalyses electron transport from NADPH to a heme group. Three isoforms have been identified, two of which are constitutively expressed. These are nNOS (or NOS1) and eNOS (or NOS3) the third type being iNOS (NOS2) which is synthesised in the vascular wall during inflammation and produces significantly higher levels of NO than either nNOS or eNOS.

Relaxation of the blood vessel is essential for healthy blood flow and maintenance of vascular homeostasis and in 1980 Furchgott and Zawadzki used a rabbit aortic model to show that vascular endothelial cells were necessary to induce acetylcholine mediated relaxation, work that ultimately led to a Nobel Prize in Medicine for Furchgott in 1998 (Furchgott and Zawadzki, 1980). The mediator of this endothelium dependent relaxation was initially named endothelium derived relaxing factor (EDRF), and subsequently identified as nitric oxide. Nitric oxide is a short lived free radical that is highly diffusible in the body and acts as an intercellular signalling molecule with a wide range of biological effects. It is ubiquitous in the body and its roles include inhibition of vascular smooth muscle cell migration and proliferation (a common factor in vascular pathology), attenuation of platelet activation and adhesion and reduction of vascular inflammation (Schmidt and Walter, 1994). With increased cardiovascular risk factors like hypertension, hypercholesterolemia, smoking and diabetes, endothelium-dependant relaxation is impaired and reduced NO bioavailability is implicated (Dzau, 1990). Indeed, the importance of NO in endothelium-dependent relaxation has been illustrated by the finding that such relaxation could be enhanced by eNOS gene transfer to endothelial cells (Kullo et al., 1997).

Nitric oxide is synthesised from L-arginine, with L-citrulline being produced as a by-product, the reaction being catalysed by the NOS enzymes. Concerning the three isoforms of the NOS enzyme, the constitutively expressed forms of endothelial (eNOS)

and neuronal (nNOS) have molecular weights of 135 kDa and 150-160 kDa respectively, and the inducible form (iNOS) is a molecule of about 130 kDa. The constitutive forms are regulated by intracellular calcium fluxes or exogenous calmodulin and are permanently active, generating low concentrations of NO (in the nmol/litre range). iNOS is induced by a variety of cytokines, growth factors and inflammatory stimuli acting on target cells and produces much higher levels of NO ($\mu\text{mol/L}$ range). All three isoforms are an active form of homodimers consisting of two domains: a C-terminal reductase domain and an N-terminal oxygenase domain. The reaction catalysed by the NOS enzyme occurs at the site of the terminal guanidino nitrogen atom of arginine. Here, the guanidino nitrogen accepts five electrons in an oxidation process requiring molecular oxygen, resulting in formation of NO and citrulline. Another essential substrate is nicotinamide adenine dinucleotide phosphate (NADPH) and it is this that donates the electrons necessary for oxidation of arginine. This is facilitated by calcium-loaded calmodulin binding between the reductase and oxygenase domains whereupon the electrons donated by NADPH are passed through the calmodulin-binding domain toward the heme-containing oxygenase domain making calmodulin and heme essential co-factors for formation of NO (Abu-Soud and Stuehr, 1993). NO is labile with a short half-life of less than 4 seconds in biological situations. It is rapidly oxidised to nitrite and then to nitrate by oxygenated haemoglobin and is then excreted into the urine.

In addition to NADPH and calmodulin, the co-factors flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD) and tetrahydrobiopterin (BH_4) must be present for NO formation. NADPH, FMN and FAD bind to the NOS reductase domain, while the binding sites for BH_4 and L-arginine are found on the oxygenase domain.

Many biological effects of NO such as control of vascular tone and platelet function are mediated by the second messenger cyclic guanosine-3',5-monophosphate (cGMP). Intracellular cGMP is increased after NO diffuses across the endothelial cell membrane and enters smooth muscle cells resulting in activation of guanylate cyclase.

The importance of NO is further demonstrated by findings of reduced levels in states of atherosclerosis, restenosis, transplant vasculopathy and bypass graft disease and eNOS expression has been shown to be elevated early in the development of experimental atherosclerosis (Minor et al., 1990). Associated with impaired NO activity can be observed dysregulation of cell growth, cell death, cell migration, inflammation and extracellular matrix leading to pathological vascular remodelling but even before these changes occur, impairment of endothelium dependent relaxations in the vessel can be seen. Atherosclerotic states can also be associated with augmented production of superoxide due to a switching of the product of eNOS to the O_2^- free radical or to H_2O_2 rather than NO resulting in oxidative stress. This can lead to a further breakdown of NO by superoxide giving a resulting impairment of endothelium dependent relaxations. This change in activity is known as NOS uncoupling, where there is an uncoupling of NADPH oxidation and superoxide is generated rather than NO (Pou et al., 1992). This may be due to a relative dearth of a substrate or co-factor. For example, if there is an abundance of both arginine and BH_4 , eNOS produces NO. However, if the concentration of one is relatively low eNOS can generate superoxide (Vasquez-Vivar et al., 1998). Similar behaviour has been seen with nNOS (Vasquez-Vivar et al., 1999) and iNOS has been found to be able to produce superoxide via its reductase domain (Xia et al., 1998). Increased oxidative stress can lead to an inactivation of NO which can form the toxic end product peroxynitrite (Ohara et al., 1993). This uncoupling has been confirmed in cardiovascular disease including hypercholesterolaemia, diabetes, smoking, nitrate tolerance and hypertension (Heitzer et al., 2001, Hink et al., 2001, Landmesser et al., 2003, Laursen et al., 2001). Contribution by eNOS to oxygen derived, free radical formation in vascular pathological conditions may be due to low levels of essential cofactors such as BH_4 (Stroes et al., 1997), illustrating how NO may have beneficial or detrimental effects on local vascular function depending on its interactions and balance with cofactors and pathophysiological mediators.

1.5.2 Nitric oxide and wound healing

All 3 isoforms of NOS are expressed in skin tissue (Frank et al., 2002) and eNOS can be detected in keratinocytes of the basal epithelial layer as well as dermal fibroblasts, endothelial capillaries and eccrine glands and NO in the skin is involved in the regulation of circulation, sunburn erythema and maintenance of the protective barrier against microorganisms (Luo and Chen, 2005). Enhancement of collagen deposition and increased wound strength has been shown as a result of increased intake of dietary L-arginine (Barbul et al., 1990) suggesting an important role for NO in healing and a decrease in collagen formation is one method by which inhibition of NOS may delay wound healing. Nitrate (NO_3) and nitrite (NO_2) have been found at increased levels in the fluid of subcutaneous wounds (Schaffer et al., 1996) and increased urinary nitrate excretion has been observed after excisional wounding (Luo and Chen, 2005) indicating increased metabolism of NO due to wounding. The NO metabolites are also associated with increased collagen deposition (Schaffer et al., 1996, Schaffer et al., 1997a, Schaffer et al., 1997b) as are treatment with NO donors, increased dietary L-arginine and gene therapy strategies to overexpress iNOS (Shi et al., 2000, Schaffer et al., 1996, Thornton et al., 1998).

NO involvement can also be seen in the inflammatory and proliferative stages of wound healing as it modulates chemoattractant cytokines which lead to infiltration of monocytes and neutrophils which produce $\text{TNF-}\alpha$ and IL-1, both of which have roles in wound healing (Schwentker et al., 2002, Belenky et al., 1993). NO is also involved in keratinocyte recruitment, proliferation, differentiation and apoptosis as monocyte and neutrophil produced-IL-1 is a potent attractor of keratinocytes (Schwentker et al., 2002, Seo et al., 2002) and iNOS inhibition has been shown to have a debilitating effect on keratinocyte proliferation, thus delaying re-epithelialisation and slowing healing (Stallmeyer et al., 1999, Opal and DePalo, 2000).

Another important function of NO at the wound site is its involvement in angiogenesis. Aside from its role in monocyte induced angiogenesis (Leibovich et al., 1994) it stimulates proliferation of endothelial cells and provides protection from apoptosis as well as NOS mediating VEGF production which can positively feedback to stimulate eNOS activity further increasing NO production (Hood et al., 1998, Gelinis et al., 2002,

Ziche et al., 1997). In addition, inhibition of NOS has been found to impair angiogenesis in granulation tissue during gastric ulcer healing (Konturek et al., 1993). The beneficial effects of NO on healing of wounds may extend to influences on inflammation, cell proliferation, matrix deposition and remodelling (Luo and Chen, 2005).

All 3 isoforms of NOS are involved in the dermal repair process. iNOS and nNOS mRNA and protein expression as well as eNOS protein and enzymatic activity are upregulated at the wound site (Frank et al., 1998, Boissel et al., 2004, Luo et al., 2004). In both eNOS and iNOS knockout mice delay in healing of excisional wounds was measured at 30% over wildtype littermates (Lee et al., 1999, Yamasaki et al., 1998) and gene therapy strategies to deliver iNOS and eNOS have shown an increase in NO production and an improvement in healing rates (Luo et al., 2004, Yamasaki et al., 1998).

Delayed healing in the diabetic milieu has long been of major concern and this effect may also be attributable, at least in part, to insufficient NO activity. Both eNOS and NO levels have been seen to be reduced in an induced model of type I diabetes and sustained hyperglycemia can be accompanied by an increase in superoxide levels which can inactivate NO (Schaffer et al., 1997b, Stallmeyer et al., 2002, Kim et al., 2002, Hink et al., 2001). This effect of glucose concentration has been seen in a murine model of type I diabetes (Luo et al., 2004) and diabetes impaired wound healing with reduced epithelialisation has been associated with reduced eNOS protein (Stallmeyer et al., 1999). Conversely, in studies where levels of superoxide were reduced, an accompanying increase in NO levels was observed along with increased NO-mediated vasodilation and accelerated wound healing (Luo et al., 2004, Nishikawa et al., 2000, Kim et al., 2002). Wound healing has also been enhanced by L-arginine supplementation even in the diabetic setting (Stallmeyer et al., 1999, Arana et al., 2004).

Formation of blood vessels in the granulation tissue of healing wounds is an essential part of the healing process and this is underscored by more recent evidence showing the importance of endothelial progenitors and of nitric oxide in this process. Nitric oxide is a potent stimulator of angiogenesis and its effects in wound healing appear to be at least in part attributable to its effect on EPCs (Fukumura et al., 2001) with improvement being

seen after EPC mediated vascular remodelling (Asai et al., 2006b) and delayed wound healing due to ischaemia has been associated with reduced bone-marrow derived EPCs being found in the granulation tissue compared to normal controls (Bauer et al., 2006). Transgenic eNOS deficient mice have reduced levels of EPCs mobilised in response to VEGF and lower levels of MMP-9, both essential factors in blood vessel development in healing wounds. Overall in vivo angiogenesis in response to hindlimb ischaemia is compromised with eNOS appearing to act downstream of VEGF in progenitor cell mobilisation (Aicher et al., 2003b, Murohara et al., 1998). The inhibition of EPC differentiation, survival and normal function by C-reactive protein is partly mediated by a reduction in eNOS expression (Verma et al., 2004) with eNOS mediating EPC mobilisation after treatment with oestrogen (Iwakura et al., 2003). The effect of oxLDL on eNOS is a major factor in its adverse effects on EPC survival, adhesion, migration and tube formation (Ma et al., 2006) while retroviral transduction of EPCs allowing overexpression of eNOS showed an enhancement of re-endothelialisation and a reduction in the formation of neointimal thickening after arterial balloon injury (Kong et al., 2004b). Moreover, in diabetes, NO mediated mobilisation of EPCs is impaired (Gallagher et al., 2007). It has also been shown that eNOS is required for adequate endothelial cell migration, proliferation and differentiation as well as capillary growth and lack of eNOS delays wound closure and leads to a reduced tensile strength (Lee et al., 1999) and a similar effect has been observed with EPCs. Observing these factors together it has been seen that increased levels of NO increase EPC release into the circulation and enhance wound healing (Goldstein et al., 2006).

1.6 Gene Therapy

1.6.1 Gene therapy overview

Broadly speaking, the overall goal of gene therapy is the introduction of a functional gene of interest into a target cell where protein production has been disrupted or is absent altogether due to a genetic disorder. Thus the inserted sequence would modify the genetic material of the living cells with the gene acting as a drug. Currently only somatic gene therapy is being used and here the inserted gene would not be passed on to the patient's progeny. Gene therapy is also being widely explored as a means to overexpress a therapeutic gene of interest for therapeutic benefit. It is clear therefore, that the success of this approach is dependent on the efficient transfer of the gene of interest. Methods of gene delivery can be divided roughly into two categories: synthetic systems depending on direct delivery of genetic information to the target cell such as direct injection of naked DNA or encapsulation of DNA with cationic lipids (liposome complexes) and viral systems which use attenuated viruses to deliver genetic information to the host cell. Although synthetic systems exhibit low toxicity, they are generally less efficient than viral systems and often gene expression is transient.

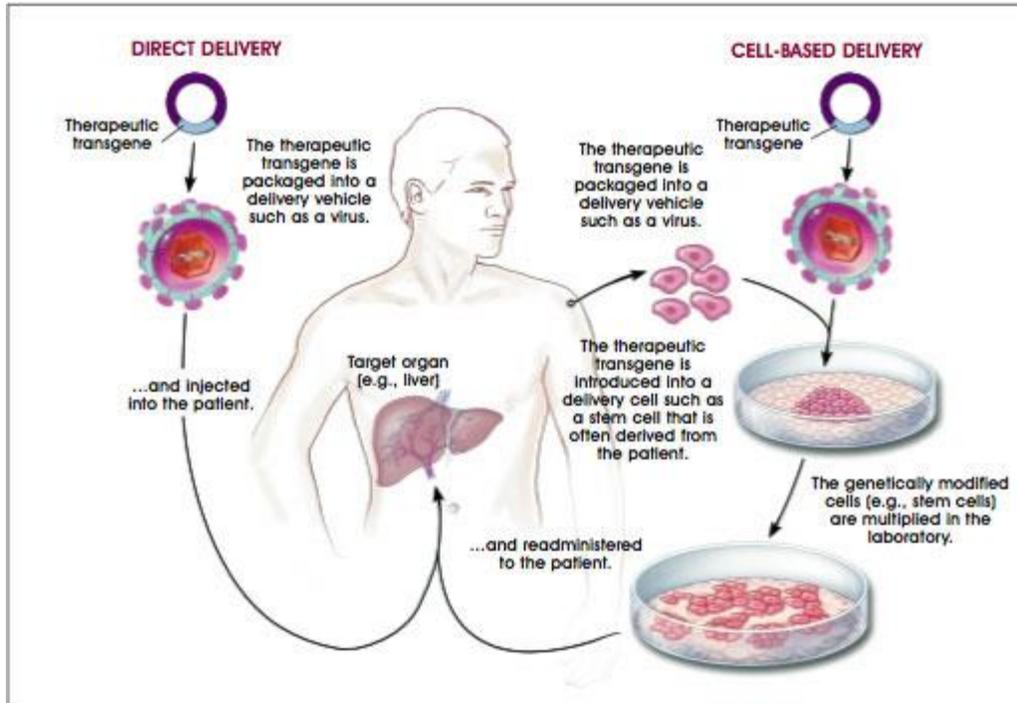


Figure 1.3: Overview of the basic methodology of gene therapy. The therapeutic gene may be directly introduced to the target site or carried by transplanted cells. Taken from <http://stemcells.nih.gov/info/scireport/chapter11.asp>

1.6.2 Viral and Non-Viral Vectors

The innate ability of viruses to deliver genetic information to a host cell can be utilised as the basis for viral gene therapy. Vector systems can be constructed based on replicating viruses but the viral genome is attenuated to eliminate the ability of the virus to replicate. Viral genomes contain both coding regions and *cis*-acting regulatory elements. The genetic information coding for the viral structural and regulatory proteins required for propagation of the virus is enclosed in the coding sequences while the *cis*-acting sequences are essential for packaging of viral genomes and integration into the host cell. In order to construct a replication deficient viral vector the coding regions are replaced with the gene of interest which is to be delivered to the target cells. The viral vector is then introduced into producer cells which provide the structural proteins in *trans* and newly formed viral particles are released on cell lysis.

1.6.2.1 Adenovirus

Adenoviral (Ad) vectors can efficiently transduce dividing as well as non-dividing cells. The double stranded DNA remains episomal and high levels of transgene expression have been observed.

Adenoviral particles are icosahedral particles consisting of a viral capsid enclosing a viral core where the large 36kb DNA genome is located. This linear double stranded genome is flanked by inverted terminal repeats (ITRs) and contains 5 early transcription regions: E1A, E1B, E2, E3 and E4. Also included is the late transcription region from which are generated 5 families of late mRNAs (L1-5). The viral capsid packages the genome and is composed of 3 proteins: hexon, penton base and knobbed fibres (Fields virology).

The replication cycles can be divided into both early and late phases. In the early stage the viral DNA is transported to the nucleus and transcription of the early viral genes is initiated. The products of these early genes interfere with the host antiviral mechanisms and lead to the initiation of the host cell cycle resulting in transcription and DNA replication, the latter of these two processes being initiated by the E2 gene product and leading to commencement of the late replication stage. A high concentration of structural proteins is produced by expression of mRNA regulated by late promoter activity. These proteins assemble with the viral genomes in the nucleus and newly synthesised virions are released following cell lysis (Fields virology).

The first recombinant adenoviral vectors were constructed by deletion of the E1 and/or E3 regions and replacement of these with a promoter and transgene of 6.5-8.3 kb in size (Berkner and Sharp, 1983, Haj-Ahmad and Graham, 1986). In this construct DNA replication and production of structural proteins is limited due to the restriction of transcription of the E2 genes which is itself a result of the deletion of the E1 region. Such vectors can, however be propagated in 293 cells which express Cre recombinase and compensate for E1 deletion (Graham et al., 1977). Packaging capacity could be increased by engineering deletions in the E2 and/or E4 regions but this approach required the development of suitable complimentary cell lines expressing E2 and/or E4 (Wang and Finer, 1996, Yeh et al., 1996).

One of the principal concerns with the use of adenoviral recombinant vectors is the possibility of recombinant events between the viral sequences leading to a replication competent virus. Such a virus expressing Ad proteins would illicit a severe cellular and humoral immune response (Kafri et al., 1998) which, quite apart from the safety issues it raises, would lead to a transient expression of the transgene as transduced cells as well as the transgene are eliminated by the immune response. Following clearance of the transduced cells by cytotoxic T cells the humoral response renders re-administration of the vector ineffective. However, long-term gene expression can be achieved by the removal of viral genes from the vector thus preventing the induction of an immune response with significantly reduced inflammatory responses and cellular infiltration observed (Morsy et al., 1998). These so-called gutless vectors have been a more recent development and are deprived of nearly all viral genes. They retain only the viral ITRs and the packaging signal and require a helper virus for their replication (Parks et al., 1996). The helper virus provides the viral proteins in *trans* and is usually an E1/E3 deleted recombinant Ad vector. Packaging of the helper virus would lead to the generation of a replication competent virus and this is prevented by removing the packaging signal by Cre/*loxP* recombination in the producer cells expressing Cre recombinase. Optimum packaging size of the vector is restored by the addition of stuffer DNA to compensate for deletion of the majority of the viral coding sequences (Parks et al., 1999). Although the development of gutless vectors has circumvented the problem of elimination of transduced cells by the immune system, the vector DNA is still situated episomally and is non-replicating. Over time, therefore, transgene expression may be lost due to degradation of the episomal genome in non-dividing cells or because of a diluting out effect in replicating cells. To address this problem Ad/AAV and Ad/retrovirus hybrid vectors have been developed (Lieber et al., 1999, Zheng et al., 2000). Using these vectors low levels of transduction and integration into the host genome have been observed. Structurally, these vectors are produced by deletion of all viral genes and flanking of the transgene by AAV IRTs or retroviral LTRs which allows integration of the transgene by host cell enzymes in the absence of viral proteins commonly needed to support such activity (AAV *rep* and retro viral integrase).

The most commonly used serotype in Ad gene therapy studies is serotype 5 although use of alternative serotypes can alter the cellular tropism of the vector. This is illustrated by the enhanced tropism of Ad17 for airway epithelia (Zabner et al., 1999), the infectivity of Ad35 in hematopoietic progenitor cells (Shayakhmetov et al., 2000) and Ad subgroup D showing increased ability to infect cells in the central nervous system (Chillon et al., 1999). These differences in host range indicate affinity for more than one cellular receptor among the different serotypes used for binding of the virus but the most widely studied receptor is that which binds serotype 5: the coxsackievirus and adenovirus receptor (CAR)(Bergelson et al., 1997). This receptor is a member of the immunoglobulin superfamily and binds the virus through the knob of the fiber followed by binding of the penton to integrins $\alpha V\beta 3$ and $\alpha V\beta 5$ (Wickham et al., 1993). Virus entry then occurs through clathrin-mediated endocytosis. Although Ad receptors are ubiquitously expressed, low levels of receptor expression can be a cause of inefficient transduction in some tissues such as airway epithelia (Zabner et al., 1997). In order to permit virus entry in cells lacking the CAR receptor or to target specific cell types, retargeting of the virus using bispecific conjugates or retargeting complexes that cross-link the virus with alternate receptors has been explored. Retargeting of Ad vector via epidermal growth factor (Wickham et al., 1996, Dmitriev et al., 2000) and E-selectin (Harari et al., 1999) have been achieved with retargeting complexes designed using either Ad neutralizing antibodies chemically linked to ligands or antibodies specific for cellular receptors (Wickham et al., 1996, Harari et al., 1999, Miller et al., 1998) or fusion proteins of the ectodomain of the CAR receptor and ligands (Dmitriev et al., 2000).

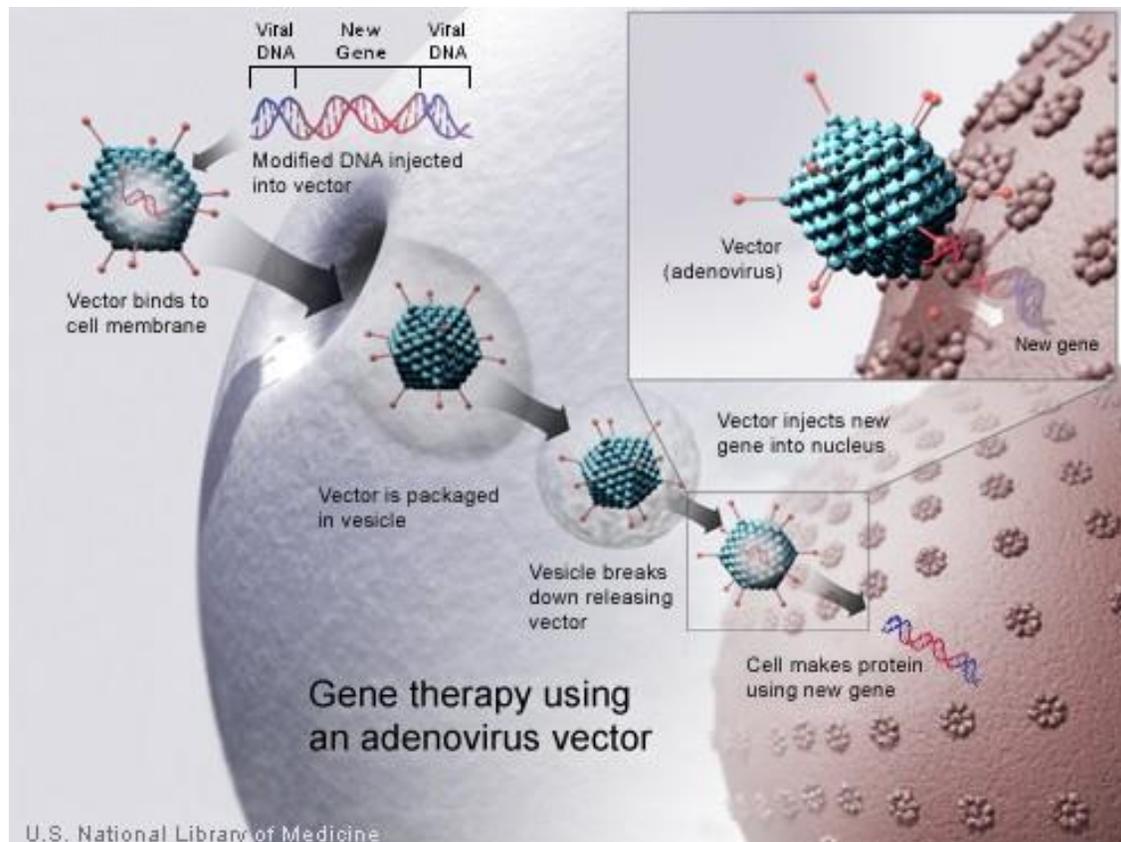


Figure 1.4: The pathway for introduction of the therapeutic gene of interest into the target cell using an adenoviral vector as the vehicle of delivery. Taken from <http://ghr.nlm.nih.gov/handbook/therapy/procedures>

1.6.2.2 Adeno-associated virus

Adeno-associate virus (AAV) is a member of the dependoviruses, a subfamily of the paroviridae. It is non-pathogenic and alone cannot replicate, requiring co-infection with a helper virus. The virus particles are small (20-25 nm) and lack an envelope. The genome is composed of 4.7 kb of linear ssDNA with two open reading frames (ORFs), *rep* and *cap*, flanked by ITRs. Of the two ORFs the *cap* sequence codes for the structural proteins making up the viral capsid and the *rep* sequence codes for the regulatory proteins (Fields virology). After binding to its receptor, the virus enters by endocytosis and is transported to the nucleus where its ssDNA is duplicated either by annealing to a complementary DNA strand from another AAV virus or by the host cell machinery. The dsDNA is then

directed to chromosome 19 by the *rep* proteins where it integrates by homologous recombination and lies dormant. The integrated viral DNA persists for the lifetime of the cell and needs the gene products of a helper virus such as Ad or herpes virus for replication. The viral proteins then regulate AAV replication from initial transcription to DNA replication. The new virions assemble in the nucleus and are released together with the helper virus on cell lysis.

Initially AAV vectors were developed by replacing the ORFs with the transgene. The *cap* and *rep* sequences were then provided on a helper plasmid and an infectious vector produced by co-infection with Ad (Hermonat and Muzyczka, 1984). However, this still leaves over 400 bps of viral genome which can recombine with homologous sequences in the helper construct and the viral vector to produce wild type AAV. In the next generation of vector only the ITRs and 45 adjacent bps essential for virus production and integration remain of the AAV genome (Samulski et al., 1989). These constructs basically consisted of only promoter and transgene flanked by ITRs which eliminated the possibility of replication competent AAV formation. *Rep* and *cap* proteins are provided in *trans* but coinfection with Ad to provide the Ad proteins necessary for initiation of replication allowed the possibility of coproduced wild type Ad contamination. A more recent vector producing system, free of contamination Ad, has been developed (Xiao et al., 1998). Here the Ad proteins E2A, VA and E4 are provided on a second helper construct in 293 cells, which provide the E1A and E1B gene products (Graham et al., 1977). Using this system vector production is also improved by reducing cytotoxicity in the packaging cells by a reduction of *rep* production from the helper construct.

In order for the transgene to be expressed efficiently the ssDNA vector genome must be converted to a stable dsDNA or it is lost rapidly. For this to occur, AAV relies solely on cellular conditions as transduction occurs in the absence of helper virus and *rep* proteins. While transduction can occur in non-dividing cells, efficiency is markedly increased in cells in S-phase (Russell et al., 1994) and activation of the cellular DNA repair machinery supports second strand synthesis, thus improving efficiency (Alexander et al., 1994, Russell et al., 1995). The transgene appears to be predominantly expressed from episomal forms and expression may decline over time due to degradation of the episomal genome. However, the major disadvantage of AAV vectors is the small packaging capacity. To

overcome this limitation, promoter and transgene sequences can be split over two AAV vectors (Duan et al., 2000, Sun et al., 2000) although using this system transduction efficiency was seen to be reduced compared to single vector transduction. Although AAV was initially thought to illicit no immune response, it has been found that a strong humoral response can be raised against the viral capsid which presents difficulties for re-administration of the vector (Brockstedt et al., 1999). In addition, the majority of the population is believed to have circulating neutralizing antibodies against AAV as a result of natural infection (Brockstedt et al., 1999). The use of AAV vectors with *cap* proteins form different serotypes may overcome this problem.

Of the 5 serotypes identified AAV-2 is the most commonly used and with a broad host range it shares the natural tropism of the wild type virus. The primary receptor of AAV-2 is heparin sulphate proteoglycans but internalisation by endocytosis is facilitated by fibroblast growth factor receptor-1 and integrin $\alpha_v\beta_5$ (Summerford and Samulski, 1998, Qing et al., 1999, Summerford et al., 1999). Despite its broad host range, some cell lines have been reported to be resistant to infection with AAV-2, probably due to lack of appropriate receptors. Between the 5 serotypes analysis of the *cap* protein sequences shows much diversity implying affinity for different receptors and coreceptors. There is most homology in the *cap* proteins of AAV1, 2 and 3 and these serotypes can all bind to heparin sulphate (Summerford and Samulski, 1998), while more diversity is seen in AAV4 and 5 (Chiorini et al., 1997, Chiorini et al., 1999). Differences in cellular tropism have also been observed with AAV-1 showing the highest transduction efficiency of all the serotypes when exposed to muscle and liver, AAV 5 showing high tropism for retina (Rabinowitz et al., 2002) and airway epithelia cells (Zabner et al., 2000) and AAV 3 for hematopoietic stem cells (Handa et al., 2000). These studies suggest that the use of different serotypes may allow targeting of specific tissues for transduction.

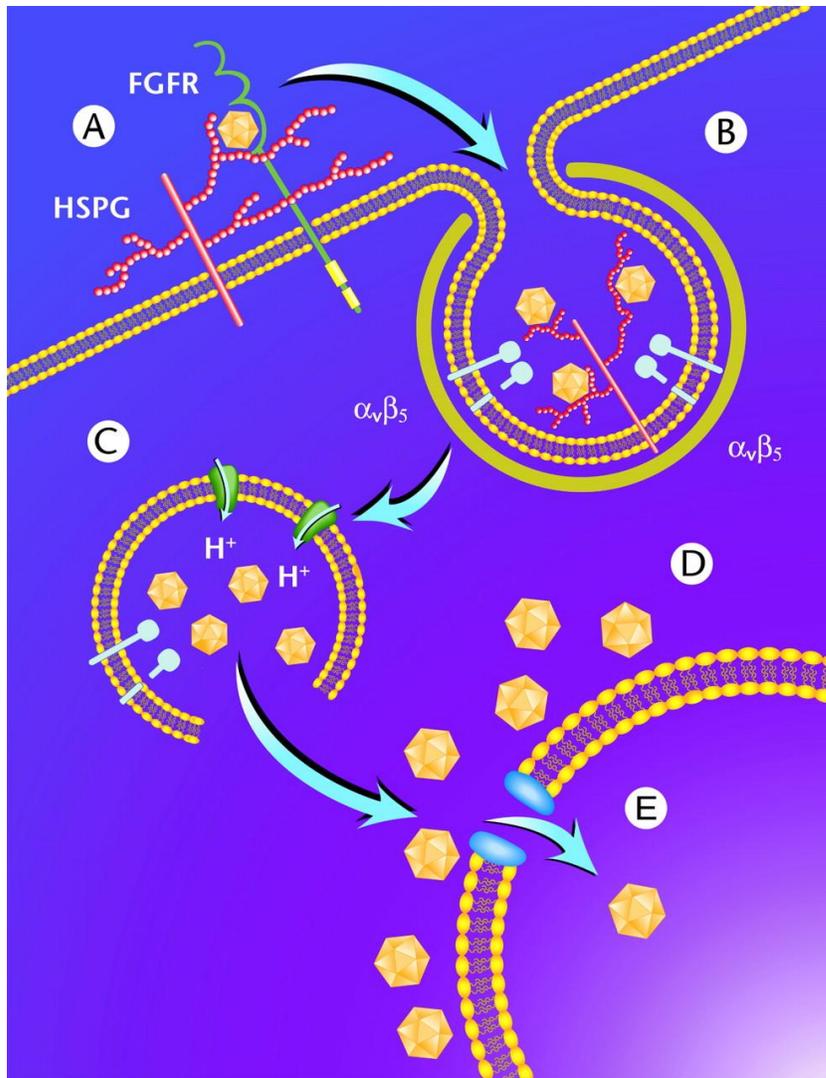


Figure 1.5: Schematic showing the mode of entry of AAV into the cell. Taken from Bartlett: Infectious entry pathway of adeno-associated virus and adeno-associated virus vectors. *J Virol* 74; 6, 2777-85

1.6.2.3 Retrovirus

Retroviruses are a large family of viruses containing 2 copies of an RNA genome surrounded by a cone shaped core within an envelope (Fields virology). The genome contains 3 essential genes - *gag*, *pol* and *env* – flanked by LTRs. The *gag* gene encodes for the core proteins capsid, matrix and nucleocapsid, the *pol* gene for the viral enzymes protease, reverse transcriptase and integrase and the *env* gene for the envelope

glycoproteins which mediate virus entry. The virus enters the host cell by a process of membrane fusion (except when pseudotyped with VSV-G when entry is via the endocytic pathway), where the viral capsid enters after binding to the appropriate receptor. The action of the viral enzyme reverse transcriptase produces double stranded proviral DNA from the viral RNA genome and this DNA translocates to the nucleus where it is integrated into the host genome through the action of viral integrase. This proviral DNA is heavily associated with viral proteins like nucleocapsid, reverse transcriptase and integrase. Transcription proceeds from the LTRs after initiation by host cell transcription factors and new particles are formed at the plasma membrane. *Gag-pol* and *gag* precursors assemble with two copies of viral RNA along with *env* glycoproteins for incorporation into the viral membrane. The virion matures after processing of the *gag* and *gag-pol* precursors by the viral enzyme protease.

Lentiviral vectors have the ability to transduce dividing as well as non-dividing cell and to integrate into the host cell genome. Their preintegrin complexes allow the virus particles to get through the membrane of the target cell nucleus. Most lentiviral vectors are based on the human immunodeficiency virus 1 (HIV-1) due to the extensive research already carried out on this virus. Besides the retroviral structural proteins, lentiviruses encode 3-6 additional proteins that are concerned with virus replication and persistence of infection for example, *tat* transactivates viral transcription (Sodroski et al., 1985, Fisher et al., 1986) and *rev* facilitates nuclear export of unspliced viral RNA (Malim et al., 1988) although development of a CMV/LTR hybrid to drive vector expression made production independent of *tat* expression (Zufferey et al., 1997).

Vector development of lentiviruses closely resembles retroviral vector design in that all viral sequences are deleted except the LTRs and packaging signal. The *rev* response region (RRE) is included to which the *rev* protein, which is provided in *trans* during production, binds. The viral genes are provided by a packaging construct which initially contained full length proviral DNA, lacking a packaging signal. The LTRs were removed from the construct and transcription driven by a CMV promoter thereby reducing sequence homology between the vector and packaging construct (Naldini et al., 1996). To improve biosafety of the vector, all non-essential genes such as accessory proteins *vif*,

vpr, *nef*, and *vpu* were removed from the packaging constructs (Zufferey et al., 1997) and subsequently vector production was made independent of *tat* with only the coding region for *gag/pol* and RRE remaining to form the 3rd generation vectors commonly used today. In order to overcome the limitations imposed by the specificity of the HIV-1 *env* glycoprotein and broaden the host range of the vector, lentiviral vectors are commonly pseudotyped with vesicular stomatitis virus glycoproteins (VSV-G) which have a wider host range. The VSV-G *env* is expressed from a different construct during vector production (Naldini et al., 1996, Dull et al., 1998).

Due to the use of HIV-1 based vectors concerns over their safety for human use has been raised. Since so much of the viral sequences have been deleted in the current system the replication capacity of the virus has been highly disabled. The risk of recombination with wild type viruses can be reduced by the use of self-inactivating (SIN) vectors, which have large deletions in the transcriptional activation units of the LTR (Zufferey et al., 1998, Miyoshi et al., 1998). These concerns may be further alleviated by the use of vectors based on other primate lentiviruses such as HIV-2 and simian immunodeficiency virus (SIV). However, these viruses are closely related to HIV-1 and so vectors based on non-primate lentiviruses such as feline immunodeficiency virus (FIV), equine infectious anemia virus and visna have been explored (Poeschla et al., 1998, Mitrophanous et al., 1999, Berkowitz et al., 2001). However, since lentiviral infection depends on cellular co-factors (Zack et al., 1992, Schuitemaker et al., 1994, Kinoshita et al., 1998, Korin and Zack, 1998, Kootstra et al., 2000) transduction efficiency may be severely impaired by the use of non-human vectors in primary human tissues.

Genome map of Lentivirus

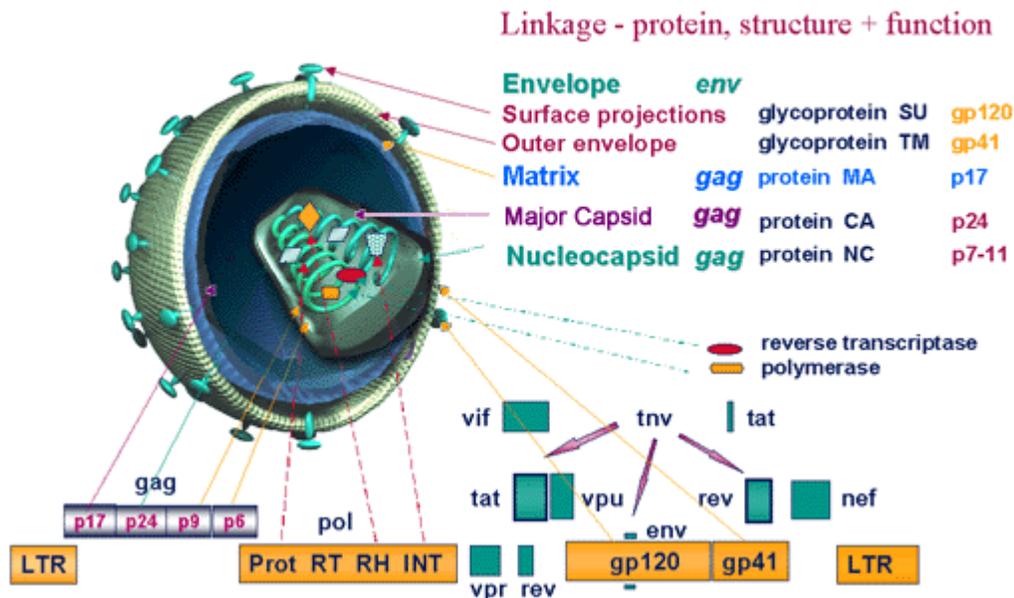


Figure 1.6: Lentiviral genome map. Taken from <http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdb/00.061.1.06.009.htm>

1.6.2.4 Liposomes

While naked DNA has been directly injected to tissues with some success, for the plasmid to reach deeper tissues a vehicle is necessary. DNA can be complexed with cationic lipids to form lipoplexes. These complexes are taken up by the target cell by endocytosis (some studies have also suggested direct penetration of the cell membrane) so that either hydrophobic or hydrophilic molecules may be delivered into the cell. Proteoglycans appear to be important for uptake and adding a targeting ligand can improve efficiency. The next barrier to efficient transfection is the translocation of the plasmid DNA from the endosome to the cytoplasm and fusion appears to be an important mechanism involved in this process. This is followed by nuclear transport of the plasmid DNA, via a pathway thought to be mediated by the nuclear localisation signal (NLS).

Electroporation 1.6.2.5

The simplest of gene delivery techniques is the introduction of unmethylated plasmid DNA which has been used to express, among other things, pharmacologically active molecules such as erythropoietin and leptin since the discovery that reporter genes could be expressed in skeletal muscle after direct injection of naked plasmid DNA (Wolff et al., 1990, Ulmer et al., 1993, Condon et al., 1996). In order to increase vector efficiency several techniques have been developed including gene gun and electroporation (Yang et al., 1990, Rols et al., 1998) which allow the plasmid to escape enzymatic degradation by allowing it to penetrate the cell membrane and bypass endosome/lysosome. The phospholipid bilayer of the cell membrane may be a barrier to gene transfer as it does not allow polar molecules such as DNA to pass freely. To overcome this barrier, temporary aqueous pores may be formed in the cell membrane by the application of a quick voltage shock. This pulse can disrupt areas of the membrane due to the relative weakness of the interactions between the hydrophobic and hydrophilic layers of the membrane. The rising electric potential across the cell membrane drives charged molecules like DNA through the pores. One of the major problems with non-viral vectors is the relatively short duration of gene expression as the transferred genetic material is not delivered into the host genome.

1.7 Hypothesis and Aims

Since diabetic ulcer healing has been long identified as an issue of significant clinical concern the aim of this work was to explore strategies for promoting and accelerating healing. Angiogenesis is integral to wound healing and a growing body of work demonstrates the contribution of EPCs in a variety of clinical and pre-clinical settings through promotion of angiogenesis. We hypothesised, therefore, that treatment of diabetic ulcers with isolated and cultured CACs would increase the rate of ulcer healing. NO has also been shown to have significant functions regarding angiogenesis and endothelial function. In addition, functional impairment of EPCs has been demonstrated in the diabetic setting and so the aim of this work was to explore whether functionality could be restored to diabetic CACs through gene modification to overexpress eNOS and the effect on diabetic ulcer healing of treatment with these modified cells with comparison to non-transduced cells. Autologous cells were deemed more clinically relevant as they circumvent the problems of cell rejection and so autologous cells were used exclusively for this study. A secondary aim was to establish the most efficient vector for transduction of these cells. Since eNOS has well documented effects on endothelial function and angiogenesis this was the gene of choice with which to modify these cells to explore the effect on angiogenesis and wound healing in the setting of diabetic ulcer healing.

2. Optimisation of Endothelial Progenitor Cell Culture and Cell Characterisation

2.1 Abstract

In order to proceed with experiments to determine the therapeutic potential of EPCs for diabetic wound healing, optimisation of the isolation and culture conditions for these cells had to be undertaken. To this end, both early and late cells, CACs and ECFCs respectively had conditions optimised for their culture and alternate cell isolation protocols were employed to attain the best yield from a particular sample. In addition, antibody and endothelial cell marker staining of these cells in order to characterise them was carried out. Although both types of cell were successfully cultured, CAC were chosen for subsequent experiments due to practical considerations relating to the model used. This chapter describes the steps taken in the optimisation procedure and results obtained relating to cell growth and characterisation.

2.2 Introduction

Due to the many different methods employed in the culture of endothelial progenitors it was necessary to optimise and standardise a culture method to be used in experiments described later in the thesis. This involved exploration of isolation method, substrate, culture volume, time of culture and plating density. Whether detachment and replating of adherent cells or culture of just the suspended cell population was of benefit in culture of EPCs was also explored.

The testing of each parameter is labour intensive as it requires the isolation of a fresh batch of cells for all conditions tested as CACs cannot be cryogenically stored and subsequently re-cultured nor can the CAC population be substantially expanded.

From 500mls of blood 3×10^8 - 7×10^8 mononuclear cells could typically be isolated although this figure could be as high as 1×10^9 . Cell number as determined by counting on a haemocytometer varied from donor to donor.

The most efficient system was selected based on cell number, morphology and viability. Adherent populations containing a greater number of elongated cells were regarded as a more differentiated population and viability could be assessed by observing the number of detached or “rounded-up” cells in the culture dish.

Due to the wide range of culture conditions that have been employed to grow EPC populations and because of the lack of a specific definition of these cells it was also necessary to do some characterisation studies on the cells obtained. Endothelial cells are known to endocytose acetylated LDL and bind UEA-1 lectin and so cells displaying these characteristics may be deemed to have endothelial features and have been designated EPCs in previous studies (Asahara, 1997; Bahlmann, 2005; Fadini, 2006). However monocytes and macrophages also display these characteristics and so further characterisation is necessary. To further confirm an endothelial phenotype, binding of endothelial specific antibodies may be seen. Cells differentiated from the MNC population of peripheral blood cells which display endothelial characteristics may be

deemed EPCs. It is more likely however that these early EPCs do not represent true EPCs but rather circulating angiogenic cells.

From a starting population of mononuclear cells isolated from peripheral blood, different populations of endothelial-like cells may be grown depending on the culture conditions. It is useful here to follow the classification method proposed by Hirschi and colleagues (Hirschi, 2008). In their review, they differentiate between colony forming unit-Hill cells (CFU-Hill), circulating angiogenic cells (CAC) and endothelial colony forming cells (ECFC). CACs are adherent mononuclear cells, cultured for 4-7 days, which typically do not display colony formation. ECFCs are cultured for 6-21 days and display a cobblestone morphology when cultures are expanded to confluence. Regarding ECFCs, clonogenic potential may be used to differentiate these cells from circulating mature endothelial cells as ECFCs can undergo more population doublings. Putative cluster-forming EPCs were described by Asahara (Asahara, 1997) and an assay for isolating such cells was refined by Hill, et al (Hill, 2003), where PBMNs were grown on fibronectin for 48 hours and the non-adherent cells then replaced. Emergence of EPC colony forming units could then be quantified and these have been referred to as colony forming unit-Hill.

For the majority of experiments in this and succeeding chapters CACs were the principal focus. Some preliminary data was collected on ECFCs as described in this chapter and CFU-Hill cells were not examined. Where the term EPC is used it applies to both CACs and ECFCs.

2.3 Methods

2.3.1 EPC culture

Whole blood was collected from patients with haemochromatosis undergoing venesection and attending the clinic at UCHG or from healthy volunteers. Donors with haemochromatosis were selected who were not taking medications that are known to interfere with EPC number or function, were not suffering from diabetes and who were on maintenance treatment and so having normal and consistent iron levels in their blood (ferritin levels of 50ng/ml (normal range 10-200)) and who were not anaemic. 500mls was collected from each donor.

Rabbit blood was collected after sedation with 0.1mls/kg of ACP and topical application of analgesic cream to the dorsal surface of the ear. The central ear artery was cannulated and blood was drawn at 8mls/kg. Following blood draw 30mls of saline was infused subcutaneously. Cells were cultured as described below and speciality media (EGM-2), containing various angiogenic cytokines was used. EGM-2 medium (Cambrex) consisted of basal media supplemented with 2% FBS, hEGF, hydrocortisone, gentamicin, amphotericin-B, VEGF, hFGF-B, R³- IGF-1, ascorbic acid and heparin.

The following protocols were used to isolate cells from patients undergoing venesection, from normal donors and from rabbits.

Standard Protocol for Isolating Endothelial Progenitor Cells from Blood

(Human or Rabbit)

Withdraw 30 ml blood (10ml/kg body weight of rabbit), and add to EDTA or heparin tubes

Chapter 2

Dilute blood (or buffy coat of human blood) in HBSS + 1mM EDTA + 5%BSA with a ratio of 1:2

Gently layer diluted blood over Ficoll Paque Plus in a ratio of 5 Ficoll: 7 blood

Spin for 30 min @ 1400 rpm at RT. Coat plates with fibronectin (50µg/ml, 1ml/well of 6 well plate @ RT for 1-2 hrs)

Remove MNC cloudy band with a pasteur pipette and transfer to a 50 ml conical tube

Spin down @ 1100 rpm for 10 mins at 4°C, remove supernatant

Add 6ml RBC lysing solution, pipette mix for 2 min (too long will damage MNC), spin for 5 min at 1100 rpm at 4°C, remove supernatant

Wash with 4 ml MCDB131 wash medium, spin for 10 min at 1100 rpm at 4°C, remove supernatant

Repeat step 8 twice, count cells

Resuspend cells in EGM-2 (4-6 ml/well) and seed cells in 6-well plates 2×10^7 /well

Change EGM-2 daily 4ml/well

MCDB Medium:

Hydrocortisone: 1µg/ml

Dibutyl cAMP: 0.5mM

Amphotericin B: 0.25µg/ml

VEGF: 10ng/ml

Heparin: 6.7U/ml

Alterations to standard isolation protocol:

No difference was seen when cell pellets were washed in PBS rather than MCDB wash medium. Washing in EGM-2 medium caused cells to adhere to the sides of the tube rather than to form distinct pellets.

Large volumes of blood (500mls) were diluted in HBSS+EDTA+BSA at a 1:1 ratio.

2.3.2 Culture conditions

For all experiments 6ml of media was added to each well of a 6 well plate and changed daily for the first 2 days. 4mls was added to each well daily thereafter. For a 12-well plate 2mls were used for the first 2 days and 1.5mls was added daily thereafter. Optimal plating concentration for all experiments was determined to be $\sim 1.76 \times 10^6$ MNCs/cm² in a 6-well culture dish or 2.41×10^6 MNCs/cm² in a 12-well culture dish.

CAC Culture: For CACs, cells were maintained in culture for 4, 7 or 10 days.

ECFC Culture: ECFCs were cultured as above initially and maintained in culture for 6-21 days and grown to confluence.

2.3.3 Alternate Isolation

In order to isolate MNCs from 500mls of whole blood large volumes must be handled which is both more costly and time consuming. It is, therefore, more desirable to isolate MNCs from a pre-prepared buffy coat. In order to do this whole blood can be centrifuged directly and the white cell portion removed.

After standing for 3 hours, a 500ml bag of whole blood was centrifuged in a Beckman large centrifuge for 10 mins at 130g. The supernatant containing plasma and white blood cells was then transferred to 2 x 50 ml tubes by inserting a site coupler into the top of the

bag without removing it from the centrifuge. The bag and the 2 tubes were then spun at 200g for an additional 10 mins. The supernatant was then removed from the tubes and the pellet resuspended in plasma and transferred to 15ml tubes. These tubes were spun at 250g and the white cells removed and plated as normal. The results are discussed below.

2.3.4 Cell staining

Cells were fixed with either 100% methanol or 2-4% paraformaldehyde following culture on chamber slides by washing with PBS and adding the fixative for 20 mins at 4°C. They were then permeabilized with 0.1% Triton X for 2 mins at room temperature. Following washing with PBS primary conjugated antibody was added at 1:200 dilution. After additional washing Hoescht stain was added for 5 mins and slides were then coverslipped with Prolong Gold antifade reagent (Molecular Probes). Where a secondary antibody was used slides were blocked with 10% normal goat serum for 30 mins at room temperature after permeabilization. In cells fixed with methanol reduced fluorescence in GFP transduced cells was observed possibly as a result of the fixation creating pores large enough for the protein to leak out and so paraformaldehyde was favoured as fixative.

Mitotracker and actin.

To stain the mitochondria, Mitotracker Red (Molecular Probes) was added to cultures before fixation at 4µl/ml of media and incubated for 1 hour at 37°. To stain actin 8µl of Phalloidin (Molecular Probes) was added to cultures after fixation for 30mins at room temperature.

2.4 Results

2.4.1 CACs

2.4.1.1 Cell Culture

Cell substrate is an essential part of EPC culture. It allows for attachment of cells and provides traction for the elongation of cells that acquire the characteristic spindle shape. Although ECFCs will adhere to plastic and may be passaged without a substrate coating after colonies have appeared, CACs will not differentiate in culture (they will survive but not acquire characteristic morphology or display markers) without substrate and thus the first colonies of ECFCs will not appear either.

In order to identify the most favourable substrate, collagen and fibronectin were both tested by coating the wells of the culture plates with solutions of either substance. After multiple batches of cells had been grown, little or no difference was seen between either substrate and fibronectin was chosen for future experiments in order to adhere more closely to the published literature (Asahara, 1997).

CACs appear to secrete cytokines into the culture medium affecting other mononuclear cells in culture in a paracrine fashion with resultant changes in growth of the entire culture. A larger culture volume then affects the concentration of cytokines in the media with larger volumes of media diluting out the effect of the secreted cytokines. As growth factors are also present in the media the volume added affects growth kinetics. For all experiments 6ml of media was added to each well of a 6 well plate and changed daily for 2 days with 4mls being added thereafter. For a 12-well plate 2mls were used for the first 2 days and 1.5mls was added daily thereafter. In addition, the size of the culture well influenced cell growth. In larger wells the cells grow and attain endothelial characteristics more readily possibly due to the effects of secretion from a larger number of cells. The initial plating density of MNCs is also a crucial factor in the development of a CAC

population. Plating at too low a concentration will impede the adherence and differentiation of these cells population. Optimal plating concentration for all experiments was determined to be $\sim 1.76 \times 10^6$ MNCs/cm² in a 6-well culture dish or 2.41×10^6 MNCs/cm² in a 12-well culture dish.

The duration of tissue culture has also varied before CAC harvest. 4, 7 and 10 day cultures were examined. After 4 days not many adherent cells had adopted the characteristic spindle morphology and by 10 days many floating cells were observed showing a reduced number of viable cells. For these reasons 7 day cultures were favoured for future experiments.

In some experiments, after 1 or 2 days the suspended fraction of cells in the culture dish was aspirated and replated on fresh substrate to assess the viability of the suspended cell fraction. However this fraction did not produce as viable a population as culturing just the adherent cells so this practice was discontinued. This practice is described by Hill et al REF and is reported as leading to growth of colonies of cells but these could not be reliably obtained.

Substrate	No Substrate	Collagen	Fibronectin <input checked="" type="checkbox"/>
Culture Volume	2mls for first 24hrs. 1.5mls daily thereafter <input checked="" type="checkbox"/> (12 well plate)	6mls for first 24hrs. 4mls daily thereafter <input checked="" type="checkbox"/> (6-well plate)	
Culture duration	4 days	7 days <input checked="" type="checkbox"/>	10 days
Cell fraction	Suspended	Adherent <input checked="" type="checkbox"/>	
Plating Density	1.76×10^6 MNCs/cm ² <input checked="" type="checkbox"/> (6-well plate)	2.41×10^6 MNCs/cm ² <input checked="" type="checkbox"/> 12-well plate	

Table 2.1: Comparison of differing culture methods tested in optimisation of CAC culture. A tick indicates the chosen method. Culture volume and plating density categories show those used for both standard 6 and 12 well plates.

2.4.1.2 Morphology

Crude identification of CACs may be made based on cell morphology. As the cells begin to acquire endothelial characteristics, adherence to the substrate allows the cells to elongate and assume a spindle shape.

CACs appear to contain a heterogeneous population containing spindle shaped and cuboid shaped cells and possibly a third population of cells with a lymphocyte-like appearance. Indeed, transduction studies described in the next chapter have identified two distinct populations based on efficiency of transgene expression.

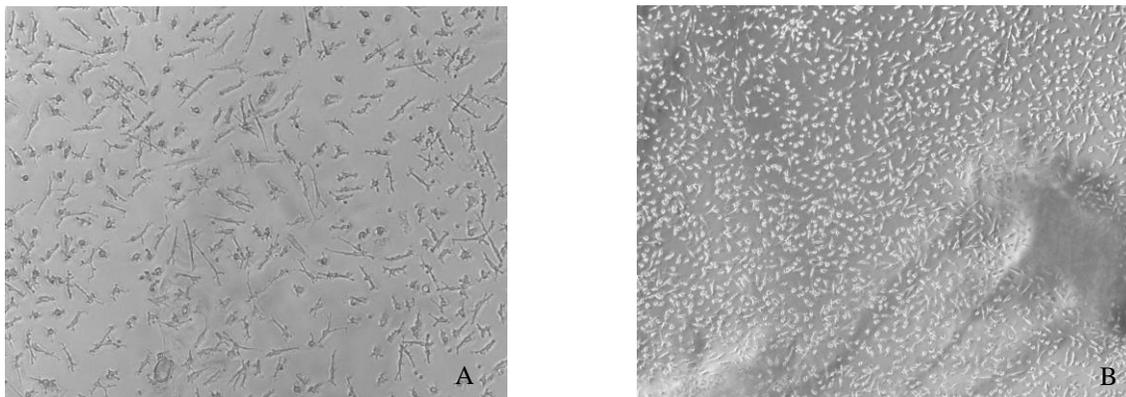


Figure 2.2: Bright field microscopy showing early EPC cultures at 7 days. A) Mag: x100. B) Mag: x40. Morphologically dissimilar cells can be seen indicating a heterogeneous population.

Another difference which may be observed between CACs and ECFCs is in the differential arrangements of actin and mitochondria. ECFCs appear to have more organised actin filaments and mitochondria predominantly arranged around the nucleus while CACs display more diffuse mitochondria with actin arranged around the periphery of the cell.

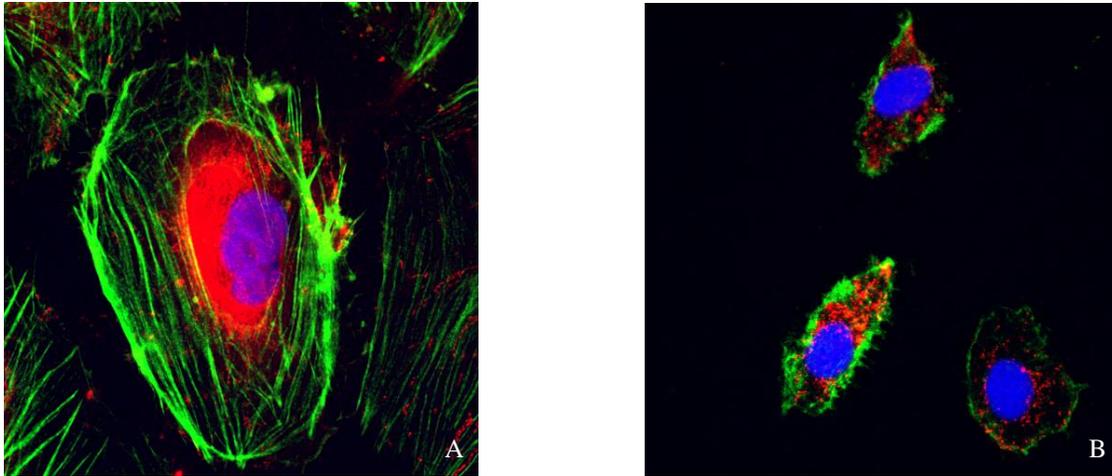


Figure 2.6: Confocal images of A) ECFC and B) CAC showing red mitochondrial stain, green actin stain and blue nuclear stain.

2.4.1.3 Staining

A commonly used criteria for characterisation of a cell as a CAC is uptake of acLDL and binding of UEA-1 lectin. Both of these stains may be seen below.

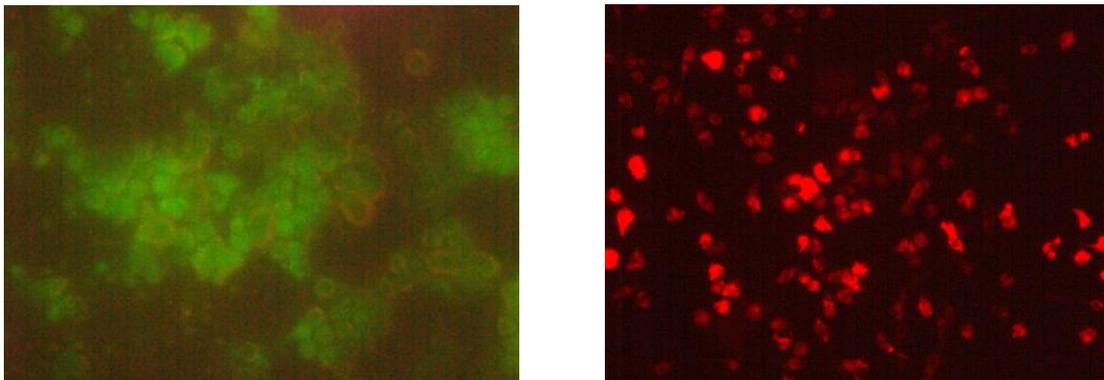


Figure 2.7: CACs displaying UEA-1 (green) and ac-LDL (red) stains.

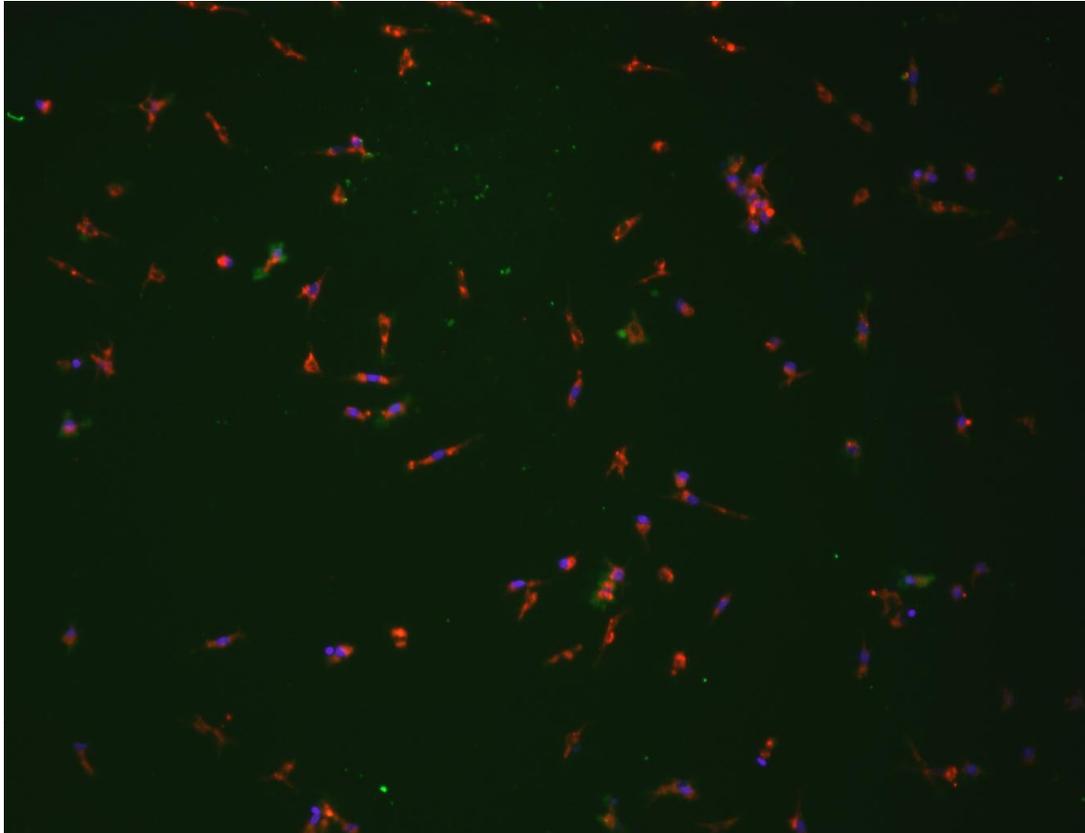


Figure 2.8: CACs dual stained with UEA-1 and ac-LDL as well as a blue nuclear stain.

However, this only indicates endothelial characteristics and while it is useful for identifying endothelial-like cells cultured from peripheral blood it does not definitively define a CAC as it does not distinguish CACs from circulating mature endothelial cells or, indeed, from myelomonocytic cells as they also take up acLDL and bind isolectin. Mature endothelial cells may also be seen to display these stains as shown in figure 2.9. Thus additional markers are needed. The stem cell marker AC133 has been used to separate out starting populations of MNCs from which may be cultured EPCs (Hristov et al., 2003) but expression of this marker diminishes and is no longer detectable after 7 days of culture. Clonogenic potential may provide a means by which ECFCs may be distinguished from mature circulating endothelial cells but this is not relevant to CACs. To date no single marker exists by which EPCs may be definitively characterised. However the endothelial character of cells cultured from MNCs may be further determined by expression of endothelial markers such as Flk-1, vWF and CD34.

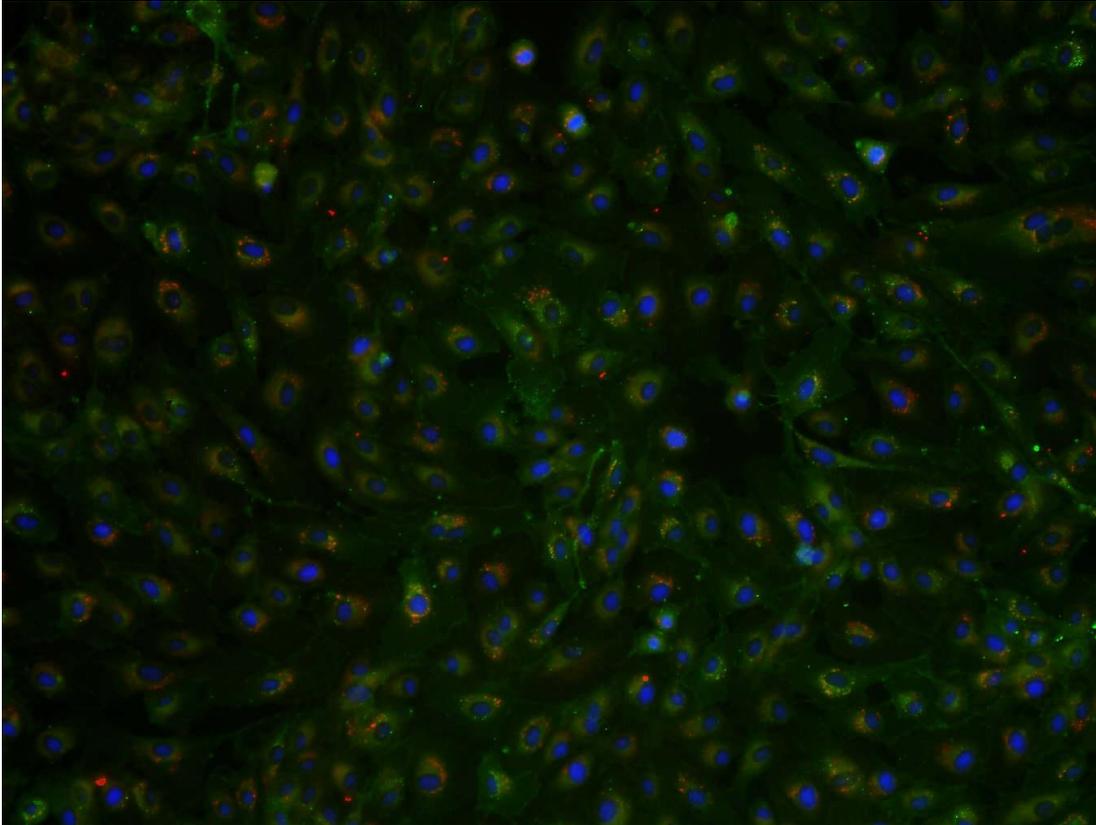
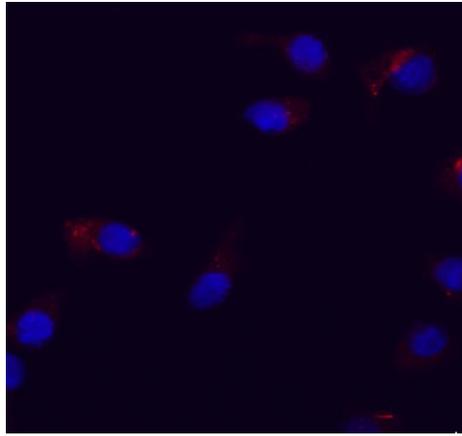
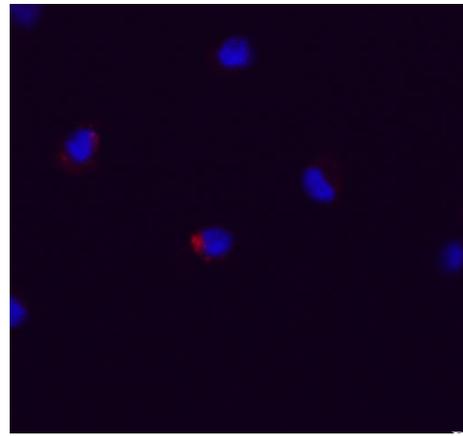


Figure 2.9: HUVECs dual stained with UEA-1 and ac-LDL. Blue nuclear stain is also apparent.

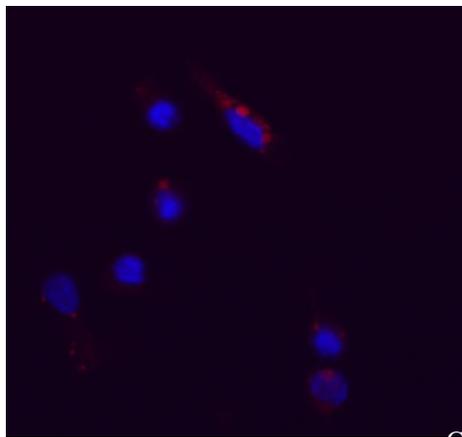
Antibody staining has also been used to characterise cells displaying endothelial-specific markers on the cell membrane and further define the CAC population. To characterise the cells in culture as having an endothelial phenotype they were stained with endothelial markers CD34 (also found on haematopoietic cells), KDR and vWF. In addition, CD133 was sought to show the stem cell nature of the cells, although this marker becomes less pronounced as the cells differentiate and the CD133 stain shown here represents 4-day cells and indeed, by day 7 this marker was not detected, consistent with previous findings (Peichev, 2000). The other stains represent 7 day cells. The CAC cells shown below show positive staining for the markers mentioned above and are as labelled. An IgG negative control is also shown.



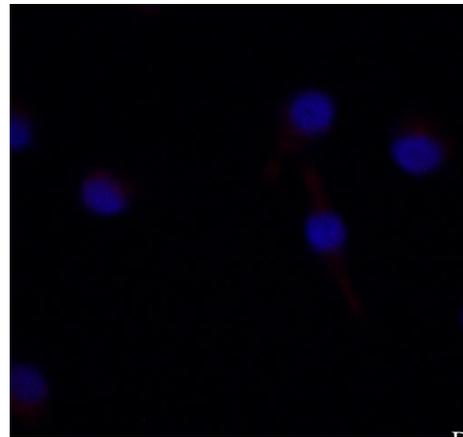
CD34



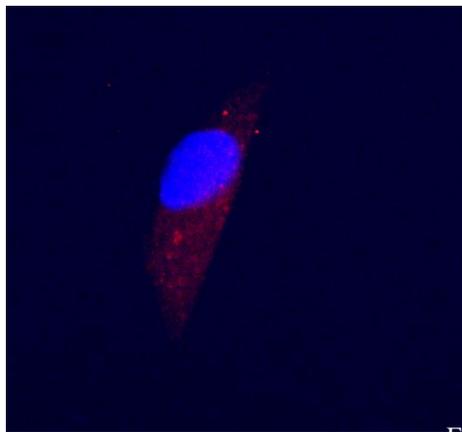
CD133



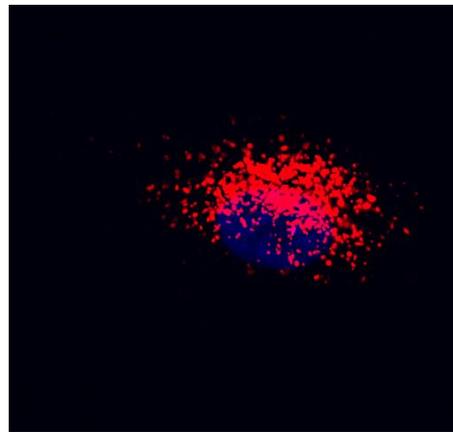
KDR



IgG



KDR



vWF

Figure 2.10: Antibody stains of early CACs. Confocal images showing red stains for A) CD34; B) CD133; C) Flk-1; D) IgG negative control; E) Higher magnification KDR; and F) vWF. These markers show development of endothelial characteristics of cultured MNCs.

2.4.2 ECFCs

2.4.2.1 Cell Culture

ECFCs were initially cultured as above for CACs. However, for ECFC culture, cells were maintained for 2-4 weeks to allow colonies to appear. Colonies appearing at 2-3 weeks appeared to have the best proliferative rate and any colonies appearing after 3 weeks tended to have low proliferative potential.

ECFCs may be passaged from a single colony and thus a pure population may be acquired. There is however variation between different colonies as to the number of times a particular clone of cells may be cultured. Although some batches of cells may be expanded indefinitely there are also colonies that appear to senesce after a certain number of population doublings and some that may not grow to confluence at all. This may reflect the clonogenic hierarchy described by Ingram et al (Ingram et al., 2004). In general, ECFCs may be identified as a homogenous population of spindle shaped cells.

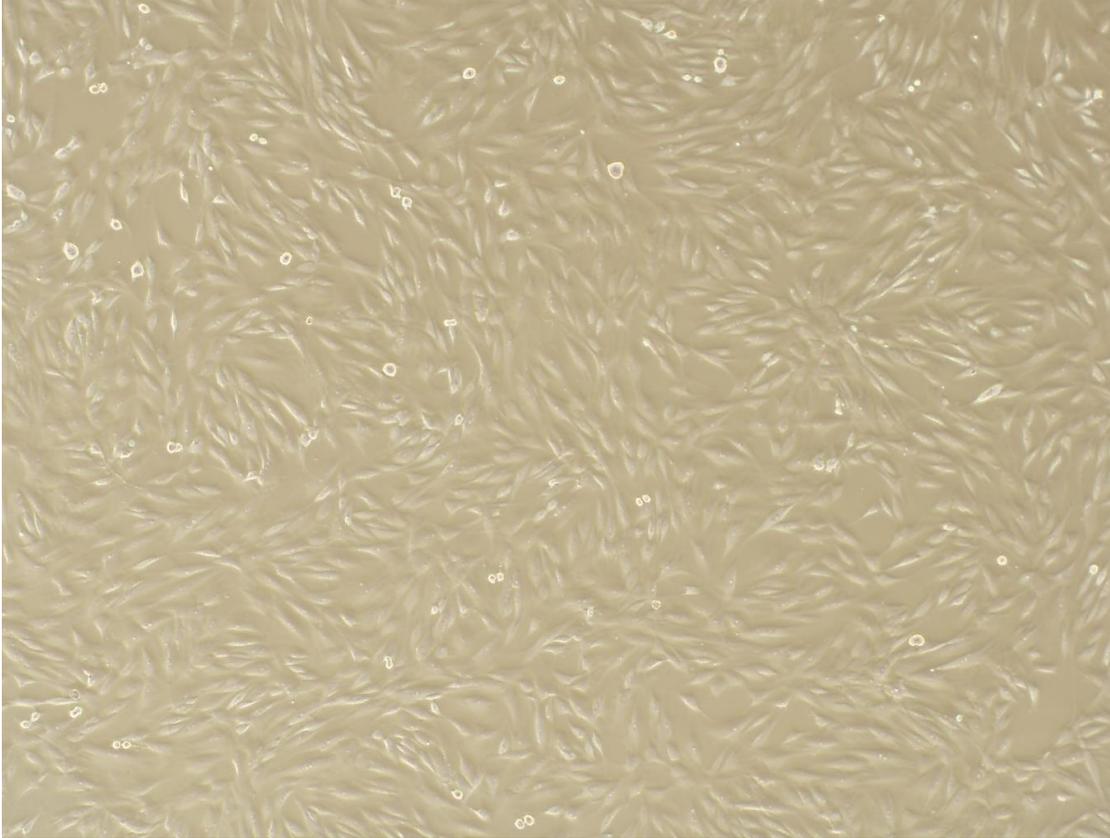


Figure 2.11: Phase contrast microscopy image showing expanded ECFC culture. These colonies could be grown to confluence and passaged numerous times. Mag: x100

As compared to CACs, ECFCs could not be as reliably cultured from small amounts of blood. This may reflect the chance of collecting clonogenic cells in a given sample of peripheral blood as small volumes would be less likely to contain these cells. Given the unreliability of growing colonies from a given sample of blood and since a rabbit model was used for subsequent experiments CACs were favoured for this work. Rabbit colonies of ECFCs are more difficult to obtain than human colonies and only about 30ml samples of rabbit peripheral blood can be taken. It is interesting that there are no papers on ECFCs from patients with diabetes published to our knowledge. This may represent the difficulty of isolating ECFCs from patients with diabetes mellitus. The volume of blood collected may also be a factor.

2.4.3 Early Cell Colonies of CFU-Hill cells

It has been noted previously that as well as ECFCs, cells cultured for a shorter time period appear to form colonies in culture (Hill et al., 2003). These are isolated in a different fashion with a subculture of loosely adherent cells incorporated – Yoder/Ingram describe CFU Hill cells, CACs (4-7 day monolayers) and ECFC. CFU-Hill cells appear to form colonies and such a colony can be observed after 2-4 days in culture and an example may be seen below. However, colonies such as are represented below were grown using the standard isolation protocol described in the methods section of this chapter using a loosely adherent subpopulation as described by Hill (Hill, 2003). Whether these are in fact true colonies grown from a single cell or are derived from initial clusters of MNCs remains unclear. It is also possible that they are the result of cells migrating toward a central stimulus such as a chemoattractant. It is interesting to note that when stained with DiI-labelled acLDL, which is a principle indicator of endothelial-like cells, there appear to be few cells staining positive within the colony while cells around the periphery appear to take up and express the stain more proficiently. Whether this indicates that the cell colony is not made up of endothelial-like cells remains unclear. Given the low proliferative nature of CACs, this cell formation may indicate another phenomenon which is distinct from colony formation, such as cells migrating towards a focal point of chemoattractant.

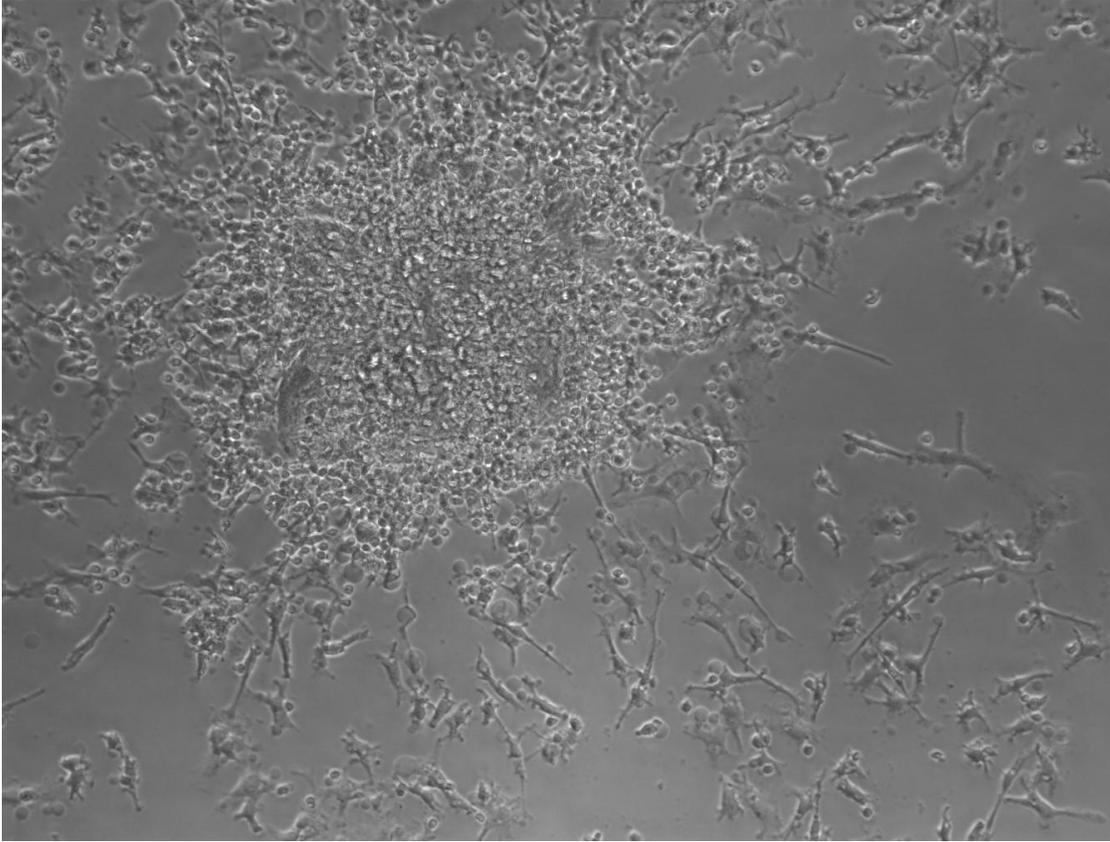


Figure 2.12: Hill CFU colony colony. Mag: x100. A central cluster of cells with surrounding adherent cells. Endothelial staining characteristics described in following figure Fig 2.13.

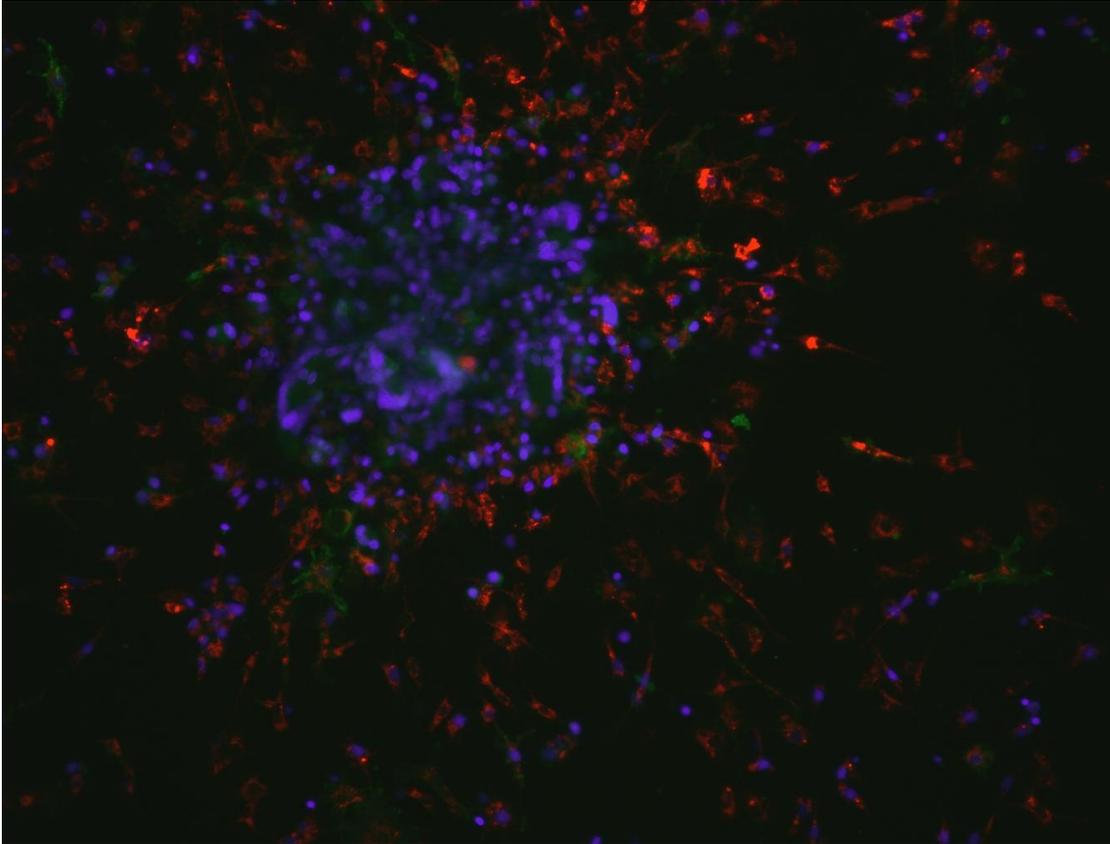


Figure 2.13: Hill CFU colony stained with DiI ac-LDL, UEA-1 lectin and Hoescht. Mag: x100. Cells located centrally within the colony do not appear to readily uptake the stains while those in the periphery and adhering around the colony do take up the stain indicating endothelial characteristics.

2.4.4 Alternate Isolation

In order to try to circumvent the technical difficulties involved with cell isolation from large volumes of whole blood an alternate protocol was explored which allowed direct isolation of a buffy coat from which expansion of an EPC population is more efficient. However, using this protocol cells isolated were morphologically too dissimilar from those obtained with the original protocol and from those in the literature and so this was abandoned.

2.5 Discussion

Before experiments directly focused on exploring the therapeutic benefit of EPCs were carried out, optimisation of cell culture conditions and characterisation of the cells had to be carried out.

However, different cell types have variously been referred to as EPCs and it is essential to identify which cell type is being referred to. The distinction made by Hirschi et al (Hirschi, 2008) is very useful here and defines three types of cell which may all fall under the umbrella term EPC. These cells are the colony forming unit-Hill (CFU-Hill) cells, the circulating angiogenic cell (CAC) and the endothelial colony forming cell (ECFC). This chapter has focussed primarily on the CAC with some work being done on ECFCs. CACs were focussed on as they were the cells which are most likely to have maximal therapeutic utility due to ease of isolation, culture and potential paracrine effects on therapeutic angiogenesis.

After optimisation of culture conditions, a population of cells expressing endothelial characteristics as shown by endothelial-specific uptake and binding of ac-LDL, UEA-1 lectin and antibodies to endothelial markers could reliably be obtained from both human and rabbit blood. However, although a profile of characteristics of EPCs has emerged there is as yet no definitive way to characterise an EPC. Since mature endothelial cells and myelomonocytic cells share many of these characteristics the possibility of contamination with these cells which may be found in low numbers in the circulation cannot be ruled out. Initially markers such as the stem cell marker CD133 or the monocyte marker CD14 may be used to exclude mature cells and, indeed, cell populations may be selected based on these markers, but their expression diminishes over 7 days of culture. Positive staining of these cells for CD34, KDR and vWF shows an endothelial-like phenotype while some remaining expression of CD133 indicates a stem cell lineage.

Regarding ECFCs, they may be differentiated from mature endothelial cells based on clonogenic or proliferative potential as ECFCs are capable of more population doublings.

In general CAC populations appear to have very low proliferation rates and begin to die off after 7 days. There have been reports of the emergence of early EPC colonies but whether these are true colonies or merely represent clusters of individual cells remains unclear. Due to the lack of endothelial staining observed in the putative colony the identity of these cells must also be questioned.

As mentioned, CACs emerged as the cell of choice to work with in future experiments. These cells could be reliably cultured from both human and rabbit peripheral blood and showed staining consistent with an endothelial-like phenotype. These cells appear to contribute to neoangiogenesis, primarily through secretion of pro-angiogenic factors rather than direct incorporation into vessels. They have limited proliferative potential and appear to exert maximum effect after 4-10 days of culture. Thus, they have previously been referred to as early EPCs.

As no definitive definition of an EPC exists it is necessary to characterise to some extent the nature of the cells under investigation. The CACs described here, which may represent a type of early EPC, have been shown to display endothelial characteristics and have been isolated from peripheral blood in a manner comparable to much of the published literature. Similarly, the ECFCs, which may represent a more homogenous cell population, have been isolated, cultured and characterised and they too display endothelial characteristics. In conclusion then, this chapter demonstrates the reliable isolation, culture and characterisation of the CAC which is comparable to early EPCs described in much of the published literature and this work validates the choice of the CAC for the work in the succeeding chapters.

3. Comparison of Viral and Non-Viral Vectors for Gene Transfer to Human Endothelial Progenitor Cells.

Kealy, B; Liew, A; McMahon, J.M; Ritter, T; O'Doherty, A; Hoare, M; Greiser, U; Maenz, M; O'Shea, C; Barry, F; O'Brien T. Tissue Eng Part C Methods. 2009 Jun;15(2):223-31.

3.1 Abstract

Background/Aims: The ability of endothelial progenitor cells (EPCs) to home to sites of neoangiogenesis makes them attractive candidates for use in the field of gene therapy as gene delivery vehicles. The efficacy of this approach depends on the efficiency of the vector used for transgene delivery.

Methods/Results: In this study we have compared the efficiency of adenovirus, 5 serotypes of AAV2, VSVG-pseudotyped lentivirus and non-viral plasmid DNA vectors to deliver the GFP reporter gene to human early EPCs to determine efficacy and vector-related cell toxicity. Adenovirus proved most effective with efficiencies of up to 80% with low levels of cell death. Lower levels of expression were seen with other vectors. Electroporation proved unsuitable at the parameters tested. We have also identified at least 2 distinct subpopulations that exist in the heterogeneous parent EPC culture, one of which is amenable to transduction with adenovirus and one which is not. In addition, adenoviral transduction did not disrupt the ability of the cells to incorporate into endothelial structures in vitro.

Conclusion: In conclusion, we have found adenovirus to be the most efficient of the vector systems tested for gene delivery to EPCs, an effect which is mediated almost entirely by one of two identified sub-populations.

3.2 Introduction

Since being first described in 1997 by Asahara et al. there has been increasing interest in the therapeutic use of endothelial progenitor cells (EPCs) in a range of models including carotid artery re-endothelialisation (Kong, 2004; He, 2004, Asahara, 1997), hindlimb ischemia (Kalka, 2000) and myocardial ischemia (Kawamoto, 2001). According to the more traditional view of blood vessel formation, new vessels sprout from existing vessels, a process termed angiogenesis. Vasculogenesis, the formation of *de novo* blood vessels, once thought to be restricted to prenatal vessel formation, has been observed in the adult with endothelial progenitor cells homing to sites of neoangiogenesis. These cells are believed to originate from bone-marrow (Asahara, 1999) and more recent evidence suggests they could also be resident in existing blood vessels (Ingram, 2005). Broadly speaking, there are two main types of EPC described: the early population which appear within the first week of culture (which may be termed circulating angiogenic cells (CACs)) and the late or outgrowth population which appear around 2-3 weeks (which may be termed endothelial colony forming cells (ECFCs))(Liew, 2006). Although *in vitro* differences have been observed between the two types, *in vivo* therapeutic effect has been shown to be comparable (Hur, 2004).

The homing ability of these cells makes them attractive candidates for use in gene therapy after they have been transduced to specifically target areas of disease and carry potentially therapeutic genes including to tumour cells which have a high rate of neoangiogenesis (Jevremovic, 2004). Their engraftment into sites of vasculogenesis could provide a more sustained level of gene expression over a longer period of time. To date various studies have been carried out in which EPCs have been genetically modified using plasmid DNA (Lin, 2002), adenovirus (Murasawa, 2002), lentivirus (Herder, 2003), but to our knowledge there has been no direct comparison of vector efficiency or resulting vector-related cytotoxicity. In addition, no consensus yet exists as to the definition of an EPC and many studies use different culture conditions, which further complicates attempts at direct comparison.

In this study we have used both viral and non-viral vectors to deliver a reporter gene to human early EPCs (CACs). These cells have comparatively low proliferation rates meaning cell numbers isolated from each donor or available for autologous transplantation may be limited, a fact that potentially makes gene transfer to these cells all the more relevant. Indeed, it has already been shown that gene transfer has substantially lessened the number of cells required *in vivo* to produce comparable therapeutic effect in a murine model of hindlimb ischaemia using CACs transduced with the VEGF 164 gene (Iwaguro, 2002). Moreover it has been shown that EPCs may be dysfunctional in disease states such as diabetes mellitus (Tepper, 2002) and, here too, gene transfer is a potential method to attenuate dysfunction in these cells for purposes of autologous transplantation.

Thus, we present a comparison of different viral and non-viral vectors for gene delivery to human early endothelial progenitor cells with consideration of the effect of the procedures on cell survival and subsequent functionality.

3.3 Methods

3.3.1 Patient characteristics

All procedures were carried out with approval from the ethics committee at University College Hospital, Galway. Peripheral blood was obtained from patients aged between 25 and 65 years undergoing venesection as part of treatment for hemochromatosis after informed consent was given. Previously published studies have used similar patient cohorts as normal controls in analysis of EPCs isolated from peripheral blood (Rad, 2007). Only non-diabetic patients who were not taking medications and who were in a maintenance treatment program to ensure uniform low levels of iron in their blood were included in the study. These patients were otherwise healthy and had ferritin levels of less than 50ng/ml (normal range 10-200) and were not anaemic. 3 donors were used for each vector and experiments were performed in triplicate for each donor.

3.3.2 EPC isolation and culture

Cells were isolated using a modification of previously described techniques (He, 2004; Kalka, 2000; Bahlmann, 2005; Michaud, 2006). Whole blood was diluted in a 1:1 ratio with HBSS (Sigma) containing 1mM EDTA and 5%BSA and peripheral blood mononuclear cells were isolated by density gradient centrifugation by gently layering diluted blood over Ficoll Paque Plus (Amersham). Mononuclear cells (MNCs) were then washed in PBS and plated on 12-well plates coated with human fibronectin (Sigma) at a density of 1×10^7 cells/well. The cells were maintained in EGM-2 medium (Cambrex) supplemented with 2% FBS, hEGF, hydrocortisone, gentamicin, amphotericin-B, VEGF, hFGF-B, R3- IGF-1, ascorbic acid and heparin and incubated at a temperature of 37°C and 5% CO₂. Medium was changed at day 2 and daily thereafter. After 7 days, cells were harvested using standard trypsinisation procedures. Endothelial phenotype was determined by uptake of acetylated LDL (Molecular Probes) and binding of UEA-1 lectin (Sigma), as previously published (Kalka, 2000; Ii, 2005; Hristov, 2004; Rehman, 2003; Suh, 2005) and expression of endothelial markers KDR and von Willebrand Factor (vWF) . Staining and analysis were carried out as previously described (He, 2004).

In this study we also transduced 10 day EPCs and endothelial colony forming cells (ECFCs) (also termed outgrowth EPCs) with adenovirus and compared MOIs of 100 and 1000 but these cells were not used for comparison of vectors. Only 7 day cells were used for this purpose. 10 day cells were isolated as above, the only difference being culture was maintained for 10 days.

ECFCs were cultured as above initially and maintained in culture for 6-21 days and grown to confluence. Cells were maintained for 2-4 weeks to allow colonies to appear. Colonies appearing at 2-3 weeks appeared to have the best proliferative rate and any colonies appearing after 3 weeks tended to have low proliferative potential.

3.3.3 Adenovirus

AdEGFP is a replication-deficient, E1- and E3-deleted adenovirus vector that expresses the enhanced green fluorescence protein (EGFP) gene under transcriptional control of the cytomegalovirus promoter (kind gift of Dr. A. Flugel, Max-Planck Institute of Neurobiology, Dept. of Neuroimmunology, Martinsried, Germany). The propagation and purification of recombinant adenovirus was performed in 911 cells as described previously (Fallaux, 1996). In brief, the 911 cells were transduced at a multiplicity of 5-10 and harvested after 36-48 h. The virus was released by five freeze-thaw cycles and purified using two CsCl-gradients. The banded virus was recovered, desalted over sephadex columns (Pharmacia) and stored in virus storage buffer after addition of 10% Glycerol at -80°C . Titration of the virus concentration after elution from the column was performed by plaque assay on the 911 cells (plaque forming units, pfu).

MNCs were seeded in 12 well plates at a density of 1×10^7 cells/well in 2 ml of EBM-2 medium with growth factors. After 7 days culture medium was removed and cells were incubated with AdEGFP at different virus/cell ratios (multiplicity of infection, MOI) of 100, 500 or 1000 in 500 μl medium for 1 hour at 37°C . Untreated cells served as control. Then supernatant was removed and cells were further incubated in fresh medium for 24 hours at 37°C . To enhance transduction efficiency AdEGFP was spun onto the cells by centrifugation for 60 min, at 2000g, 37°C . Then supernatant was removed and cells were further incubated in fresh medium for 24 hours at 37°C .

3.3.4 Adeno-Associated Virus

Pseudotyped AAV vectors (AAV2 genome) were generated using a dual plasmid transfection of HEK 293T cells with a recombinant plasmid containing the GFP transgene within AAV2 ITRs under control of the CMV promoter (pTRUF-2) (Zolotukhin 1999) and the appropriate plasmid for the various serotypes AAV-1, -2, -4, -5 and -6 (Grimm, 2003; Grimm 1998), using established protocols (Harris, 2003). Briefly, equimolar amounts of plasmid were transfected by calcium phosphate precipitation of 293T cells. After 48 hours, cells were harvested and lysed, releasing the

virus, followed by treatment with endonuclease (Sigma) to degrade non-encapsulated DNA. The clarified lysate was further purified by density centrifugation in iodixanol (Axis-Shield), crude virus was removed and applied to prepared affinity chromatography columns (Heparin or Q-Sepharose columns; Amersham). Purified virus was eluted and concentrated in a centrifugal filter device (Millipore) and stored at -70°C. Titration was performed by real-time PCR of viral samples, expressed as DNase-resistant particles (drp). \square 1-1, using a probe for the CMV promoter (Operon).

At day 7, medium was aspirated off cultured EPCs and the equivalent of either 5×10^4 or 2×10^5 drp/cell of pseudotyped AAV2/1, AAV2/2, AAV2/4, AAV2/5, or AAV2/6 in 1ml serum-free medium was added to each well. Plates were centrifuged at 1,200g for 90minutes at 34°C and replaced in a 37°C incubator with 5% CO₂ and 95% humidity. After 12-16 hours, 2ml complete EPC medium was added, without aspirating off the viral load. Cells were analysed for GFP expression at 3 days post transduction.

3.3.5 Lentivirus

Two rHIV-1-based lentiviruses were used in this study. Both were pseudotyped with VSVG envelope and encoded the GFP gene. PPT-PGK-GFP-WPRE, a 3rd generation vector containing the PGK promoter was produced by Genethon (Genethon ref: pG3.22116). PWPT-GFP, a 2nd generation vector containing the EF1- \square promoter, was produced in-house using the pWPT-GFP, pRSV-rev, pMD2.G and pMDLg/pRRE plasmids which were a kind gift from Didier Trono, Lausanne. Both virus vectors were produced using standard four-plasmid calcium phosphate transfection on 293T cells. Viral titres were determined by transduction levels in HeLa cells and correlated to ELISA for p24 viral protein. EPC culture medium was aspirated off and cells were transduced at MOIs of 1, 10 and 50 in 500 μ l of fresh media before being replaced at 37°C and 5% CO₂ for 1 hour. After 1 hour, 1ml of fresh media was applied and media was replaced after 24 hours. The cells were then maintained in culture for a further 48 hours before analysis.

3.3.6 Liposomes

EGFP-C1 plasmid (Clontech), which contains the GFP gene under the control of the CMV immediate-early promoter, was prepared using a Qiagen Endofree™ plasmid preparation kit, as per manufacturer's instructions.

Liposome complexes were formed using EGFP-C1 plasmid and Invitrogen Lipofectin reagent (Lipofectamine 2000). Prior to transfection, 10µl lipofectin reagent was mixed with serum-free media and 1µg plasmid was mixed with serum free media. These were incubated for 45 min before forming complexes. When lipid and DNA was mixed, it was left for a further 15 min. Media was removed from cells and complexes were mixed in serum-free media and overlaid on cells. Following incubation (6-24 h), complexes were removed and serum-containing medium was placed on cells for a further 48h. Cells were then harvested and fixed and analysed using Guava Expressplus assay.

3.3.7 Electroporation

EGFP-C1 plasmid (as described above) was used for the electroporation experiments. Cells were prepared from whole blood as per standard protocol and plated onto 12-well plates. Non-adherent cells were pipetted off and retained and adherent cells were harvested from well and added to stock of non-adherent cells. These were spun at 1500rpm for 5min.

Cells were resuspended in 400ml of basal EBM medium containing 20µg plasmid and placed in 0.4cm electroporation cuvettes. Cells were electroporated (according to parameters below) and allowed to sit at room temperature for 10min. Electroporated cells (and non-electroporated controls) were then plated onto fibronectin-coated 12-well plates, supplemented with EBM (complete) and monitored for expression at 24 and 48h.

Electroporation was carried out on a Biorad Gene Pulser Electroporator and electroporation parameters were chosen with reference to previously described techniques (Wu, 2001; Ear, 2001).

3.3.8 Analysis of gene expression and cytotoxicity

All cytometric analysis was carried out using the Guava Express Plus program on the Guava EasyCyte (Guava Technologies Inc, CA, USA) except the population separation experiments which were carried out on the FACS Canto flow cytometer (BD). Cells were harvested by trypsinisation and an equal volume of complete EGM-2 medium was added to stop the reaction. The cells were then centrifuged for 5 minutes at 300g and the pellet was resuspended in 250µl of PBS and 4µl of 7-amino actinomycin D (7AAD) fluorescent dye (Cell Technology Inc, CA, USA). This dye stains apoptotic and dead cells by permeating compromised membranes and binding to DNA (Philpott, 1996). Cells were again washed prior to fixation in 4% paraformaldehyde (250µl) at 4°C for 20 minutes. The cells were centrifuged again as described above and resuspended in 200µl of cell wash buffer (BD biosciences) for analysis of GFP expression and 7-AAD binding.

3.3.9 Matrigel tubule assay

Matrigel (Sigma) was thawed on ice and placed onto a 4-chamber glass slides (Nalgene Nunc) at 37°C for 30 minutes to allow solidification. 2×10^4 DiI-acLDL labelled-EPCs (control or transduced with adenovirus at MOI of 500) were co-cultured with 4×10^4 HUVEC (Passage 3) on the pre-plated matrigel. The number of DiI-acLDL EPCs incorporated to the tubule was determined in 5 random high power fields in duplicates. A tubule was defined as a structure exhibiting a length 4 times its width.

3.4 Results

3.4.1 EPC culture

After 7 days in culture, a monolayer of cells exhibiting heterogeneous morphology developed on the fibronectin substrate (Figure 3.1). These cells were found to stain positively for uptake of DiI-labeled acetylated LDL and UEA-1 Ulex lectin (Figure 3.2) and were deemed to be early EPCs (CACs) as per convention (Vasa, 2001). Positive staining was also observed on the cell surface for the endothelial marker KDR and the haematopoietic marker CD34 (Figure 3.3).

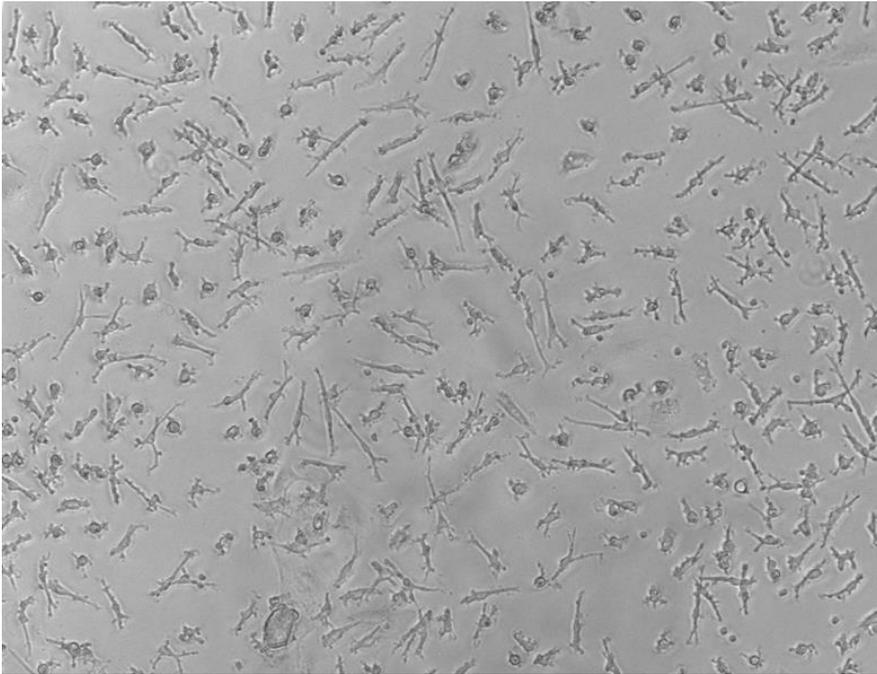


Figure 3.1: Phase contrast images of hEPCs at 7 days displaying heterogeneous morphology. 1×10^7 peripheral blood mononuclear cells were plated on a fibronectin substrate and maintained in culture for 7 days. Mag: $\times 100$.

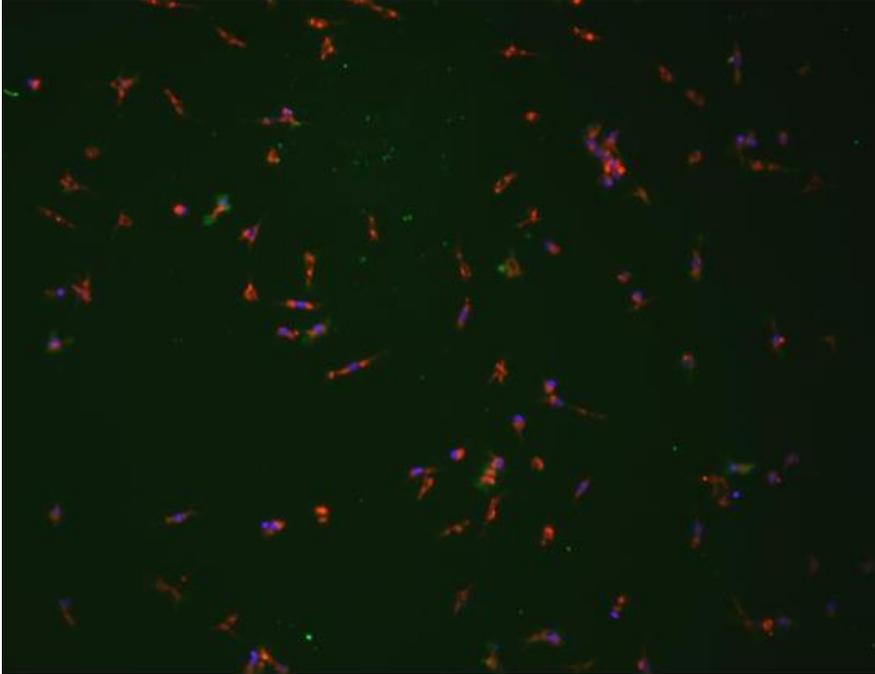


Figure 3.2: 7 day EPCs showing binding of UEA-1 lectin and incorporation of DiI-labeled ac-LDL.

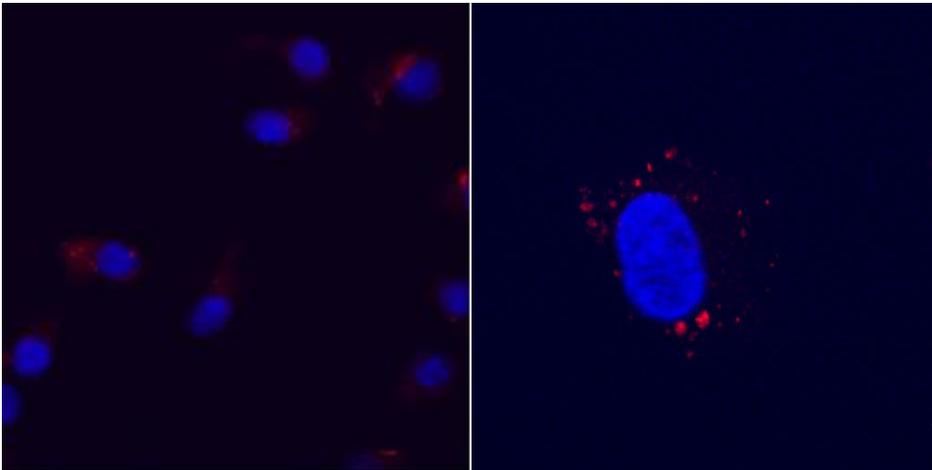


Figure 3.3: Immunofluorescence confocal images of EPCs after 7 days in culture displaying positive staining for A) anti CD-34 and B) anti KDR after antibody labelling. Nuclei counterstained with Hoechst.

3.4.2 Adenovirus

Of all vectors tested in this study adenovirus proved the most efficient at transducing human EPCs. GFP expression was observed in cells using direct fluorescent microscopy (Figure 3.4) as well as flow cytometry with efficiencies of up to 80% and very low levels of cell death (<1%). Using standard transduction methods, cells expressed GFP at levels up to about 15% (average 12.6 +2.3%) at MOI of 1000 with a dose dependent response relating to different MOIs. However the transduction efficiency was increased 5-fold with an average of 54.4 (+13.3)% by the incorporation of a centrifugation step which also largely abrogated the dose dependant response (Figure 3.5). Data represents combined results of experiments carried out in triplicate with n=3 per experiment for each MOI.

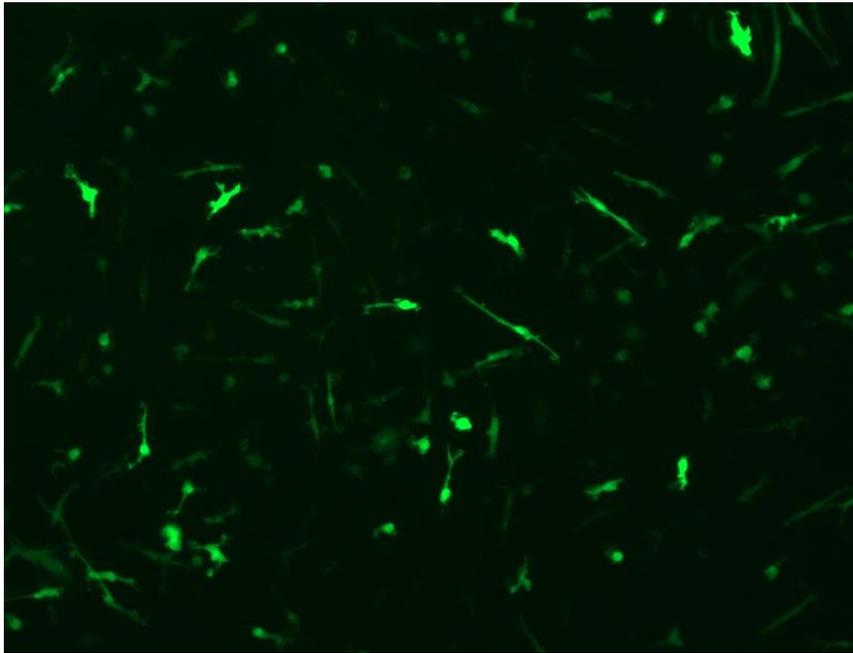


Figure 3.4: EPCs transduced with adenovirus (MOI of 500) expressing GFP after correction for autofluorescence from negative controls. Mag: x100.

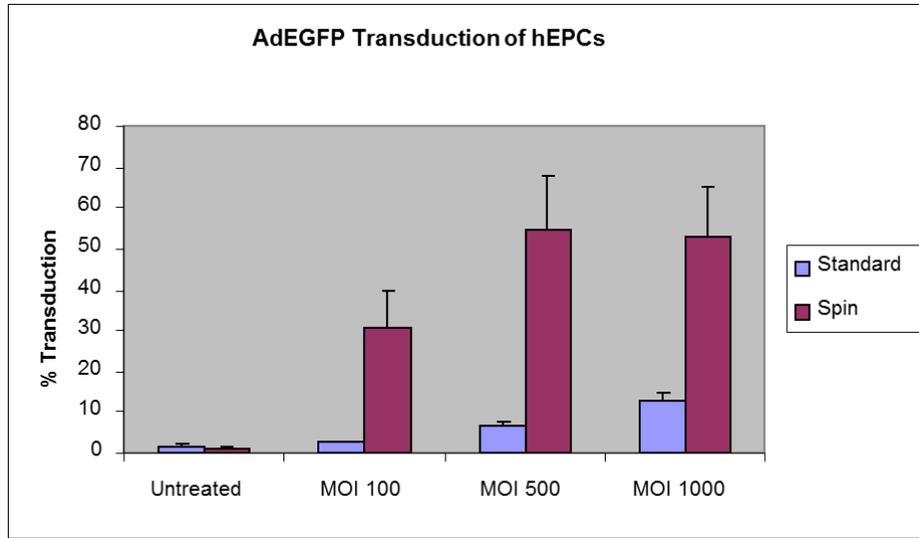


Figure 3.5: Comparison of percentage of hEPCs expressing transgene after adenoviral transduction using standard or spin protocol. GFP expression is shown for both MOI of 500 and 1000 with comparison between standard transduction protocol and protocol including centrifugation for 1h at 2000g. Representative of experiments where n=3 performed in triplicate with error bars expressed as standard error of the mean

3.4.3 Cell Subpopulations

In addition, 2 sub-populations of cells were identified in the original parent population, separated on the basis of cell size. Transduction efficiencies of these sub-populations were investigated using adenovirus as it proved to be the optimal gene transfer protocol. Of these subpopulations, one population (designated as sub-population 1 and which comprised about 31 (+2.1)% of the total population) showed remarkable resistance to transduction with adenovirus, with less than 1% of cells expressing the transgene (Figure 3.6a) while the second population (sub-population 2) was very effectively transduced by adenovirus showing up to 87.7 (+2.2)% expression at an MOI of 500 (Figure 3.6b) with minimal cell death comparable to sub-population 1.

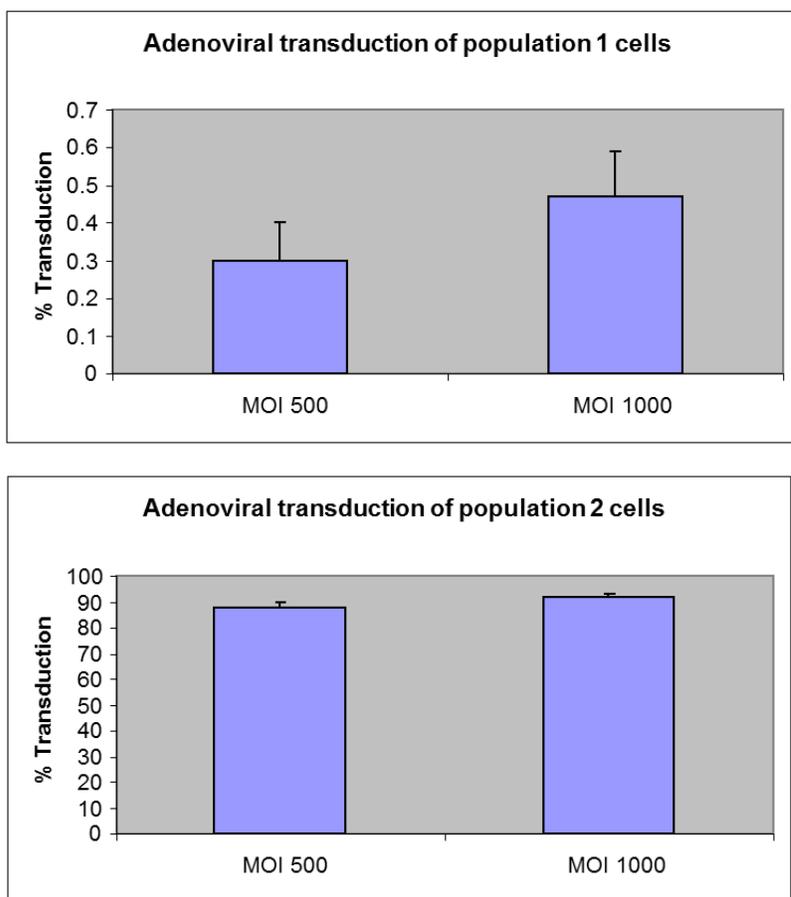


Figure 3.6: Comparison of transduction efficiencies for 2 separate subpopulations in parent EPC population after cytometric sorting based on cell size. A) Percentage transduction of sub-population 1 cells after adenoviral transduction. B) Percentage transduction of sub-population 2 under similar conditions. n=3 for each MOI and all experiments performed in triplicate. Error bars represent standard error of the mean.

3.4.4 Adeno-associated virus

Surprisingly, we observed substantial variation in AAV efficiencies between repeated experiments with this pattern being displayed by all serotypes tested. However, this variation was much more pronounced over experiments involving different donors. Intra-experimental variation, where 3 samples were generated from the one donor, was greatly reduced in comparison. The greatest variation observed was in AAV2/1 experiments with inter-experimental standard error being as high as 10.5%. However, over 3 different experiments the average intra-experimental standard error was much lower at 3.3(+0.3)%. Overall, the highest expression obtained for this vector was using AAV serotype 2 with efficiency up to a maximum of 38.89% with the average over 3 series of experiments (n=3 for each experiment) being 25.32 (+ 7.29)% with DNase resistant particles (drp) of 200,000. Increases in drp from 50,000 to 200,000 did not show proportional increases in GFP expression levels although some increase was seen with serotypes 2, 5 and 6 (5.68, 5.86, 8.09% respectively) (Figure 3.7).

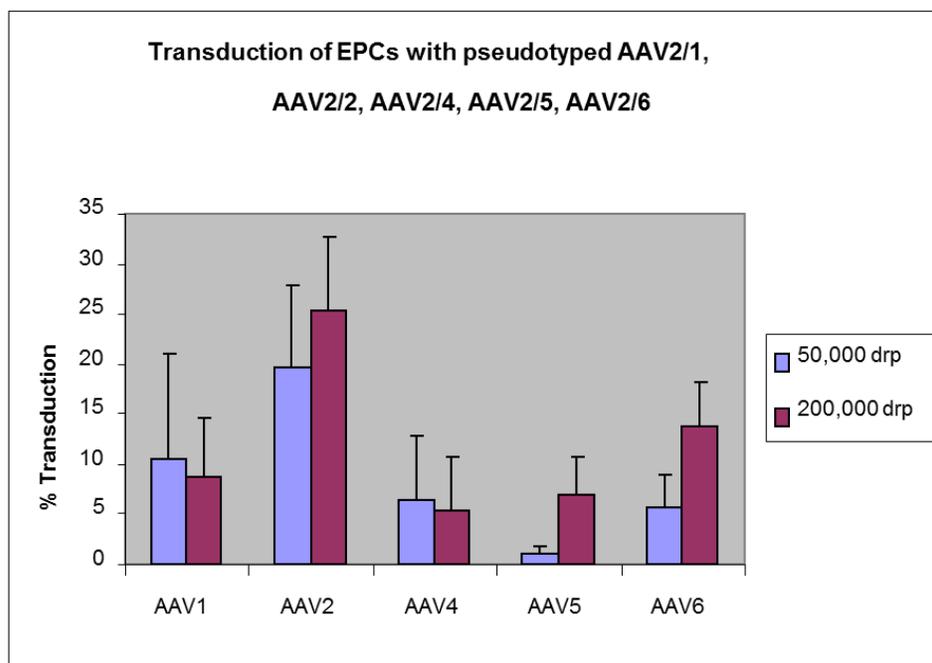


Figure 3.7: Percentage of hEPCs expressing transgene after transduction with two different titres of AAV (50,000 and 200,000 drp) for AAV serotypes 1, 2, 4, 5 and 6. All experiments performed n triplicate with n=3 per experiment. Error bars represent standard error of the mean.

3.4.5 Lentivirus

The HIV-1-based, VSV-G pseudotyped lentiviruses used in this study showed relatively low levels of transduction with maximum expression at an MOI of 50 and little improvement at higher levels. Using a PGK promoter driven construct, a dose dependent response was seen with increasing virus titres of 1, 10 and 50 with cell death levels being minimal (<1% due to exposure to vector). The maximum expression seen in a single experiment was 36.9% with the average result from 2 different experiments being 25.8 ±(11.1)%. Here, again, substantial variation was seen over the entire series of experiments. At an MOI of 50, standard error was as high as 11.1% across 3 different experiments while the average standard error within these experiments was 2.28% (Figure 3.8). Using the EF1 α promoter almost no gene expression was measurable, this result being confirmed by direct fluorescence microscopy. However, in HeLa cells transduced with this virus, efficiencies of up to 100% were observed at an MOI of 10 (data not shown).

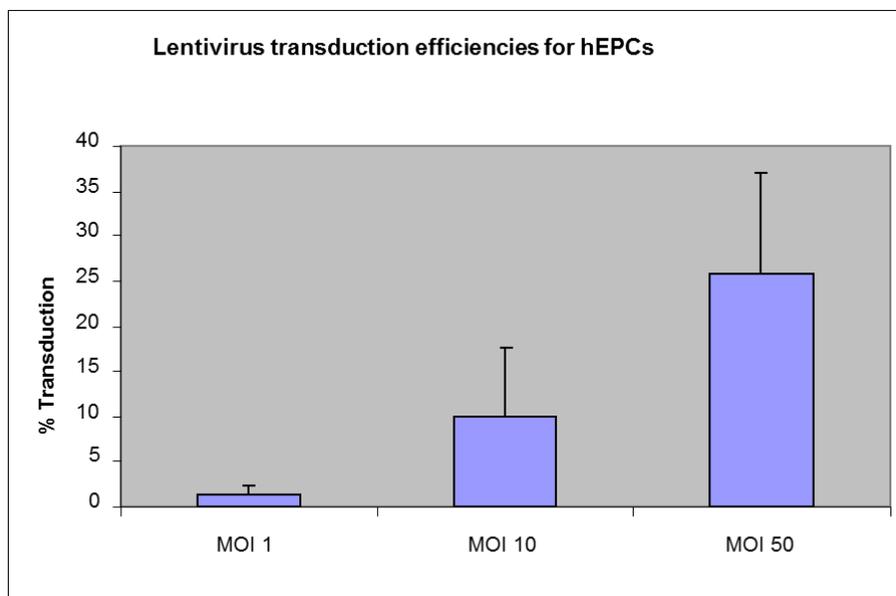


Figure 3.8: Percentage of transgene positive hEPCs after transduction with increasing titres of VSVg pseudotyped lentivirus. Error bars show standard error of the mean. All experiments performed in triplicate. n=3 per experiment.

3.4.6 Liposomes

Liposomal gene delivery reliably transfected cells although at low levels. Expression was evident in 29.2+(1.1)% of cells after 6 hours with similar levels being observed after 24 hours (Figure 3.9). Uptake of plasmid alone was negligible when compared to levels achieved with the use of Lipofectin.

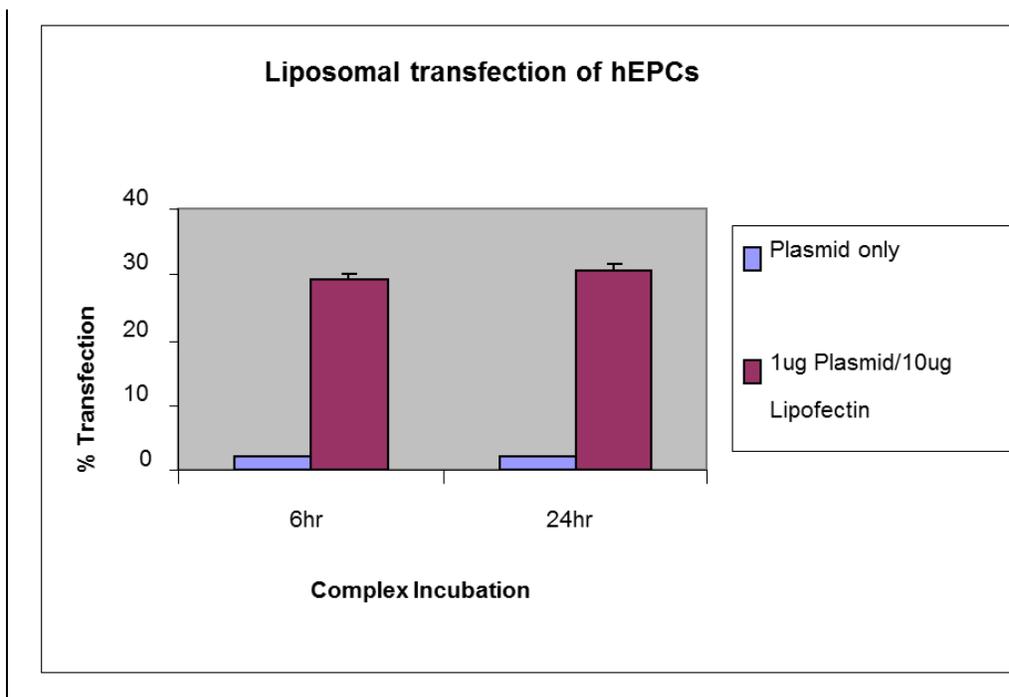


Figure 3.9: Percentage increase of hEPCs expressing GFP transgene after plasmid gene transfer using lipofectin vs plasmid alone.

3.4.7 Electroporation

Electro-transfer techniques were found to be unsuitable for the cell populations studied. Increasing voltages led to high cell death with very little transgene expression observed in any remaining, viable cells (data not shown).

3.4.8 Endothelial tubule formation

When examined for ability to incorporate into endothelial tubules, there was no statistical difference between the mean number of incorporated EPCs in control group and group transduced with adenovirus at MOI of 500 (Mean \pm SEM: 10.9 \pm 0.6 vs 11.1 \pm 1.0; p=0.87) suggesting that the transduced EPCs did not exhibit in vitro functional impairment (Figure 3.10). Figure 3.11 shows GFP positive EPCs incorporating into endothelial tubules after co-culture with HUVECs at a ratio of 1:2.

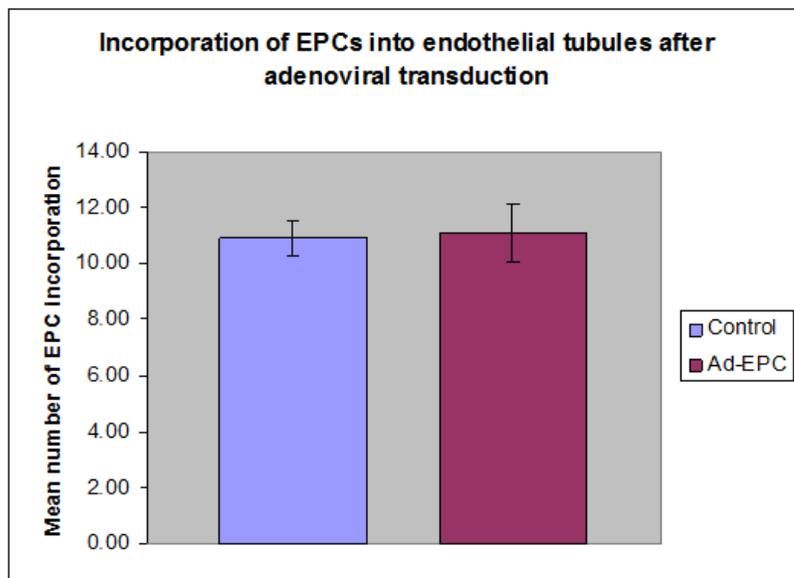


Figure 3.10: Comparative incorporation of transduced and non-transduced EPCs into endothelial tubules formed on Matrigel by HUVECs after EPCs were exposed to adenovirus at MOI of 500.

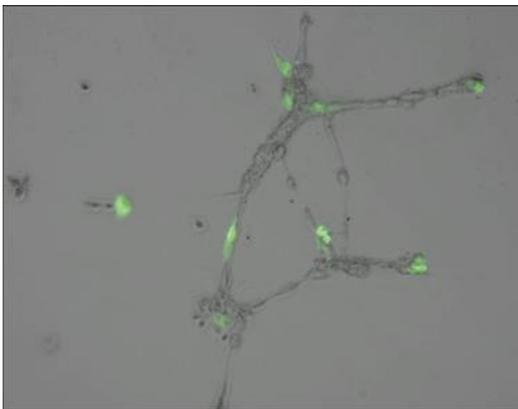
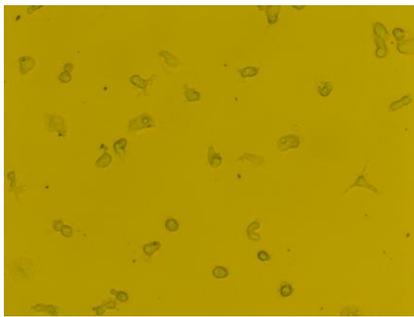


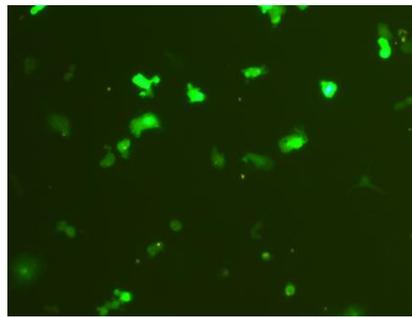
Figure 3.11: Incorporation of EPCs expressing GFP into endothelial tubules after co-culture with HUVECs in Matrigel. EPCs were transduced with adenovirus at an MOI of 500.

3.4.9 10-day EPC Transduction

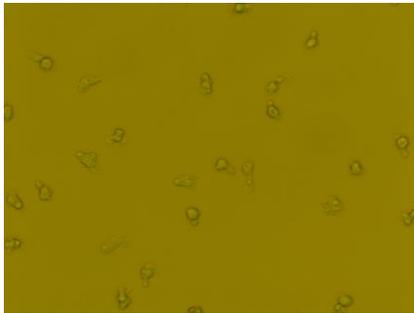
In order to determine the optimal time for transduction of early EPCs different time-points were explored. Although efficient transduction was seen at 10 days when cells were transduced with adenovirus at MOIs of 100 and 1000 the morphology of the cells raised questions about their viability and indeed, the efficiency of transduction may have been associated with compromised membrane integrity. 7 day cells were used for transduction studies as discussed in the next chapter.



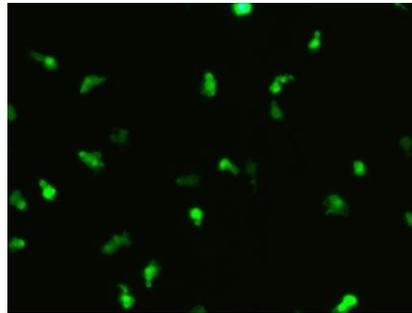
A: 100 MOI x200



100MOI x200 GFP



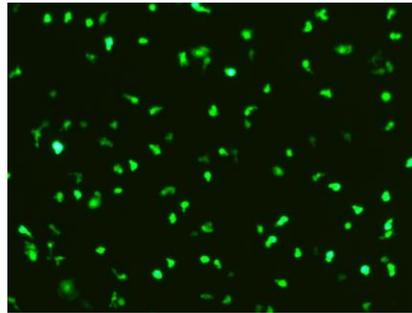
B: 1000 MOI x200 GFP



1000 MOI x200



C: 1000 MOI x 100



1000 MOI x 100 GFP

Figure 3.12: 10-day CACs expressing GFP after adenoviral transduction at MOIs along with background bright fields images. A) MOI 100 at Mag: x200, B) MOI 1000 at Mag: x200, C) MOI 1000 at Mag 100. Images with and without fluorescent filters show good transduction efficiencies, however the morphology of the cells suggests that the cell membranes may be compromised which could lead to artificially high transduction rates.

3.4.10 ECFC Transduction

Adenoviral transduction of ECFCs at MOIs of 50 and 100 showed efficiencies of close to 100% with low cell cytotoxicity. As previously mentioned we used CACs for the remaining experiments in this thesis due to the relative uncertainty of expanding ECFC populations from a given sample of blood, particularly with small volumes. These cells appear to circulate at low concentrations in unmobilised peripheral blood and so the probability of isolating clonogenic cells from a given sample of blood made them unattractive candidates for use in the pre-clinical model in the rabbit. In addition, expansion of colonies of ECFCs appears particularly difficult from rabbit species compared with porcine, murine or human. Mobilisation of blood with cytokines such as G-CSF was not employed as this would lead to ambiguity as to whether the applied treatment or endogenous cell mobilisation from the bone marrow was responsible for any benefit seen in the *in vivo* model.

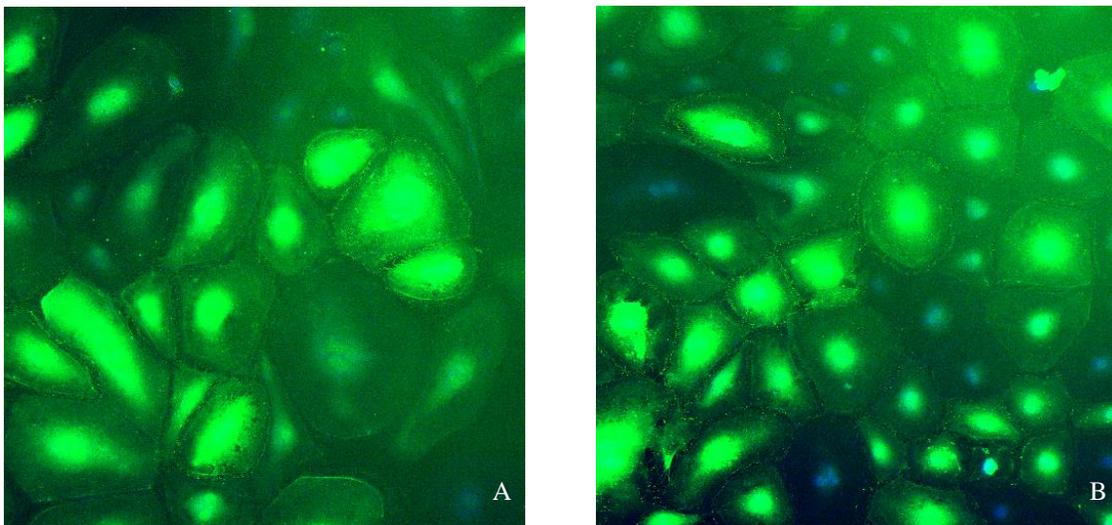


Figure 3.13: ECFCs overexpressing GFP after adenoviral transduction at MOI A) 100, B) 50.

3.5 Discussion

Gene therapy has, for some time, been the subject of intensive research in a variety of areas but one of the major challenges remaining is the identification of the most suitable and efficient delivery vehicle for the transgene of interest. This will vary depending on the cell one is attempting to transduce. Ex vivo gene therapy has been used in human conditions such as SCID. Culture of CACs from diseased individuals may allow restoration of cell function or indeed augmentation of therapeutic efficacy if the cells are transduced with a therapeutic gene. The homing abilities of stem cells have made these cells attractive candidates in this regard and embryonic (Strulovici, 2007), hematopoietic (Doering, 2007) and mesenchymal (McMahon, 2006) stem cells have all been considered. However, these cells may be lacking in specific targeting ability when introduced in vivo when the transgene and its products are required at certain locations. Progenitor cells, on the other hand, have already undergone some degree of differentiation and have been shown to home quite specifically. In particular, endothelial progenitor cells have been found to have the ability to home directly to areas of endothelial and vascular damage and neoangiogenesis (Kawamoto, 2002), making them particularly attractive for use as agents to deliver gene products after being genetically modified when only selected areas are to be targeted.

While there have been studies carried out to investigate the use of EPCs overexpressing a transgene of interest (Lin, 2002; Murasawa, 2002; Iwaguro, 2002; Zhao, 2005) there has not, to our knowledge, been a direct comparison of different vectors to determine the most effective method of genetic modification of these cells. The field is further confounded by the lack of a consensus on the exact nature of these cells and the different culture conditions used for propagation. Efficient gene transfer with minimal damage to the cells must be considered when looking at EPCs in terms of a gene delivery vehicle and in this study we have compared both viral and non-viral gene delivery systems to deliver the GFP reporter gene to cells cultured under identical conditions. Different transduction protocols have been used for different vectors based on preliminary studies to determine the optimum MOI ranges and time-points for analysis for each vector used.

These protocols differ owing to the fact that different MOIs are required to achieve similar transduction levels when using different vectors in a particular cell type. From the results obtained, adenovirus seems to be the most efficient vector with efficiencies as high as 80% observed. Previous reports have suggested the need for the use of high MOIs (up to 1000) (Iwaguro, 2002) but these results are consistent with results we obtained using a standard transduction procedure in which a dose dependent response was seen with efficiency levels only rising to about 15% at high MOIs (1000). However, when a centrifugation step was introduced efficiency levels rose dramatically averaging about 50% across a series of experiments with the dose dependent response not being seen above an MOI of 500, and with minimal levels of cell death. It is possible that the use of the centrifugation step allows a physical proximity between virus and cell which bypasses the reliance on high levels of CAR receptor expression on the EPC. This receptor is used by the virus for loose binding to the cell surface (Bergelson, 1997), which is provided for by the physical force of the centrifugation, thus making the adenovirus vector more reliant on the integrin family of cell surface heterodimers (Wickham, 1993) for internalisation. However, this is speculative as we are not aware of any study examining the expression levels of CAR on EPCs. In addition, the low proliferation rate of these cells negates one of the inherent disadvantages of using adenoviral mediated genetic modification in other more proliferative cell types. Since nucleic acid delivered via adenoviral vectors remains extrachromosomal it offers more control over production of transgene-encoded protein as it does not involve genetic modification of the chromosomes which could be passed on in dividing cells. However, this may prove to be disadvantageous in the use of adenovirus for gene therapy as it can lead to a “diluting out” effect of the transgene which can prove to be undesirable if rapidly dividing cells are targeted but this problem is not of such concern in cells that do not rapidly proliferate such as early EPCs.

Lentivirus has been shown to efficiently transduce non-dividing cells (Trono, 2000), which makes it seem an attractive candidate for gene transfer to low proliferative, early EPCs. However, our results have shown lower transduction efficiencies using this vector than with adenovirus. We have shown transduction levels of up to 37% at an MOI of 50 with minimal cell death due to the virus seen. However, overall efficiency of lentivirus

transduction was inconsistent with fluctuating levels between different experiments and overall levels being in the region of 25% at an MOI of 50. In contrast to the study published by Liu et al. we observed a higher efficiency of transduction using the PGK promoter than with the EF1 α promoter (Liu, 2006). In this study a centrifugation step was included in the transduction protocol which may account for the higher overall gene expression rates observed. We did not include such a step as prior experiments with rat MSCs showed that this technique conferred no advantage with lentiviral vectors (data not shown). This raises interesting questions concerning the method of entry of lentivirus vectors to different cell lines from different species.

AAV has many advantages for gene transfer such as absence of vector toxicity and prolonged transgene expression. AAV mediated gene delivery to EPCs showed a large degree of variation in a series of experiments, with AAV 2/2 showing the highest levels at around 25%. An increase in *drp* made little or no difference with serotypes 1, 2 and 4. In addition, we observed a significant degree of variability in transduction efficiency using this vector but this variation was much more pronounced when cells from different donors were studied. In repeat experiments using cells from the same donor, efficiency of transgene expression was more consistent. Although a degree of species specificity has been shown using AAV (McMahon, 2006) the reason for this variation using cells from different donors is unclear. It is possible that it is due to low receptor expression as the same degree of variation was not seen with all serotypes tested. Indeed, it has been suggested that low levels of receptor expression account for variability observed between different donors in experiments to transduce hematopoietic progenitor cells (Ponnazhagan, 1997).

Although viral vectors are traditionally regarded as being more effective than non-viral vectors, we found comparable transgene expression efficiency in cells transfected using lipofectin for 6 hours to those transduced with lentivirus or AAV. Liposome mediated gene delivery to CACs resulted in transgene expression in up to 35% of cells after 24 hours accompanied by low rates of cell death. In future studies the use of nucleofectin could be explored to improve efficacy.

Enhancement of plasmid uptake by electroporation proved unsuitable for this cell type. Minimal gene transfer was observed at the parameters tested with high levels of cell

death. Membrane pore formation is influenced by the radius of curvature of a cell and more asymmetric cells have been found to be more amenable to permeabilization than more regular cells (Somari, 2000). CACs tend to attach strongly to a substrate such as fibronectin when in culture, allowing them to develop varied morphologies. However, when detached from the surface on which they have been growing, they adopt a much more uniform and rounded appearance. This may affect the ease with which the membrane can be permeabilized by an electric field with the voltages required to porate them being too high for the cell to tolerate. In addition, it has been shown that cells derived from a monocytic lineage were difficult to electroporate (van Tendeloo, 1998). CACs are also suspected to be of monocytic origin as evidenced by their expression of CD14 (Gulati, 2003). These monocytic characteristics may also go some way towards explaining the relatively low levels of cell death observed when using viral vectors and liposome-mediated gene transfer. If these cells are from a lineage concerned with defence *in vivo*, a certain level of robustness could be expected.

In terms of functionality, exposure of the cells to adenovirus appears to have little or no effect on ability of cells to incorporate into a 3-D endothelial structure as seen after co-culture of transduced CACs with mature endothelial cells growing in Matrigel. No difference was observed in levels of incorporation over untreated controls after transduction with the most efficient vector identified in this study. Proliferation of cells following gene transfer must also be considered. However, this mainly applies to actively proliferating cells. Early EPCs do not proliferate rapidly and indeed, after 7 days start to die off. Exposure to the gene delivery vehicle did not appear to increase toxicity but the proliferation of cells was not relevant to this cell type. Cells that undergo many divisions after exposure to gene delivery vehicles may experience a “diluting out” effect depending on the vehicle used which may essentially render transformation of the cells temporary. This is not of great concern with the early EPC population as transformation is likely to be for the duration of the life of the cell. This may have implications *in vivo* but this was not within the scope of the current study.

Although some variation in transduction efficiencies was observed using some of the vectors as described above (most notably AAV and lentivirus, with much less variation seen using adenovirus), it is interesting to note that these differences occurred between

experiments carried out using cells from different donors. Due to the low proliferation of early EPCs and the fact that they tend to die off after about 7 days, it was necessary to use successive cell preparations, isolated from different donors. However, cell samples from each donor were analysed in triplicate and data collected from only one donor shows much lower variation, suggesting donor variability is an important factor in CAC transduction. Although differences in EPC number and function have been reported to correlate with cardiovascular risk factors, for example, and to be impacted on by conditions such as CAD and diabetes (Tepper, 2002; Vasa, 2001; Hristov, 2006), the exclusion of such patients from this study reduce the likelihood of variation in behaviour of cells being due to such clinically relevant factors.

Basic morphology observed in a monolayer of cultured early EPCs suggests a heterogeneous population. However, no reliable markers have so far been identified to separate out different populations. In this study we have identified at least two different populations which we have separated by flow cytometry based on cell size. Morphologically, a spindle shaped cell type and a more cuboid cell type may be observed in the parent CAC population but whether these different types correspond to the different sized populations seen with flow analysis is unclear. As adenovirus had been found to be the most efficient vector for CAC transduction it was used to transduce both populations using the same protocol as previously described, and we noted a marked difference in their transduction efficiencies. It is clear from our data that one population, making up about 70% of the total population, is responsible for almost all observed expression of the transgene, while the second population does not appear to be easily transducible. In the absence of a specific marker to facilitate separation of these populations, the differing transduction efficiencies may provide a means by which they may be separated for further and individual analysis. These findings raise interesting questions concerning the relative characteristics of these different subpopulations as well as the differing transduction potentials but this was not the focus of the current study.

Viral transduction of CACs using adenovirus to transfer the GFP gene was examined at both 7 and 10 days and good efficiencies were found for both. However, at 10 days cells appear more rounded and less viable and it is possible that the high transduction efficiency is related to a compromised cell membrane. In addition, paraformaldehyde was

used as the fixative of choice as a decreased GFP signal was seen after methanol fixation which may have been associated with loss of the protein due to membrane damage after transduction. Use of day 10 cells was therefore abandoned for this study in favour of 7 day cells.

ECFC transduction efficiency was high and cells also showed very high viability making these cells excellent candidates for gene transfer. Of course, depending on the vector used, the gene of interest may not be integrated into the host genome and so the high proliferative rate of these cells must be taken into account when assessing gene expression as expression of the gene of interest may diminish with population doublings. Further gene transfer studies were not performed using these cells however, because they cannot be isolated with the same reliability as CACs. In addition there appears to be some inter-species variability regarding ease with which colonies may be obtained with rabbit cells being more difficult to isolate than human. There is also some doubt about the feasibility of growing robust colonies from diabetic donors. These considerations made these cells unsuitable for the proposed animal model described in following chapters.

In summary, this study has shown by comparison of both viral and non-viral vectors, that human early EPCs are most readily transduced by adenovirus. We have also shown that transduction and reporter gene expression do not have a detrimental effect on the ability of these cells to incorporate into an endothelial monolayer. Furthermore, we have identified 2 distinct populations within the heterogeneous parent population that show markedly different capacities for adenoviral transduction. This provides a useful procedure to facilitate the use of this cell type in conjunction with gene therapy in a range of studies.

4. Development of a Skin Ulcer Model in the Alloxan Induced Diabetic Rabbit

4.1 Abstract

In order to explore the therapeutic effects of EPCs on wound healing a suitable animal model is needed. The model chosen was the diabetic rabbit ear ulcer model. In order to develop this model many aspects had to be optimised including induction and confirmation of diabetes mellitus, creation of the wound itself as well as aftercare and deciding on the healing parameters which would be used for measurement. In this chapter epithelial height and thickness were the parameters under examination. Epithelial height and thickness were found to be unsuitable as there was a very large variability, meaning that a prohibitively large sample size would have been required, and other parameters were preferred as described later in this thesis. This chapter details the development of the model and outlines the methods used.

4.2 Introduction

The delayed healing characteristic of diabetic ulcers is a serious problem both in terms of increasing the length of hospital stay and in increased pressure on health care budgets (Harding, 2002). Strategies to overcome this have mainly centred on improved wound care and management but application of therapeutic products such as growth factors like PDGF, VEGF and bioengineered skin equivalents have also been investigated (Mustoe, 1991; Galiano, 2004). We have focused on a cell based therapy exploring the potential benefits of CACs or CACs transduced with Ad/eNOS, as described in the next chapter. In the current chapter we describe the development of an animal model which could be used subsequently to determine the effects of diabetes mellitus on wound healing.

In order to explore the utility of a cell therapy based approach to wound healing, a reliable pre-clinical model was needed. We used a model developed by Mustoe et al which resembles the human venous leg ulcer (Mustoe 1991). This was the rabbit ear model which also has benefits for investigation of wound healing due to the fact that healing occurs exclusively from the periphery rather than from underlying tissue and thus quantification of healing parameters is made easier. In addition, this model is non-contractile, a situation more analogous to lower extremity ulcer healing in humans which reduces variability and facilitates analysis of healing.

In establishing this model a number of issues were considered: how the wound model would be tolerated by the experimental animal; precautions necessary to ensure minimal interference with wound healing; mode of delivery of cells; processing of tissue; and induction of diabetes mellitus. Also to be determined were the optimal healing parameters to be used. It was necessary to have a model in which the wound site would not be disturbed by the animal but which would restrict as little as possible the normal healing process.

Healing was analysed both macroscopically and histologically seven days after the creation of the wound. A pilot study was carried out to determine the number of sections

needed to be analysed to produce meaningful results. As the strength of the healed epithelium is of great clinical importance, the epithelial thickness and epithelial volume relative to the volume of underlying tissue were measured. Wound height, that is the distance between the cartilage and the surface of the epithelium, was also measured and wound area was measured macroscopically.

This chapter describes the process of development and optimisation of the wound model as well as the previously mentioned pilot study to determine the most useful parameters by which to measure the degree of healing and the assessment of those parameters.

4.3 Methods

Male New Zealand White rabbits weighing 2.5-3 kilograms were used and maintained with standard laboratory chow and water *ad libitum*. After delivery, animals were allowed one week to acclimatise before any procedures were carried out. All procedures were approved by the animal ethics committee of the National University of Ireland, Galway.

4.3.1 Induction of diabetes mellitus

In order to induce diabetes, 150mg/kg of alloxan (Sigma) in solution was infused intravenously over 30 minutes at a rate of approximately 1ml/min. Only animals showing a sustained elevated blood glucose level of greater than 12mmol/L were used in the study.

4.3.2 Blood collection

After sedation with acepromazine (Novartis, 0.1ml/kg) the dorsal surface of the ear was shaved and Emla cream or Xylocaine spray containing lidocaine (AstraZeneca) was applied to minimise discomfort. The ear was then disinfected with an alcohol swab. The central ear artery was cannulated and blood collected into 6ml heparinized tubes (Beckton Dickenson). Pressure was then applied to the point of entry until bleeding stopped. Normal saline was then administered to prevent hypovolaemia.

For terminal bleeds the animal was anaesthetized using ketamine (Narketan 10, Vetoquinol; 0.35ml/kg) and Xylazine (Xylapan Vetoquinol; 0.25ml/kg) and exsanguinations performed by cannulation of the carotid artery. Incision was made using a scalpel along the midline of the throat and the skin pulled and clamped open. The artery was dissected out from the connective tissue and sutures placed around the proximal end from the point of entry to control flow. Clamps were placed at both ends before the

cannula was inserted. The proximal clamp was then released and blood collected into 6ml heparinized tubes. After blood flow stopped the animals were euthanized by administration of 2mls of sodium pentobarbital into the lateral ear vein or direct cardiac injection.

4.3.3 Wound Induction

Animals were anaesthetized as above and placed on isoflourine gas for the duration of the surgery. All wounds were made using a disposable 6mm sterile biopsy punch (trephine, Kai Medical and Kruuse). Three wounds per ear were made by cutting down to the cartilage and the overlying skin removed using a scalpel and forceps. Excessive bleeding was taken as a sign of injured cartilage and skin was not removed from these wounds nor were they used for the study. The wounds were photographed and covered with Opsite (Smith and Nephew) film dressing which was secured with silk tape. Initially, cotton swabs were inserted to add support and ears were bandaged using elastic tape to prevent interference during healing. However, the tape appeared to restrict blood flow to the ear and on harvesting of the tissue ischaemia was observed. Therefore the practice was discontinued and thereafter opposing edges of the ear were then sutured together using 2-3 sutures per ear. Following surgery carprofen (Rimadyl, Pfizer; 0.08ml/kg) was administered to alleviate any post-recovery pain. Animals were then returned to their housing and allowed to recover on vedbed bedding. After recovery collars were placed around their necks to minimise interference.

4.3.4 Tissue Harvesting

Animals were anaesthetised with xylazine (0.25ml/kg) and ketamine (0.35ml/kg) and then euthanized by administration of 2mls of sodium pentobarbital either into the lateral ear vein or by direct intra-cardiac administration. Wounded tissue sections were then cut out from surrounding tissue and the dorsal skin peeled away from the cartilage. Tissue

samples were then mounted on cardboard and immediately fixed in 4% paraformaldehyde and kept at 4°C until processing.

Processing was done in a Leica ASP 300 processing machine on the routine overnight setting. Samples were then embedded in paraffin wax 5µm sections were cut on a Leica microtome after being chilled on a coldplate.

4.3.5 Measurement of healing parameters

In order to measure epithelial height a grid was placed randomly over an image of a tissue section and the height measured perpendicularly from the cartilage to the outer surface of the epithelium at regular intervals. The same technique was employed to measure epithelial thickness with the perpendicular measurement being taken between the upper and lower edges of the epithelium. Grid squares were used to measure the relative areas of epithelium and connective tissue per field. Each section was divided into regions which were adjacent to the healing edges of the wound or outside the wound area as normal controls.

Cumulative frequencies of epithelium and connective tissue were calculated and from these the cumulative ratios were obtained. A range was then described being +/-5% of the cumulative ratio. The minimum number of fields per section needed to be measured could then be determined as the number whose cumulative ratios lay outside this range.

Wound area was measured by capturing digital images of fixed tissue sections and tracing around the wound margin to obtain an area for the healing wound. Tracings on paper or dressing cover were found to be inferior to scanning of wound tissue and digital tracing of the wound margin.

4.4 Results

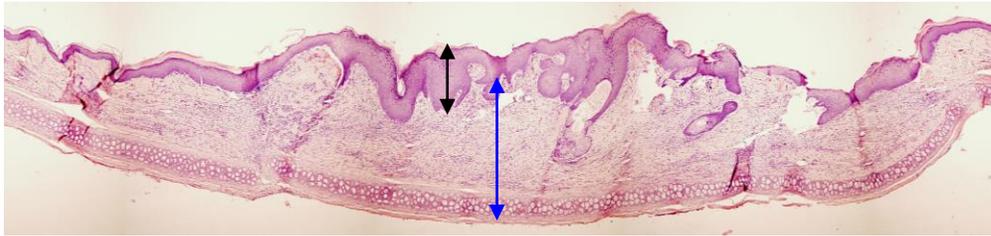


Figure 4.1: Transverse tissue section of rabbit ear. Black line indicates epithelial thickness. Blue line indicates epithelial height. A plot of the cumulative means for epithelial thickness and height were produced and indicated a prohibitively high number of samples would be needed, and interpretation of results rendered difficult due to high variability within these parameters.

Figure 4.2: Sample of rabbit ear tissue showing 3 wounds after 7 days healing.

4.4.1 Preliminary Healing Analysis

In order to assess the healing of a wound there are a number of parameters that may be considered including epithelial thickness and height, degree of inflammation and inflammatory cell infiltration, total wound area, angiogenesis and collagen deposition among others. It is through assessment of a combination of these factors that an overall picture of the healing state of the wound may be made.

A pilot study was carried out to determine the optimal parameters to use. Epithelial height and thickness were assessed along with epithelial volume relative to total wound area in section. Figure 4.1 above shows one representation of a measure of epithelial height (blue line) and one of epithelial thickness (black line).

Across multiple fields for each region of the tissue sample cumulative frequencies of epithelium and connective tissue were calculated and from these the cumulative ratios were obtained. A range was then described being $\pm 5\%$ of the cumulative ratio. The

minimum number of fields per section needed to be measured could then be determined as the number whose cumulative ratios lay outside this range, and a graph plotted using these cumulative means. Stabilisation of the graph represents the number of fields needed to ensure a large enough sample has been analysed to yield reliable results (Figure 4.3). For this pilot study an initial large sample size was necessary to ensure the point at which the graph stabilised was included. Due to the high number of fields found to be necessary using this method, it could be seen that epithelial measurements represented a noisy parameter which would result in difficulties with interpretation.

Since the parameters of epithelial height, thickness and volume proved unsuitable for measurement of healing, macroscopic wound area together with epithelial gap and tissue regeneration were preferred as described in the next chapter.

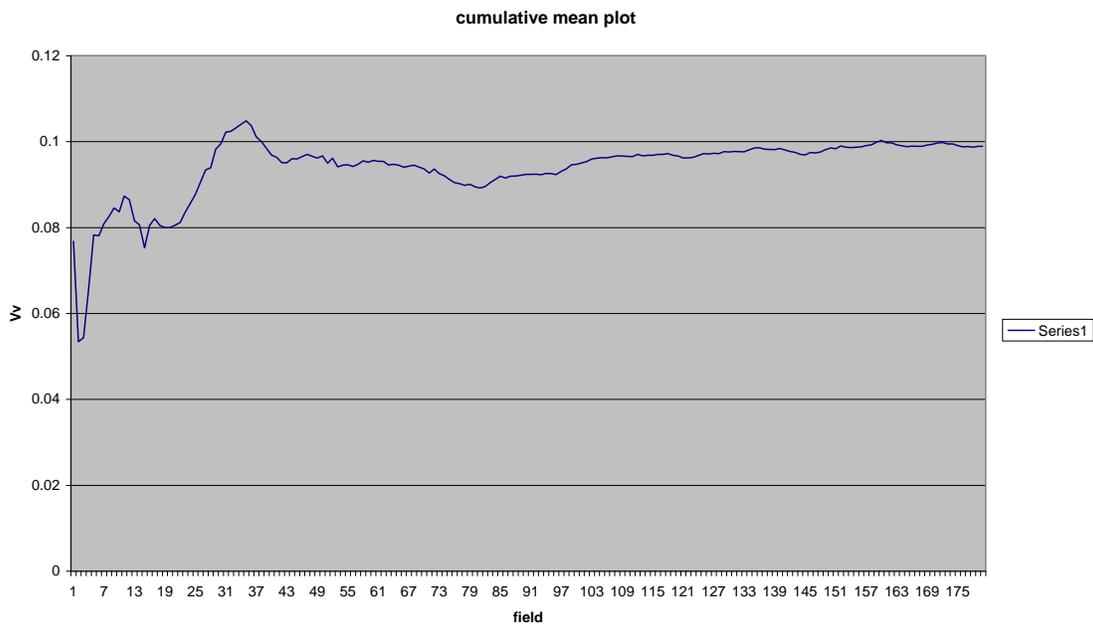


Figure 4.3: Plot of ratio of cumulative mean of epithelial volume and cumulative mean of connective tissue. 42 sections needed to be analysed to achieve stability on the graph, with 4 fields per section and 5 measurements per field being taken.

4.5 Discussion

Wound healing is a multifactorial process and assessment of healing is best made using an amalgamation of results of measurements of different parameters. The re-epithelialisation of a wound is essential to provide strength and protection of the area to help prevent recurrence of the wound. It also prevents infection. Newly formed epithelium shows marked thickening compared to adjacent normal tissue and thickness and relative volume of this epithelium may be measured as an assessment of healing. While it may be reasonable to assume that epithelial height, thickness and volume are all important in the strength of a healed wound, it must be feasible to accurately measure these parameters so comparison may be made to controls and other test groups. In this study, epithelial thickness and volume were found to be very noisy parameters showing great variation even within one group. As may be seen from the results showing the cumulative mean of these parameters, for the study to be sufficiently powered to facilitate meaningful statistical analysis, measurement from 42 sections would have been necessary from each wound. This makes the analysis of these parameters problematic as that number from each would increase the workload so as to make it unfeasible from a logistics point of view. This does not mean that these parameters are not good measures of healing, but rather that there exists so much variation between sections analysed that it was found to be substantially more time-efficient to focus on alternative parameters that displayed less variability. For this reason these parameters were not deemed suitable as the number of sections calculated to be necessary to provide meaningful data was prohibitively high compared to other parameters. Thus alternative and more robust measures were sought for this study. These were wound area, epithelial gap and degree of tissue regeneration and are described in the next chapter.

Healing in the rabbit ulcer model proceeds exclusively from the wound periphery and so total wound area may be measured to assess the degree of healing. However, macroscopic measurement of wound area is rendered difficult by indistinct edges and scar tissue. Wounds may completely re-epithelialise and a colour gradient may be all that

is remaining to examine the degree of healing using this method. Despite this, measurement of wound area was felt to be a very clinically relevant endpoint.

Another problem which we identified in the pilot development study was that the rabbit ears had been bandaged tightly to prevent interference during healing. This bandaging resulted in ischemia with some necrotic tissue being evident on harvesting. Furthermore, in relating this model to the clinical setting, a degree of ischaemia may not be entirely unhelpful as it could reflect the ischaemia which may be associated with peripheral artery disease, a well recognised complication of diabetes. However, should such a parameter be introduced into a study of this nature it would need to be standardised, possibly by Doppler flow measurements.

In this chapter then, the steps taken to establish a working model of the diabetic ulcer are described and the best parameters for measurement of healing were elucidated by this work.

5. Effects of duration of hyperglycaemia on diabetic wound healing

5.1 Abstract

The duration of diabetes is a parameter that shows little consistency and standardisation across a range of studies using diabetic animal models. Studies frequently use animal in whom diabetes has been induced for a very short time and these models more accurately represent an investigation of acute hyperglycaemia on the endpoint of interest. In this chapter we show a delay in healing of rabbit ear ulcers in diabetic rabbits compared to non-diabetic controls. Moreover, this delay is shown to be increased with a longer duration of diabetes. Healing was measured histologically by re-epithelialization and tissue regeneration at the wound site and while a trend towards delayed healing was seen when wounds were induced 1 week after infusion of alloxan, statistical significance was not seen until diabetes had been induced 4 weeks previously. This result has implications for work done using diabetic animal models.

5.2 Introduction

In using an animal model to examine the consequences of diabetic complications the model must be as clinically relevant as possible. A range of animal models of diabetes and wound healing have been used including genetically diabetic strains and those in which diabetes was chemically induced (Brown et al., 1994, Junod et al., 1969, Lenzen and Panten, 1988) by destruction of pancreatic cells. However, despite the importance of the duration of hyperglycaemia seen in the clinical setting, no consensus appears to exist in the literature regarding the time-points post-induction of diabetes at which work was commenced nor has there been much exploration of the severity of the effects of diabetes in different models which vary by the duration of diabetes mellitus. In a range of studies done using the alloxan induced diabetic rabbit model a range of time-points were adopted from 3 days to 7 months from induction of diabetes to commencement of experimental work (Weekers, 2003; Alnaeb, 2007; Lau, 2007; Vogel, 1988; Winiarska, 2010). A similar situation exists when other agents are used such as streptozotocin or where other animal models are used. In a study by Wang et al (Wang, 2010) wound healing was measured at 2 weeks and at one year with a significant delay being seen at the one year time-point but not at two weeks compared to non-diabetic controls. However, most studies are conducted within a much shorter time course and in this study we have examined the difference within this much shorter time-frame. Since the duration of a hyperglycaemic state influences the diabetic complications which may occur, both in type and severity, we have explored healing in this model at different time-points after induction of diabetes by alloxan infusion. Both acute and chronic hyperglycaemic states have been shown to impair endothelial function (Williams et al., 1998, Makimattila et al., 1996) and we have explored how this may translate into the healing of ulcers. In order to do this a short term hyperglycaemic state maintained for 1 week was compared to a longer term of 4 weeks and in this study we show a significant difference within this time-frame. Healing was measured by different parameters including, wound area, re-epithelialization, tissue regeneration and angiogenesis. In many animal models wound closure occurs mainly through contraction of the wound rather than through formation of granulation tissue and migration of epithelium. The rabbit ear ulcer provides a model that

is non-contractile with a full thickness excisional wound extending to the cartilage which ensures healing is exclusively from the wound margin thus more closely resembling the human ulcer and making analysis easier which makes it an attractive model for the study of wound healing.

5.3 Methods

5.3.1 Induction of diabetes mellitus

In order to induce diabetes, 150mg/kg of alloxan (Sigma) in solution was infused intravenously over 30 minutes at a rate of approximately 1ml/min. Only animals showing a sustained elevated blood glucose level of greater than 12mmol/L were used in the study.

5.3.2 Wound Induction

All procedures were approved by the ethics committee of the National University of Ireland, Galway. Male New Zealand White rabbits were anaesthetized using ketamine (Vetoquinol; 0.35ml/kg) and xylazine (Vetoquinol; 0.25ml/kg) and placed on isoflourine gas for the duration of the surgery. 4 animals were assigned per group with 3 wounds induced on each ear giving n=24 wounds per group. All wounds were made using a disposable 6mm sterile biopsy punch (trephine, Kai Medical and Kruuse). Three wounds per ear were made by cutting down to the cartilage and the overlying skin removed using a scalpel and forceps. Excessive bleeding was taken as a sign of injured cartilage and skin was not removed from these wounds nor were they used for the study. The wounds were photographed and covered with Opsite film dressing (Smith & Nephew) which was secured with silk tape. Opposing edges of the ear were then sutured together using 2-3 sutures per ear to prevent interference. Following surgery carprofen (Pfizer; 0.08ml/kg) was administered to alleviate any post-recovery pain. Animals were then returned to their housing and allowed to recover on vedbed bedding. After recovery, collars were placed around their necks to minimise interference with the wound site.

5.3.3 Tissue Harvesting

Animals were anaesthetised with xylazine (Vetoquinol, 0.25ml/kg) and ketamine (Vetoquinol, 0.35ml/kg) and then euthanized by administration of 2mls of sodium pentobarbital either into the lateral ear vein or by direct intra-cardiac administration. Wounded tissue sections were then cut out from surrounding tissue and the dorsal skin peeled away from the cartilage. Tissue samples were then mounted on cardboard and immediately fixed in 4% paraformaldehyde and kept at 4°C until processing. Samples were then scanned using a Canon CanoScan 3200F to capture digital images for analysis. Processing was done in a Leica ASP 300 processing machine on the routine overnight setting. Samples were then embedded in paraffin wax and 5µm sections were cut on a Leica microtome after being chilled on a coldplate. These were then stained with haematoxylin and eosin.

5.3.4 Healing Measurements

The overall reduction in wound area was measured using the Image J program to trace around the wound margin and measure the area on tissue samples photographed using a Canon scanner. For histological analysis sections of 5µm thickness were cut and digital images captured for analysis. Epithelial gap was measured using Image J to draw a straight line between the advancing edges of epithelium, parallel to the cartilage (Figure 5.1). For tissue regeneration measurements, sections were assigned a score from 1-5 based on amount of tissue at the wound site with 5 representing complete re-epithelialisation. Angiogenesis was measured by counting vessel intersections of a cycloid arc placed randomly over the section to calculate surface density. The endothelial cells were identified morphologically and confirmation was by matching to sections stained with CD31 antibody. Wounds were analysed one week after induction.

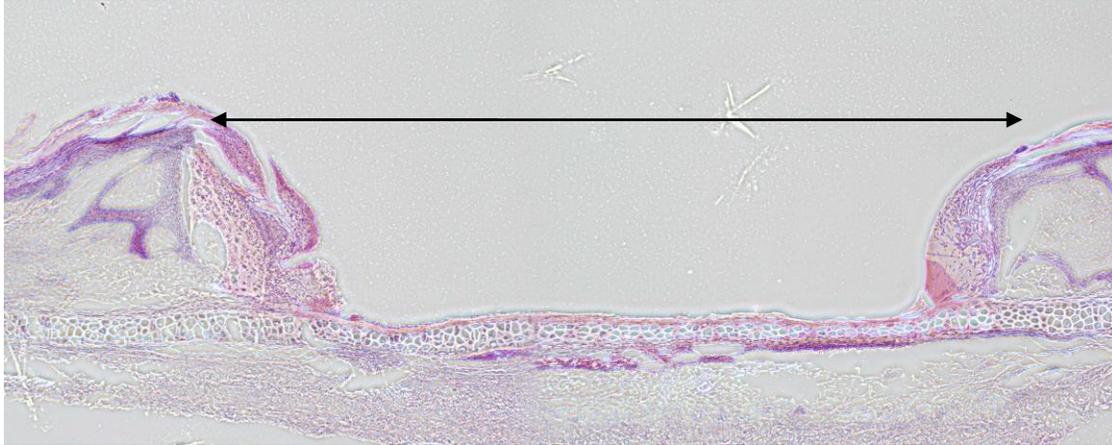


Figure 5.1: Section of rabbit ear wound site. Arrow indicates space between advancing edges of epithelium. Exposed cartilage can be seen with granulation tissue forming at the wound periphery.

5.3.5 Statistical Analysis

Statistical analysis was carried out with the aid of SigmaStat Version 3.0 software (SysStat Inc). Differences in data sets were determined by carrying out using either Student's t-test, one way analysis of variance (ANOVA) or Kruskal-Wallis (non-parametric) followed by multiple-paired comparisons. Choice of post-test depended on whether data was normally distributed or not and this was determined by the Advisor Wizard function in the SigmaStat software. All data were presented on charts as mean values, with error bars representing standard error of the mean, and statistical test p values of <0.05 were deemed to be significant.

5.4 Results

5.4.1 Healing Measurements

5.4.1.1 Wound Area

When healing in non-diabetic controls, healing after 1 week of hyperglycaemia and healing after 4 weeks of hyperglycaemia were compared by macroscopic analysis of the entire wound area, no significant difference was seen between the groups (Figure 5.2).

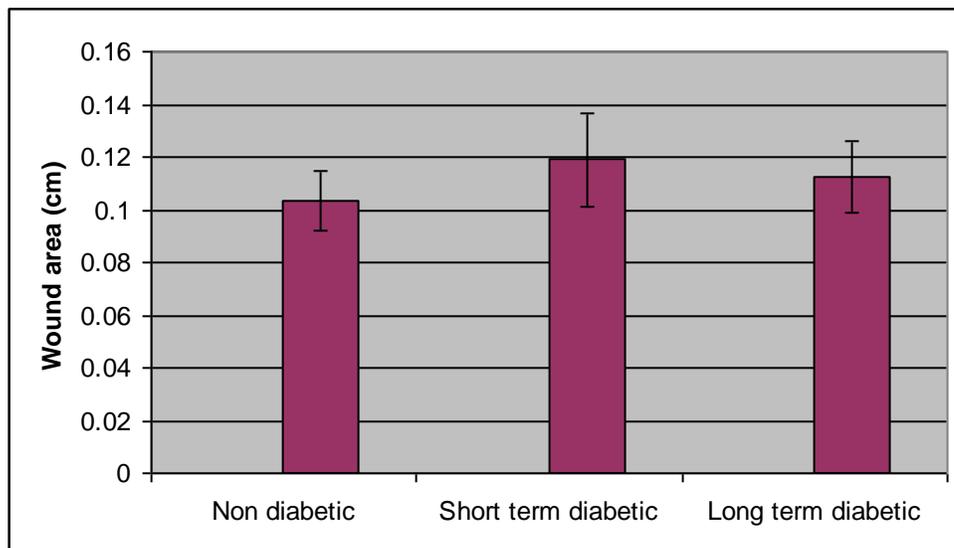


Figure 5.2: Macroscopic measurements (in cm^2) of total wound area comparing groups with short term (1 week) duration and long term (4 weeks) duration of diabetes against non-diabetic controls. $n=24$ wounds per group.

5.4.1.2 Epithelial Gap

The degree of re-epithelialisation is a good measure of the progress of the healing of skin. When healing of non-diabetic controls, healing after 1 week of hyperglycaemia and healing after 4 weeks of hyperglycaemia were compared in terms of advancement of epithelium a definite trend was seen with short term hyperglycaemia delaying epithelial advance and long term hyperglycaemia delaying it even further. This delay showed statistical significance when the long term hyperglycaemic and the normal control groups were compared (Figure 5.3)

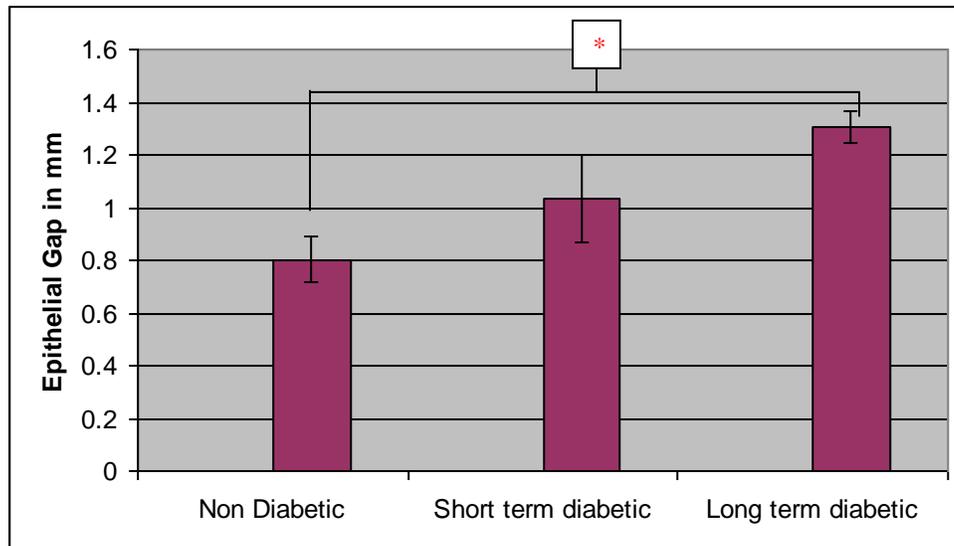


Figure 5.3: Comparison of epithelial gap between non-diabetic, short term diabetic (1 week) and long term diabetic (4 weeks) groups. * $P < 0.05$. $n = 24$ wounds per group.

5.4.1.3 Tissue Regeneration

When the amount of tissue present over the previously exposed cartilage and between the wound edges was assessed between the control and both hyperglycaemic groups a trend correlating with that of re-epithelialisation was seen. Less tissue had regenerated in the short term hyperglycaemic group compared with normal controls with less still being seen in the long term hyperglycaemic group with the difference between the normal and long term groups reaching statistical significance (Figure 5.4).

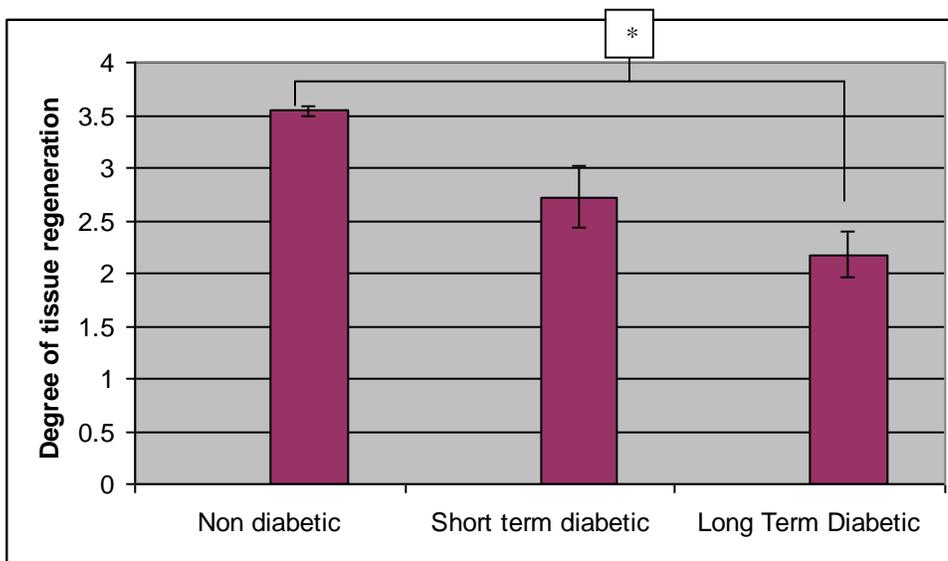


Figure 5.4: Comparison of amount of tissue regeneration between non-diabetic group and groups with short term (1 week) and long term (4 weeks) duration of diabetes.
* $P < 0.05$. $n = 24$ wounds per group.

5.4.1.4 Angiogenesis

When measured as surface density of blood vessels on each section, no significant differences were seen between the three groups of differing glycaemic states (Figure 5.5) Angiogenesis is known to be a major contributing factor to the healing of wounds and yet was not found to correlate with other parameters of healing measured. This, however, is not without precedent and serves to highlight the fact that healing is a multifactorial process with many pathways acting synergistically to give the overall healing effect (Stepanovic et al., 2003).

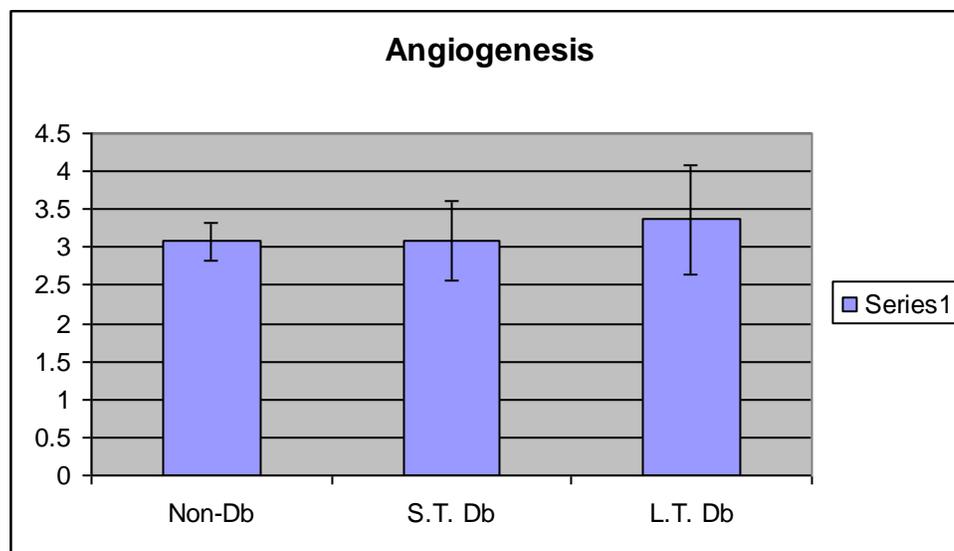


Figure 5.5: Blood vessel density in non-diabetic (Non-Db), short term diabetic (S.T. Db, 1 week duration of hyperglycaemia) and long term diabetic (L.T. Db, 4 weeks duration of hyperglycaemia). n=24 wounds per group.

5.5 Discussion

The observation that diabetes mellitus compromises the healing of wounds has long been established. In this study we examined the effect of the duration of hyperglycaemia on the rate of healing by measuring the amount of granulation tissue formed and the rate of re-epithelialisation both 1 week and 4 weeks after induction of diabetes by alloxan infusion using the rabbit ear ulcer model. It is to be acknowledged that both time points represent acute hyperglycaemia but the study was done as time points such as this are frequently used but little attention has been paid to the effect of the duration of hyperglycaemia on wound healing. Both granulation tissue deposition and epithelial closure tended to be restricted in animals experiencing a state of hyperglycaemia for 1 week compared to non-diabetic controls with this difference reaching statistical significance between the controls and the 4 week hyperglycaemic group. Although the fact that diabetes restricts wound healing is widely accepted the question of what effect the duration of hyperglycaemia has on healing remains unclear. It may be expected that a longer duration of hyperglycaemia may result in increased endothelial damage and the detrimental effects of reduced circulation may be more pronounced in later stages of diabetes. However, in experimental studies, methods of induction and duration of hyperglycaemia are far from standardised (Sivan-Loukianova et al., 2003, Komesu et al., 2004, Witte et al., 2002, Schaeffer et al., 2006, Luo et al., 2004). In a range of studies using the alloxan diabetic rabbit model a variety of time-points were chosen as to when to start experimental work after induction of diabetes. In some instances, these were based on measurement and stabilization of blood glucose but often no rationale for the choice of time-point is offered. A study by van Golde et al. (van Golde, 2008) on collateral vasodilatation and remodelling chose an 8 week time-point while Lau et al. (Lau, 2007) randomised groups 6 months after alloxan infusion to explore the effect of a PD5 type 5 inhibitor on renal function. Alnaeb et al. used a 7 month time point (Alnaeb 2007), Schiller et al. used 7 months (Schiller, 1999) and Wang et al. used 2 weeks (Wang, 2010). Lengyel et al. used 3 weeks (Lengyel, 2008) while Winiarska et al. used 3 days (Winiarska, 2011). Other studies have variously used 10 day (Pradhan, 2011), 1 week (Breen 2008), 8 week (Zagon, 2010), and 2 month time-points (Akar, 2011) with one

study using 10-16 week and 7-9 week time points based on the experimental model used (Vogel, 1988). Although different treatments may be compared using one pre-determined time point to obtain relative values, the question remains as to whether different absolute values would be seen depending on the time point chosen. Moreover, this may have clinical implications for outcome depending on how soon in the illness a particular treatment is administered.

Mir et al. have shown that glucose levels rose throughout a 26 week study period with attendant progressive histopathological changes (Mir, 2009) and Wang et al. have shown delayed healing of wounds in diabetic rabbits at one year post induction of diabetes compared with 2 weeks (Wang, 2010). However in this study we show significant delay at much shorter time-points and these time points are much closer to those commonly used in the literature.

In this study we directly compared pre-surgery durations of hyperglycaemia of 1 week and 4 weeks. The parameters chosen for measurement were the degree of re-epithelialization, tissue regeneration and neo-vascularization as well as macroscopic wound area. Re-epithelialization is an important parameter for determining the tensile strength of the wound site and for estimating the risks of recurrence of the ulcer while tissue regeneration scores give information not only on recovery and granulation tissue formation but also on whether necrotic tissue is present. Although a trend towards delay in re-epithelialisation of wounds and tissue regeneration were seen after a week in the diabetic setting compared to non-diabetic controls, this reduction in healing did not reach statistical significance until the state of hyperglycaemia had continued for 4 weeks. With this in mind it may be speculated that a treatment that is effective against relatively recent onset diabetes may be less effective against more established disease. Our measurements included both macroscopic and microscopic measurements of wound healing. The rabbit ear ulcer model we chose exhibits little or no wound contraction due to the adherence of the skin to the underlying cartilage which confers a significant advantage over some other models in terms of wound measurement. However, even without contraction, macroscopic measurements of wound area proved difficult to interpret due to subtle

variations in variables such as colour and margin, amongst others, which combine to give an overall impression of healing. The macroscopic analysis of wound areas requires consideration of a number of factors. A wound margin may be difficult to clearly delineate as subtle colour changes, rather than a clearly demarcated edge can give a more accurate indication of the degree of healing. Complete re-epithelialisation may not be immediately apparent macroscopically leading to an underestimation of healing. In addition, scar tissue formation may further confound clear location of the wound edges. For these reasons it was desirable to place emphasis on histological measurements which proved more suitable for data collection.

The reasons for the discrepancies seen when re-epithelialization and tissue regeneration were examined are likely multifactorial due to the complex nature of wound healing and tissue repair but since angiogenesis is of such paramount importance in this process we measured surface density of blood capillaries within the wound site. However, no significant difference was observed between any of the groups. This result would perhaps not be initially expected but is not without precedent. It has been previously found that angiogenesis and rate of wound healing are not always linked (Stepanovic et al., 2003). Since wound tissue was harvested at 1 week post wound induction, inflammatory cells featured very prominently in sections analysed. This may indicate that the tissue repair process was still in a relatively early phase and endothelial cell migration and vessel formation had not reached their peak and this raises interesting questions concerning what effect the phase of wound healing has on the rate of healing.

In this chapter a statistically significant difference has been demonstrated in healing rates of diabetic ulcers depending on length that the state of hyperglycaemia had persisted when the wound was induced. Because of this, different time-points were also examined in subsequent work when therapeutic interventions were explored using circulating angiogenic cells delivered to the wound using a collagen scaffold and also with these cells after transduction with the eNOS gene.

Since these cells were cultured for 7 days and applied to the wound site at the time of wound induction, the time-point after induction of diabetes that the cells were isolated at

was necessarily different in the different time-point groups. As these cells have been shown to have impaired functionality in the diabetic setting (Fadini et al., 2005) it may be that a longer duration of diabetes, as well as further delaying healing of the wounds, also has greater impact on the functionality of the cells isolated after longer duration of hyperglycaemia.

In conclusion, our results show that the duration of hyperglycaemia affects the rate of healing of dermal ulcers as measured by re-epithelialization and tissue regeneration. Moreover, this delay could be seen within 4 weeks which has implications for comparison of studies using diabetic models.

6. Treatment of diabetic ulcers in a rabbit model using eNOS overexpressing circulating angiogenic cells

6.1 Abstract

In this chapter we explored the therapeutic potential of CACs for diabetic ulcer healing by applying cells directly to a rabbit model of wound healing. In order to do this a collagen scaffold was employed to apply the cells. In addition to autologous CACs, CACs that had been transduced with the bovine eNOS gene were applied in a similar manner with an unseeded scaffold serving as a control. Wounds were induced at 1 week and 4 weeks after induction of diabetes and healing was measured in terms of re-epithelialization, tissue regeneration, wound area and angiogenesis. However, no differences were seen in any of the parameters tested when the treatment groups were compared with untreated controls

6.2 Introduction

The progression and healing of ulceration is a multifactorial process. This healing involves a complex series of events including proliferation and migration of various cell types as well as extra cellular matrix deposition with all necessary cytokines and a cascade of signalling pathways. Formation of granulation tissue is a key sign of the progression of healing. It forms as new blood vessels infiltrate into the wound area, first as thin capillaries, providing nutrients to facilitate the accumulation of fibroblasts and ground substance at the wound site. Production of nitric oxide by migrating endothelial cells among others is another process of paramount importance in the healing of wounds. In the human diabetic setting, delayed healing of wounds has long been established. The healing milieu of diabetic wounds is complex with over 100 physiologic factors contributing to wound healing deficiencies in the diabetic setting (Brem, 2007). Peripheral neuropathy is a major contributor to diabetic ulcer formation due to decreased sensation and lessened protective response to pain as well as lack of correction of limb positioning that causes pressure sores to develop. A chronic wound may be defined as one which takes upwards of 8 weeks to heal or recurs. Figures for the annual incidence of foot ulcers range from 1-4.1% and 15% of diabetic patients will develop an ulcer at some point (Bartus and Margolis, 2004, Armstrong and Lavery, 1998). This leads to prolonged hospital stays as well as increased costs and with estimates predicting a rise in worldwide diabetic patients from 171 million in 2000 to 366 million in 2030, this problem can be expected to worsen (Wild et al., 2004). Among the vascular complications of diabetes is the accelerated onset of peripheral arterial disease (PAD). Vascular insufficiency is among the most prevalent causes of leg ulcers with reduced arterial flow being implicated in the aetiology of 20% of diabetic lower extremity ulcers (Leung, 2007, Tam and Moschella, 1991, Reiber et al., 1999). Indeed, abnormal angiogenesis is associated with diabetes and a reduced wound healing activity (Martin et al., 2003). A variety of strategies to improve healing have been employed including the application of growth factors which may be delivered using a bioengineered scaffold (Mustoe et al., 1991, Yao et al., 2006) or cell therapy (Fathke et al., 2004, Ichioka et al., 2005).

In recent years much interest has emerged in the therapeutic use of endothelial progenitor cells (EPCs) for cardiovascular conditions such as PAD. A subset of EPCs, the so-called circulating angiogenic cells (CACs) can be isolated from peripheral blood. These cells have been shown to incorporate into sites of neoangiogenesis and promote blood vessel growth at least partially by secretion of pro-angiogenic cytokines (He et al., 2004b). In discussing the cell types we will use the nomenclature proposed by Hirschi (Hirschi et al., 2008) to describe the cells, using CAC to describe the cell of choice for this work. However, in discussing the literature it is often unavoidable to use the term EPC in order to maintain consistency with the original author's work as culture conditions and characterization of these cells has not been standardised and no consensus exists in the literature and so the term EPC will be used of necessity where deemed appropriate. Angiogenesis is an integral part of the wound healing process and the presence of nitric oxide has also been shown to be of significant importance. In this study we have examined the combined effects of topically applied CACs and eNOS on the healing of diabetic ulcers by using a collagen scaffold to deliver autologous CACs and autologous CACs virally transduced with the eNOS gene to rabbit ear ulcers.

The mode of delivery of cells was also of paramount importance. Although direct injection of cells has proven effective in other studies using different models (Sivan-Loukianova, et al) the relative paucity of tissue overlying the cartilage of the rabbit ear made this unfeasible and application of a bleb containing cells as well as systemic injection also proved unsuitable. To contend with this issue we isolated mononuclear cells as described in chapters 2 and 3 and seeded the cells on a collagen scaffold. The cells could then be cultured on the scaffold and applied directly to the wound site.

Having determined the number of histological sections required by means of a pilot study described in the preceding chapter, the effects of the applied cells were analysed. We compared the effects on wound area, epithelial gap, tissue regeneration, and angiogenesis, of cells seeded on the collagen scaffold and the effect of the scaffold alone when applied to diabetic wounds compared to an untreated diabetic control. We also compared the effect on these parameters of application of the scaffold seeded with

autologous CACs that had been transduced with the eNOS gene against untreated diabetic ulcers.

6.3 Methods

6.3.1 Cell Isolation

After sedation with acepromazine (Novartis, 0.1ml/kg) the dorsal surface of the ear was shaved and Emla cream or Xylocaine spray containing lidocaine (AstraZeneca) was applied to minimise discomfort. The ear was then disinfected with an alcohol swab. The central ear artery was cannulated and blood collected into 6ml heparinized tubes (Beckton Dickenson). Pressure was then applied to the point of entry until bleeding stopped. Mononuclear cells were isolated as previously described (Kealy et al., 2009). Briefly, blood was diluted in a 1:1 ratio with HBSS (Sigma) containing 1mM EDTA and 5% BSA. The mononuclear fraction was isolated by density gradient centrifugation using Ficoll Paque Plus (Amersham). MNCs were washed in PBS and plated on collagen scaffolds in standard 12-well plates and maintained in EGM-2 media (Cambrex) for 7 days. Media was changed at day 2 and daily thereafter.

Endothelial phenotype was determined by uptake of acetylated LDL (Molecular Probes) and binding of UEA-1 lectin (Sigma), as previously published (Kalka, 2000; Ii, 2005; Hristov, 2004; Rehman, 2003; Suh, 2005) and expression of endothelial markers KDR, CD 34 and von Willebrand Factor (vWF) . Staining and analysis were carried out as previously described (He, 2004) and detailed in chapter 2.

6.3.2 Cell transduction

Cells were transduced with the bovine eNOS gene using adenovirus as described in chapter 2 with the viral solution being applied directly to cells growing on the collagen scaffold. Briefly, a replication-deficient, E1- and E3-deleted adenovirus vector (adGFP)

that expresses the enhanced green fluorescence protein (EGFP) gene under transcriptional control of the cytomegalovirus promoter (kind gift of Dr. A. Flugel, Max-Planck Institute of Neurobiology, Dept. of Neuroimmunology, Martinsried, Germany) was propagated and purified in 911 cells as described previously (Fallaux et al., 1996). In brief, the 911 cells were transduced at a multiplicity of 5-10 and harvested after 36-48 h. The virus was released by five freeze-thaw cycles and purified using two CsCl-gradients. The banded virus was recovered, desalted over sephadex columns (Pharmacia) and stored in virus storage buffer after addition of 10% Glycerol at -80°C . Titration of the virus concentration after elution from the column was performed by plaque assay on the 911 cells (plaque forming units, pfu). A recombinant adenovirus encoding the bovine eNOS gene driven by the cytomegalovirus was also generated as previously described (Cooney et al., 2007).

MNCs were seeded in 12 well plates at a density of 1×10^7 cells/well in 2 ml of EBM-2 medium with growth factors on the collagen scaffold. After 7 days culture medium was removed and cells were incubated with AdENOS at a multiplicity of infection (MOI) of 500 in 500 μl medium for 1 hour at 37°C . Untreated cells or cells transduced with AdEGFP at the same MOI served as controls. Supernatant was then removed and cells were incubated in fresh medium for a further 24 hours at 37°C . To enhance transduction efficiency virus was spun onto the cells by centrifugation for 60 min, at 2000g, 37°C . Then supernatant was removed and cells were further incubated in fresh medium for 24 hours at 37°C .

To assess transduction efficiency RNA was extracted from the cells after exposure to the virus using the RNeasy kit (Qiagen) and real time PCR was performed using the Applied Biosystems Step One Plus processor. RNA levels were normalized using hypoxanthine phosphoribosyltransferase (HPRT) expression and compared to levels from AdEGFP transduced cells. PCR primers were designed to amplify bovine eNOS without amplifying endogenous rabbit orthologs. Bovine eNOS (NM 181037) 5'-GAGAGGCTGCATGACATTGAGA-3' and 5'-GGTAGAGATGGTTCGAGTTGGGA-3', expected product size 94bp. Primers were also designed for the rabbit HPRT gene 5'-

CTCAACCTTAACTGGAAAGAATGTC-3' and 5'-CCTTTTCACCAGCAGGCT-3', expected product size 135 bp.

All efforts were made to design primers with limited crossreactivity with rabbit eNOS. To determine the level of gene expression real time PCR was performed. Samples from transduced rabbit cells were analysed with and without a reverse transcription step to allow for viral DNA in the sample and compared to cells exposed to adenovirus carrying the GFP gene. All samples were normalised against the HTTP housekeeping gene.

6.3.3 Cell Delivery

2 different methods of cell delivery were explored. Firstly, after staining of CACs with DiI-labeled ac-LDL, cells were trypsinised, washed and resuspended in PBS. Both immediately and one day after wound induction the central ear artery was clamped at both ends and the cell suspension was directly injected into the artery and allowed to dwell for 20 mins. Tissue was then sectioned on a cryostat and mounted.

In the alternate method, a collagen scaffold was investigated as a vehicle. CACs were isolated from rabbit peripheral blood as described in a chapter 2. 1×10^7 MNCs were placed on a dry collagen disc situated at the bottom of a standard 12-well plate and cultured as normal in EGM-2 media (Cambrex).

6.3.4 Collagen Scaffold

The collagen used for the scaffold was extracted from bovine Achilles tendon and prepared by freeze drying in 24-well plates. Collagen purity was about 90%. The scaffolds used were prepared by Dr. Li Yao of the Biomechanical Engineering Department, NUI Galway (Figure 6.1).

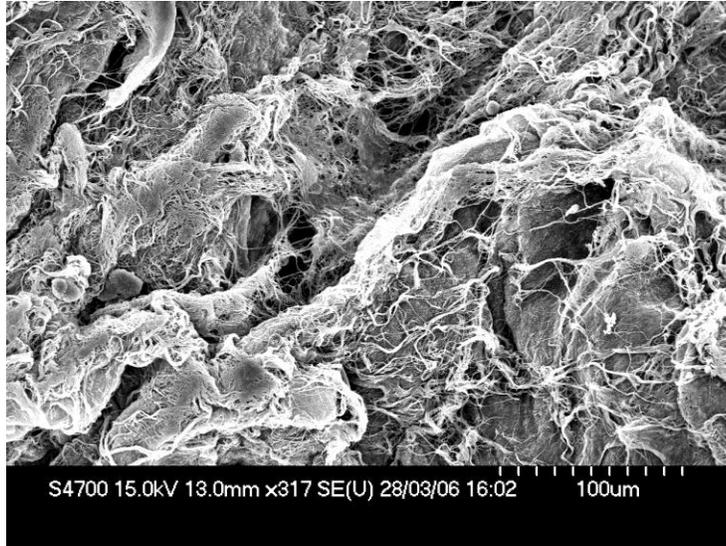


Figure 6.1: SEM of collagen scaffold showing structure of collagen in scaffold.

6.3.5 Wound Induction

All procedures were approved by the ethics committee National University of Ireland, Galway. Male New Zealand White rabbits were anaesthetized using ketamine (Vetoquinol; 0.35ml/kg) and xylazine (Vetoquinol; 0.25ml/kg) and placed on isoflourine gas for the duration of the surgery. 4 animals were assigned per group with 3 wounds induced on each ear giving n=24 wounds per group. Wounds were induced at 1 week and 4 weeks post induction of diabetes. All wounds were made using a disposable 6mm sterile biopsy punch (trephine, Kai Medical and Kruuse). Three wounds per ear were made by cutting down to the cartilage and the overlying skin removed using a scalpel and forceps. Excessive bleeding was taken as a sign of injured cartilage and skin was not removed from these wounds nor were they used for the study. The wounds were photographed and covered with Opsite film dressing (Smith & Nephew) which was secured with silk tape. Opposing edges of the ear were then sutured together using 2-3 sutures per ear to prevent interference. Following surgery, carprofen (Pfizer; 0.08ml/kg) was administered to alleviate any post-recovery pain. Animals were then returned to their housing and allowed to recover on vedbed bedding. After recovery collars were placed around their necks to minimise interference with the wound site. Wounds were analyzed one week after induction.

6.3.6 Tissue Harvesting

Animals were anaesthetised with xylazine (Vetoquinol, 0.25ml/kg) and ketamine (Vetoquinol, 0.35ml/kg) and the euthanized by administration of 2mls of sodium pentobarbital either into the lateral ear vein or by direct intra-cardiac administration. Wounded tissue sections were then cut out from surrounding tissue and the dorsal skin peeled away from the cartilage. Tissue samples were then mounted on cardboard and immediately fixed in 4% paraformaldehyde and kept at 4°C until processing. Samples were then scanned using a Canon CanoScan 3200F to capture digital images for analysis. Processing was done in a Leica ASP 300 processing machine on the routine overnight setting. Samples were then embedded in paraffin wax and 5µm sections were cut on a Leica microtome after being chilled on a coldplate. These were then stained with haematoxylin and eosin.

6.3.7 Healing Measurements

The overall reduction in wound area was measured using the Image J program to trace around the wound margin and measure the area on tissue samples photographed using a Canon scanner. For histological analysis sections of 5µm thickness were cut and digital images captured for analysis. Images were taken before and after marking the wound edges with india ink to improve accuracy.

Epithelial gap was measured using Image J to draw a straight line between the advancing edges of epithelium, parallel to the cartilage (Figure 6.2). For tissue regeneration measurements sections were assigned a score from 1-5 based on amount of tissue at the wound site with 5 representing complete re-epithelialisation. Angiogenesis was measured by counting vessel intersections of a cycloid arc placed randomly over the section to calculate surface density. The endothelial cells were identified morphologically and confirmation was by matching to sections stained with CD31 antibody. Wounds were analysed one week after induction.

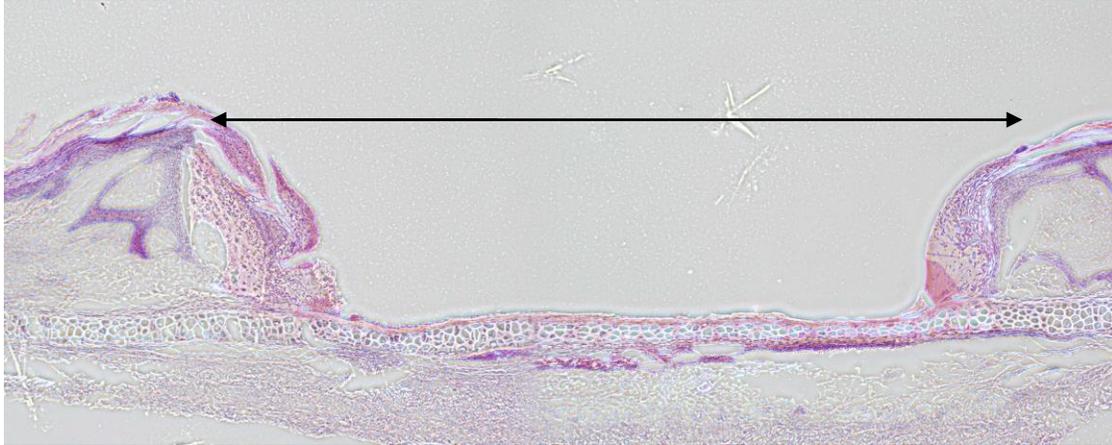


Figure 6.2: Section of rabbit ear wound site. Arrow indicates space between advancing edges of epithelium. Exposed cartilage can be seen with granulation tissue forming at the wound periphery.

6.3.8 Statistical Analysis

Statistical analysis was carried out with the aid of SigmaStat Version 3.0 software (SysStat Inc). Differences in data sets were determined by carrying out using either Student's t-test, one way analysis of variance (ANOVA) or Kruskal-Wallis (non-parametric) followed by multiple-paired comparisons. Choice of post-test depended on whether data was normally distributed or not and this was determined by the Advisor Wizard function in the SigmaStat software. All data were presented on charts as mean values, with error bars representing standard error of the mean, and statistical test p values of <0.05 were deemed to be significant.

6.4 Results

6.4.1 Cell Delivery

Due to the relative dearth of soft tissue in the rabbit ear direct injection of cells to the wound site tissue was not feasible. Therefore an alternative means of delivery was required. Initially cells were stained and injected into the central ear artery after the artery had been clamped off to allow the cell suspension to dwell for 20 minutes. On examination of sections from these samples however, no red fluorescence was detected on microscopic examination.

Based on the knowledge that CACs will differentiate on a collagen substrate, a collagen scaffold was investigated as a vehicle with cells being cultured and stained directly on the scaffold. Under bright field microscopy cells were detected both on the scaffold surface and having migrated deeper into the collagen. Dil-labeled cells could also be detected showing endothelial differentiation. Morphologically, the cells had a rounded appearance and whether this was due to compromised cells or simply due to the relative instability of the collagen scaffold as a substrate remains unclear.

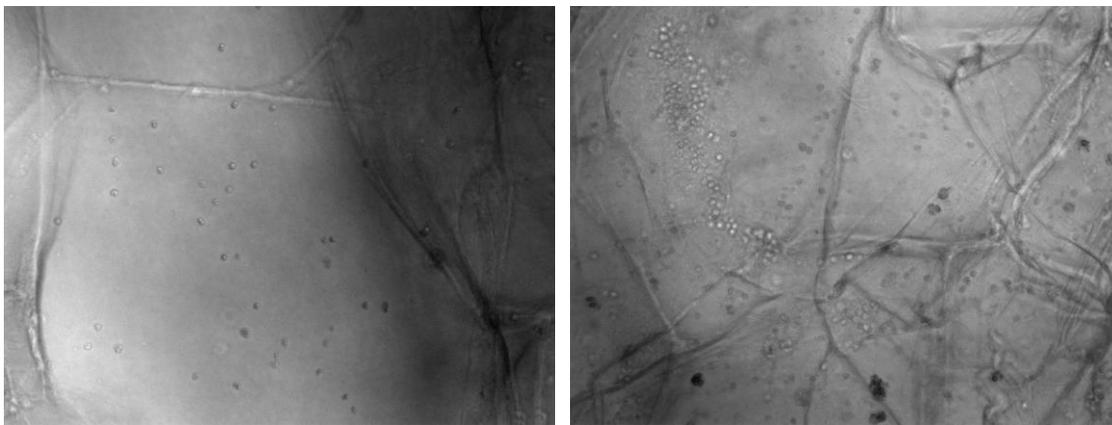


Figure 6.3: Phase contrast images of cells seeded on collagen scaffold after 7 days culture.

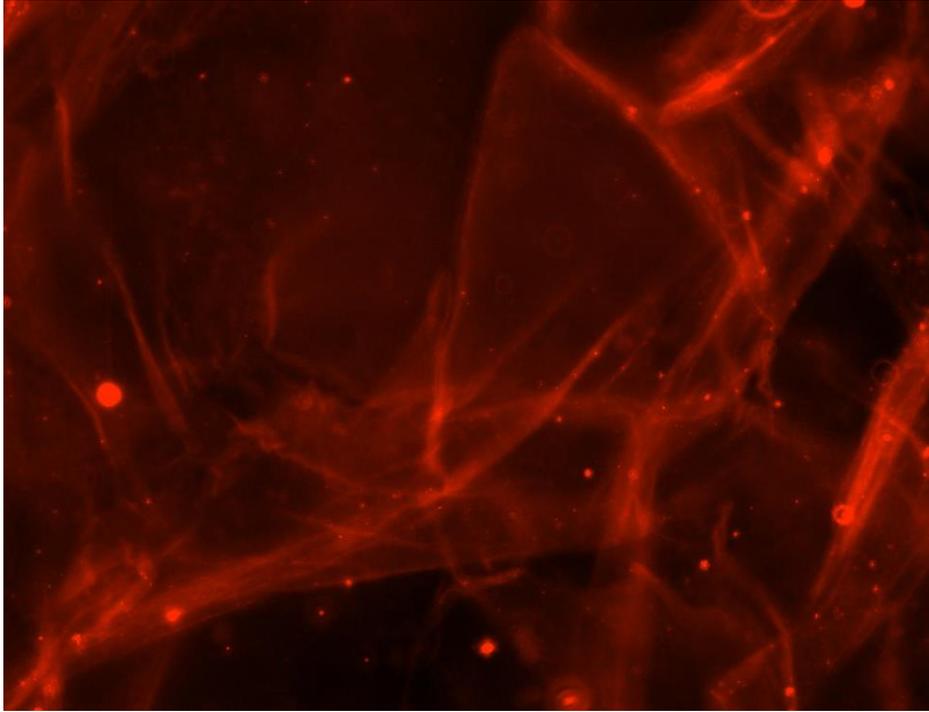


Figure 6.4: Cells stained with DiI-labeled ac-LDL after culture for 7 days on collagen scaffold.

6.4.2 Viral Transduction

In order to determine efficacy of transduction real time PCR was carried out. The samples run without the reverse transcriptase step showed some background eNOS DNA but this would be expected from the viral genome and did not approach the levels found with the RT step included. After normalisation the virally transduced cells showed greater than a 3000-fold increase in eNOS RNA expression than the GFP-transduced controls (Figures 6.5 and 6.6).

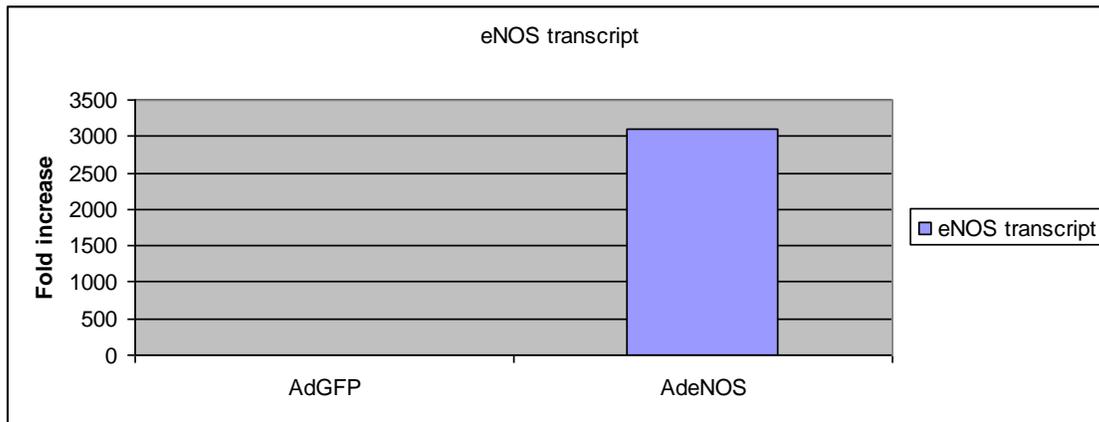


Figure 6.5: Adenoviral transduction of rabbit CACs. Fold increase in eNOS transcript detected over AdEGFP transduced cells is shown.

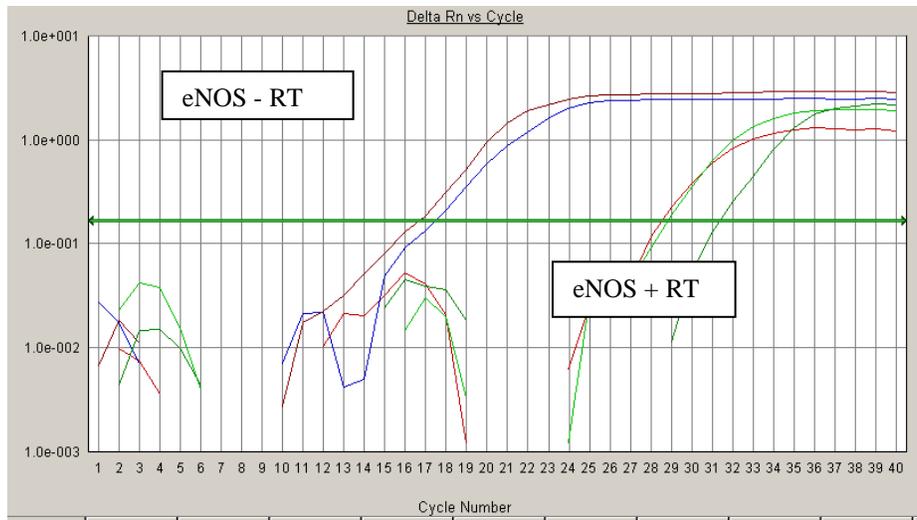


Figure 6.6: Real time PCR data showing increase in transgene expression in with reverse transcription step (RT) in transduced rabbit CACs after adenoviral transduction. Transgene expression was also greatly increased in cells transduced with Ad/eNOS compared with cells transduced with Ad/GFP.

6.4.3 Wound Area

When wound area was measured macroscopically no significant differences were seen between any of the untreated, the collagen, the CAC or the transduced CAC treatment groups whether the wounds were induced at the 1 week or 4 week time-points (Figure 6.7).

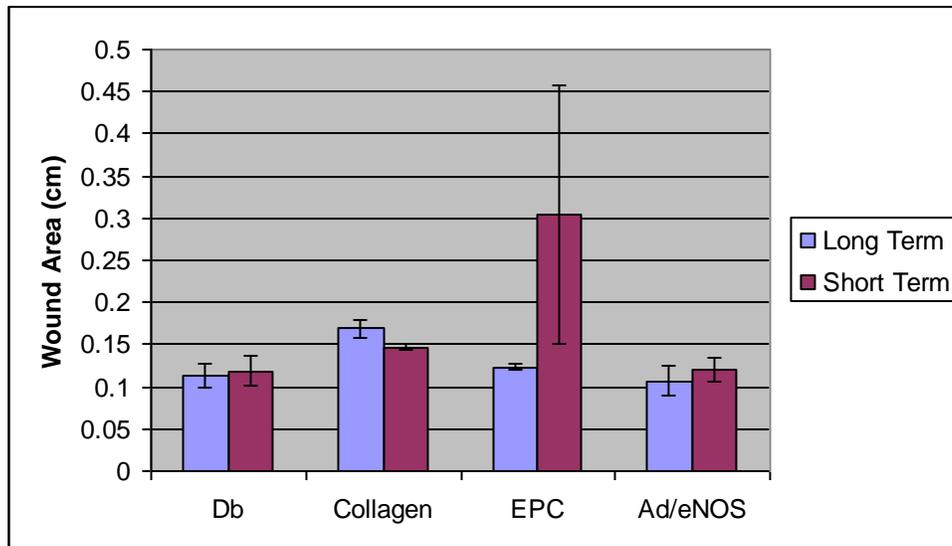


Figure 6.7: Comparison of total wound area between diabetic groups treated with: collagen scaffold alone (collagen), collagen scaffold containing CACs (EPC), collagen scaffold containing CACs transduced with AdeNOS (Ad/eNOS) and untreated diabetic group (Db). 1 week pre-surgery duration of diabetes (short term) and 4 week duration (long term) are also compared. n=24 wounds per group.

6.4.4 Epithelial Gap

No significant differences were seen when epithelial gap was measured between any of the treatment groups or at either time point (Figure 6.8).

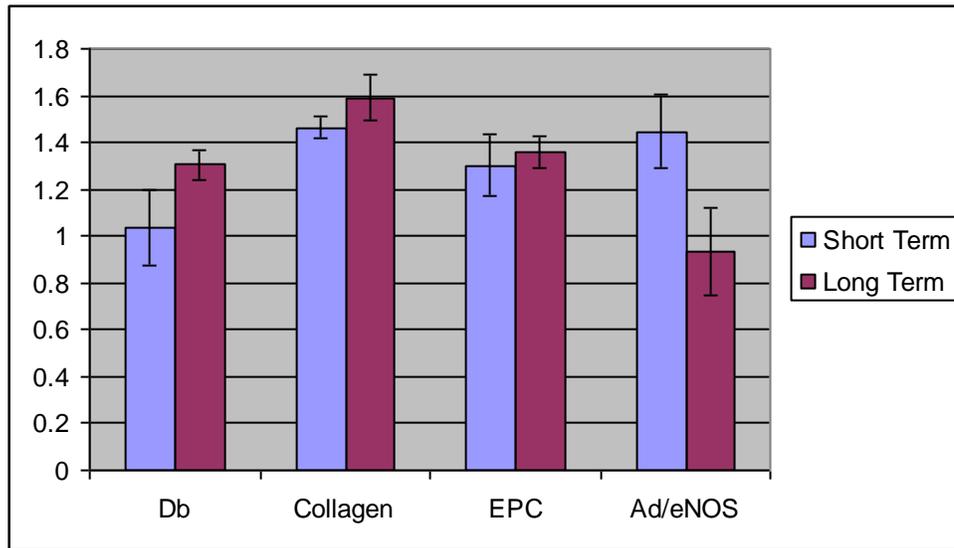


Figure 6.8: Comparison of epithelial gap in untreated diabetic group and diabetic groups treated with collagen scaffold alone (collagen), collagen scaffold containing CACs (EPC) and collagen scaffold containing CACs transduced with AdeNOS (Ad/eNOS). Short term duration of diabetes (1 week) and long term duration (4 weeks) are also compared. n=24 wounds per group.

6.4.5 Tissue regeneration

Consistent with the results for the other parameters differences were again not in evidence when the treatment groups were compared at either time-point (Figure 6.9).

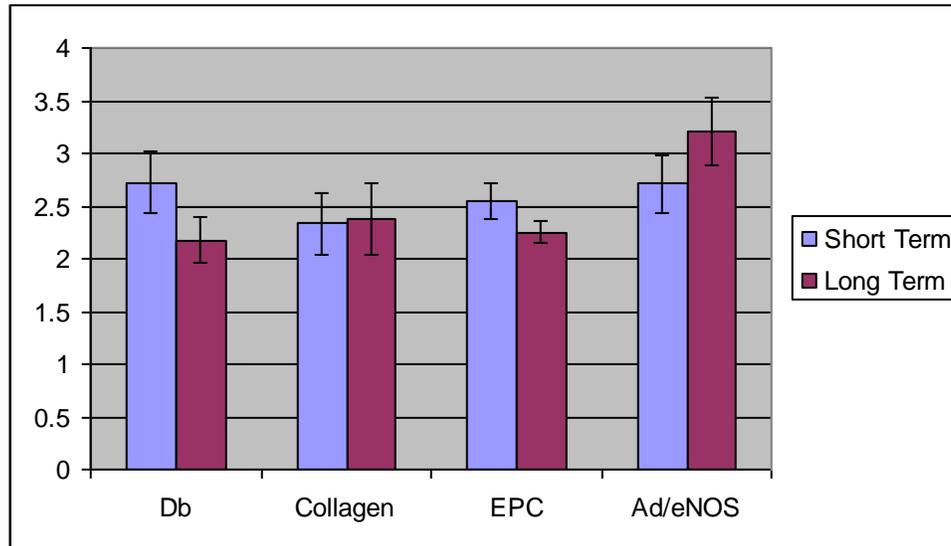


Figure 6.9: Comparison of degree of tissue regeneration between untreated diabetic group and diabetic groups treated with collagen scaffold alone (collagen), collagen scaffold containing CACs (EPC) and collagen scaffold containing CACs transduced with AdeNOS (Ad/eNOS). Short term duration of diabetes (1 week) and long term duration (4 weeks) are also compared. n=24 wounds per group.

6.4.6 Angiogenesis

Angiogenesis measurements also failed to show any differences between the treatment groups or at either time-point (Figure 6.10).

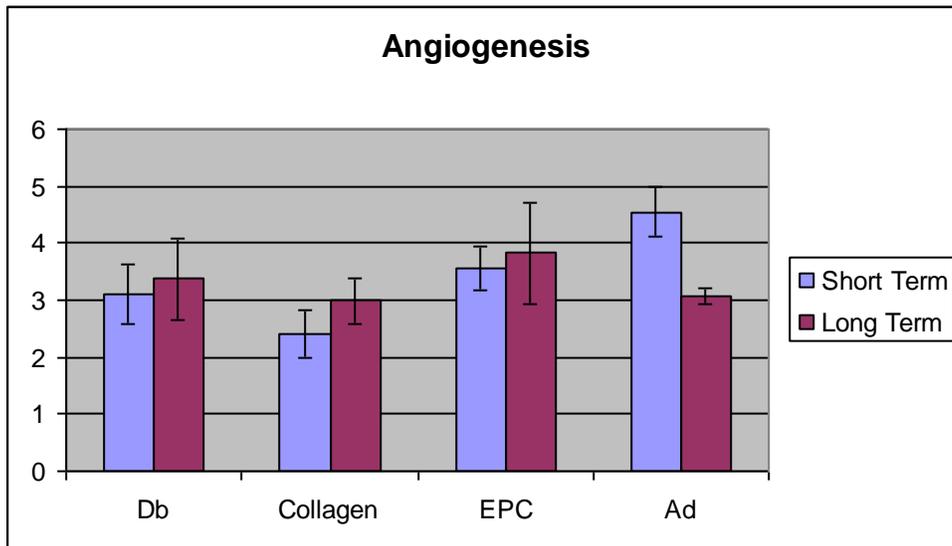


Figure 6.10: Comparison of blood vessel formation between untreated diabetic group and diabetic groups treated with collagen scaffold alone (collagen), collagen scaffold containing CACs (EPC) and collagen scaffold containing CACs transduced with AdeNOS (Ad/eNOS). Short term duration of diabetes (1 week) and long term duration (4 weeks) are also compared. n=24 wounds per group.

6.5 Discussion

In this chapter we have demonstrated the feasibility of CAC delivery and genetically modified CAC delivery to the surface of diabetic wounds. In addition, in this study we have examined the effects of transplanted circulating angiogenic cells on diabetic ulcer healing using a collagen scaffold to apply cells to the wound site. In addition, given the contribution of nitric oxide to angiogenesis, CACs transduced by adenovirus carrying the bovine eNOS gene and which greatly overexpressed the gene were applied to examine potential effects of increased nitric oxide production on the rate of healing. In one group a collagen scaffold without cells was also applied as a control.

In the diabetic setting CACs have been shown to be functionally impaired (Fadini et al., 2004) and in this study eNOS was chosen as the transgene to explore its effects on improving functionality and accelerating wound healing. This gene was chosen due to its well documented pro-angiogenic effects.

Several studies to date have also looked at the feasibility of genetic modification of EPCs. Adenoviral transfer of the manganese superoxide dismutase gene has been found to increase wound healing in a mouse model of type 2 diabetes (Marrotte et al., 2010) and overexpression of PDGFR- β in EPCs accelerated re-endothelialisation after carotid artery injury (Wang et al., 2016). Adenoviral transfer of VEGF and HIF 1 α genes to EPCs was found to enhance neovascularisation and resulted in significant reduction of limb necrosis in murine ischaemia models (Iwaguro et al., 2002, Jiang et al., 2008). Retroviral transduction of human EPCs with eNOS and transplantation of these cells to rats after balloon injury inhibited neointimal hyperplasia and enhanced endothelium-dependent vasodilation (Cui et al., 2010). Retroviral vectors have also been used to modify EPCs to overexpress VEGF which enhanced the incorporation of transplanted EPCs into the neovasculature (Yu et al., 2009) and a lentiviral vector has been developed for EPCs to endow these cells with anti-tumour properties (Yang et al., 2015). EPCs genetically modified with human telomerase reverse transcriptase have also been used in a rat model to treat diabetes- induced erectile dysfunction (Zhang et al., 2016). EPCs transfected with the haptoglobin gene which potentiated gene expression of various pro-angiogenic factors, increased in vitro tube formation and improved blood perfusion and recovery

from ischaemic injury (Park et al., 2009). Additionally, transfection of EPCs with plasmid for SDF-1 resulted in enhanced tissue perfusion in hindlimb ischaemia (Kuliszewski et al., 2011) and CXCR4 gene transfer has been shown to contribute to the enhanced reendothelialization capacity of EPCs (Chen et al., 2010). In a rat model of balloon-induced vascular injury, EPCs transfected with hepatocyte growth factor and transplanted were associated with reduced neointimal formation and increased re-endothelialisation as well as enhanced proliferative, migratory and angiogenic capabilities (Song et al., 2009). Calcitonin gene-related peptide transfected EPCs have been found to exert reversal effects on pulmonary vascular remodelling and attenuate established pulmonary hypertension and intravenous transplantation of bone morphogenic protein 2-transduced EPCs have been shown to attenuate induced lung injury in rats (Zhao et al., 2007, Yin et al., 2015) and EPCs were seen to phagocytose plasmid DNA-gelatin complexes allowing a highly efficient, non-viral gene transfer (Nagaya et al., 2003). Angiogenesis has long been associated with wound healing and numerous studies have demonstrated the contribution of EPCs to blood vessel formation (Asahara et al., 1997, Asahara et al. 1999, He et al., 2004, Kalka et al., 2000). However, due to the relative dearth of tissue on the rabbit ear, direct injection of cells is not feasible. After clamping of the ear artery and injection of cell into the artery, no cells were identified on subsequent histological analysis of the tissue sections. This method was therefore abandoned. It was therefore necessary to apply the cells topically and a collagen scaffold was employed as a vehicle for this purpose as these cells have been shown to adhere to and differentiate on a number of substrates including collagen. This allowed for a stable platform for the cells to adhere to which facilitated keeping the cells at the wound site and allowed direct contact between the cells and the wound bed. Paracrine effects of pro-angiogenic substances is likely an important method of action influencing angiogenesis by these cells and this method of delivery also facilitated this.

Healing was measured using macroscopic wound area, epithelial gap, tissue regeneration within the wound site and angiogenesis. However, no significant differences between treatment groups in any of the parameters tested were observed. The reason for the absence of a therapeutic effect is unclear but may be due to diabetes induced cell

dysfunction in an autologous transplantation setting. In fact, autologous transplantation may lack efficacy due to disease induced cellular dysfunction. This may be overcome by allogeneic transplantation which is not possible with CACs or by augmentation of cell function by genetic modification. In this chapter we tried to accomplish this with eNOS overexpression. Unfortunately this strategy did not result in a positive therapeutic outcome. The reason for this is also unclear but could include the fact that this strategy does not reverse diabetes induced cellular dysfunction. Diabetic EPCs have been shown to be functionally impaired (Tepper, 2002), so whether this could account for the difference warrants further study. Stepanovic *et al* suggested that the use of diabetic progenitor cells may in fact impair angiogenesis but still have a positive effect on wound healing and therefore it would seem the cells must be contributing in a manner distinct from direct incorporation (Stepanovic et al., 2003). These cells are known to produce various cytokines and changes in the inflammatory response are a possible mechanism by which wound healing may be altered. A central question arising from our data is whether incorporation into a collagen scaffold limits the cell's ability to differentiate or alters the production of cytokines. Cell viability is, of course, of paramount importance for this study. Phase contrast microscopy reveals cells which take up ac-LDL adhering to the collagen scaffold but these cells do not exhibit the typical CAC morphology having a more rounded appearance. Whether this is a reflection of compromised cells or simply of the fact that the gel-like scaffold provides less purchase and support than the plastic of a tissue culture plate for adherent cells to elongate is unclear. A more stable substrate may be necessary for cells to take on the characteristic CAC morphology but whether this affects functionality warrants further study. A study carried out subsequent to this work by O'Loughlin et al. using a collagen scaffold as a vehicle for CAC delivery first seeded MNCs on a fibronectin coated plate and transferred the cultured CACs to the collagen scaffold 24 hours prior to administering it to the wound site with metabolic activity of the cells confirmed (O'Loughlin et al., 2013). Although trypsinising and replating these cells may result in some loss of cells as CACs appear to be a relatively delicate cell type as evidenced by their low proliferative rates (Yoder et al., 2007) and difficulty with passaging and cell destruction with electroporation compared to ECFCs, this strategy does appear to have yielded favourable results. Assessment of metabolic activity of

MNCs seeded directly on the collagen scaffold and cultured as per CAC culture protocol as pursued here would yield some interesting information. Assessment of cell viability could be carried out in future studies and a functional assay such as a cytokine array or protein analysis for secreted pro-angiogenic cytokines to measure the secretory activity of these cells would be very useful as CACs are thought to play an important role in augmenting angiogenesis and arteriogenesis via a paracrine role (Yoon et al., 2005, Sieveking et al., 2008).

Another possibility for the negative results with AdeNOS transduced cells is the possibility that although eNOS RNA was detected that the transgene did not result in functionally active eNOS and increased NO production. In terms of nitric oxide production, although expression of the eNOS transgene was greatly increased after transduction of the cells and bovine eNOS mRNA could be detected in large quantities by RT PCR functional eNOS protein or increased NO generation was not verified. Another possibility is an interaction between NO and superoxide which may be present in increased amounts in the wound. Tetrahydrobiopterin (BH₄) is decreased in diabetes and low levels of BH₄ results in eNOS uncoupling and production of superoxide anion (O₂⁻) instead of NO (Channon, 2004) and BH₄ overexpression has been shown to accelerate wound healing in diabetic mice by enhancing constitutive NOS (cNOS) activity and suppressing oxidative stress (Tie et al., 2009). Thus, even with production of sufficient eNOS protein, the potential lack of BH₄ in the diabetic milieu could lead to production of the superoxide anion rather than NO. Hyperglycaemia leads to increased reactive oxygen species (ROS) and cellular damage and augments the superoxide anion in skin tissue resulting in delayed wound healing in diabetic mice (Brownlee, 2001, Rolo et al., 2006, Luo et al., 2004). Accumulation of ROS leads to widespread cellular damage and poor neovascularization (Tie et al., 2009). In addition, EPC function including migration, adhesion, and tube formation are impaired in diabetes (Asai et al., 2006, Gallagher et al., 2007, Tepper et al., 2002). High glucose levels have also been shown to elevate oxidative stress and decrease EPC survival by inhibiting cell proliferation, NO production and MMP-9 activity (Krankel et al., 2005, Balestrieri et al., 2008). Normal EPCs have been shown to express intrinsically high levels of the antioxidant enzyme manganese

superoxide dismutase (MnSOD), which has a key function in EPC resistance to oxidative stress via scavenging mitochondrial ROS (He et al., 2004, Dernbach et al., 2004, Chen et al., 2006). Decreased levels of MnSOD expression and elevated levels of oxidative stress have been demonstrated in diabetic EPCs and gene therapy of MnSOD for diabetic EPCs prior to transplantation onto diabetic wounds has been shown to restore their angiogenic functions in vivo with accelerated wound repair (Marrotte et al., 2010). Duration of hyperglycaemia has been shown in the preceding chapter to affect the rate of wound healing and may also affect the relative impairment of EPC function.

In summary, we have been unable to demonstrate a beneficial effect of autologous transplantation of CACs with or without eNOS overexpression on wound healing in the setting of 1 or 4 weeks of hyperglycaemia. This may be due to autologous cells dysfunction and will need to be explored in future studies and promising research has recently been done using CACs treated with osteopontin delivered using a collagen scaffold, which showed augmented healing of diabetic wounds (O'Loughlin, 2013). These results do not preclude the value of eNOS as a therapeutic gene of interest as many factors need to be considered in the complex diabetic milieu and further study would clarify the contributing roles and associations of elements of this setting.

7. Concluding Comments

7.1 SUMMARY AND DISCUSSION

The healing of diabetic ulcers remains one of the most important aspects in the management of the complications of diabetes. It constitutes a major area of healthcare expense with inpatient stays being extended considerably, as well as being a source of patient distress and a risk factor for infection and amputation. Along with peripheral neuropathy, poor angiogenesis in the diabetic limb contributes greatly to the formation and deficiency in healing of these ulcers. Lack of vessel formation means generation of granulation tissue is compromised as is epithelial coverage. This compromises the strength of the tissue in wounds that do heal and in those that do not there is a risk of necrosis. Accordingly, strategies to improve and accelerate wound healing are of great clinical significance. In this work we explored such a strategy using circulating endothelial cells, a type of cell that may be referred to as a type of endothelial progenitor cells. These cells are bone marrow derived cells which can be isolated from peripheral blood and may be seen to develop endothelial characteristics and participate in angiogenesis. Because these cells have been shown to be functionally impaired and reduced in number in the diabetic setting, strategies to improve or restore function are also of importance clinically. Here we used a gene transfer approach and after identifying the most efficient vector for gene transfer, we transduced these cells with the eNOS gene. This enzyme catalyses the production of nitric oxide, which is well known to have pro-angiogenic effects. These cells were seeded on a collagen scaffold and applied directly to a rabbit ulcer model to explore their effect on healing.

Since being first described in 1997, there has been considerable interest in endothelial progenitor cells for promotion of blood vessel formation in a range of ischaemic conditions. These cells have mostly been isolated from peripheral blood but cells

designated EPCs have also been isolated from bone marrow and cord blood. Two types of cell which have previously been designated early EPCs and late or outgrowth EPCs have been identified. There is little consensus on the precise phenotype of these cells and varying culture techniques have been used to isolate cells which have then been called EPCs. However, broadly there do seem to be two types of cell, one which may be cultured from 4-10 days which shows low proliferative rates and a second type which appear after 2-3 weeks of culture and which have greater proliferative potential. These have been referred to as early and outgrowth EPCs but we have used the nomenclature circulating angiogenic cells (CACs) and endothelial colony forming cells (ECFCs). While these cells may operate differently, both have been shown to have angiogenic properties in a variety of settings. This study was undertaken to determine the effects of endothelial progenitor cells on the healing of diabetic ulcers. Cells isolated, first from human peripheral blood and then from rabbit blood, were characterised and used in an in vivo model of diabetic ulceration. Results in relation to the healing of the wound itself and the degree of angiogenesis were then examined.

The role of nitric oxide in the enhancement of angiogenesis has also generated much interest in recent years. With this in mind we also examined the effects of CACs overexpressing endothelial nitric oxide synthase in terms of additional benefits for wound healing over non-transduced cells. In addition to transplantation of autologous cells, cells that had been genetically modified by transducing them with bovine eNOS gene were examined regarding their potential to affect a range of parameters related to healing. This necessitated exploring the potential of these cells for transduction as well as identifying the most efficient vector. Furthermore, an appropriate mode of delivery had to be designed to apply the cells to the wound area.

Currently, no definitive marker to characterise an EPC exists. A range of studies using cells isolated from peripheral blood, bone marrow and cord blood have designated the cell population that they used as EPCs although no consensus has been reached to define an EPC and differing characterising features were used. The first step in this study was, therefore, to define and characterise the population we were using. Chapter

2 describes the process of optimisation of the culture and characterisation of the CACs. Culture conditions were optimised for both CACs and ECFCs and characterisation studies had to be carried out as well as preliminary exploration of transduction efficiencies.

Since being first described, EPCs have been characterised according to markers displayed by cells and by their staining properties. The cells we have isolated from peripheral blood show uptake of ac-LDL and UEA-1 lectin, both of which are known to stain cells of endothelial lineage. In addition, the cells have stained positive for the haematopoietic marker CD34 and the stem cell marker CD133. They have also stained positive for endothelial specific markers including KDR and vWF. These results give a picture of a cell isolated from peripheral blood which displays properties of endothelial cells, stem cells and shows haematopoietic lineage which have all been associated with purported endothelial progenitor cells.

4-day, 7-day and 10-day CACs were cultured for comparison. The 4-day cells did not show the same degree of morphological differentiation as seen in the literature and as more data exists for 7-day cells, work with 4-day cells was discontinued. Transduction efficiencies of day 10 EPCs were analysed but the morphology of the cells was inconsistent with a healthy population, as many rounded cells could be observed and, although transduction efficiencies appeared high, this may have been in part due to compromised membrane integrity. OECs also showed high transduction efficiencies but due to the relative difficulty and unreliability of growing these cells from small volumes of peripheral blood, the use of these cells for further experiments was decided against as results from these experiments were to be used as a basis for the animal studies which were to be subsequently carried out. This decision was informed by the relatively small volumes of blood which would be available. With these factors in mind it was decided to use 7-day EPCs for subsequent experiments.

Chapter 3 describes the experiments undertaken to choose the most efficient vector for transduction of CACs. The 3 viral vectors, adenovirus, adeno-associated virus and

lentivirus were examined as well as 2 non-viral vectors, namely liposomes and electroporation. Electroporation proved unsuitable for these cells as cell death was high and of the other 4 vectors adenovirus was shown to be the most efficient. This was the vector then used in the remaining experiments. Morphologically, it may be observed that there are clear differences between the cells found in a culture of 7-day EPCs. This heterogeneity may be one reason why it is difficult to arrive at a consensus as to the definition of an EPC. This may also lead to differences in culture that are grown from a parent population sorted by flow cytometry. Of note in our transduction studies 2 distinct populations, separated on the basis of cell size, emerged which showed markedly different transduction efficiencies. One of these populations was responsible for practically all observed transduction and subsequent transgene expression while the other showed remarkably limited capacity for transgene expression. This finding may provide a basis by which EPCs populations may be further refined. To our knowledge, no other direct comparison of different vector types for transduction of CACs has been carried out. There have been a number of studies carried out using a variety of viral and non-viral vectors for EPCs but due to the aforementioned difficulties with a lack of standardisation of culture conditions and characterisation of these cells, a comparison of vectors using cells cultured and characterised in an identical manner, as shown here, represents a robust model to identify the most efficient vector for this cell type.

Having optimised culture conditions and established the most suitable vector for cell transduction, the CACs were then used in a pre-clinical model. Chapters 3 and 4 describe animal experiments which were carried out to determine the effects of the transduced CACs on the healing of diabetic ulcers. The rabbit ear ulcer model was used in both the diabetic and non-diabetic settings as well as with non-transduced cells and cells transduced with bovine eNOS. Various models have been used to examine wound healing. The rabbit ear ulcer model provides significant advantages for this work in that it is non-contractile. This differs from the mouse model, for example, which has a sub-cutaneous contractile layer which is designed to aid in wound healing. The non-contractile model is more relevant clinically and the rabbit ear model also heals from

the periphery of the wound, with granulation tissue advancing from the edges, rather than from the base of the wound, which greatly facilitates analysis of healing.

In addition, wounds induced at 2 different time-points after induction of diabetes were examined and healing was measured by a variety of parameters. Wound area, epithelial gap and tissue regeneration were all used to assess healing as well as blood vessel formation. Cells were delivered to the wound area by means of a collagen scaffold. Due to the relative dearth of tissue on the rabbit ear, direct application of cells was non-feasible. Additionally, since these cells appear to exert their angiogenic effects largely through paracrine action rather than direct incorporation, a stable platform was required. Since these cells may be cultured on collagen-coated plates, a collagen scaffold was used as the delivery vehicle of choice.

Although no differences were seen in terms of angiogenesis, delayed healing was observed when wounds were created at 4 weeks after induction of diabetes compared to 1 week after diabetes induction. In terms of the methodology for studies of this nature the fact that the duration of diabetes had significant effects on healing raises interesting questions. Different studies have used differing lengths of time as well as different agents of induction of diabetes. Since this appears to be an important parameter perhaps a more standardised approach would have benefits for relating different studies to one another. The fact that 2 different time-points were used after the induction of hyperglycaemia may also have effects on the cells used. Since cells were isolated and cultured 7 days prior to wound induction, the state of hyperglycaemia had existed for an additional period prior to cell isolation. Since these cells are known to be impaired in functionality and number, the possibility that cells used for the later time-point may have been more impaired than those from the earlier time-point should also be considered. Interestingly, although no differences were detected when angiogenesis was measured between either the treatment groups or the different duration of diabetes groups, a difference was seen in the degrees of healing between the latter 2 groups. This raises questions about the fundamental mode of action by which diabetes compromises the healing of ulcers.

However, when wounds treated with collagen scaffold alone, collagen scaffold containing CACs or CACs transduced with adenoviral vectors to overexpress the eNOS gene were examined no differences were seen between any of the treatment groups in terms of angiogenesis or the healing parameters examined.

One possible factor contributing to this may be the method of cell delivery. Since CACs are thought to mediate their effects largely through the paracrine effects of angiogenesis-promoting cytokines rather than direct cell incorporation, a cytokine array or protein analysis would be useful to assess the levels of cytokines being produced by the cells. Subsequent work has shown positive effects on angiogenesis and wound healing when CACs were applied to wounds using a collagen scaffold when the cells were grown in culture plates and transferred to the scaffold 24 hours before application to the wound site rather than being seeded directly on the scaffold after isolation. However, different measures of angiogenesis and different parameters of healing were examined in these studies so direct comparison is difficult. Although PCR studies showed a high level of bovine eNOS mRNA production, production of increased levels of viable eNOS protein were not verified. A protein assay for the eNOS protein would provide useful additional information. Additionally, insufficient levels of BH₄, the substrate from which NOS catalyses NO have been shown to lead to production of the superoxide anion rather than NO and this may also account for an apparent lack of effect of NO on angiogenesis and wound healing in this study. In addition, since diabetic EPCs are known to be compromised it may also be desirable to try the transduction studies with non-diabetic EPCs as this may yield more favourable results in terms of cytokine and protein production. This however has the drawback of not being directly applicable to allogenic transplant in the diabetic model. The use of SCID mice may circumvent this problem but a model of allogenic transplant would seem to be more directly relevant to the clinical setting.

Additionally, many studies have used a macroscopic measure of wound healing as their primary parameter of change. The results from this study suggest that the most

sensitive measures of healing are histological and such measurement may further refine such studies in future.

Some of the difficulties in the field of EPC/CAC biology may be addressed by standardising the culture and characterisation methods of these cell types. The cells need to be defined in terms of cell phenotype and function as well as cytometric, transcriptomic, proteomic and metabolic evaluations. It should also be noted that *in vitro* culture may be associated with changes in phenotype and increased risk of cell senescence. Clinical trial using these cells may be confounded by inconsistent cell definitions, different number of cardiovascular risk factors in different patient populations and interaction of EPCs/CACs with other haematopoietic progenitor cells, inflammatory cells and platelets. Factors affecting the validity of these studies include small numbers of patients enrolled in studies, variation in cell phenotype, different administration methods and lack of long-term follow up results. Difficulties may also be encountered in attempts to expand suitable numbers of cells. Reported changes in cell number and function may be influenced by methodological approaches such as timing and methods of taking samples, detection methods, panel of antibodies used for phenotypical evaluation, age of patients and their clinical conditions and ethnicity of populations studied. Clinical considerations include transplantation modality, delivery systems, grafting, rejection biology, clinical outcomes and device technology.

In conclusion, this study set out with two broad aims. To assess the optimal transduction criteria for EPCs and to use these cells, after being transduced to overexpress eNOS, to explore their effects on diabetic ulcer healing. Regarding the first aim we have identified adenovirus as the most suitable vector for cell transduction although similar experiments may be of benefit with other cell types such as outgrowth EPCs which show potential for use in *in vivo* studies.

In terms of the second aim, no improvement was seen in healing parameters measured when either non-transduced early EPCs or those transduced to overexpress the bovine eNOS gene. This may reflect a need for further studies to optimise the cell delivery or a

deficiency downstream of eNOS mRNA production by the cells. Of note the duration of diabetes prior to wound induction proved a significant parameter and this effect appears to be independent of angiogenesis. This does not preclude the use of eNOS as a potential target for genetic modification of these cells. The diabetic milieu is a very complex environment and many factors must be considered when examining this area. These include production of sufficient amounts of eNOS protein and sufficient substrate for it to act on. In addition, cell viability and activity could be explored further, particularly regarding use of autologous cells in the diabetic setting and further exploration regarding optimisation of cell delivery may prove beneficial. Additionally, further measurement of angiogenesis such as length density, surface area and volume of vessels could yield additional information.

This thesis outlines the development of a useful model for the study of diabetic ulcers and the use of CACs in this field as well as giving insight into the mechanism of action and effects of diabetes on healing of these ulcers. It also provides a valuable tool in the area of gene therapy studies on CACs and contributes to the classification, characterisation and identification of these studies. It is our hope that this work can be built on to further expand the body of knowledge in this area.

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