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THE ROLE OF ENDOTHELIAL PROGENITOR CELLS IN ACUTE VASCULAR INJURY IN MAN

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To Leigh, for her love and patience

ABSTRACT

Percutaneous coronary intervention (PCI) acutely improves coronary blood flow and myocardial perfusion but at the expense of endovascular laceration and endothelial denudation. PCI associated vascular injury is associated with intense inflammation and a loss of vascular function that may lead to significant in-stent restenosis (ISR), and the potentially catastrophic, acute stent thrombosis. Re-endothelialisation is essential to the restoration of normal homeostasis and facilitating vascular healing. Attention has recently focused on a novel mechanism of re-endothelialisation mediated by bone marrow-derived precursor or stem cells: endothelial progenitor cells (EPC). EPC are thought to home to, and re-endothelialise sites of endothelial denudation, and therefore offer the potential to provide exciting new developments in the management of cardiovascular disease. Understanding the role of EPC following vascular injury may help us to enhance vascular repair following PCI.

The following studies were performed to clarify the relationships between putative EPC and vascular injury associated with PCI. In studies of patients undergoing elective PCI for stable anginal symptoms I found that concentrations of traditional circulating phenotypic EPC expressing $CD34^+VEGFR\text{-}2^+$ were unaffected, unlike $CD34^+CD45^-$ cell concentrations, which were transiently increased six hours following PCI, subsequently returning to normal by 24 hours, notably without an increase in $CD34^+$ adhesion molecule expression or VEGF-A production. However, the purported progeny of $CD34^+VEGFR\text{-}2^+$ cells, endothelial cell-colony forming units (EC-CFU), were mobilised at 24 hours, commensurate with a systemic inflammatory response. Interestingly the concentration of circulating

$CD34^+VEGFR\text{-}2^+$ cells and EC-CFU were unrelated to each other, emphasising the distinction between these two cell populations. Although EC-CFU contained proliferating cells and exhibited some endothelial characteristics, EC-CFU predominantly expressed the leukocyte antigen CD45 in addition to the lymphocyte markers CD4 and CD8, and most intensely, the surface markers CD68 and CD105, epitopes commonly expressed on macrophages. Notably, EC-CFU were a potent stimulus for the migration of mononuclear cells. However, despite being mobilised in the context of an acute systemic inflammatory response and being composed of leukocytes, isolated systemic inflammation in healthy volunteers (induced by *Salmonella Typhus* vaccination) in the absence of vascular injury did not cause selective mobilisation of EC-CFU or indeed of putative phenotypic EPC. It is therefore likely that EC-CFU mobilisation is a relatively specific inflammatory response to cardiovascular injury.

In a cohort of 201 patients undergoing coronary angiography, traditional circulating phenotypic EPC ($CD34^+VEGFR\text{-}2^+$ and $CD34^+VEGFR\text{-}2^+CD133^+$) were very rare indeed and were not increased in response to an acute coronary syndrome (ACS). Furthermore traditional EPC concentrations bore no relation to atheroma burden or clinical outcome. In contrast, concentrations of $CD34^+CD45^-$ cells were increased in patients with coronary artery disease compared to those with normal coronary arteries and were increased in association with more severe coronary disease. Increased concentrations of circulating $CD34^+CD45^-$ cells were also associated with a shorter cumulative event-free survival. Both EC-CFU and

angiogenic monocytes expressing Tie-2 and VEGFR-2 were increased following acute myocardial infarction but did not relate to coronary atheroma or clinical outcome.

These studies examine the behavior of putative EPC in response to both discrete vascular injury and myocardial infarction, and isolated inflammation in the absence of vascular injury. I have identified novel characteristics of the EC-CFU assay and determined that specific factors associated with cardiovascular injury likely trigger EC-CFU mobilisation. The clinical relevance of the traditional phenotypic EPC population is uncertain, but a novel $CD34^{+}CD45^{-}$ population is mobilised acutely following discrete vascular injury and is significantly associated with coronary atheroma and clinical events. It is probable that the circulating $CD34^{+}CD45^{-}$ concentration reflects vascular injury and atheroma burden, and I suggest that $CD34^{+}CD45^{-}$ cells are released directly from the vessel wall following PCI, and do not reflect a reparatory response. In order to determine the impact of EPC populations on vascular healing, prospective studies examining the impact of peri-procedural EPC concentrations on vascular healing following PCI are required.

CONTENTS

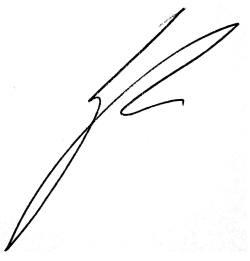
Chapter 2:

DECLARATION

This thesis represents research undertaken in the Centre for Cardiovascular Sciences, University of Edinburgh, and the Department of Cardiology of the Royal Infirmary of Edinburgh. These studies were supported by a British Heart Foundation Clinical Research and Training Scholarship (SS/CH/92101) awarded to myself, and project grants awarded by the British Heart Foundation (07/017/22405) and Chest, Heart and Stroke (RES07/A106). I was personally involved in the conduct of all of the following studies and the data analysis presented in this thesis. Assistance with these studies was gratefully received from Dr Mark De Belder's group at James Cook University Hospital in Middlesborough and in the supervision of the laboratory assays as acknowledged.

Chapters one, three, four, five, six and selected data from chapter eight have been published in peer-reviewed journals. Chapter seven is presently a manuscript under review. Copyright permission has been obtained for inclusion of the printed journal manuscripts within this thesis. This thesis has not been accepted in any previous applications for a degree and all sources of information have been acknowledged. All studies were undertaken in accordance with the regulations of the Regional Ethics Board of Lothian and London Research Ethics Committees and with the Declaration of Helsinki of the World Medical Association. The written informed consent of each subject or patient was obtained before entry into these studies.

Dr Gareth John Padfield



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microscopy. I am indebted to Gillian Kidd from the graphics lab at the Western General Hospital, Edinburgh who helped me produce some of the figures that greatly facilitated the communication of some of the hypotheses presented within this work. The staff of the Wellcome Trust Clinical Research Facility (supported by NHS Research Scotland through NHS Lothian) were instrumental in the organisation of blood sample processing and storage and facilitated the vast majority of the work presented here. I also received statistical guidance at the Wellcome trust at the Western General Hospital, Edinburgh from Dr Cat Graham and help with the use of SPSS from Dr David McAllister.

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I place a huge amount of value on the selfless contributions made by the patients and healthy volunteers who participated in these studies, many of whom underwent considerable inconvenience; returning on several occasions for uncomfortable blood tests; receiving vaccinations; and undergoing potentially dangerous invasive procedures without immediate benefit to themselves. Without the generous sacrifices made by people who recognise the importance of clinical research, studies such as these would grind to a halt.

Finally I would like to thank my partner Leigh, for the love and patience she has shown me throughout my PhD studies, and my parents, who have provided me with so much good fortune.

ABBREVIATIONS

ACE	=	angiotensin converting enzyme
ACS	=	acute coronary syndrome
ANOVA	=	analysis of variance
ARB	=	angiotensin receptor blocker
AUC	=	area under curve
BrdU	=	bromodeoxyuridine
CABG	=	coronary artery bypass grafting
CAC	=	circulating angiogenic cell
CFU	=	colony forming unit
CRP	=	C-reactive protein
CV%	=	Coefficient of variation
DAPI	=	4',6-diamidino-2-phenylindole
DES	=	drug eluting stents
EC	=	endothelial cell
EC-CFU	=	endothelial cell colony forming unit
ECFC	=	endothelial colony forming cell
EOC	=	endothelial outgrowth colony
EPC	=	endothelial progenitor cell
FITC	=	fluorescein isothiocyanate
FSC	=	forward scatter
GFP	=	green fluorescent protein
GTN	=	glyceryl trinitrate
ICAM	=	inter-cellular adhesion molecules
IL	=	interleukin
IQR	=	interquartile range
ISR	=	in-stent restenosis
IVUS	=	intra-vascular ultrasound
LPS	=	lipopolysacharride
MAB	=	monoclonal antibody
MCP-1	=	monocyte chemoattractant protein – 1
MLD	=	minimal luminal diameter
MRC	=	medical research council
NSTEMI	=	non-ST elevation myocardial infarction
PCI	=	percutaneous coronary intervention
PCR	=	polymerase chain reaction
PSI	=	pounds per square inch
QCA	=	quantitative coronary analysis
RNA	=	ribonucleic acid
SCD/NOD	=	severe combined immunodeficiency/non-obese diabetic
SD	=	standard deviation
SDF-1	=	stromal derived factor-1
SEM	=	standard error of the mean
SSC	=	side scatter
STARS	=	steroids against restenosis trial
STEMI	=	ST-elevation myocardial infarction
UEA-1	=	*Ulex europeaus* agglutinin-1

CHAPTER ONE

INTRODUCTION

UNDERSTANDING THE ROLE OF ENDOTHELIAL PROGENITOR CELLS IN ACUTE VASCULAR INJURY AND PERCUTANEOUS CORONARY INTERVENTION

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1.1 OVERVIEW

Although percutaneous coronary intervention (PCI) improves myocardial perfusion and clinical outcomes in patients with ischaemic heart disease, the treated coronary artery segment inevitably undergoes significant mechanical trauma. Endothelial denudation and endovascular laceration by rigid stent struts and high-pressure balloon inflations disturb vascular function and initiate an intense local inflammatory response. As a consequence of this, vascular injury is invariably associated with a degree of neo-intimal hyperplasia, which if severe may lead to clinically significant in-stent restenosis (ISR). The thrombotic milieu resulting from PCI underlies the potentially catastrophic complication of acute stent thrombosis. Re-endothelialisation is a critical component of vascular healing necessary for restoring vascular homeostasis. Re-endothelialisation was previously thought to occur purely through the migration and proliferation of mature endothelial cells adjacent to regions of endothelial denudation [Risau, 1995]. However, attention has recently focused on a novel mechanism of vascular repair involving a bone marrow-derived precursor or stem cell: the endothelial progenitor cell (EPC). It has been proposed that EPC are mobilised in response to vascular injury, and can home to sites of endothelial denudation [Asahara *et al.*, 1997] in order to facilitate re-endothelialisation [Shi *et al.*, 1998]. The discovery of the EPC launched a new field of cardiovascular research, changed our understanding of the mechanisms involved in vascular repair, and has offered exciting and novel developments in the management of cardiovascular disease and the complications associated with modern revascularisation strategies.

1.2 PERCUTANEOUS CORONARY INTERVENTION AND VASCULAR INJURY

Adverse cardiac events following PCI were initially very common, with major complications occurring in up to 20% of patients undergoing stent implantation [Serruys *et al.*, 1994]. Technological advances have improved patient outcomes, though angiographic restenosis following PCI remains significant, occurring in approximately 11% of cases, although the incidence can approach 30% in higher risk populations using bare metal stents [Mauri *et al.*, 2008]. Stent thrombosis, although a relatively rare complication of PCI, still occurs in up to 2% of cases and has potentially devastating and fatal consequences [de la Torre-Hernandez *et al.*, 2008]. These complications occur, in part, as a consequence of the vascular injury that occurs during PCI [Farb *et al.*, 1999]. High-pressure balloon inflations and the forceful apposition of rigid stent struts against the vessel wall which occur during PCI invariably cause laceration of the tunica intima and the disruption of endothelial continuity. Endovascular laceration may extend through the media to involve the external elastic lamina, and even vessel rupture is an uncommon but well recognised complication of PCI. Disruption of endogenous fibrinolytic and vasomotor function, combined with the exposure of underlying collagen and tissue factor, leads to activation of platelets and the coagulation cascade which may lead to acute or sub-acute stent occlusion [Mak *et al.*, 1996]. An intense local [Farb *et al.*, 1999] and systemic inflammatory response [Almagor *et al.*, 2003] is generated, involving a rapid influx of neutrophils, and later monocytic cell populations into the vessel wall. The endothelium normally provides a protective barrier for smooth muscle cells against inflammatory cytokines and growth factors, and also secretes a number of cytostatic factors that prevent smooth muscle cell proliferation. In the absence of a functional

endothelium fibroblastic activation and smooth muscle hypertrophy are potentiated, leading to in-stent restenosis and myocardial ischaemia [Wilcox *et al.*, 2001]. Rapid re-endothelialisation is therefore important in the restoration of normal vascular function, reduction of vascular inflammation and the prevention of adverse remodelling following PCI [Kipshidze *et al.*, 2004].

The process of stent integration into the vessel wall has been elegantly characterised using electron microscopy [Grewe *et al.*, 2000]. During the first 6 weeks following PCI a thin multi-layered thrombus is present on the endovascular surface and progressive smooth muscle hyperplasia and deposition of extracellular matrix occurs. From 6 to 12 weeks following PCI the thrombus resolves and endothelial cells begin to cover the stented segment. At approximately three months, re-endothelialisation is complete, commensurate with a diminution in the quantity of smooth muscle cells. The use of drug eluting stents (DES) has dramatically reduced the incidence of ISR [Mak *et al.*, 1996], however rather than encouraging re-endothelialisation, this approach is based on the suppression of cellular proliferation. Re-endothelialisation is therefore also suppressed [Hofma *et al.*, 2006] and the thrombotic risk is increased as a consequence. The use of DES therefore demands a prolongation of anti-platelet therapy, which unfortunately is associated with an increased risk of bleeding complications. A means of encouraging re-endothelialisation is therefore highly attractive. EPC may comprise an important component of the cellular response to vascular injury and are therefore a potential therapeutic target through which re-endothelialisation following PCI can be enhanced.

1.3 HISTORICAL PERSPECTIVE OF ENDOTHELIAL PROGENITOR CELLS

The concept that naïve precursor cells with the capacity to differentiate into mature cell types exist within the adult circulation is supported primarily by studies of bone marrow transplant recipients, within whom exist appropriately differentiated cells of donor origin, integrated into host structures such as the heart [Quaini *et al.*, 2002], lung [Suratt *et al.*, 2003] and vasculature [Jiang *et al.*, 2004]. The formation of blood vessels by naïve precursor cells in the adult is contrary to the traditional paradigm of re-endothelialisation or neovascularisation, whereby new endothelial cells are generated through the proliferation and migration of mature endothelial cells [Risau, 1995]. Although the recipient of enormous interest in the last decade, the concept of circulating vascular progenitor cells is not so novel. In the 1930s, the formation of vascular structures arising from peripheral blood cultures was observed in vitro [Hueper WC, 1932; Parker, 1933], and in the 1960s, 'islands' of endothelium were observed developing on the surface of implanted devices and intravascular prostheses [Florey *et al.*, 1961; Mackenzie *et al.*, 1960; Poole *et al.*, 1962; Stump *et al.*, 1963]. The presence of circulating progenitors capable of re-endothelialisation was later suggested by Scott, *et al.*, in 1994 following the successful re-endothelialisation of a vascular graft that was suspended within the aorta of a dog [Scott *et al.*, 1994].

In 1997 Asahara *et al*, popularised the term EPC by isolating cell populations from peripheral blood which were capable of homing to regions of ischaemia and facilitating vascular regeneration [Asahara *et al.*, 1997]. EPC have been recognised as circulating [Asahara *et al.*, 1997], bone marrow [Peichev *et al.*, 2000] or tissue resident [Zengin *et al.*, 2006] cells, that are mobilised in response to tissue ischaemia

or vascular perturbation, and possess the capacity to home to regions of injury and differentiate into a mature endothelial cells, or adopt an 'endothelial like' phenotype and participate in vascular repair. However, the term EPC is ambiguous, having been used to define a variety of different cell populations. Whilst our understanding is incomplete it is likely that the various populations so far identified fulfil different components of a vascular repair system. Broadly speaking EPC have been identified either phenotypically using flow cytometry, or 'functionally' using cell culture assays whereby the presence of an EPC is inferred through the demonstration of the evolution of mature endothelial characteristics in a population of cultured heterogeneous cell types.

1.4 IDENTIFYING ENDOTHELIAL PROGENITOR CELLS BY SURFACE PROTEINS

Through the close spatial relationship observed between endothelial and haematopoietic cell lineages in developing embryos, it is evident that a common progenitor, the haemangioblast, gives rise to both the vascular and haematopoietic systems [Sabin, 1920]. This is supported by gene deletion studies that identified the existence of common cell surface proteins necessary for vascular and haematopoietic development [Cheng *et al.*, 1996; Shalaby *et al.*, 1995]. Whilst previously thought to be specific to the developing embryo, there is now evidence to support the existence of a post-natal haemangioblast in man [Bailey *et al.*, 2003; Ribatti, 2008]. Efforts to define the haemangioblast have helped shape our understanding of the EPC. A variety of haematopoietic and endothelial surface markers have been used to identify putative EPC (Table 1.1).

Endothelial progenitor cells have been predominantly defined by the expression of CD34 and the extracellular domain of vascular endothelial growth factor receptor-2 (VEGFR-2), as these cell surface markers are thought to indicate cellular naivety and a vascular phenotype respectively. The surface receptor CD34 is often regarded as a 'stem cell' marker and is widely used as an indicator of cellular naivety. CD34 is a cellular adhesion molecule necessary for normal haematopoiesis [Cheng *et al.*, 1996], and is also expressed on vascular endothelium [Fina *et al.*, 1990]. VEGFR-2 is one of a family of transmembrane cell surface receptors for vascular endothelial growth factor, a pro-angiogenic cytokine. VEGFR-2 is considered as a marker of endothelial lineage, being widely expressed on mature endothelial cells and is necessary for the normal development of the vascular system and haematopoiesis *in-utero*. Upon differentiation toward a haematopoietic lineage the VEGF and CD34 receptors are lost, whilst those committed to an endothelial lineage retain expression [Civin *et al.*, 1984; Matthews *et al.*, 1991].

Asahara *et al*, demonstrated that both $CD34^+$ and $VEGFR\text{-}2^+$ fractions isolated from mononuclear cells when cultivated under angiogenic conditions up-regulated endothelial surface antigens (CD31, Tie-2, E-Selectin, VEGFR-2), and were capable of homing to regions of ischaemia and appeared to participate in neovascularisation in an animal model of hind limb ischaemia [Asahara *et al.*, 1997]. Whilst fuelling an intense period of research this study was far from definitive and indeed raised more questions than it answered. The purity of the cell populations used in the study was poor (CD34 ~ 15.7% and VEGFR-2 ~ 20%), and the infused cells were not specifically dual positive. CD34 and VEGFR-2 co-expression is also a

characteristic of mature endothelial cells therefore additional surface markers have been used in an attempt to distinguish EPC from mature endothelial cells sloughed from the vessel wall by vascular stress [Dignat-George *et al.*, 2000]. The cell surface protein CD133 is a relatively novel surface receptor with unknown function that is expressed on most $CD34^+$ cells. CD133 is quickly down regulated on differentiation and is therefore a reliable indicator of cellular naivety [Miraglia *et al.*, 1997]. Whilst CD133 is generally considered to be a marker of haematopoietic lineage, on account of the ability of $CD133^+$ cells to provide haematopoietic reconstitution by transplantation, $CD133^+$ cells have also been reported to give rise to cells with an endothelial phenotype in culture [Gehling *et al.*, 2000]. The co-expression of all three receptors has been used to identify a population of mononuclear cell capable of developing a mature phenotype [Peichev *et al.*, 2000]. CD133 expression either in isolation, or in combination with CD34 and VEGFR-2 has been used in variety of studies to define EPC [Friedrich *et al.*, 2006; Gill *et al.*, 2001; Grisar *et al.*, 2005; Kanayasu-Toyoda *et al.*, 2003; Kipshidze *et al.*, 2004; Mauro *et al.*, 2007; Palange *et al.*, 2006; Quirici *et al.*, 2001; Valgimigli *et al.*, 2005; Yang *et al.*, 2004].

$CD34^+CD133^+VEGFR2^+$ cells are however extremely rare in the peripheral circulation and reliably measuring them in clinical studies is extremely difficult and it is questionable that they could be used in clinical practice. Furthermore although the expression of CD133 is a reliable indicator of cellular naivety, it still fails to adequately distinguish a vascular progenitor from a haematopoietic progenitor. Case *et al*, specifically compared the behaviour of $CD34^+CD133^+VEGFR2^+$ cells when used in two widely employed endothelial colony forming assays, early and late EPC

(discussed below), and a haematopoietic colony-forming assay [Case *et al.*, 2007]. $CD34^+CD133^+VEGFR2^+$ cells were incapable of forming colonies in the culture assays previously used to define EPC, but consistently formed colonies in haematopoietic assays. The majority of $CD34^+CD133^+VEGFR2^+$ cells in fact expressed the common leukocyte antigen CD45, and the capacity to form an endothelial phenotype was confined to those $CD34^+$ cells that were $CD45^-$. These findings have been supported by Timmermans *et al*, who also demonstrated that $CD45^-CD34^+CD133^-VEGFR\text{-}2^+$ cells are capable of forming late outgrowth endothelial colonies (see below), and that a separate $CD45^+CD34^+CD133^+VEGFR\text{-}2^-$ population, formed haematopoietic colonies [Timmermans *et al.*, 2007]. $CD45^+$ is a powerful discriminator of pure haematopoietic and non-haematopoietic fractions and is beginning to be used to discriminate populations of EPC in clinical studies. It may transpire that the absence of CD45 may define a population of progenitor cell that ultimately forms true endothelial cells, as $CD34^+CD45^-$ cells generate colonies that more closely resemble mature endothelial cells both functionally and phenotypically. However, as mature endothelial cells are $CD45^-$ and may also express CD34 [Delia *et al.*, 1993; Fina *et al.*, 1990], the detection of $CD34^+CD45^-$ cells in the peripheral circulation may simply represent the presence of circulating mature endothelial cells rather than bone marrow derived progenitor cells. Late outgrowth colonies may represent the same phenomenon [Case *et al.*, 2007].

Just as endothelial function is integral to the maintenance of normal vascular

homeostasis [Ross, 1990], EPC regulation appears to be similarly important. Concentrations of circulating $CD34^+VEGFR\text{-}2^+$ cells are reduced in patients with traditional cardiovascular risk factors including cigarette smoking, elevated LDL cholesterol [Vasa *et al.*, 2001b], diabetes mellitus [Fadini *et al.*, 2005] and hypertension [Pirro *et al.*, 2007]. $CD34^+VEGFR\text{-}2^+$ cell concentration is markedly reduced in patients with overt atherosclerotic disease of the coronary and peripheral circulation [Chironi *et al.*, 2007]. Consistent with their putative vasculoprotective function, a high circulating concentration of $CD34^+VEGFR\text{-}2^+$ cells is associated with a lower risk of myocardial infarction, hospitalisation, revascularisation and cardiovascular death in patients with coronary artery disease [Schmidt-Lucke *et al.*, 2005; Werner *et al.*, 2005]. $CD34^+VEGFR\text{-}2^+$ [Vasa *et al.*, 2001b], $CD133^+VEGFR\text{-}2^+$ [Thum *et al.*, 2007b], and $CD133^+CD34^+$ populations fall with advancing age, and mobilisation in response to cardiovascular stress is reduced in older patients [Scheubel *et al.*, 2003]. Matching for biological characteristics however, in particular angiographic severity of coronary artery disease, diminishes the importance of chronological age as a determinant of EPC concentration [Pelliccia *et al.*, 2009], however EC-CFU are significantly higher in children compared to adults [Fabbri-Arrigoni *et al.*, 2012]. EPC biology is in part inherited, with apparently healthy offspring of patients with coronary artery disease having reduced levels of $CD34^+VEGFR\text{-}2^+$ cells compared with the offspring of healthy controls [Whittaker *et al.*, 2008].

Even in healthy people $CD34^+$ cells comprise a mere 0.05% of circulating

leukocytes. Circulating sub-populations co-expressing CD133 and VEGFR-2 are even more rare, and the capacity of such a small population to provide significant endovascular repair is questionable. CD14 is a membrane bound receptor primarily responsible for the detection of lipopolysaccharide [Wright *et al.*, 1990]. It is expressed in abundance on monocytes, and also at low levels on mature endothelial cells [Jersmann *et al.*, 2001]. $CD14^+$ cells are involved in the response to vessel injury and new vessel formation, and share many antigenic characteristics with mature endothelial cells in culture. Importantly, $CD14^+$ cells are approximately 10 times more abundant in the peripheral circulation than $CD34^+$ cells. There has therefore been much speculation and investigation into whether $CD14^+$ cells may be a source of clinically useful EPC [Rehman *et al.*, 2003; Rohde *et al.*, 2007; Yoder *et al.*, 2007; Zhang *et al.*, 2006]. Monocytes are highly plastic, having the capacity to differentiate into a variety of mature phenotypes depending on environmental cues [Zhao *et al.*, 2003]. In particular, monocytes will up-regulate a variety of endothelial characteristics in culture [Fernandez Pujol *et al.*, 2000; Harraz *et al.*, 2001; Rehman *et al.*, 2003; Schmeisser *et al.*, 2001; Urbich *et al.*, 2003; Zhang *et al.*, 2005; Zhang *et al.*, 2006; Zhao *et al.*, 2003], and will form vascular structures in vitro under appropriate angiogenic stimulation [Rohde *et al.*, 2007; Schmeisser *et al.*, 2001; Zhang *et al.*, 2005]. Activated monocytes accumulate at sites of new vessel formation and adhere to injured endothelium, and under experimental conditions $CD14^+$VEGFR-2^+ and $CD14^+$Tie-2^+ cells accelerate re-endothelialisation with demonstrable improvements in endothelial function by enhanced acetylcholine-mediated vasodilatation in a NO-dependent manner [Elsheikh *et al.*, 2005; Nowak *et al.*, 2004]. Such VEGFR-2 and Tie-2 expressing monocytes are also known to facilitate neo-angiogenesis in the

context of neoplasia [De Palma *et al.*, 2005; Murdoch *et al.*, 2007; Venneri *et al.*, 2007]. Numerous studies however indicate that whilst monocytes are pro-angiogenic, they do not incorporate directly into the vasculature [De Palma *et al.*, 2005; O'Neill *et al.*, 2005; Zentilin *et al.*, 2006; Ziegelhoeffer *et al.*, 2004]. $CD14^+$ cells most likely accelerate vascular repair through the secretion of angiogenic growth factors rather than by differentiating into true endothelial cells [Rehman *et al.*, 2003].

Both $CD34^+$ and $CD14^+$ populations therefore appear important in the regeneration of diseased and damaged arteries and it is likely that populations bearing the two receptors interact. $CD34^+$ cells for instance augment the incorporation of $CD34^-CD14^+$ cells into the endothelium of blood vessels in mouse ischemic limbs [Harraz *et al.*, 2001]. Drawing a firm distinction between $CD14^+$ and $CD34^+$ cells however is an over simplification, as several lines of evidence indicate that a proportion of $CD14^+$ cells are in fact derived from the more naïve $CD34^+$ population. $CD34^+$ cells cultured on fibronectin in the presence of VEGF and basic fibroblastic growth factor differentiate into a $CD14^+$ population before developing endothelial characteristics *in-vitro*, including the expression of Von Willebrand factor (vWF), eNOS and CD144 [Nakul-Aquaronne *et al.*, 2003]. A study using a $CD34^+$ acute myeloid leukemia cell line MUTZ-3, interrogated the differentiation of $CD34^+CD14^-CD11b^-$ progenitors through a $CD34^-CD14^-CD11b^+$ stage, into non-proliferating $CD14^+CD11b^{hi}$ progeny [Santegoets *et al.*, 2006]. The presence of $CD34^+CD14^+$ cells has been confirmed by Romagani *et al*, who used highly-sensitive

antibody-conjugated magneto-fluorescent liposomes to isolate a subset of $CD14^+$ cells co-expressing CD34 [Romagnani *et al.*, 2005]. In peripheral blood these $CD14^+CD34^{low}$ cells constitute 0.6% to 8.5% of all peripheral-blood leukocytes and are the dominant population among circulating VEGFR-2^+ cells. In the bone marrow virtually all $CD14^+$ cells were $CD14^+CD34^{low}$ double positive. Circulating $CD14^+CD34^{low}$ cells, exhibit high expression of the embryonic stem cell markers such as Nanog and Oct-4, and are capable of differentiating into endothelial cells. Taken together these studies suggest that $CD14^+$ cells are derived from $CD34^+$ cells and have the ability to develop endothelial characteristics depending on the action of external cues. The interactions between these cell types require further investigation.

Table 1.1: Haematopoietic and endothelial surface markers

Identifier	Distribution	Function	
CD45	Leukocytes	A signalling molecule regulating leukocyte differentiation and proliferation	**HAEMATOPOIETIC**
CD14	Monocytes Macrophages Some neutrophils	Endotoxin receptor regulating inflammatory cytokine production such as TNF by monocytes	
CD 115	Monocytes Macrophages	Receptor for macrophage-CSF regulating myeloid proliferation and differentiation	
CD117 (c-Kit)	Haematopoietic stem and progenitor cells	Receptor for stem cell factor. Stimulates cellular proliferation.	
CD133	Haematopoietic stem and progenitor cells	Unknown	
CD34	Haematopoietic stem and progenitor cells Capillary endothelium	Intracellular adhesion molecule. Binds E and L-Selectins and is thought to regulate leukocyte/endothelial interactions	
CD31	Leukocytes Platelets Endothelium	Adhesion molecule thought to be important for trans-endothelial cellular migration to sites on acute inflammation.	
Ac-LDL Uptake	Macrophages Monocytes Endothelium	N/A - Phagocytic process occurring in myeloid and endothelial cells	
Ulex binding	Macrophages Monocytes Endothelium	N/A - Histochemical stain	
CD105	Endothelium Activated macrophages Smooth muscle	Constituent of transforming growth factor-beta receptor 1. Important regulator of angiogenesis.	
CD 141	Endothelium, Smooth Muscle, Monocytes and Neutrophils	Binds thrombin and activates protein C and initiates anticoagulant pathways	
Von Willebrand Factor	Endothelium Platelets	Haemostasis	
Tie 2 (CD202)	Endothelium Monocytes Stem cells	Angiopoietin 1 receptor. Regulates vessel remodeling and maintains vascular integrity	
CD 146	Endothelium Melanoma cells Dendritic cells	Intra-cellular adhesion molecule.	
E-Selectin (CD62E)	Endothelium	Adhesion molecule regulating leukocyte/endothelial interactions and cell trafficking to sites of inflammation	
CD 144	Endothelium	Intracellular adhesion molecule regulating endothelial permeability and proliferation	
VEGFR–2 (CD309, KDR, Flk1)	Endothelium	Regulation of endothelial adhesion and signalling. Essential for embryonic vascular development	
eNOS	Endothelium	Enzymatic generation of nitric oxide	**ENDOTHELIAL**

Ac-LDL = Acetylated low density lipoprotein; CD = cluster of differentiation; TNF = tumour necrosis factor; CSF = colony stimulating factor; eNOS = endothelial nitric oxide synthase; VEGFR-2 = vascular endothelial growth factor receptor-2

1.5 CULTURING ENDOTHELIAL PROGENITOR CELLS

A widely used but largely inferential approach to the isolation and quantification of EPC has been through demonstrating the development of mature endothelial characteristics in mononuclear cells following a period of culture. Broadly speaking, there are 3 populations of cultured EPC or 'functional' EPC.

1) Endothelial Cell - Colony Forming Unit (EC-CFU), also referred to as EPC-CFU, and as early outgrowth colonies on the basis of the time at which they appear in culture (five days).
2) Circulating angiogenic cells (CAC), also at times referred to as early outgrowth colonies.
3) Endothelial Colony Forming Cells (ECFC) or late outgrowth colonies based on their appearance in culture at two to three weeks. These have also been referred to as late outgrowth EPC, endothelial outgrowth colonies, and blood outgrowth endothelial cells.

1.5.1 Endothelial Cell - Colony Forming Unit

The EC-CFU or early outgrowth colony assay is a modified version of the method originally used by Asahara [Asahara *et al.*, 1997]. Colonies are generated in endothelial growth medium on fibronectin. A pre-plating step at 2 days excludes initially adherent cells, in an attempt to remove circulating endothelial cells from the assay. Mature colonies form at around day 5, and are comprised of clusters of small round cells with peripheral spindle shaped cells (Figure 1.1) that express endothelial characteristics such as CD146, CD31, Tie-2, VEGFR-2, CD34, E-Selectin and

eNOS expression, *Ulex Europeus* agglutinin-1 (UEA-1) binding and acetylated low density lipoprotein uptake. Consistent with a vasculoprotective role, EC-CFU concentration is associated with improved brachial reactivity [Hill *et al.*, 2003] and are increased in response to tissue ischaemia and vascular injury such as that occurring in patients undergoing coronary artery bypass surgery [Roberts *et al.*, 2007], the presence of myocardial ischaemia [George *et al.*, 2004] or infarction [Massa *et al.*, 2005; Shintani *et al.*, 2001], and in patients undergoing angioplasty [Banerjee *et al.*, 2006; Bonello *et al.*, 2006; Chen *et al.*, 2008; Marboeuf *et al.*, 2008]. Impaired mobilisation of EC-CFU in response to ischaemic injury is also associated with an adverse outcome [Sobrino *et al.*, 2007]. EC-CFU concentrations are lower in association with adverse cardiovascular risk profiles, such as in patients with type I [Loomans *et al.*, 2004] and type II diabetes mellitus, hypercholesterolaemia [Hill *et al.*, 2003], hypertension [Delva *et al.*, 2007], coronary artery disease [Heeschen *et al.*, 2004], cerebrovascular disease [Ghani *et al.*, 2005], COPD [Palange *et al.*, 2006], rheumatoid arthritis [Grisar *et al.*, 2005], and heart failure [Valgimigli *et al.*, 2004], although notably not in non-ischaemic cardiomyopathy [Zhou *et al.*, 2008].

1.5.2 Circulating Angiogenic Cells

Unlike EC-CFU, CAC are derived from an adherent population of mononuclear cells and do not form discrete colonies. They are thought to be derived from the bone marrow and have been defined as EPC on the basis of the expression of endothelial characteristics; UEA-1 binding and uptake of Ac-LDL, and expression of vWF, CD31, VEGFR-2, CD144, Tie-2 [Asahara *et al.*, 1999; Dimmeler *et al.*, 2001; Dimmeler *et al.*, 2000; Gehling *et al.*, 2000; Kalka *et al.*, 2000b]. They probably

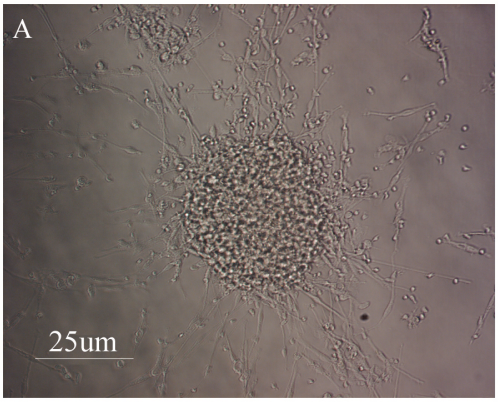
represent a population of adherent monocyte. Similar to EC-CFU, CAC and are depressed in cardiovascular diseases such diabetes, coronary artery disease and rheumatoid arthritis [Adams *et al.*, 2004; Grisar *et al.*, 2005; Tepper *et al.*, 2002; Vasa *et al.*, 2001b], and are mobilised in response to acute stressors such as myocardial ischaemia [Shintani *et al.*, 2001] and coronary artery bypass grafting [Roberts *et al.*, 2007]. They promote neovascularisation in animal models of acute myocardial infarction [Kawamoto *et al.*, 2001], although they probably do this through the secretion of angiogenic cytokines as they have little proliferative capacity of their own [Kalka *et al.*, 2000b; Kawamoto *et al.*, 2001; Rehman *et al.*, 2003].

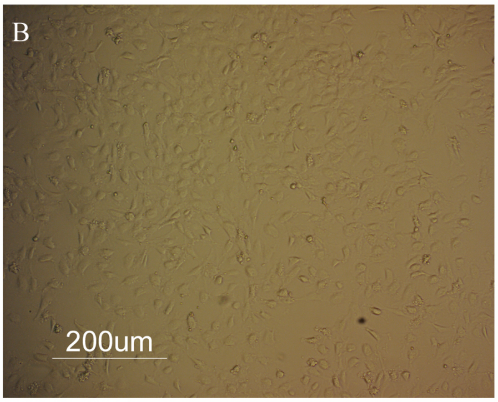
1.5.3 Endothelial colony forming cell or late outgrowth colonies

This population of cells is also generated from the culture of peripheral mononuclear cells, but in contrast to EC-CFU, endothelial colony forming cells (ECFC) are derived from an adherent fraction, grown on type I collagen. ECFC are morphologically described as flat clusters of cells with a cobblestone appearance (Figure 1.1). Whilst ECFC share many phenotypic characteristics with EC-CFU [Ingram *et al.*, 2004], they do not express the haematopoietic markers CD45 or myeloid/macrophage markers such as CD115, and do not exhibit phagocytic function. ECFC arise from non-haematopoietic cells negative for CD133 and the pan leukocyte marker CD45, but positive for CD34 and VEGFR-2 [Timmermans *et al.*, 2007]. Enrichment of unsorted mononuclear preparations for $CD45^{-}CD34^{+}$ cells increases the frequency of ECFC by some 400% compared to an abolition of ECFC growth in the $CD45^{+}$ fraction [Timmermans *et al.*, 2007]. Of the populations so far identified, the $CD45^{-}CD34^{+}$ derived ECFC more closely fulfil the characteristics of a

true EPC. They conform to an endothelial phenotype and morphology, are derived from the bone marrow [Lin *et al.*, 2000], have robust proliferative potential [Gulati *et al.*, 2003a; Ingram *et al.*, 2004], and are capable of forming perfusing vessels *in vivo* [Yoder *et al.*, 2007]. ECFC are mobilised within the first few hours following myocardial infarction [Huang *et al.*, 2007; Massa *et al.*, 2009] and have been positively correlated with the severity of coronary artery disease in patients undergoing coronary angiography [Guven *et al.*, 2006]. However, as previously discussed, mature endothelial cells may also express CD34 [Fina *et al.*, 1990] and are also $CD45^-$. The growth of late outgrowth colonies from the peripheral blood may therefore represent the presence of circulating mature endothelial cells sloughed from the vasculature, rather than circulating bone marrow derived progenitor cells. This important distinction has yet to be established.

Unfortunately, across hundreds of different studies, the term EPC has been used interchangeably with reference to several different cell populations, bearing various permutations of surface markers and cultured under different conditions, and this has made it difficult to make reliable comparisons between studies. Erroneous conclusions regarding the phenotype of cell populations in culture as a result of the low specificity of given endothelial characteristics may provide some explanation for the diversity of cells labeled as EPC.





Characteristic	EC-CFU (A)	ECFC (B)
Adhesive Protein	Fibronectin	Collagen
Precursor	Monocytes/Lymphocytes $CD14^{+}$ & $CD2^{+}$	Late outgrowth EPCs $CD45^{-}CD34^{+}KDR^{+}$
Appearance in culture	5 -7 days	14 – 21 days
Morphology	Mass of round and spindle shaped cells	Flat cobblestone appearance
CD45 expression	Yes	No
Proliferative Potential	Low	High
Phagocytic function	Yes	No

Figure 1.1 Endothelial cell-colony forming units (EC-CFU) and endothelial colony forming cells (ECFC): (A)EC-CFU are generated from a non-adherent population of mononuclear cells cultured on fibronectin and appear some 5-7 days into culture. EC-CFU exhibit endothelial surface characteristics but are composed of monocytes and angiogenic lymphocytes with little capacity to form perfusing vessels or incorporate directly into vascular structures. EC-CFU exhibit phagocytic activity and avidly secrete angiogenic growth factors. **(B)** ECFC are generated from adherent mononuclear cells grown on type I collagen and appear after 2-3 weeks of cell culture. ECFC are morphologically indistinguishable from mature endothelial cells and are derived from non-haematopoietic ($CD45^{-}$) cells expressing CD34. ECFC have robust proliferative potential and the capacity to form perfusing blood vessels *in vitro*.

1.6 ENDOTHELIAL PROGENITOR CELLS AND IATROGENIC VASCULAR INJURY

Using various animal models of vascular injury pre-clinical studies have amassed a significant body of evidence to indicate that bone marrow derived cells localise to regions of vascular injury and accelerate vascular healing by restoring endothelial function and the attenuation of neo-intimal hyperplasia (Table 1.2) [Kong *et al.*, 2004a; Takamiya *et al.*, 2006; Walter *et al.*, 2002; Werner *et al.*, 2003; Werner *et al.*, 2002; Yoshioka *et al.*, 2006]. Several clinical studies have specifically addressed the role of putative EPC populations following angioplasty (Table 1.3). Most of these studies have examined the behaviour of EC-CFU. These studies have been small, and have often lacked appropriate control groups for comparison. A study of patients undergoing peripheral angioplasty detected an increase in circulating mature endothelial cells immediately after angioplasty, followed by a two to three fold increase in EC-CFU at 24 hours [Bonello *et al.*, 2006]. These findings were confirmed by Marboeuf *et al*, who also correlated EC-CFU with plasma C-reactive protein (CRP) concentrations following angioplasty [Marboeuf *et al.*, 2008]. Garg *et al*, described EC-CFU mobilisation following PCI in patients with acute coronary syndromes. However, it is impossible to determine whether EC-CFU were mobilised in response to myocardial infarction or discrete vascular injury in this study given the absence of a control group of patients (i.e. patients not undergoing PCI) [Garg *et al.*, 2008a]. However mobilisation of EC-CFU type cells within the first 24-hours of angioplasty has been observed, suggesting a role for these cells in the immediate response to vascular injury [Banerjee *et al.*, 2006; Bonello *et al.*, 2006; Garg *et al.*, 2008a; Marboeuf *et al.*, 2008].

Few studies have addressed whether EC-CFU mobilisation influences the development of ISR and a need for target vessel revascularisation and the available data are poor. In a retrospective study, George *et al.*, demonstrated that in patients presenting with proliferative, as opposed to focal ISR, EC-CFU were reduced both in number and adhesive capacity, suggesting that a deficiency in EC-CFU might predispose to aggressive neointimal hyperplasia [George *et al.*, 2003]. This study did not detect a difference in the number of EC-CFU between those patients with and without angiographic restenosis, however subsequently two other groups have reported a reduction in EC-CFU in patients presenting with restenosis, and these patients had reduced EC-CFU proliferative and migratory capacity as well as increased cellular senescence [Lei *et al.*, 2007; Matsuo *et al.*, 2006].

A strong association exists between reduced $CD34^+VEGFR\text{-}2^+$ cells and those risk factors predictive of ISR, i.e. cigarette smoking [Vasa *et al.*, 2001b], diabetes mellitus [Fadini *et al.*, 2005], and endothelial dysfunction [Herbrig *et al.*, 2006]. Two small clinical studies demonstrated a fall in $CD34^+VEGFR\text{-}2^+$ cells within the first few hours of coronary angioplasty, although interpretation of these studies is limited as neither used a control group [Lee *et al.*, 2009; Thomas *et al.*, 2008a]. In a moderate sized study of 102 patients, $CD34^+CD133^+$ concentrations were significantly lower in patients undergoing PCI for stable anginal symptoms than in matched controls without angina, however the peripheral concentration was similar at 24 hours following PCI [Arao *et al.*, 2010]. Egan *et al.*, measured a variety of surface markers expressed on mononuclear cells including CD34, CD133, VEGFR-2, CD117, CD31 and $CXCR4^+$ in patients undergoing PCI, diagnostic

coronary angiography alone and healthy controls. In this small study, patients with CAD had lower resting levels of $CD34^+$, $CD133^+$, $CD117^+$, $CD34^+CD117^+$, $CD34^+CD31^+$ and $CXCR4^+$ cells compared to healthy controls. Following PCI, they observed an increase in those cells expressing CD133, CD117, CD34/CD31, CD34/CD117 and CXCR4 within 6-12 hours [Egan *et al.*, 2009]. As CXCR4 is thought to be integral to EPC homing and integration, it is of interest to note that reduced levels of CXCR4 correlated with the incidence of angina at one year. Whilst underpowered to address clinical outcomes, this study suggests a role for CXCR4 in EPC homing and integration at the site of vascular injury following PCI.

Mobilisation of ECFC and $CD45^-CD34^+$ has been detected within hours of acute myocardial infarction in small preclinical and clinical studies. One may speculate that PCI would exert a similar effect although no studies have specifically examined ECFC or $CD45^-CD34^+$ cells following PCI. Several studies have documented an increase in $CD34^+CD45^+$ cells following PCI [Bonello *et al.*, 2006; Inoue *et al.*, 2007; Schober *et al.*, 2005]. Importantly there is an indication that this might be associated with the development of neo-intimal hyperplasia. Schober *et al.*, found that $CD34^+CD45^+$ mobilisation following PCI was significantly correlated with, and independently predictive of, late lumen loss at 6 months [Schober *et al.*, 2005]. Inoue *et al.*, performed a similar study and combined coronary and peripheral venous sampling to detect a local inflammatory response within the coronary circulation in response to PCI [Inoue *et al.*, 2007]. The magnitude of inflammation within the coronary circulation correlated with $CD34^+CD45^+$ mobilisation, and

both were significantly greater in patients who subsequently developed ISR. Interestingly the use of DES in this study was associated suppression of both coronary inflammation and circulating $CD34^{+}CD45^{+}$ cell concentrations, suggesting that in addition to a local anti-proliferative action, DES may also reduce ISR by suppressing inflammatory signalling to the bone marrow. Inoue *et al.*, demonstrated that circulating mononuclear cells of patients with ISR exhibited a propensity to develop a smooth muscle phenotype over that of an endothelial phenotype in culture. The potential role of circulating smooth muscle progenitor cells (SMPC) in the development of ISR has been supported in other pre-clinical studies [Sata *et al.*, 2002]. It is interesting to note that SMPC are also reported to express CD34 and VEGFR-2 [Simper *et al.*, 2002]. Furthermore both CD34 and CD133 are expressed at higher concentrations in the neo-intima of re-stenotic lesions compared to de-novo lesions [Skowasch *et al.*, 2003]. These populations may indeed potentiate neo-intimal hyperplasia if such a response is dictated by an inflammatory microenvironment in a given patient.

Table 1.2. Preclinical studies of putative EPC following vascular injury

Reference	Model	Number	Putative EPC	Effect
[Walter *et al.*, 2002]	Murine Tie2/lacZ BM transplant recipients subjected to balloon mediated arterial injury and pretreatment with simvastatin or placebo	18 placebo 34 simvastatin	Dil-Ac-LDL$^+$lectin$^+$ MNCs	Simvastatin enhanced EPC mobilisation following vascular injury and increased their adhesive capacity. Re-endothelialisation was accelerated by BM derived cells and neo-intimal hyperplasia was reduced.
[Werner *et al.*, 2002]	Murine GFP BM transfection followed by wire mediated arterial injury and pretreatment with rosuvastatin or placebo	5 placebo 4 rosuvastatin	Sca 1$^+$KDR$^+$ cells of BM origin	Rosuvastatin enhanced BM derived EPC mobilisation following vascular injury. Re-endothelialisation was accelerated by BM derived cells and neo-intimal hyperplasia was reduced.
[Werner *et al.*, 2003]	Intravenous cell therapy following wire mediated murine arterial injury	6 vascular injury	Spleen derived Dil-Ac-LDL$^+$lectin$^+$ MNCs with or without a period of culture in endothelial growth medium	Cell therapy enhanced re-endothelialisation in splenectomised animals and was associated with a reduction of neointima formation but MNC were more effective than EPC.
[Fujiyama *et al.*, 2003]	Intravenous cell therapy vs saline following balloon mediated murine arterial injury	12 vascular injury 12 controls	BM CD14$^+$CD34- CD14-CD34$^+$ PB CD14$^+$CD34-	Compared to saline placebo, BM derived CD34$^+$ cells and both PB and BM derived CD14$^+$ cells upregulated endothelial markers, accelerated neo-endothelialisation and inhibited neo-intimal hyperplasia following activation with MCP-1
[Kong *et al.*, 2004a]	G-CSF vs control prior to balloon mediated murine arterial injury	5 G-CSF 5 control	CD34$^+$KDR$^+$	G-CSF enhanced EPC mobilisation following vascular injury. Re-endothelialisation was accelerated by BM derived cells and neo-intimal hyperplasia was reduced.
[Nowak *et al.*, 2004]	Intravenous cell therapy following balloon injury in mice using CD14/CD11b cells expressing, Tie 2$^+$, Tie2-, KDR$^+$, KDR-, or saline control	6 cell therapy 6 controls	Myeloid cells (CD14, CD11b) expressing KDR$^+$ or Tie2$^+$	CD14$^+$KDR$^+$ and CD14$^+$Tie-2$^+$ displayed endothelial characteristics in culture and enhanced re-endothelialisation of denuded arteries with an associated reduction in neo intimal hyperplasia.
[Elsheikh *et al.*, 2005]	Intravenous cell therapy using GFP transduced cells following balloon mediated arterial injury in mice	10 CD14$^+$KDR$^+$ 10 CD14$^+$KDR$^-$ 6 controls	CD14$^+$KDR$^+$ CD14$^+$KDR-	Unlike CD14$^+$KDR- cells, CD14$^+$KDR$^+$ cells exhibited an endothelial phenotype in culture and contributed to neo-endothelialisation.
[Yoshioka *et al.*, 2006]	G-CSF following wire mediated arterial injury in mice Treatment pre- and post-arterial injury vs post-arterial injury alone	22 G-CSF 20 controls	CD34$^+$KDR$^+$	G-CSF reduced neo-intimal hyperplasia in association with mobilisation of bone marrow derived EPC and accelerated re-endothelialisation compared to control. The effect was enhanced if administered prior to injury. Few BM derived cells contributed to neo-endothelium.
[Takamiya *et al.*, 2006]	1) G-CSF prior to balloon mediated arterial injury in rats 2) GFP BM transfected mice subjected to balloon mediated arterial injury	10 G-CSF 5 placebo	CD117$^+$KDR$^+$	1) G-CSF enhanced EPC mobilisation following vascular injury. Re-endothelialisation was accelerated and neo-intimal hyperplasia was reduced. 2) GFP expressing BM derived cells contribute to neo-endothelialisation.

BM = bone marrow; Dil-Ac-LDL= Di acetylated low-density lipoprotein; EPC = endothelial progenitor cell; G-CSF = granulocyte-colony stimulating factor; GFP = green fluorescent protein; KDR = kinase domain receptor; MCP-1 = monocyte chemoattractant protein – 1; mononuclear cells = MNCs.

Table 1.3. Clinical studies of putative EPC following PCI

Reference	Study design	Number	Putative EPC	Effect of Angioplasty
[George *et al.*, 2003]	Retrospective Restenosis vs no restenosis	16 restenosis 11 no restenosis	EC-CFU	No difference in EC-CFU in those presenting with or without angiographic restenosis, but EC-CFU lower in those presenting with diffuse vs focal restenosis and reduced adhesive capacity in restenosis.
[Schober *et al.*, 2005]	Prospective Elective PCI with no control group	17 PCI	$CD34^+$	Mobilisation of $CD34^+$ following PCI predicts restenosis and correlates with late lumen loss.
[Bonello *et al.*, 2006]	Prospective Elective PCI with no control group	15 PCI	CEC $CD45^+CD34^+$ EC-CFU	CEC were increased 6.-fold at 6 hours, $CD45^+CD34^+$ cells increased 2.6-fold at 6hrs and EC-CFU increased 2.6-fold at 24 hours.
[Matsuo *et al.*, 2006]	Retrospective Restenosis vs no restenosis	16 restenosis 30 no restenosis	EC-CFU	EC-CFU 2-fold greater in patients without ISR and increased senescence in EC-CFU of patients with ISR.
[Banerjee *et al.*, 2006]	Prospective PCI in elective and ACS patients with diagnostic angiography control group	20 elective PCI 10 ACS PCI 8 controls	EC-CFU $CD34^+CD31^+$	No change in $CD34^+CD31^+$with PCI in ACS or diagnostic angiography but 1.4-fold increase in EC-CFU with elective PCI.
[Lei *et al.*, 2007]	Retrospective Angiographic restenosis vs no restenosis	15 restenosis 17 no restenosis	EC-CFU $CD34^+VEGFR\text{-}2^+$	EC-CFU 3.5 fold greater in patients without ISR. Reduced proliferative and migratory capacity in EC-CFU of patients without ISR but no difference in adhesive capacity.
[Inoue *et al.*, 2007]	Prospective Elective PCI with no control group	40 PCI	$CD34^+$	$CD34^+$ increased 3-fold following BMS at 7 days, but decreased by 30% following DES at 7 days.
[Marboeuf *et al.*, 2008]	Prospective Peripheral arterial intervention with no control group	14 angioplasty	EC-CFU	EC-CFU increased 2.5-fold at 24 hours.
[Garg *et al.*, 2008b]	PCI in NSTEMI with no control group	20 PCI	EC-CFU	EC-CFU increased 1.3-fold at 24 hours.
[Thomas *et al.*, 2008a]	Prospective Elective PCI with no control group	20 PCI	$CD34^+$, $CD34^+CD45^+$, $CD133^+$, $CD34^+CD133^+$, $CD34^+VEGFR\text{-}2^+$ & $CD133^+VEGFR\text{-}2^+$	Transient fall in all populations at 6 hours post PCI.
[Egan *et al.*, 2009]	Prospective Elective PCI vs diagnostic angiography.	10 PCI 13 controls	$CD34^+$, $VEGFR\text{-}2^+$, $CD133^+$, $CD117^+$, $CD34^+VEGFR\text{-}2^+$, $CD34^+CD117^+$, $CD34^+CD31^+$, $CXCR4^+$	Compared to angiography, $CD133^+$, $CD117^+$, $CD34^+CD117^+$, $CD34^+CD31^+$, and $CXCR4^+$cells increased 6–12 hours following PCI. No difference in $CD34^+$, $VEGFR\text{-}2^+$, or $CD34^+VEGFR\text{-}2^+$ cells. CXCR4 expression correlated with freedom from angina at 1 year.
[Lee *et al.*, 2009]	Prospective Elective PCI in diabetics No control group	8 patients	$CD34^+VEGFR\text{-}2^+$	Transient fall of ~50% in first 4 hours following PCI.

ACS = acute coronary syndrome; CD = cluster of differentiation; CXCR-4 = chemokines receptor 4; EC-CFU = endothelial cell colony forming unit; NSTEMI = non-ST-segment elevation myocardial infarction; PCI = percutaneous coronary intervention

1.7 THERAPEUTIC ENDOTHELIAL PROGENITOR CELLS FOR PCI ASSOCIATED VASCULAR INJURY

Current strategies to reduce the incidence of complications following percutaneous intervention are based on suppressing neo-intimal cellular proliferation rather than enhancing endovascular repair. Drug-eluting stents have dramatically reduced the incidence of early in-stent restenosis, but local anti-proliferative therapy may interfere with vascular healing and prevent formation of a functional endothelial layer [Muldowney *et al.*, 2007]. Therapies designed to mobilise endothelial progenitors or to increase their ability to home to the site of stent implantation and facilitate vascular repair are attractive, and have the potential to improve clinical outcomes following PCI. These approaches fall into three broad categories; pharmacological, stent-based and cellular therapies.

1.7.1 Pharmacological mobilisation of endothelial progenitor cells

Mobilisation of precursors from the stem cell niche occurs via phosphatidylinositol-3-kinase/Akt/eNOS (PI3K/Akt/eNOS) activation. The PI3K/Akt/eNOS pathway is responsible for the regulation of cellular apoptosis, proliferation, and migration in a variety of biological systems, including the cardiovascular system [Shiojima *et al.*, 2002]. Activation of the PI3-Akt pathway by angiogenic factors such as VEGF, fibroblastic growth factor and angiopoietin stimulates nitric oxide synthesis by bone marrow stromal cells [Dimmeler *et al.*, 2001; Llevadot *et al.*, 2001]. Increased nitric oxide bioavailability leads to cleavage of intra-cellular adhesions between stem cells and stromal cells of the bone marrow by proteinases, such as elastase, cathepsin G, and matrix metalloproteinases [Heissig *et*

al., 2002]. A high stromal derived factor -1 (SDF-1) gradient across the bone marrow generated by bone marrow stromal cells acts through its cognate receptor CXCR-4 to force mobilised stem cells into the peripheral circulation such that they may home to regions of vascular injury [Asahara *et al.*, 1999; Seeger *et al.*, 2009; Yla-Herttuala *et al.*, 2007]. Mobilisation of EPC is therefore heavily reliant on nitric oxide synthesis via PI3K/Akt/eNOS activation [Aicher *et al.*, 2003; Landmesser *et al.*, 2004]. Reduced NO bioavailability is evident in patients with cardiovascular risk factors and is integral to the development of atherosclerosis and disordered function of eNOS is thought to be in part responsible for impaired mobilisation of EPC in these patients [Huang, 2009; Thum *et al.*, 2007a]. Bone marrow cells treated with an eNOS transcription enhancer in order to increase eNOS activity exhibit enhanced migratory and neovascularisation capacity and when administered in a model of hind-limb ischaemia can improve organ function. Furthermore this beneficial effect is reversed by the use of an eNOS inhibitor, strongly implicating nitric oxide in the process of vascular repair and neovascularisation [Sasaki *et al.*, 2006]. Detailed mechanistic data regarding EPC mobilisation in the context of discrete vascular injury are limited, although there are numerous factors thought to induce EPC mobilisation including VEGF, fibroblastic growth factor, growth hormone, insulin-like growth factor, angiopoietin, SDF-1, erythropoietin, eostrogens, granulocyte colony-stimulating factor, statins, angiotensin receptor blockers, peroxisome proliferator-activated receptor antagonists and physical exercise. Several of these have been examined in the context of discrete vascular injury as potential therapeutic strategies for the mobilisation of EPC.

3-Hydroxy-3-methylglutaryl coenzyme-A reductase inhibitors

3-Hydroxy-3-methylglutaryl coenzyme-A reductase inhibitors (statins) mobilise $CD34^+$ and $VEGFR\text{-}2^+$ cells to the peripheral circulation in a dose-dependent manner via the PI3/Akt pathway [Leone *et al.*, 2008; Park *et al.*, 2008; Vasa *et al.*, 2001a]. Statins also stimulate EC-CFU and ECFC formation and augment the quantity, migratory and proliferative capacity of a variety of putative EPC in culture [Deschaseaux *et al.*, 2007; Landmesser *et al.*, 2004; Llevadot *et al.*, 2001; Walter *et al.*, 2002; Walter DH, 2001], and also induce differentiation of $CD14^+$ and $CD34^+$ cells toward an endothelial phenotype [Dimmeler *et al.*, 2001]. The reduced rate of ISR following PCI in patients treated with statins may in part be explained by enhanced mobilisation and function of EPC [Walter *et al.*, 2001b]. Walter *et al.*, demonstrated an accelerated rate of re-endothelialisation and a reduction in neo-intimal hyperplasia in rats treated with simvastatin subjected to balloon mediated arterial injury, associated with an increase in quantity and adhesive capacity of Ac-LDL^+BS-1-$Lectin^+$ cells in culture. Although this definition of EPC is non-specific, the neo-endothelium in statin treated animals was confirmed to be of bone marrow origin by the use of a Tie2/LacZ bone marrow transplant model [Walter *et al.*, 2002]. Werner *et al*, obtained similar results using rosuvastatin [Werner *et al.*, 2002]. Fluvastatin ameliorates impaired re-endothelialisation caused by sirolimus coated stents, however this effect does not appear to be mediated through mobilisation of bone marrow derived progenitors, rather by enhancing the traditionally recognised mechanism of re-endothelialisation; proliferation of mature endothelial cells adjacent to the stented segment [Fukuda *et al.*, 2009]. The stimulatory effects of statins on re-endothelialisation may not be sustained. Hristov *et*

al., demonstrated a reduction of both EC-CFU and $CD34^{+}VEGFR\text{-}2^{+}$ cells in patients with coronary artery disease on chronic statin therapy [Hristov *et al.*, 2007]. The authors speculated over the possible exhaustion of EPC reserves, desensitisation of the bone marrow to the effects of statins, or possible enhanced recruitment of circulating EPC to peripheral sites of endothelial disrepair. Whilst another small study concurred with the finding of reduced colony formation (similar to EC-CFU) on chronic statin therapy, ECFC were increased [Deschaseaux *et al.*, 2007]. Stimulation of EC-CFU may therefore represent an initial haematopoietic effect of statin therapy as opposed to the long-term angiogenic effects of chronic therapy reflected by the increased quantity of ECFC. Statin therapy is of course already well established as a treatment for primary and secondary prevention for atherosclerotic events, however novel effects of statins on enhanced EPC mobilisation and function may support an argument for intensive statin therapy prior to PCI.

Renin-angiotensin-aldosterone antagonism

Activation of the renin-angiotensin-aldosterone system (RAAS) occurs in a variety of cardiovascular disorders, with associated deleterious long-term consequences, and antagonism of the RAAS is recognised to be beneficial in the setting of conditions such as heart failure, myocardial infarction, atherosclerosis and hypertension. The mechanism of action is thought to be partly via amelioration of the endothelial dysfunction that characterises these conditions, and hypothetically such benefits may occur through enhanced endogenous vascular repair via EPC. Treatment with ACE inhibitors augments the mobilisation of bone marrow-derived progenitors and enhances the proliferative and migratory capacity of CAC in vitro

[Muller *et al.*, 2009]. Similarly, treatment with angiotensin receptor blockers causes circulating EC-CFU to increase [Bahlmann *et al.*, 2005], probably through a reduction in cellular senescence by reducing oxidative stress caused by angiotensin II [Imanishi *et al.*, 2005]. Paradoxically, angiotensin II has also been shown to stimulate VEGF receptor expression in a dose-dependent manner, and increase proliferation of EC-CFU and tube formation in Matrigel® when co-treated with VEGF [Imanishi *et al.*, 2004]. Aldosterone itself has an inhibitory effect on the progenitor cell maturation in bone marrow cells ex-vivo in a PI3K/Akt-dependent manner [Marumo *et al.*, 2006] and aldosterone antagonism with either spironolactone and eplerenone increase circulating EPC with an associated increase in capillary density in animal models of ischaemia [Kobayashi *et al.*, 2010; Ladage *et al.*, 2011]. The effect of antagonising the RAAS on EPC mobilisation and function remains poorly understood, however the behaviour of putative EPC populations in response to RAAS antagonism in small experiments has largely mirrored the beneficial effects observed in clinical practice.

Granulocyte colony stimulating factor

Granulocyte colony stimulating factor (G-CSF) has no established role in the treatment of cardiovascular disease but is routinely used to mobilise progenitor cells in the context of bone marrow transplantation. G-CSF induces release of elastase and cathepsin G from neutrophils, leading to the release of progenitors from the stem cell niche into the blood stream. G-CSF mobilises a variety of EPC phenotypes including CAC [Honold *et al.*, 2006], EC-CFU [Korbling *et al.*, 2006; Powell *et al.*, 2005], $CD34^+CD133^+$ and $CD133^+VEGFR\text{-}2^+$ [Powell *et al.*, 2005], c-$Kit^+VEGFR\text{-}2^+$

[Takamiya *et al.*, 2006], $CD34^+VEGFR\text{-}2^+$ [Yoshioka *et al.*, 2006] and $CD34^+CD133^+VEGFR\text{-}2^+$ [Korbling *et al.*, 2006]. Several studies have demonstrated a positive effect of G-CSF on re-endothelialisation [Kong *et al.*, 2004a; Mei *et al.*, 2008; Shi *et al.*, 2002; Takamiya *et al.*, 2006; Yoshioka *et al.*, 2006]. Kong *et al*, treated rats with G-CSF and demonstrated accelerated re-endothelialisation and reduced neointimal hyperplasia following endovascular balloon injury, associated with increased expression of CD34, eNOS, VEGFR-2, stem cell factor receptor-c-kit, and E-selectin on circulating MNC [Kong *et al.*, 2004a]. Takamiya *et al* showed similar effects of G-CSF on arterial repair associated with the mobilisation of $c\text{-}Kit^+VEGFR\text{-}2^+$ cells and enhanced endothelial coverage [Takamiya *et al.*, 2006]. A green fluorescent protein bone marrow transplant model was used to confirm that a significant proportion of the neo-endothelium was derived from the bone marrow. This contrasts with the findings of Yoshioka *et al*, who found that although G-CSF mobilised $CD34^+VEGFR\text{-}2^+$ cells and accelerated re-endothelialisation, very few BM derived EPC contributed to re-endothelialisation [Yoshioka *et al.*, 2006]. The mechanism through which G-CSF reduces neo-intimal hyperplasia is therefore unclear. Yoshioka *et al*, speculate that G-CSF may increase proliferation and migration of adjacent endothelial cells either directly or through stimulation by attaching EPC. Even a marginal increase in attaching EPC might be enough to increase the secretion of angiogenic factors [Gulati *et al.*, 2003b; Kocher *et al.*, 2001]. Shi *et al.* used G-CSF to accelerate endothelialisation of Dacron grafts implanted in the aortas of dogs, but despite enhanced endothelialisation, animals treated with G-CSF had considerably more neo-intimal formation than controls [Shi *et al.*, 2002]. Concordant with this pre-clinical study, intra-coronary G-CSF administered to

patients with myocardial infarction was associated with an increased incidence of ISR, despite improvements in left ventricular ejection fraction (LVEF%) [Kang *et al.*, 2004]. This effect may have been mediated through G-CSF's non-specific pro-inflammatory actions and the mobilisation of smooth muscle progenitors from the bone marrow. The non-specific nature of G-CSF's effects hampers its translation into a targeted therapy for vascular repair. Ongoing studies using G-CSF in combination with sitagliptin, an anti-diabetic dipeptidylpeptidase IV –inhibitor, known to cleave SDF-1 and augment progenitor cell mobilisation will address whether the effects of G-CSF may be honed and make it clinically useful in the context of cardiovascular disease [Theiss *et al.*, 2010].

Peroxisome proliferator–activated receptor agonists

Peroxisome proliferator–activated receptor (PPAR) agonists inhibit vascular smooth muscle proliferation and migration, and accelerate re-endothelialisation and improve endothelial function. This can attenuate neo-intimal formation in mice subjected to femoral angioplasty. Wang *et al.* attributed this effect to the ability of roziglitazone to drive pleuripotent bone marrow-derived vascular progenitors toward an endothelial phenotype, and away from a smooth muscle phenotype [Wang *et al.*, 2004]. PPAR agonists may therefore have a role to play in augmenting a maladaptive response to iatrogenic vascular injury although concerns remain regarding the safety profile of these drugs in patients with ischaemic heart disease [Singh *et al.*, 2007].

Erythropoietin

The effects of erythropoietin on the response to discrete vascular injury are similarly favorable in terms of re-endothelialisation. Erythropoietin significantly increases $CD34^+VEGFR\text{-}2^+$, and enhances neovascularisation in response to ischaemia cells in mice. Serum erythropoietin concentration correlates tightly with progenitor cells expressing $CD34^+VEGFR\text{-}2^+$, $CD34^+CD133^+$ $CD34^+Lin^+$ and cultured EPC similar to CAC in patients with coronary artery disease [Heeschen *et al.*, 2003], and Erythropoietin therapy causes acute mobilisation of $CD34^+VEGFR\text{-}2^+$ [Bahlmann *et al.*, 2004] and increases the proliferative and adhesive capacity of cultured EPC in a PI3-Akt dependent manner [George *et al.*, 2005]. Chronic erythropoietin therapy in patients with congestive cardiac failure (CCF) however had no effect on circulating $CD34^+$, $CD34^+CD45^+$, $CD34^+CD133^+$, $CD34^+VEGFR\text{-}2^+$ or $CD34^+CD133^+VEGFR\text{-}2^+$ progenitor cells. The acute effects of erythropoietin may therefore be attenuated over time, although bone marrow suppression related to CCF may explain these differences [Westenbrink *et al.*, 2010]. In an animal model of endovascular injury, erythropoietin treatment enhanced proliferation of resident endothelial cells and reduced apoptosis of the injured artery, in association with eNOS dependent mobilisation of $Sca\text{-}1^+VEGFR\text{-}2^+$ cells [Urao *et al.*, 2006]. Erythropoietin has also been associated with an increased incidence of neo-intimal proliferation despite adequate re-endothelialisation in animal studies, possibly as a result of the non-specific mobilisation of smooth muscle progenitors and pro-inflammatory effects [Reddy *et al.*, 2007]. In the HEBE-III trial, a single dose of erythropoietin therapy had a favourable impact on clinical outcomes following acute

myocardial infarction, however the mechanism of this benefit is uncertain as this effect appeared to be independent of improvement in LV function [Voors *et al.*, 2010]. Similarly in the REVEAL trial patients with AMI treated with erythropoietin showed now improvement in infarct size however of concern, sub-group analyses in older patients suggested an increased infarct size and erythropoietin therapy was associated with increased adverse events [Najjar *et al.*, 2011]. Whether erythropoietin has a role in cardiovascular remodelling remains unknown.

Oestrogens

Oestrogens have also been shown to enhance vascular repair in models of arterial injury, in part through eNOS dependent mobilisation and proliferation of bone marrow-derived EPC which exhibited enhance migratory and mitogenic activity [Iwakura *et al.*, 2003], and reduced apoptotic signaling through the actions of caspase-8 [Strehlow *et al.*, 2003].

Physical exercise

Physical exercise is a known stimulus of EC-CFU [Rehman *et al.*, 2004], and using running wheels has been shown to enhance vascular repair in mice subjected to vascular injury in association with enhanced progenitor cell mobilisation [Laufs *et al.*, 2004].

1.7.2 Stent-based Therapy

Technological advances and the evolution of intra-coronary stents provide a potential vehicle to deliver novel therapies directly to the site of vascular injury.

Attempts to coat intra-coronary stents with endothelial mitogens, such as VEGF, have not been encouraging in terms of re-endothelialisation [Swanson *et al.*, 2003]. However, gene-eluting stents directly delivering naked plasmid DNA encoding for VEGF-2 can accelerate re-endothelialisation and reduce lumen loss in animal models [Walter *et al.*, 2004]. The Genous bioengineered R-stent is coated with monoclonal antibodies directed against CD34 and designed to attract EPC and encourage re-endothelialisation. Genous stents have already progressed to phase II and III clinical trials and have been deployed in over 5,000 patients. Preliminary data from small registries reported major adverse cardiovascular event (MACE) rates ranging from 7.9% to 13% [Aoki *et al.*, 2005; Co *et al.*, 2008; Duckers *et al.*, 2007; Lee *et al.*; Miglionico *et al.*, 2008]. The e-HEALING registry involving 4,939 patients, reports target vessel failure rate of 8.4% with a target lesion revascularisation rate of 5.7% and a MACE rate of 7.9%, with a low incidence of stent thrombosis (1.1%) [Silber *et al.*]. Although underpowered, the HEALING II registry reported that patients with normal $CD34^{+}VEGFR\text{-}2^{+}$ titers had lower rates of in-stent restenosis in comparison to those patients with reduced circulating EPC (late luminal loss 0.53 ± 0.06 versus 1.01 ± 0.07 mm). Furthermore, a subgroup of 30 patients in this non-randomised study underwent serial evaluation using intravascular ultrasound, and regression of neointimal volume was observed in patients with higher concentrations of EPC [Duckers *et al.*, 2007]. The first randomised controlled trial in a small cohort of patients with ST-segment elevation myocardial infarction reported a trend toward increased restenosis with the Genous stent when compared to a standard chromium-cobalt stent [Cervinka, 2009]. The Tri-stent Adjudication Study (TRIAS) compared the Genous stent with tacrolimus eluting, Taxus stents in 193 patients with lesions

carrying a high risk of restenosis, i.e chronic total occlusions, small caliber vessels (<2.8mm), long lesions (>23mm) or diabetic patients. At 1 year there was a highly significant increase in restenosis in the Genous stent group. Target vessel failure was also increased (17.3% *versus* 10.5%) although this was not statistically significant, possibly due to the relatively small number of patients in the trial. The Genous stent does however have a favorable safety profile with respect to stent thrombosis; no stent thromboses occurred in the Genous stent group compared to 4 stent thromboses in the Taxus stent group and this was despite a significantly lower use of clopidogrel in patients randomised to receive the Genous stent [Beijk *et al.*, 2009]. It is difficult however to envisage a role for the Genous stent, as the early discontinuation of clopidogrel it allows, which would be favourable in patients at higher risk of developing bleeding complications, is provided to a similar extent by bare metal stents, yet BMS may have more favourable profile with respect to ISR.

Restenosis may be increased with CD34 capture stents for a variety of reasons. As discussed CD34 is not specific for EPC, being common to a number of progenitors including smooth muscle progenitor cells [Simper *et al.*, 2002]. Circulating smooth muscle progenitor cells are poorly characterised, but are thought to contribute to neo-intimal hyperplasia [Sata *et al.*, 2002]. Both CD34 and CD133 are detected at increased levels in the neo-intima of re-stenotic lesions compared to de-novo lesions [Skowasch *et al.*, 2003]. The effect of indiscriminate binding of $CD34^+$ cells to intra-coronary stents could theoretically amplify the re-stenotic effect due to proliferation of smooth muscle progenitors, particularly if exposed to the pro-inflammatory micro-environment of patients susceptible to ISR. As our

understanding of EPC biology improves, so too will our ability to use intra-coronary stents to modulate the cellular response to vascular injury and directly enhance re-endothelialisation following iatrogenic vascular injury through gene and drug delivery or progenitor cell capture.

1.7.3 Infusion of endothelial progenitor cells in acute vascular injury

As an alternative to pharmacological mobilisation, direct intravenous, and also intra-arterial infusion of 'EPC' have also been used in an attempt to accelerate vascular healing in the context of discrete vascular injury. In animal models of endovascular injury, transfusion of mononuclear cells cultured in angiogenic medium to produce endothelial like cells have successfully accelerated re-endothelialisation and attenuated neointimal hyperplasia [Griese *et al.*, 2003; Gulati *et al.*, 2003b; Werner *et al.*, 2003; Zhao *et al.*, 2008]. Generally these studies have used unselected cell populations as 'EPC', so it is therefore uncertain through what mechanism infused cells exerted their effect. Beneficial effects may have been mediated through the paracrine influence of angiogenic monocyte and lymphocyte populations. The restoration of endothelial function may be best imparted by the synergy exists between unselected mononuclear cell sub-populations [Wassmann *et al.*, 2006].

Concerns that infusion of mononuclear cells may increase inflammatory signalling and smooth muscle proliferation are well founded theoretically and there are some reports of an increased incidence of coronary events including restenosis and thrombosis following such treatment. Circulating progenitor cells have also been implicated in the progression of atherosclerotic plaques in the context of ischemia

[Silvestre *et al.*, 2003]. Mononuclear cells cultured in endothelial growth medium genetically engineered to over express endothelial nitric oxide synthase (eNOS) were effective in accelerating re-endothelialisation with an associated reduction of neo-intimal hyperplasia and thrombosis [Kong *et al.*, 2004b]. There have been no clinical studies using infused progenitor cells specifically to influence restenosis. Data regarding the transfusion of progenitor cells in human studies are derived largely from studies examining the effect of mononuclear cell infusion on left ventricular dysfunction and myocardial ischaemia in the context of acute myocardial infarction [Padfield, 2012].

1.8 AIMS AND HYPOTHESES

Delayed re-endothelialisation following vascular injury permits persistent vascular inflammation and the potentiation of neo-intimal hyperplasia that may lead to restenosis and myocardial ischaemia. Circulating EPC may play a critical role in vascular healing following PCI however putative EPC expressing $CD34^{+}VEGFR\text{-}2^{+}$ are not known to be mobilised by discrete vascular injury or specifically affect vascular healing following PCI. The EC-CFU assay was once widely regarded as a measure of circulating EPC, but emerging evidence would suggest that EC-CFU are in fact composed of activated leukocytes, providing possible explanation for their association with acute systemic inflammation. Although systemic inflammation is closely associated with a maladaptive response to vascular injury, the impact of systemic inflammation on the various putative EPC populations remains poorly understood. A better understanding of the role of putative EPC following vascular injury may allow a targeted cellular approach to reduce the complications of PCI.

This thesis aims to determine whether putative EPC are related to atherosclerotic vascular injury and whether they are mobilised in response to iatrogenic vascular injury and how systemic inflammation impacts on EPC mobilisation and function.

I hypothesise that EC-CFU and bone marrow derived vascular progenitors will be mobilised in response to PCI mediated vascular injury in order to contribute to effective re-endothelialisation and the restoration vascular homeostasis. A robust response by these populations may therefore lead to rapid reconstitution of the endothelial monolayer thereby facilitating rapid recovery of normal endothelial function and vascular homeostasis (Figure 1.2). Mobilisation of EPC following vascular injury is likely to be mediated by cytokines and growth factors induced by endothelial denudation and vessel injury. However, vascular injury is also associated with a more generalised acute phase systemic inflammatory response. I wish to establish whether EPC mobilisation occurs as part of a non-specific inflammatory response or is a specific response to vascular endothelial injury. Rather than mobilising EPC following PCI, systemic inflammation may exert deleterious effects on EPC function, and impair progenitor cell engraftment at the site of vascular injury. In addition I aim to relate circulating concentrations of putative EPC populations to the severity of coronary atheroma to further interrogate the relationship between circulating progenitors and vascular health.

HYPOTHESES

The following hypotheses will be addressed:

1. Vascular injury during PCI mobilises putative EPC (Chapters three and seven).
2. EC-CFU are composed of leukocytes, formed through the proliferation and migration of peripheral blood monocytes and lymphocytes (Chapter four).
3. Acute systemic inflammation will not mobilise putative EPC in the absence of vascular injury in healthy volunteers (Chapter five).
4. Circulating putative EPC are diminished in patients with coronary artery disease but are mobilised following acute myocardial infarction (Chapter six).
5. Increased concentrations of EPC are associated with less atheroma and better clinical outcomes (Chapter six).

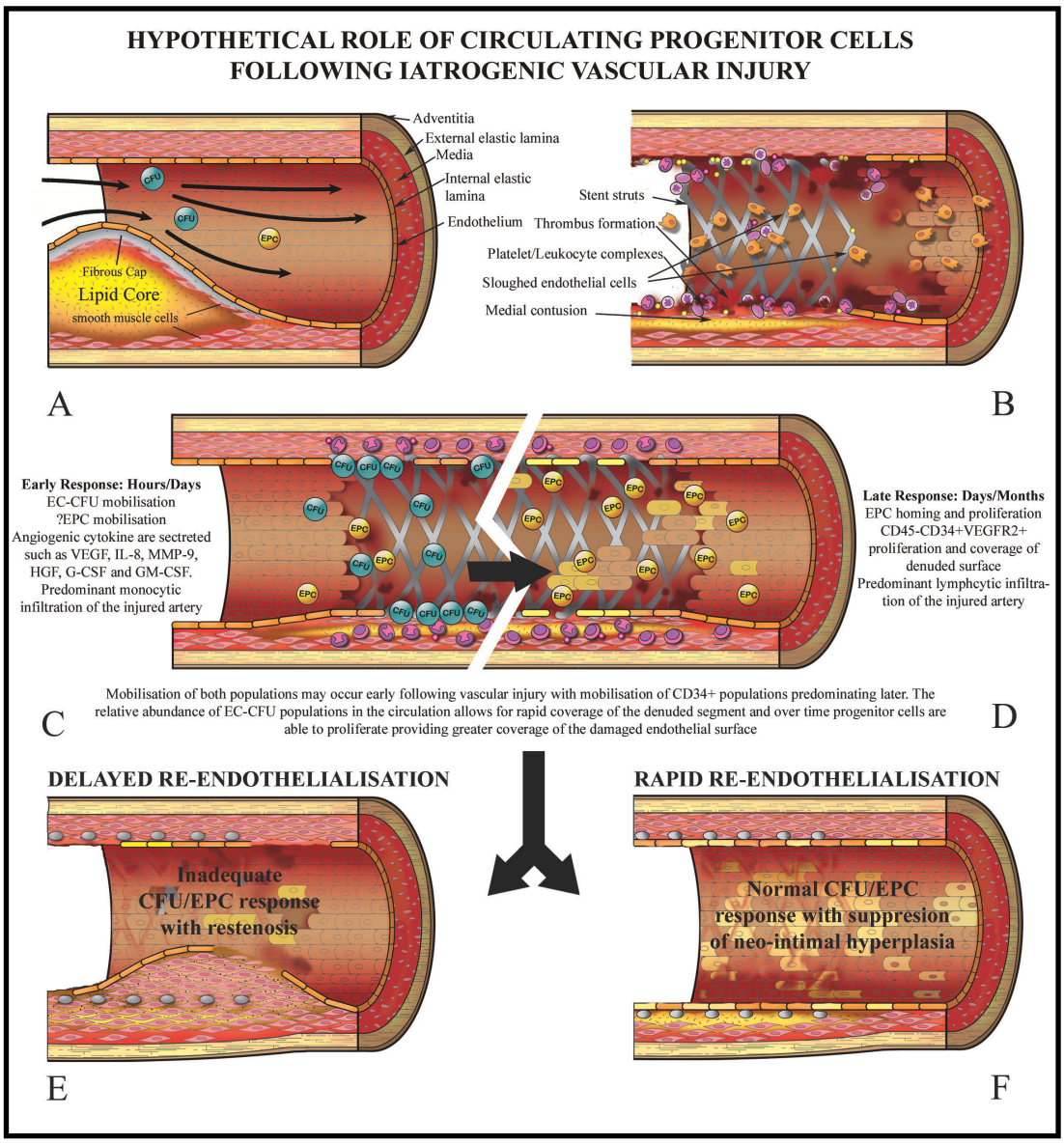


Figure 1.2 Hypothetical role of circulating progenitor cells following iatrogenic vascular injury: (**A**) Under resting conditions circulating concentrations of EC-CFU precursors and circulating EPC are low. (**B**) Intra-coronary stent deployment relieves obstruction, but damages the endothelium and underlying artery, exposing collagen and tissue factor, activating platelets and the coagulation cascade. In the absence of an intact endothelium, local platelet/platelet and platelet/leukocyte complexes form and intense local inflammatory infiltrate ensues with detectable systemic inflammation. I hypothesise a biphasic response to vascular injury caused by PCI. (**C**) The early response consists of mobilisation of the precursors of EC-CFU involving angiogenic monocytes and lymphocytes. EC-CFU precursors home to the site of injury and avidly secrete angiogenic factors encouraging resident endothelial cell proliferation and migration, and the mobilisation and homing of bone marrow derived and local endothelial progenitors to the site of vascular injury. (**D**) Subsequently progenitor cells proliferate and contribute to effective re-endothelialisation and the restoration vascular homeostasis. (**E**) An inadequate EC-CFU/EPC response following PCI may lead to delayed re-endothelialisation and persistent inflammation, smooth muscle hypertrophy and extra-cellular matrix deposition leading to restenosis and symptoms of myocardial ischaemia. (**F**) A robust response by the bone marrow leads to rapid homing of EC-CFU precursors to sites of vascular injury with recruitment and integration of EPC to the endothelial monolayer, facilitating rapid re-endothelialisation and recovery of normal endothelial function.

CHAPTER TWO

METHODOLOGY

FLOW CYTOMETRIC ANALYSES, CELL CULTURE ASSAYS, INVASIVE VASCULAR PROCEDURES AND CLINICAL FOLLOW-UP

2.1 GENERAL

2.1.1 Ethical Considerations

All studies were undertaken in accordance with the regulations of the Lothian and London Research Ethics Committee, and with the Declaration of Helsinki of the World Medical Association. Specific ethical approval was obtained from Lothian local research ethics committees and research and development office for each of the clinical studies that follows (Chapter four, 06/S1102/42; Chapter five & seven, 06/S11ADMIN/108; Chapter six, 06/S1103/44) and all subjects partaking in the following studies provided written consent. For all participating subjects, the registered General Practitioner and the responsible Consultant Cardiologist was informed of the patient's participation in the study.

2.1.2 Subject Recruitment

Healthy Volunteer studies

Healthy non-smoking male volunteers (aged between 18 and 35 years) were recruited through an advertisement distributed using the University of Edinburgh's electronic mailing list. Subjects were required to have no significant past medical history or an inter-current illness likely to be associated with an inflammatory response. Subjects were excluded if they had previously undergone typhoid vaccination, or had received any vasoactive or non-steroidal anti-inflammatory drugs in the week before the study. Suitable volunteers were provided with an information sheet.

Patients undergoing coronary angiography and angioplasty

Elective patients

Patients undergoing elective coronary angiography for the investigation of suspected angina or as part of an evaluation prior to surgery for valvular heart disease were identified prospectively from elective angiography lists at the Royal Infirmary of Edinburgh by members of the research team in conjunction with the express permission of the physician clinically responsible for the patients care. Patients were then contacted by a member of the research team (GJP or LF) by means of an invitation letter and a patient information sheet posted to their home address. This was followed by a telephone call in order to further elaborate on the study details and answer any questions prospective participants might have.

Patients with acute coronary syndrome

Patients undergoing coronary angiography following an acute coronary syndrome (ACS) were identified by daily review of patients scheduled for coronary angiography at Edinburgh Royal Infirmary (ERI) with either an increase in plasma troponin concentration above 0.2ng/mL or evidence of myocardial ischemia on a 12-lead electrocardiogram in the context of unstable anginal symptoms. Patients were provided with a brief description of the study rationale and protocol and were provided with an information leaflet and were given at least 24 hours to consider participation in the study. Patients were excluded given the presence of a significant co-morbid illness such as any haematological or internal malignancy, acute ST-segment elevation myocardial infarction, significant ventricular arrhythmia or haemodynamic instability, hepatic or renal failure, intercurrent infection or a clinically active inflammatory disorder. Patients were excluded in the event of

systemic corticosteroid treatment within 30-days of study enrolment. All volunteers underwent baseline evaluation including documentation of clinical characteristics, cardiovascular risk factors, and medication. Hyperlipidemia and hypertension were defined as either a documented history or the use of a lipid lowering or anti-hypertensive medication respectively.

2.1.3 The Steroids Against ReStenosis - STARS trial

In the absence of an intact endothelium persistent inflammation encourages smooth muscle hypertrophy and in-stent restenosis [Toutouzas *et al.*, 2004]. Elevated pre-procedural serum CRP concentrations predict adverse outcome after coronary stent implantation and are associated with a greater degree of angiographic restenosis. *In vitro* data supports the hypothesis that CRP is detrimental to EPC function [Fujii *et al.*, 2006]. This would suggest that systemic inflammation inhibits vascular repair and is associated with an adverse proliferative response [Walter *et al.*, 2001a].

In collaboration with Dr Mark de Belder at James Cook University Hospital Middlesbrough (JCUH), we are participating in the STeroids Against Re-Stenosis (STARS) Trial, 2x2 randomised controlled trial examining the effect of oral Prednisolone and two different types of BMS design (Zeta™ or Vision™; Abbott Vascular; UK) on the development of in-stent restenosis following PCI. This study population will undergo protocol driven repeat coronary angiography and intravascular ultrasound at six months following stent insertion, and therefore provides the ideal vehicle to prospectively test the hypothesis that increased circulating numbers of EPC and mobilisation following angioplasty will encourage

early endothelialisation and prevent persistent vascular inflammation and in-stent restenosis. This study also provides an opportunity to explore the effect of anti-inflammatory treatment on EPC mobilisation and function.

2.1.4 Randomisation procedures

Healthy volunteer studies

In a double-blind fashion, subjects were randomly allocated by the nursing staff of the Wellcome Trust Clinical Research Facility, Edinburgh Royal Infirmary to receive intramuscular *Salmonella Typhus* polysaccharide vaccination 0.025 mg (Typhim Vi, Aventis-Pasteur MSD, UK) or saline placebo given by intramuscular injection. Subjects received the alternate treatment following a two-week wash out period.

Patients undergoing coronary angiography

Patients undergoing coronary angiography with a view to follow on PCI underwent baseline evaluation of clinical, biochemical and putative EPC concentration. The decision to proceed to coronary intervention in all cases was determined by clinical need at the operator's discretion. For studies using PCI as a model of vascular injury discussed in chapters three and seven, potential recruits undergoing coronary angiography were selected by an augmented method of minimisation in order to minimise between group differences and potential confounding effects between those undergoing PCI or diagnostic angiography alone [Treasure *et al.*, 1998]. For example, if during recruitment the mean age of one group were to be become significantly different from the other this would be taken into

account when inviting subsequent patients to take part in the study. Age, gender, and traditional risk factors for CAD; diabetes; smoking history; hypercholesterolaemia and hypertension were taken into consideration.

The STARS trial

Although the results of the STARS trial are not reported within this thesis, the patients recruited for chapter 6 comprise the STARS cohort recruited at ERI. Follow up data is not yet available for these patients, as the data regarding their treatment allocation remains blinded. However, the STARS trial is discussed here and in further detail in chapter eight.

Following informed consent, patients undergoing coronary angiography with a view to follow on PCI underwent baseline evaluation of clinical, biochemical and putative EPC concentration. Patients were then randomised in a double blind fashion to receive oral prednisolone 40 mg or placebo in addition to empirical proton pump inhibition with lansoprazole 30mg, 24 hours (no earlier than 8 hours) prior to angiography. The study medications were prepared at James Cook University Hospital (JCUH) Pharmacy, and were presented as reddish brown capsules in plain white containers identifiable only by a study reference code. In the event of the decision to proceed to treatment with a BMS, of a de novo lesion affecting a native coronary artery with a reference diameter of > 3mm (single or multiple PCI to simple or ostial/bifurcation lesions included), patients were then randomised by sealed envelope in a single blind fashion to receive either Zeta or Vision BMS. Patients were excluded in the event of the use of drug eluting stent, primary PCI for AMI, re-stenotic lesions, vein graft lesions and any unprotected left main stem PCI. Oral

prednisolone 40mg daily, was continued for a total of 28-days, and aspirin 75mg and clopidogrel 75 mg daily, were given to all patients for at least three months following PCI. Prescription of other pharmacological agents was at the discretion of the attending physician. Patients were re-evaluated at 24 hours and at one and four weeks in order to assess clinical status and perform serial assays of haematological and biochemical assays and EPC measures. Patients returned at 6-months for repeat coronary angiography and intra-vascular ultrasound examination. Patients treated with drug eluting stents (DES), or those not undergoing PCI received no further study medication, but were invited to return for further phlebotomy for assessment of putative EPC measures at 24 hours. The protocol is summarised below (Figure 2.1).

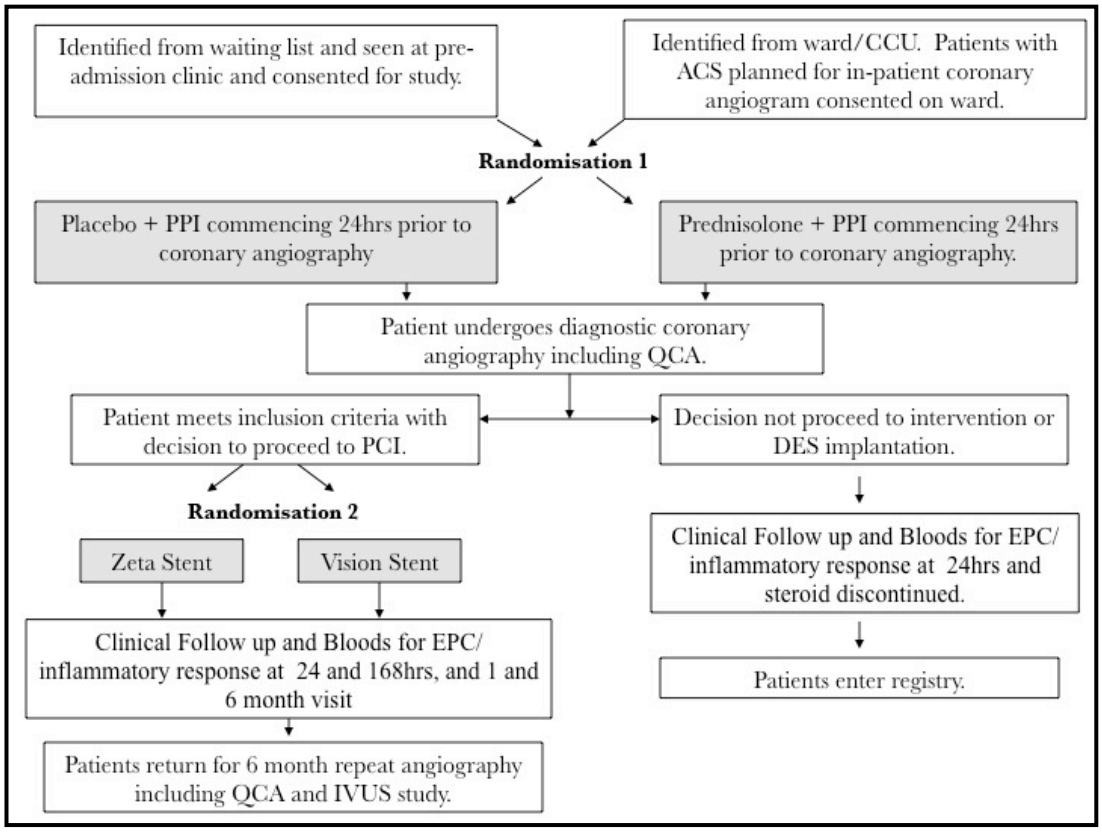


Figure 2.1. Protocol for patients enrolled in to the STARS trial at ERI

2.2 INVASIVE VASCULAR PROCEDURES

All invasive procedures took place in the cardiac catheterisation laboratory at ERI. Baseline procedures formed part of the patient's routine clinical management and were conducted by the responsible Physician caring for the patient.

2.2.1 Diagnostic coronary angiography

Diagnostic coronary angiography was performed via a 6 French sheath placed in the femoral or radial artery using appropriate 6 French arterial catheters following the administration of 200μg of intra-coronary glyceryl trinitrate (GTN). Standard angiographic projections were employed in addition to specific evaluation of target lesions in at least two orthogonal views, before and immediately following PCI.

2.2.2 Percutaneous coronary intervention.

PCI was performed using standard techniques on flow limiting coronary stenoses (>50%) thought to be clinically relevant at the operator's discretion, given ECG changes at presentation, and lesion characteristics. Briefly, an appropriate guide catheter is advanced to the coronary ostium. A guide wire of suitable weight is then advanced through the obstructive lesion. Balloon angioplasty is performed in order to optimise stent delivery at the operator's discretion. A balloon-mounted intracoronary stent of a suitable length and diameter necessary to effectively address the obstructive lesion is then advanced to the point of maximal stenosis and deployed at a pressure necessary to evenly appose the stent with the vessel wall. Further dilatation of the stent by balloon angioplasty in order to optimise stent deployment is performed at the operator's discretion. Artery closure is performed by manual

pressure, or by device closure (Radistop™ or Angioseal™) at the operator's discretion.

2.3 IMAGE ANALYSIS AND GENSINI SCORING

The severity of coronary disease was quantified offline by observers blinded to the patient's clinical profiles through visual inspection of the angiographic appearance of each epicardial arterial segment. Coronary artery disease severity was graded using a modified version of the Gensini scoring system [Gensini, 1983; Ringqvist *et al.*, 1983]. Fourteen pre-determined arterial segments of the major epicardial arteries are graded according to a visual estimate of severity of stenosis and allocated a weighting score according to the lesion's location (Table 2.1; Figure 2.2). The original Gensini score does not discriminate between minor grades of atheroma, i.e. normal, plaque disease and stenoses of <25% which all score 0. In order to discriminate between the presence of minor degrees of atheroma and normal arteries the score was modified to discriminate between these gradations of atheroma (table 2.1). This provides a continuous variable quantifying atheroma burden accounting for proximal *versus* distal disease. The median intra-observer variability as assessed in twenty randomly selected coronary angiograms was 11% (IQR=7-32%).

Table 2.1: Gensini scoring system.

Disease severity	Segment scores	Arterial Segment	Weighting
Occluded	32	Left main stem	5
Sub-total occlusion	16	Proximal left anterior descending	2.5
Stenosis >95%	8	Mid left anterior descending	2.5
Stenosis 75-95%	4	Distal left anterior descending	1
Stenosis 50-74%	3	First diagonal	1
Stenosis 25-50%	2	Second diagonal	1
Stenosis <25%	1	Proximal circumflex	2.5
Plaque Disease only	0.5	Mid and atrioventricular circumflex	1
Nil	0	First obtuse marginal	1
		Second obtuse marginal	1
		Proximal right coronary artery	1
		Mid right coronary	1
		Distal right coronary	1
		Posterior descending artery	1

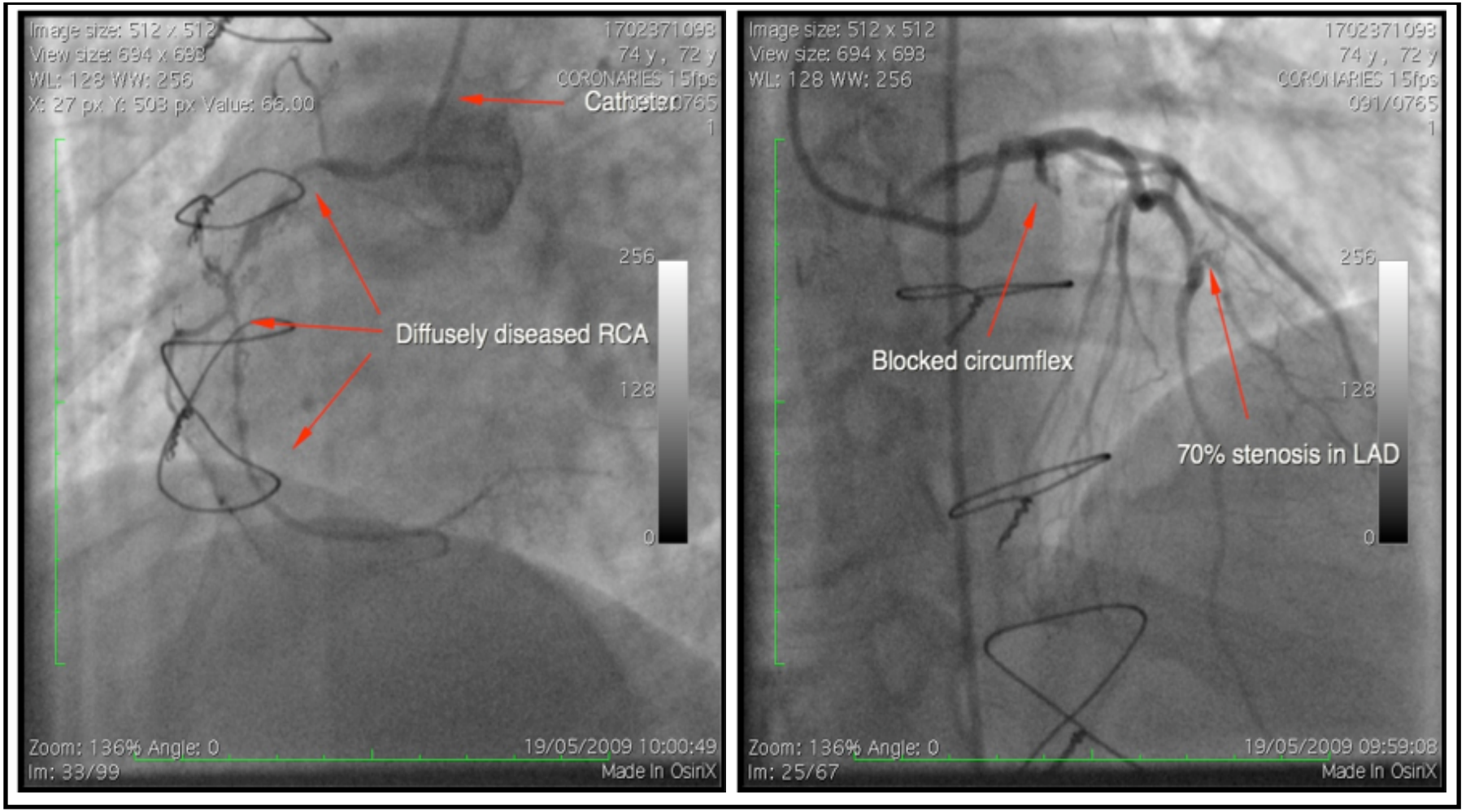


Figure 2.2 Gensini scoring: Diagnostic angiograms of the left and right coronary are shown. In the example above we have a blocked proximal circumflex, a 70% stenosed mid LAD and a diffusely diseased RCA with a critically proximal stenosis. This would therefore score: Cx (32x2.5)+LAD(2x2.5)+RCA((8x1)+(8x1))=101.

2.4 CLINICAL FOLLOW UP

Clinical event monitoring and follow up

The nature and timing of clinical events were determined by reference to medical records using the TrakCare® software application (InterSystems Corporation, Cambridge, MA, USA), an electronic patient record system used by the Acute Hospitals Division of Lothian National Health Service (NHS) Health Board, United Kingdom. Deaths due to cardiovascular causes included any sudden death, and death from acute myocardial infarction, coronary artery disease, or congestive heart failure. Acute myocardial infarction was defined as any increase in plasma concentration above the 99th centile in the presence of symptoms or ECG changes indicative of myocardial ischaemia. Revascularization procedures included all percutaneous or surgical coronary artery procedures. Hospitalisation for cardiovascular causes included any planned or unplanned hospitalisation for acute coronary syndromes, heart failure, stroke or uncontrolled arrhythmia, or any coronary or peripheral vascular revascularization occurring from the date of enrollment. Major adverse clinical events were a composite of cardiovascular death, recurrent myocardial infarction, revascularization or hospitalisation for a cardiovascular cause.

2.5 FLOW CYTOMETRIC ANALYSES

All laboratory bench procedures were performed in the Centre for Regenerative Medicine at the Little France site, Edinburgh Royal Infirmary or at King's Buildings, Edinburgh University in class II laboratories by trained laboratory personnel adhering to standard safety procedures (GJP, EF, MW, OT-C).

2.5.1 Immunostaining of putative EPC and angiogenic monocytes:

Using Gilson® PIPETMAN Classic™ pipettes, and disposable pipette tips, 5μL of antibody solution at the dilutions described (Table 2.2) were transferred to polyurethane tubes according to the following table (Table 2.3). Optimal antibody dilutions were determined by titration of antibody concentrations to achieve the lowest possible concentration producing maximal separation of populations of interest with minimal non-specific staining. Compensation was performed using commercially available flow cytometry compensation beads (Miltenyi Biotec, UK).

Table 2.2: Immunofluorescence staining materials for flow cytometric analyses

Antibody	Manufacturer	Dilution
CD3-PerCP	R&D systems, Minneapolis, USA	1:10
CD14-FITC	Caltag Systems, Buckingham, UK	1:10
CD14-PE	Caltag Systems, Buckingham, UK	1:10
CD18-PE	R&D systems, Minneapolis, USA	1:10
CD19-APC	R&D systems, Minneapolis, USA	Undiluted
CD34-FITC	Becton Dickinson, Oxford, UK	Undiluted
CD45-PerCP	Becton Dickinson, Oxford, UK	1:3
CD133-PE	Miltenyi Biotec Ltd, Surrey, UK	1:2
CXCR-4-APC	R&D systems, Minneapolis, USA	1:10
Tie-2-APC	R&D systems, Minneapolis, USA	1:5
VEGFR-2-APC	R&D systems, Minneapolis, USA	1:2
VEGFR-2-PE	R&D systems, Minneapolis, USA	Undiluted

APC = allophycocyanin; CD = cluster of differentiation; FITC = Fluorescein isothiocyanate; PBS = phosphate buffered saline; PE = phycoerythrin; PerCP=Peridinin-chlorophyll-protein complex; VEGFR-2 = vascular endothelial growth factor receptor-2

200μL of peripheral blood was then transferred to each tube, with care taken to avoid smearing of blood on the walls of the tube. All tubes were then briefly agitated with a mechanical vortex mixer and incubated at room temperature in the dark. At

20 minutes, according to the manufacturer's instructions, 200μL of lysing reagent A, from the Utilyse™ (Dako/BioStat; UK) kit was transferred to each tube followed again by a brief agitation. A further period of incubation at room temperature in the dark for 10 minutes was performed at which time 2mL of lysing reagent B was added

Table 2.3: Flow cytometric protocol

Tube	Antibodies			
1	Primary negative control			
2	CD45-PERCP			
3		CD34-FITC		
4	CD45-PERCP	CD34-FITC		
5	CD45-PERCP	CD34-FITC	VEGFR-2-APC	
6	CD45-PERCP	CD34-FITC	VEGFR-2-APC	CD133-PE
7	CD45-PERCP	CD14-FITC		
8	CD45-PERCP	CD14-FITC	Tie-2-APC	
9	CD45-PERCP	CD14-FITC	Tie-2-APC	VEGFR-2-APC
10	CD45-PERCP	CD34-FITC	CD18-PE	
11	CD45-PERCP	CD34-FITC	CD18-PE	CXCR4-APC

APC = allophycocyanin; CD = cluster of differentiation; FITC = Fluorescein isothiocyanate; PBS = phosphate buffered saline; PE = phycoerythrin; PerCP=Peridinin-chlorophyll-protein complex; VEGFR-2 = vascular endothelial growth factor receptor-2

followed by a final agitation. Erythrocyte lysis was evident by the solution becoming transparent. In the event of an incomplete lysis, as evidenced by persisting opacification of the solution, a further period of incubation was occasionally required to ensure complete lysis of red blood cells and avoid contamination of the sample with excessive cellular debris. Balanced samples were then centrifuged for 10 minutes at 250 x g in a desktop centrifuge (Jouan BR3-11). The supernatant was then discarded and the samples subjected to two further washing steps entailing suspension of the cell residue in 2mL of phosphate buffered saline solution (PBS; pH7.4; Sigma-Aldrich; UK), and centrifugation for 10 minutes at 250 x g. The cell

residue was finally resuspended in 450μL of CellFix™ (Becton Dickinson; UK), cell fixative agent at a 1:10 dilution in PBS and analyzed immediately.

2.5.2 Flow cytometric data analyses.

Flow cytometry was performed on a Becton Dickinson FACS Caliber™ using Cellquest™ software on an Apple Macintosh desktop computer. Standard maintenance procedures were performed prior to and following analysis of cell samples to ensure proper working order of the FACS Caliber™. Acquisition gates were applied to encompass the total leukocyte population and specifically the lymphocyte population according to the characteristic forward side scatter appearance. For each sample, 80,000 events were acquired in the lymphocyte region, equating to approximately 500,000 total events. Data were analysed using FlowJo (Treestar, USA). Analyses involved first identifying leukocytes by their characteristic forward and side scatter profile. For $CD34^+$, $CD45^+$, $CD133^+$, VEGFR-2^+, $CD18^+$, and CXCR-4^+ quantification, the proportion of leukocytes cells bearing each epitope was determined individually by using the side scatter profile plotted against the appropriate fluorescence channel. Permutations of co-expression were determined automatically using Boolean principles (AND, NOT, OR etcetera.). Although opinion remains divided, iso-type controls are considered by many to be unnecessary in flow-cytometric analyses, particularly for rare event haematopoietic populations [Keeney *et al.*, 1998]. I therefore used the autofluorescence of unstained samples to establish positive stain boundaries (the fluorescence minus one technique) as depicted below Figure 2.5) [Tung *et al.*, 2004]. Control gates were set within the leukocyte population of an unstained sample to include events with autofluorescence in the

appropriate channel at an arbitrarily predefined level (0.005% for CD34 and 0.01% for other populations. $CD14^+$ cells were gated and VEGFR-2 and Tie2 expression was assessed using quadrant analysis (Figure 2.5). Quadrants were set on unstained negative controls to include approximately 0.5% autofluorescence. The absolute concentration of cells per mL of blood was calculated by equating the total number of events in the lymphocyte, monocyte, neutrophil and eosinophil regions by forward and side scatter to the total leukocyte count from the full blood count. The intra-assay coefficients of variation for the phenotypic markers used are tabulated below (Table 2.4).

Table 2.4 Intra-assay co-efficient of variation for the phenotypic markers used to identify EPC and angiogenic monocytes

Phenotypic marker	Intra-assay co-efficient of variation
CD45	0.1%
CD34	9%
CD133	6%
VEGFR-2	8%
$CD34^+CD45^-$	19%
$CD34^+VEGFR\text{-}2^+$	25%
$CD34^+CD133^+VEGFR\text{-}2^+$	39%
CD14	1%
Tie-2	15%
$CD14^+KDR^+$	19%
$CD14^+Tie\text{-}2^+KDR^+$	45%

Values expressed are the median CV% for duplicate samples where CV% is calculated as the standard deviation of 20 duplicate measures / mean x 100%.

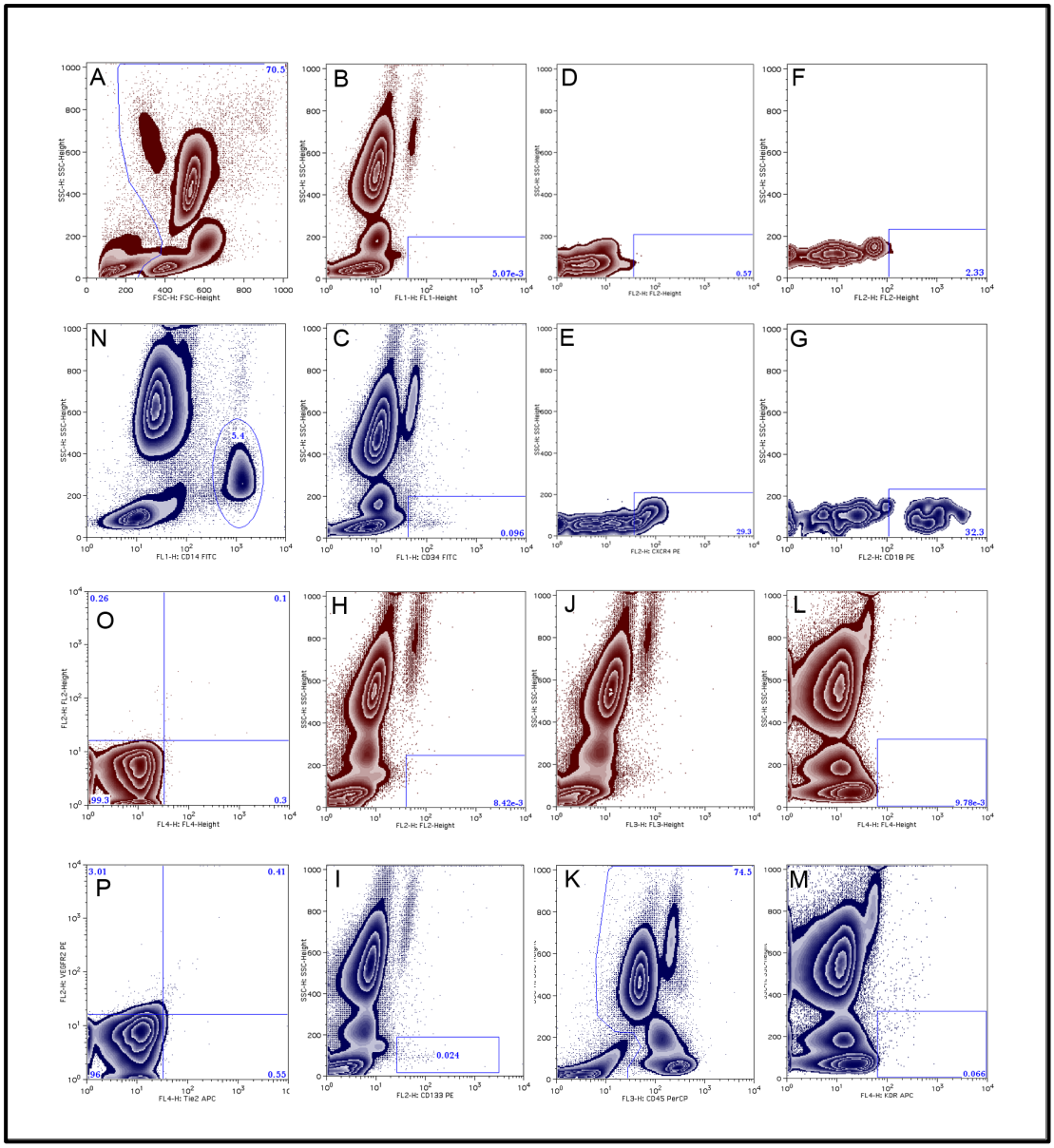


Figure 2.5 Flow cytometric analysis of putative progenitor cells and angiogenic monocytes: Representative dot plots of negative controls and stained samples are shown in red and blue respectively. First leukocytes were identified on the basis of their characteristic forward and side scatter profile (**A**). CD34-FITC (**B&C**) expression and the proportion of $CD34^+$ events expressing CD18 (**D&E**) and CXCR-4 (**F&G**) were determined. Similarly CD133-PE^+ (**H&I**), CD45-$PercP^+$ (**J&K**), and VEGFR-2-APC^+ (**L&M**) events were identified. Co-expression of surface markers was determined using Boolean principles. In separate analyses $CD14\text{-}FITC^+$ events were identified (**N**), and those expressing Tie-2-APC and or VEGFR-2-PE were determined using quadrant analysis (**O&P**). Gates were set on single or unstained stained negative controls where appropriate.

2.5.3 Cell sorting

In order to specifically isolate monocytic and lymphocytic fractions of a mononuclear preparation and specifically interrogate the behaviour of these cells when placed in close proximity to maturing EC-CFU, mononuclear preparations underwent fluorescent activated cell sorting (FACS). Sixty mL of peripheral blood was collected into ethylene-diamine tetra-acetic acid (EDTA) and diluted 3:1 with Ca^{2+} and Mg^{2+} deficient PBS (2% fetal calf serum). Diluted blood was layered on 10mL of Histopaque-10771 (Sigma-Aldrich; UK) in 50mL falcon tubes (Beckton Dickinson; UK). Density gradient centrifugation was performed at 1000 x g for 25 minutes. The 'buffy coat' of mononuclear cells was aspirated and washed three times with PBS. Approximately $8x10^7$ cells were obtained and resuspended in 4mL of 2% FCS and then stained using antibodies directed against the T-cell marker CD3 pre-conjugated to PE, the B-cell marker CD19 pre-conjugated to APC, and the monocyte marker CD14 pre-conjugated to PE (Invitrogen, UK) for 20 minutes at 37°C, 5% CO_2. Cell clumps were removed by agitation followed by sedimentation at 37°C, 5% CO_2. The single cell suspension was transferred to a fresh tube, centrifuged at 300 x g for 5 minutes, and re-suspended in

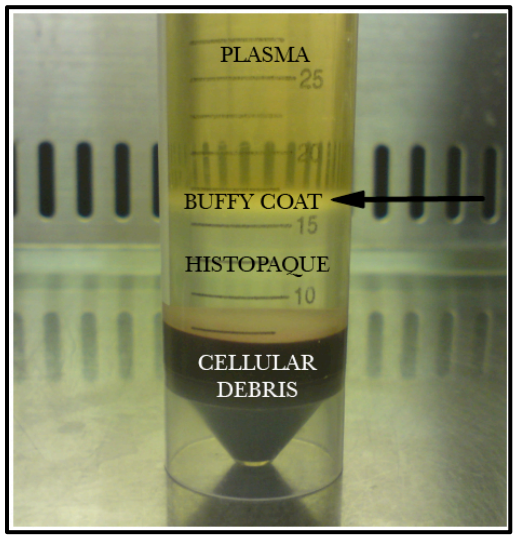


Figure 2.6 Isolation of mononuclear cells: Whole blood collected in EDTA is diluted with PBS and layered on Histopaque and centrifuged. Mononuclear cells collect on the surface of the histopaque to form a 'buffy coat' that is then aspirated with a Pasteur-pipette.

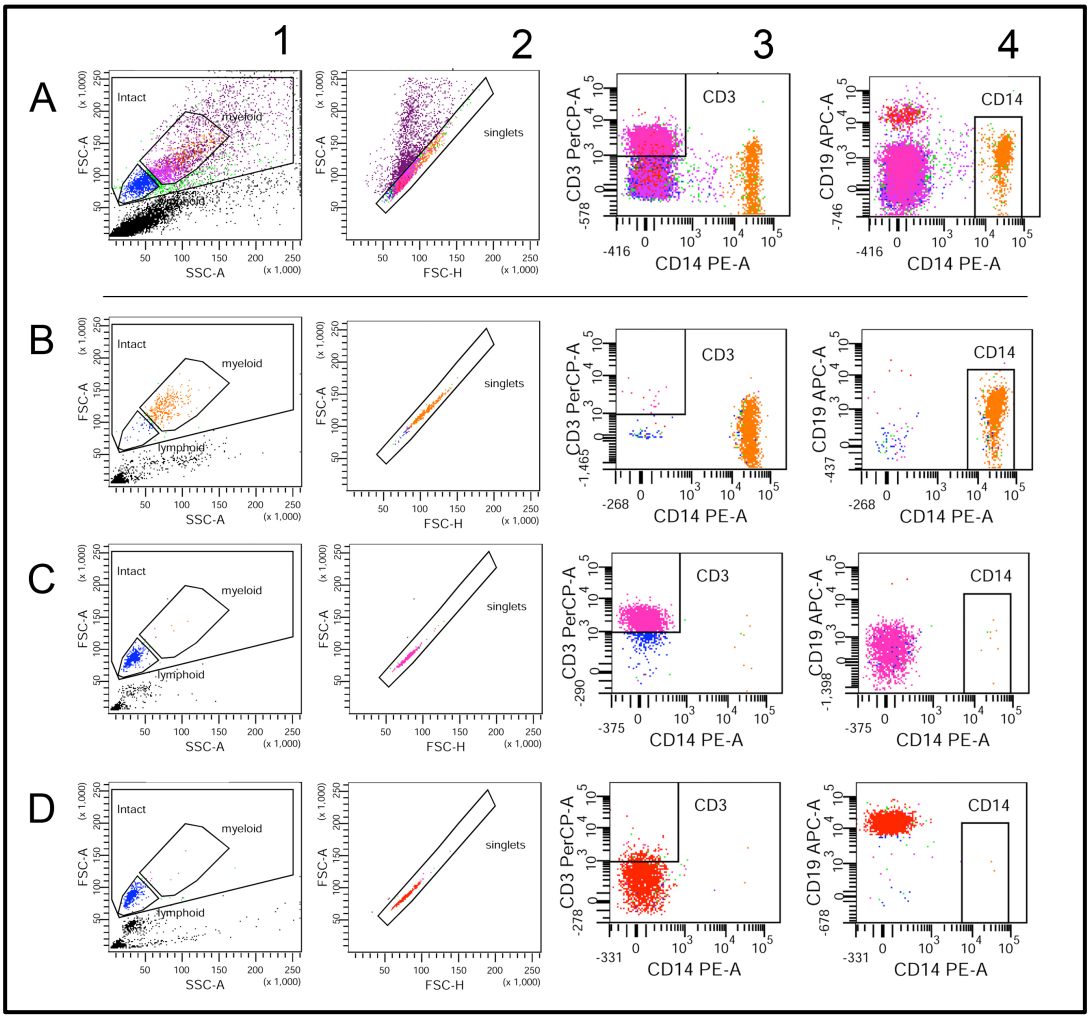


Figure 2.7 Fluorescence activated cell sorting: Mononuclear cells were considered 'intact' on the basis of their FSC-A against side scatter-area (SSC-A) appearance **(A1)**. To minimize cellular aggregates populations were then selected on the basis of their FSC-A against FSC-H profile **(A2)**. Populations were sorted on the basis of their fluorescence in the relevant channel **(A3&A4)**. Following cell sorting samples were reacquired to confirm purity **(B-D)**.

fresh culture medium supplemented with 100U/mL penicillin and 100g/mL streptomycin. Data were acquired on a FACSAria cell sorter (Becton-Dickinson, UK) equipped with an argon laser and standard filter set using a 70-µm nozzle at a sheath pressure of 70 PSI and flow rate setting of 1. The sort rate was less than 1000 events/sec. Cell separation and analyses were performed using CellQuestPro software (Becton Dickinson; UK). Acquisition gates were set to exclude cellular debris on the basis of the characteristic FSC against SSC profile. In order to avoid cellular

aggregates, events were included on the basis of a forward scatter – area (FSC-A) against FSC-width bivariate plot [Tzur *et al.*, 2011]. Myeloid and lymphoid gates were set using forward and side scatter. From these sub-populations of viable events, lymphocyte (CD3 and CD19) and monocytic (CD14) populations were collected using gates set on the basis of immunofluorescence in the appropriate channel corresponding to the fluorochrome (Figure 2.7). Cells were collected into 5mL polystyrene sterile tubes (Falcon; UK), primed with 1mL of endocult medium and antibiotics (penicillin 100U/mL and streptomycin 100g/mL). An aliquot of each sorted sample was reacquired in order to assess purity. Finally, recovered cells were resuspended in complete medium.

2.6 CELL CULTURE AND FUNCTIONAL ANALYSES

2.6.1 EC-CFU generation

Endothelial cell colony forming units were generated using a commercially available assay. Whole peripheral blood collected in EDTA was diluted with PBS at 1:1 dilution and layered on 10mL of Histopaque 10771 (Sigma-Aldrich; UK) in 50ml falcon tubes (Beckton Dickinson; UK). Density gradient centrifugation was performed at 1000 x g for 25 minutes. The resulting mononuclear buffy coat was aspirated and transferred to fresh tube and the volume made up to 50ml in PBS. The cell suspension was centrifuged at 200 g for 10 minutes and the supernatant discarded. The pellet was transferred to a 15mL centrifuge tube and subjected to two further washing steps using PBS and centrifugation at 200g. This was repeated and

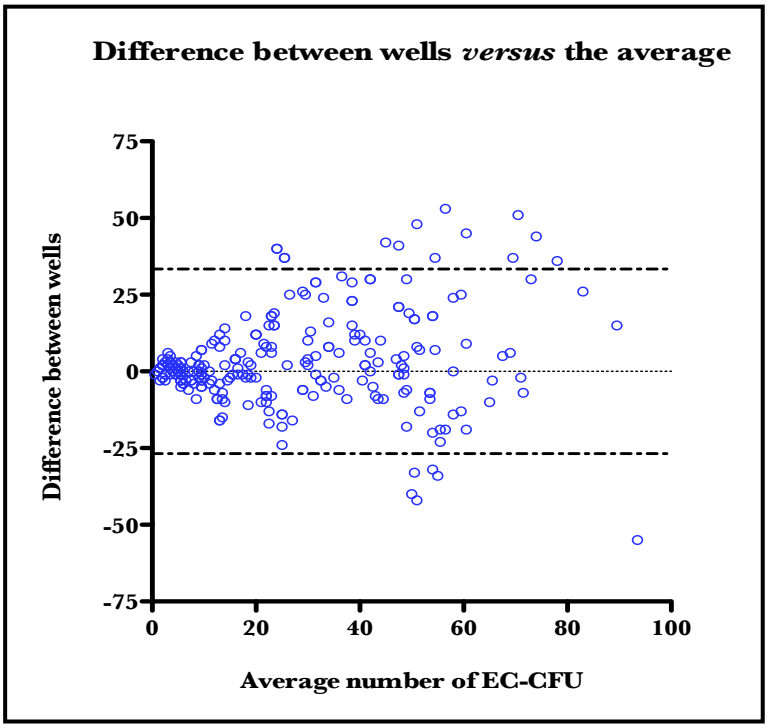


Figure 2.8 Bland-Altman plot of inter-well agreement: Bland-Altman plot demonstrating the level of agreement between 260 pairs of wells from healthy volunteers. The level of disagreement tended to increase with greater EC-CFU concentrations. Level of bias = 3.26 ± SD 15.35. 95% CI = -26.8 to 33.34 (dashed lines).

the remaining cell residue was supended in 2mls of PBS. Using a Multisizer™ 3 Coulter counter® (Beckman-Coulter; UK) the cellular concentration was determined, and 5 x 10^6 mononuclear cells were transferred to 6 well fibronectin coated plates. Two mL of complete endothelial culture medium, Endocult™ (Stem Cell Technologies) were added to each well and plates were incubated at 37°C and 5% CO_2. After two days, non-adherent cells were removed and three to four aliquots of 8x10^5 cells were replated, each in 1mL of complete Endocult™ on fibronectin. EC-CFU colonies were defined as a central core of round cells with associated elongated spindle-like cells at the periphery (Figure 1.2A). Colonies were counted by visual inspection using an inverted light microscope on day five.

The quantity of EC-CFU colonies generated by an individual at any given time point was determined from three to four separate wells. Variation between wells is displayed in the form of a Bland-Altman plot (Figure 2.8) demonstrating the level of

agreement between 260 pairs of wells from healthy volunteers. The level of disagreement increased with greater EC-CFU concentrations. Level of bias = 3.26 ± SD15.35 (95% CI = -26.8 to 33.34). The median co-efficient of variation between wells was 33% (IQR: 20-61%).

2.6.2 EC-CFU characterisation

Much of the debate regarding the phenotype of EC-CFU, has revolved around their haematopoietic, versus the endothelial characteristics. I wished to verify the phenotypic characteristics of EC-CFU that I would be using in these studies. EC-CFU were therefore characterised using a battery of surface and intracellular stains (Table 2.5) described as follows.

Stains and cellular markers

Acetylated low-density lipoprotein (Ac-LDL) and *Ulex Uropeaus* binding are classical endothelial characteristics but both also identify phagocytic cells such as macrophages. VEGFR-2 is considered a marker of endothelial lineage, being widely expressed on mature endothelial cells and is necessary for the normal development of the vascular system. Similarly the tyrosine kinase receptor Tie-2 is expressed on mature endothelial cells and is necessary for normal vascular development. CD146 and CD144 are endothelial intercellular adhesion molecules that facilitate the formation of a confluent layer of endothelial cells. CD105, or endoglin is a constituent of the transforming growth factor-beta receptor is thought to be limited to proliferating cells and is expressed on activated endothelium and smooth muscle cells but also extensively on macrophages. Endothelial nitric oxide synthase is an enzyme

responsible for the production of nitric oxide by endothelial cells in response to sheer stress. Von Willebrand factor (vWF) allows intercellular adhesion, primarily between platelets and exposed collagen following tissue injury to facilitate coagulation, and receptors for vWF are expressed on activated endothelial cells. CD31 is a platelet endothelial cell adhesion molecule also expressed on activated endothelial cells. CD45, also known as the common leukocyte antigen, is a protein tyrosine phosphatase expressed on all haematopoietic cells and leukocytes. CD4 is expressed primarily on helper T-cells and is broadly a lymphocyte marker but it is also expressed to an extent on myeloid cells. CD8 is another glycoprotein expressed on T-cells. CD68 is a surface protein instrumental in phagocytosis and is expressed strongly on macrophages. The lipopolysaccharide receptor CD14 is widely expressed on monocytes.

Colony immunostaining method

Day five EC-CFU grown on fibronectin-coated chambered cover slips (BD Biosciences) were fixed with ice-cold methanol, permeabilised with 0.02% MP40 (Sigma-Aldrich; UK) and blocked with blocked with 10% Goat serum, 1% bovine serum albumin (BSA), prior to immunostaining with the primary antibody. After three washes, cells were incubated with the secondary antibody and incubated for an hour, for stains where the primary step used a murine monoclonal antibody (MAB), the secondary step involved incubation with a biotinolated anti-mouse MAB directed against the primary antibody. A tertiary step using Streptavadin, pre-conjugated to a fluorochrome excitable at 546nm, directed against biotin was then employed. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and incubated in the

dark for 10 minutes and then washed twice in PBS. Colonies were then mounted with PermaFluor™ (Thermo Scientific). After three final washes, cells were photographed using confocal microscopy. Secondary antibodies were titrated such that controls lacking primary antibodies were routinely negative.

Positive and negative control cell populations

Human umbilical vein endothelial cells (HUVEC) served as positive controls for endothelial markers. Having undergone no more than three passages, HUVEC were cultured in EGM-2 (Lonza; UK) on glass chamber slides until a confluent cell monolayer was achieved. Mononuclear cell (MNC) preparations served as positive controls for haematopoietic markers. 100μl of MNC at a density of $4x10^5$ cells/mL in 1% BSA solution were spun onto glass slides and fixed with methanol for five minutes before being stained as above. The human adenocarcinoma cell-line, HT-29 (courtesy of Dr S. Bader, ERI) served as a universal negative control population. HT-29 cells were cultured in RPMI (Gibco/Invitrogen; UK) supplemented with 5% FCS until confluency was achieved. Cells were fixed and stained as described above.

Confocal microscopy and image analysis

Stained EC-CFU, MNC preparation, HUVEC and HT-29 were all visualized using a Zeiss 'LSM 510 Meta' confocal fluorescent microscope at x40 or x20 magnification with an oil immersion objective lens. Fluorescent intensity on EC-CFU was qualitatively compared against the positive and negative controls.

Table 2.5: Immunofluorescent staining materials for EC-CFU characterisation

Primary Antibodies	Manufacturer	Dilution in PBS
Polyclonal rabbit/antihuman		
eNOS	BD Transduction Laboratories	1:500
CD146	Springbioscience	1:200
CD34	BD Biosciences; Santa Cruz	1:100
Tie-2	BD Transduction Laboratories	1:200
CD8	Springbioscience	undiluted
Monoclonal mouse/anti-human		
CD4	BD Transduction Laboratories	1:500
CD45	BD Transduction Laboratories	1:500
CD14	Caltag	1:100
CD31	BD Transduction Laboratories	1:200
CD68	Dako	1:200
CD105	BD Transduction Laboratories	1:200
CD144	BD Transduction Laboratories	1:200
VEGFR-2	R&D	1:200
vWF	Dako	1:200
Secondary Antibodies		
Biotinolated anti-mouse	Dako	1:500
Streptavadin 546	Molecular Probes	1:500
Anti-rabbit Alexafluor 488	Invitrogen	1:200
Nuclear stain		
DAPI	Sigma	1:1000

Primary antibodies are directed against the relevant cellular component. Alexafluor 488 is a monoclonal antibody (MAB) directed against rabbit primary antibodies pre-conjugated to a fluorochrome excitable at 488nm. Streptavadin was pre-conjugated to a fluorochrome excitable at 546nm, and binds to biotinolated anti-mouse MABs directed against the primary antibody. CD = cluster of differentiation; eNOS = endothelial nitric oxide synthase; VEGFR-2 = vascular endothelial growth factor receptor-2

2.6.3 EC-CFU functional assays

Proliferation

Cellular proliferation within EC-CFU was assessed by the detection of incorporation of the thymidine analogue bromodeoxyuridine (BrdU) (Sigma-Aldrich; UK). Mononuclear cells replated on fibronectin at day two were incubated with BrdU at a 100 μmol concentration. On day five colonies were fixed with 4% paraformaldehyde at room temperature for 30 minutes and permeabilised using 0.25% tween 20 (Caltag) for 12 hours. Colonies were then incubated with DNAse (Perbio; UK) at 500Uml^{-1} in 50mM Tris buffered deionised water, $MgCl_2$10mM, BSA 100ugml^{-1} pH 7.4) at 37°C for 30 minutes. Colonies were rinsed well with PBS

and incubated with anti-BrdU conjugated to alexafluore-488™ (Invitrogen) at a 1:100 dilution. Colonies were examined with an inverted microscope at 488nm. Proliferation was expressed as the proportion of BrdU positive cells per unit volume of colony. Colony volume (V) was calculated using the formula: $V = (\pi r^2 h)/3$. Where r = the radius of the base of the colony and the height (h) of the colony is assumed to approximate r.

Migration

The ability of EC-CFU to migrate toward a high VEGF concentration was assessed using 8μm pore transwell plates (Costar). On day five of culture EC-CFU were rinsed twice with Hepes buffered calcium and magnesium free saline solution (Sigma-Aldrich; UK) and then detached from the wells using non-enzymatic dissociation solution (Sigma-Aldrich; UK) at 37°C for 5-10 minutes. Complete growth medium was returned to the well and the cell suspension was pipetted repeatedly to dissociate cellular aggregates. Recovered cells were washed, resuspended in 1mL of serum free medium at a concentration of $2x10^6$ cells/mL using a coulter counter (Beckman Coulter; UK). Using five wells of a plastic transwell migration kit (Costar; UK), 600μL of serum free medium was placed in the lower chamber of each transwell, with VEGF (Peprotech; UK) at a concentration of 50ng/ml. Transwell chambers were gently placed into the wells with the porous membrane just submerged in the medium. $2x10^5$ cells (100μL of $2 x10^6$/mL) were then pipetted into each transwell. Using a Beckman Coulter counter the cell concentration in wells 1-5 were then counted at three hours

2.7 VENOUS SAMPLING AND LABORATORY ASSAYS

2.7.1 Peripheral Venous Sampling

Venous blood was withdrawn from the antecubital fossa in a standard fashion collected into trisodium citrate for cytokine analysis, potassium EDTA for mononuclear cell preparation, flow cytometric analysis and full blood count, and serum gel tubes for CRP and clinical biochemistry (Monovette®, Sarstedt, Nümbrecht, Germany).

2.7.2 Sample Preparation

Citrate and acidified buffered citrate samples were centrifuged at 2,000 g for 30 minutes at 4°C, EDTA samples at 1000g for 10 minutes at 20°C. Serum samples were centrifuged at 2,000 g for 20 minutes after being allowed to clot on ice. Platelet free plasma or serum was decanted and stored at -80°C before assay.

2.7.3 Cytokine and circulating biomarker detection

Plasma troponin I concentrations were measured using an automated immunometric assay; ARCHITECT *STAT* troponin I assay (Abbott Laboratories, Chicago, IL). Plasma IL-6, vascular endothelial growth factor A (VEGF-A), measured with commercially available enzyme linked immunosorbant assays (ELISAs) (Quantikine, R&D Systems; UK). Intra-assay, and inter-assay coefficients of variability were 5.25% and 9.5% for IL-6 and 2.4% and 7.6% for plasma VEGF-A respectively. Serum CRP concentrations were determined using a validated, highly sensitive assay (Department of Clinical Biochemistry; Fife NHS Trust, UK), using the method of particle-enhanced immunonephelometry (Behring BN II nephelometer,

Dade Behring Inc.). Intra-assay and inter-assay coefficients of variability for hs-CRP were 3.7% and 4.2% respectively. All assays were performed in duplicate and the mean value taken. Differential leukocyte count, haemoglobin and standard routine biochemical assays were undertaken on fresh venous samples (Departments of Haematology and Clinical Biochemistry, Lothian NHS University Hospitals Trust; UK).

2.7.4 RNA extraction and quantitative real-time polymerase chain reaction

Total leukocyte ribonucleic acid (RNA) extraction from 1mL of whole blood was performed using QIAGEN's RNeasy Mini Kit (QIAGEN Ltd., Crawley, UK). One microgram of total RNA was transcribed into cDNA in each reverse transcription reaction with 200U of M-MLV reverse transcriptase for 60 minutes at 37^{o}C in 20μL reactions containing 1μL (0.5μg/μL) of random hexamer primers, with 0.625μL (40U/μL) of RNAse inhibitor, 5μL of dNTP mix, and 5μL of 5X RT reaction buffer. Real-time polymerase chain reaction (PCR) was carried out using the ABI Prism® 7900HT system (Applied Biosystems, Warrington, UK) to determine the relative quantity of mRNA. PCR primers and probes for amplification of cDNA derived from CD34 and CD14 transcripts were obtained from Applied Biosystems (Foster City, CA, USA). Each assay contained forward and reverse PCR primers and one Taqman MGB probe. In each PCR reaction, 4μL of the reverse transcription reaction was analysed. The PCR reactions were run in triplicate. Analysis was performed using ABI 7900HT SDS software in order to obtain the relative quantities of mRNA compared to a calibrator.

2.8 DATA ANALYSIS AND STATISTICS

Flow cytometric analysis, EC-CFU enumeration, and Gensini scoring were performed by an observer blinded to the patients' clinical profiles. Statistical analyses were performed with SPSS version 17 (SPSS Inc, Chicago, USA). The D'Agostino & Pearson test was used to test for normality of distribution. Continuous variables are reported as the mean ± standard error, or the median and inter-quartile range where appropriate. Independent two-tailed Student's *t*-test, Mann-Whitney U tests and Pearson's Chi-Square tests were used for comparison between groups where appropriate. Spearman's test was used to test for correlation between variables. For partial correlation analyses controlling for covariates, data were normalised where appropriate. For the evaluation for associations between the various cell populations and clinical endpoints, cell concentrations were categorised into tertiles after natural logarithmic transformation. Multivariate Cox-regression analysis was performed to determine associations between progenitor cells and event-free survival with adjustment for the diagnosis of ACS on enrollment and cardiovascular risk factors including; age, gender, diabetes, hypertension, hyperlipidemia, a history of myocardial infarction, left ventricular systolic dysfunction, previous revascularisation, the severity of coronary artery disease, and use of cardiac medication. Hazard ratios represent the predicted change in the hazard between the lowest and the highest tertiles. Statistical significance was taken at a two-sided P value <0.05.

CHAPTER THREE

DISSOCIATION OF PHENOTYPIC AND FUNCTIONAL ENDOTHELIAL PROGENITOR CELLS IN PATIENTS UNDERGOING PERCUTANEOUS CORONARY INTERVENTION

Published by Mills NL, Tura O, Padfield GJ, Millar C, Lang NN, Stirling D, Ludlam C, Turner ML, Barclay GR & Newby DE

in *Heart.* 2009 Dec; 95(24): 2003-8.

3.1 SUMMARY

Endothelial progenitor cells are circulating mononuclear cells with the capacity to mature into endothelial cells and contribute to vascular repair. The aim of this study was to assess the effect of PCI related local vascular injury on putative circulating EPC in patients with coronary artery disease. Putative phenotypic EPC were quantified by flow cytometry ($CD34^+VEGFR\text{-}2^+$) complemented by real-time PCR, and the endothelial cell colony forming unit (EC-CFU) assay, before and during the first 24 hours after elective diagnostic angiography (n=27) or PCI (n=27). Percutaneous coronary intervention, but not diagnostic angiography, caused a systemic inflammatory response, increasing the circulating neutrophil ($P<0.001$) and C-reactive protein concentrations ($P=0.001$) without causing significant myocardial necrosis. Twenty-four hours after PCI, EC-CFU increased by three fold (median [IQR], 4.4 [1.3-13.8] *versus* 16.0 [2.1-35.0]; $P=0.01$), although circulating $CD34^+VEGFR\text{-}2^+$ cells (1.31 ± 0.28 *versus* 1.34 ± 0.22 $x10^6/L$; $P=0.35$) and leukocyte CD34 mRNA (relative quantity 2.3 ± 0.5 *versus* 2.1 ± 0.4; $P=0.21$) did not. There was no correlation between EC-CFU and $CD34^+VEGFR\text{-}2^+$ cells either before ($r=-0.24$, $P=0.10$) or at 24 hours ($r=-0.09$, $P=0.61$) after angiography. Therefore discrete local vascular injury inflicted by PCI causes a systemic inflammatory response and increases EC-CFU, however traditional phenotypic $CD34^+VEGFR\text{-}2^+$ putative EPC are not mobilised in the first 24 hours following vascular injury. This suggests that that unlike EC-CFU, $CD34^+VEGFR\text{-}2^+$ cells are not involved in the immediate response to vascular injury.

3.2 INTRODUCTION

Ischaemic heart disease is a major cause of morbidity and mortality worldwide. Despite advances in percutaneous coronary intervention (PCI), major adverse cardiac events occur in up to 30% of patients following balloon angioplasty and 20% following stenting [Serruys *et al.*, 1994]. Vascular trauma, induced by PCI, initiates the release of cytokines and growth factors resulting in the proliferation of smooth muscle and deposition of platelets and leukocytes at the site of injury in order to accelerate vascular repair. Endothelialisation is necessary to prevent mural thrombus formation and neointimal hyperplasia that may otherwise lead to ischaemic complications and restenosis.

The traditional paradigm of vascular repair is based on the proliferation and migration of pre-existing mature endothelial cells from the adjacent vasculature [Risau, 1995]. In an animal model of hind limb ischaemia Asahara and colleagues found that mononuclear cells from peripheral blood might have the potential to differentiate into endothelial cells [Asahara *et al.*, 1997]. Endothelial progenitor cells (EPC) have been characterised by their expression of both haematopoietic ($CD34^+$) and endothelial cell antigens ($VEGFR\text{-}2^+$), and by their ability to proliferate, migrate and differentiate into mature cell types. These putative EPC form vascular structures *in vitro* and are incorporated into the vessel wall in experimental models of neovascularisation [Murohara *et al.*, 2000]. These cells may therefore play an important role in the maintenance and repair of the vascular endothelium, and in the pathogenesis of atherosclerotic plaque formation and its consequences. Although putative EPC have been isolated and cultured from a variety of cell populations in

peripheral blood and bone marrow, the EPC remains undefined. Comparisons between clinical studies have been limited by the use of a variety of phenotypic markers to discriminate EPC and by the lack of comparable functional assays. In order to circumvent this problem, the endothelial cell colony forming unit assay (EC-CFU) was developed as an alternative means of enumerating circulating EPC [Hill *et al.*, 2003]. Although now recognised not to be a measure of 'EPC', the relationship between EC-CFU and cardiovascular stress is well recognised.

In peripheral blood $CD34^{+}VEGFR\text{-}2^{+}$ cells are infrequent, but numbers increase rapidly in response to myocardial ischaemia and acute myocardial infarction [Adams *et al.*, 2004; Massa *et al.*, 2005], and are reduced in cigarette smokers [Vasa *et al.*, 2001b], patients with diabetes mellitus [Fadini *et al.*, 2005], and in those with evidence of endothelial dysfunction [Herbrig *et al.*, 2006]. These patients are at high risk of complications following PCI. Patients with diffuse in-stent restenosis have reduced concentrations of EC-CFU like cells in comparison with matched controls at the time of presentation [George *et al.*, 2003]. Inadequate EPC number and function prior to angioplasty, as well as inadequate early and sustained EPC recruitment, may favour a maladaptive response to arterial injury and result in an increased incidence of in-stent thrombosis, restenosis and ischaemic complications. The immediate effects of local vascular injury during angioplasty and stenting on the mobilisation of EPC are unknown. The aim of this study was therefore to measure circulating phenotypic EPC and EC-CFU following PCI in patients with stable coronary heart disease.

3.3 METHODS

3.3.1 Subjects

Fifty-four patients undergoing elective coronary angiography participated in this study, which was performed with the approval of the local research ethics committee, in accordance with the Declaration of Helsinki, and the written informed consent of all volunteers. All patients were recruited following referral for angiography to investigate symptoms suggestive of stable angina. Patients with a recent acute coronary syndrome or coronary intervention (<3 months), renal or hepatic failure, or a systemic inflammatory disorder or malignancy were excluded from the study. Twenty-seven patients underwent diagnostic coronary angiography alone, and 27 required balloon angioplasty and stenting because of flow limiting luminal stenosis of a major epicardial vessel (Table 3.1).

3.3.2 Coronary angiography and PCI

All patients were treated for two weeks with 75 mg clopidogrel prior to angiography or PCI. Coronary angiography was performed via the right femoral or radial artery with 6F arterial catheters. Elective PCI was performed in all patients after the administration of 5,000 IU of intravenous heparin, and in one patient after intravenous glycoprotein IIb/IIIa inhibitor. Coronary stents (Liberté, Boston Scientific) were implanted in all patients after balloon predilatation of the lesion.

3.3.3 Blood sampling and assays

Venous blood was sampled before, immediately after and at 6 and 24 hours following the procedure. EDTA anti-coagulated blood (Sarstedt-Monovette,

Germany) was collected for flow cytometry, and for preparation of plasma for storage in all subjects. Whole blood was analysed for total cells, differential count and platelets using an autoanalyzer (Sysmex, UK). Plasma troponin I concentrations were measured using an automated immunometric assay (Ortho-clinical Diagnostics, High Wycombe, UK). Serum C-reactive protein (CRP) concentrations were measured using an immunonephelometric assay (Behring BN II nephelometer, Marburg, Germany). In 40 subjects (20 diagnostic angiograms and 20 PCI), 20 mL of whole blood was drawn at baseline and 24 hours for mononuclear cell preparation, cell culture and real-time PCR.

3.3.4 Flow cytometry

Whole blood cells were phenotyped by flow cytometry. Cells were directly stained and analysed for phenotypic expression of surface proteins using pre-conjugated anti-human monoclonal antibodies including anti-CD34-FITC, and anti-VEGFR-2-PE (R&D systems, Minneapolis, USA). Appropriate negative controls were used to establish positive stain boundaries as described. Undiluted samples (100mL) were stained with antibodies for 30 minutes in the dark. Erythrocytes were lysed (lysing solution, Becton Dickinson), and samples were centrifuged at 200 *g* for 10 minutes, washed with phosphate buffered saline (PBS), and fixed (Cell Fix solution, Becton Dickinson). For each sample, 80,000 events were acquired in the lymphocyte region (as determined by characteristic forward and side scatter profile) using a FACS Calibur flow cytometer (Becton Dickinson) and data were analysed using FCS Express (DeNovo Software). The number of leukocytes per mL of blood was measured using an automated cell counter in our regional haematology

laboratory. EPC were quantified based on the percentage of $CD34^+VEGFR\text{-}2^+$ double positive leukocytes and expressed as number of cells per mL of blood.

3.3.5 Endothelial cell colony forming units

Endothelial cell-colony forming units (EC-CFU) [Hill *et al.*, 2003] were generated from circulating mononuclear cells isolated by density gradient separation as described above (Section 2.6.1. EC-CFU were counted in a minimum of four wells on day five.

3.3.6 RNA extraction and quantitative real-time PCR

Total leukocyte ribonucleic acid (RNA) extraction and real-time polymerase chain reaction (rt-PCR) were carried out as described. Briefly, RNA extraction from 1mL of whole blood was performed and 1μg of total RNA was transcribed into cDNA. Real-time PCR was carried out to determine the relative quantity of mRNA. PCR primers and probes for amplification of cDNA derived from CD34 and CD14 transcripts were obtained from Applied Biosystems (Foster City, CA, USA). Each assay contained forward and reverse PCR primers and one Taqman MGB probe. The PCR reactions were run in triplicate. Analysis was performed using ABI 7900HT SDS software in order to obtain the relative quantities of mRNA compared to a calibrator.

3.3.7 Data analysis and statistics

Analysis was performed by an observer blinded to whether patients had undergone angiography or PCI. Statistical analyses were performed with GraphPad

Prism (Graph Pad Software, USA) using two-tailed Student's *t*-test, repeated measures ANOVA, Mann-U Whitney or Wilcoxon paired tests where appropriate. D'Agostino & Pearson omnibus normality test was used to assess whether parameters were normally distributed with continuous variables are reported as mean ± SEM or median [IQR]. Statistical significance was taken at a two sided P value of <0.05.

3.4 RESULTS

3.4.1 Subjects and procedures

Twenty-seven patients underwent diagnostic coronary angiography alone and 27 underwent PCI using intracoronary stents to treat 36 lesions with an average vessel diameter of 2.9±0.1 mm at the point of stent deployment. Patients were well matched in terms of age and sex and with respect to their cardiovascular risk profile and baseline medications (Table 3.1). There were no complications arising from angiography or PCI and all patients were discharged home 24 hours after the procedure.

3.4.2 Inflammation and myocyte necrosis

Diagnostic angiography did not increase peripheral blood leukocyte count or serum C-reactive protein concentrations (Table 3.2). Coronary intervention increased circulating neutrophil (Δ 0.9±0.3 $x10^9$/L, P<0.001) and serum C-reactive protein concentrations (Δ 1.5 [0.4-2.0] mg/L, P=0.001) at 24-hours. There was a transient reduction in monocyte count immediately following PCI (Δ -0.07±0.02 $x10^9$/L, P=0.004) and cardiac catheterisation alone (Δ -0.10±0.03 $x10^9$/L, P=0.006). Monocyte count returned to pre-procedure levels by 24 hours. There was little evidence of myocyte necrosis following diagnostic angiography or PCI, with plasma cardiac troponin I at 24 hours increased in 2 patients following angiography and 4 patients following PCI: median concentration 0.2 [0.2-0.69] ng/mL and 0.2 [0.2-1.6] ng/mL respectively (P=0.30).

3.4.3 Endothelial cell – colony forming units

Although unaffected by diagnostic angiography (median [IQR], 4.7 [0-21.5] *versus* 3.2 [1.1-9.7]; P=0.70), the number of EC-CFU increased three fold 24 hours after PCI (median [IQR], 4.4 [1.3-13.8] *versus* 16.0 [2.1-35.0], P=0.01; Figure 3.1).

3.4.4 Phenotypic endothelial progenitor cells

$CD34^+$ and $CD34^+VEGFR\text{-}2^+$ cells were similar at baseline and unaffected by either procedure (Table 3.3). There was no correlation between EPC identified by phenotype ($CD34^+VEGFR\text{-}2^+$ cells) and the number of EC-CFU either before (r=-0.24, P=0.10) or 24 hours (r=-0.09, P=0.61) after angiography. Similarly, no increase in leukocyte CD34 mRNA occurred following diagnostic angiography or PCI. There was however, a reduction in leukocyte CD14 mRNA immediately after catheterisation in both diagnostic and interventional studies that coincided with the reduction in circulating monocytes (Table 3.3). Relative quantities of leukocyte CD14 mRNA increased significantly 24 hours following PCI (P<0.05).

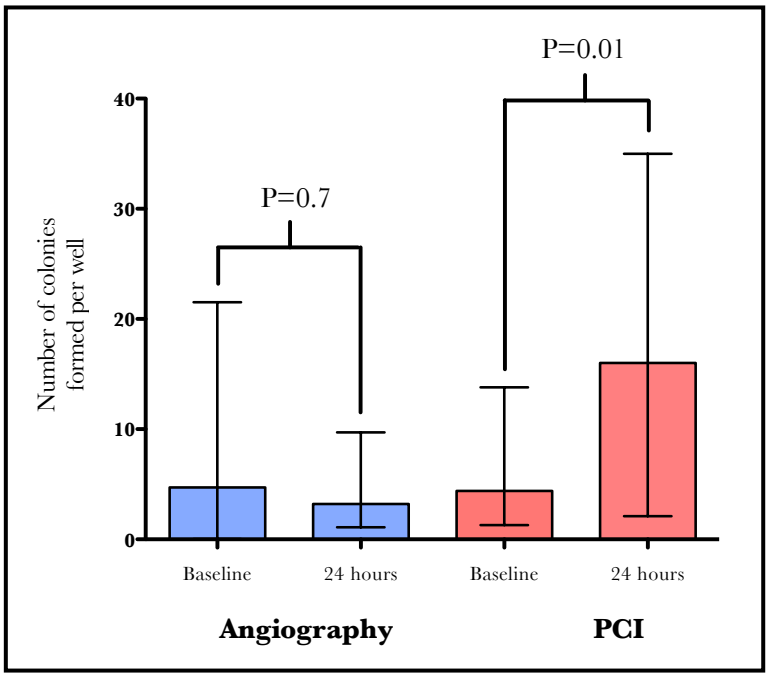


Figure 3.1 EC-CFU concentration in response to local vascular injury: The number of EC-CFU increased following PCI compared to baseline (median [IQR], 4.4 [1.3-13.8] *versus* 16.0 [2.1-35.0], P=0.01), but were not changed following diagnostic angiography alone (median [IQR], 4.7 [0-21.5] *versus* 3.2 [1.1-9.7], P=0.70).

Table 3.1. Baseline characteristics of study population

	Angiography (n=27)	PCI (n=27)	P Value
Demographics and clinical characteristics			
Age (years)	62 ± 2	61 ± 2	0.71
Male gender (%)	15/12	17/10	0.69
Cigarette smokers	5	5	0.98
Diabetes mellitus	8	3	0.18
Hypertension	15	19	0.52
Previous MI	4	6	0.69
Previous PCI	5	8	0.55
Prior CABG	4	4	0.95
Biochemistry			
Total cholesterol (mg/dL)	186 ± 11	168 ± 7	0.18
LDL-cholesterol (mg/dL)	97 ± 10	90 ± 7	0.60
HDL-cholesterol (mg/dL)	46 ± 2	42 ± 3	0.18
Triglycerides (mg/dL)	206 ± 19	193 ± 25	0.68
Medical therapy			
Aspirin	21	21	0.99
Clopidogrel	27	27	0.99
Statin	18	24	0.15
ß-blocker	18	22	0.33
ACE inhibitor/ARB	13	15	0.83
Angiographic findings			
Normal/minor disease	13	0	0.001
1 vessel disease	2	11	0.03
2 vessel disease	5	9	0.33
3 vessel disease	7	7	0.99
Stent implantation			
De novo lesions	0	24	-
Restenosis	0	3	-
Grafts	0	1	-
Stents per patient			
1 stent	0	19	-
2 stents	0	7	-
3 stents	0	1	-

Values are presented as number or mean ± SEM or number; an unpaired Student's t-test was used for comparisons involving parametric data and a Mann-U Whitney test for non-parametric data. ACE = angiotensin converting enzyme; ARB = angiotensin II receptor blocker; CABG = coronary artery bypass grafting; LDL = low density lipoprotein; HDL = high density lipoprotein.

Table 3.2. Markers of inflammation following diagnostic angiography or percutaneous coronary intervention

	Pre	Post	6 hours	24 hours	ANOVA
Angiography					
Leukocytes (x10^9/L)	7.2 ± 0.4	7.1 ± 0.3	7.6 ± 0.3	7.2 ± 0.4	0.114
Neutrophils (x10^9/L)	4.4 ± 0.3	4.6 ± 0.3	4.7 ± 0.2	4.5 ± 0.3	0.639
Lymphocytes (x10^9/L)	1.9 ± 0.1	1.8 ± 0.1	2.1 ± 0.1‡	1.9 ± 0.1	0.005
Monocytes (x10^9/L)	0.59 ± 0.03	0.49 ± 0.03	0.62 ± 0.03‡	0.58 ± 0.04	0.002
CRP (mg/L)	3.6 [2.0-4.8]	-	-	4.6 [3.4-5.8]	-
PCI					
Leukocytes (x10^9/L)	7.1 ± 0.3	6.8 ± 0.3	7.1 ± 0.2	8.0 ± 0.3*#	<0.0001
Neutrophils (x10^9/L)	4.5 ± 0.3	4.3 ± 0.3	4.3 ± 0.2	5.4 ± 0.2*#	<0.0001
Lymphocytes (x10^9/L)	1.8 ± 0.1	1.7 ± 0.1	1.9 ± 0.1‡	1.7 ± 0.1	0.007
Monocytes (x10^9/L)	0.56 ± 0.02	0.49 ± 0.03*	0.59 ± 0.02#	0.56 ± 0.02‡	0.0003
CRP (mg/L)	1.1 [0.8-1.9]	-	-	3.1 [1.2-5.6]*	-

Values are presented as mean ± SEM or median [IQR]. Normally distributed parameters were analysed using a repeated measures ANOVA with Bonferroni post-test. Wilcoxon paired test was used for non-parametric comparisons. *P<0.05, †P<0.001 pre-procedure versus time point ‡P<0.05, # P<0.001 post-procedure versus time point.

Table 3.3. Periprocedural putative EPC concentrations and leukocyte mRNA levels

	Pre	Post	6 hrs	24 hrs	ANOVA
Angiography					
$CD34^+$	4.05 [2.91-6.54]	3.83 [2.07-6.88]	4.29 [2.71-6.00]	3.38 [1.51-4.86]	0.50
$CD34^+VEGFR\text{-}2^+$	0.72 [0.37-2.01]	0.66 [0.26-1.71]	0.53 [0.24-1.90]	0.53 [0.24-1.94]	0.21
CD34 mRNA	1.7 ± 0.3	1.1 ± 0.2*	1.6 ± 0.3	2.5 ± 0.8	0.08
CD14 mRNA	3.1 ± 0.4	2.1 ± 0.3*	2.2 ± 0.3	2.9 ± 0.5	0.05
PCI					
$CD34^+$	4.89 [2.66-7.91]	5.55 [2.45-7.73]	5.04 [2.41-6.46]	4.64 [2.80-7.49]	0.79
$CD34^+VEGFR\text{-}2^+$	1.04 [0.45-1.80]	0.65 [0.24-2.81]	0.65 [0.22-1.30]	0.95 [0.26-2.11]	0.31
CD34 mRNA	2.3 ± 0.5	1.6 ± 0.5*	1.9 ± 0.4	2.1 ± 0.4	0.35
CD14 mRNA	3.3 ± 0.5	2.1 ± 0.4*	3.1 ± 0.7	4.7 ± 1.0‡	0.02

Angiogenic cell populations quantified by flow cytometry are expressed as $x10^6/L$. Values are presented as median [IQR]; comparisons made using a non-parametric repeated measures ANOVA (Freidman test). Pre and post refer to immediately before and after the procedure. mRNA values are presented as relative quantities, mean ± SEM. *P<0.05 pre-procedure versus time point. ‡P<0.05 post-procedure versus time point

3.5 DISCUSSION

This study demonstrates that PCI, but not diagnostic angiography, is associated with a systemic inflammatory response and an increase in circulating EC-CFU in the first 24 hours, however this is not associated with a change in either circulating $CD34^+VEGFR\text{-}2^+$ cell concentrations or leukocyte CD34 mRNA levels following PCI. Therefore, the immediate cellular response to vascular injury involves mobilisation of EC-CFU rather than putative $CD34^+VEGFR\text{-}2^+$ EPC. Circulating $CD34^+VEGFR\text{-}2^+$ cells may be capable of endothelial cell differentiation, but are rare in peripheral blood and are not mobilised rapidly in the early response to acute local vascular injury.

Asahara *et al.,* originally described a population of peripheral adult cells containing $CD34^+$ cells that could differentiate into cells with endothelial-like characteristics *in vitro*. 'Functional' colony assays such as the EC-CFU assay were subsequently developed in an attempt to enumerate these cell populations. However, coronary intervention increased the number of EC-CFU but was not associated with a mobilisation of $CD34^+VEGFR\text{-}2^+$ cells. These findings are superficially discordant with Asahara's original description and require exploration. The exact origin and phenotype of the progenitors identified by Asahara remains speculative in part because the purity of $CD34^+$ cells used in this initial study was only 15%, and did not employ specifically $CD34^+VEGFR\text{-}2^+$ cells [Asahara *et al.*, 1997]. Nevertheless the co-expression of transmembrane glycoproteins CD34 and VEGFR-2 has been used in an increasing number of clinical studies to phenotype and quantify circulating

EPC. Previous studies have demonstrated early mobilisation of $CD34^+VEGFR\text{-}2^+$cells following vascular injury in patients with acute myocardial infarction [Massa *et al.*, 2005], and $CD133^+VEGFR\text{-}2^+$cells following major burns and CABG surgery [Gill *et al.*, 2001]. However, these clinical events involve extensive damage to a number of tissues in addition to the vasculature that may contribute to the mobilisation of progenitors and may explain the absence of $CD34^+$ EPC mobilisation in the present study. EC-CFU are diminished in patients with cardiovascular risk factors and impaired vascular function [Hill *et al.,* 2003], suggesting they fulfil a function in the maintenance of vascular homeostasis, and protect against the development of atheroma. Mobilisation of EC-CFU in response to discrete vascular injury caused by PCI is consistent with this hypothesis, and given the endothelial phenotype of EC-CFU it is plausible that quantification of EC-CFU may provide a measure of the capacity of circulating mononuclear cells to form endothelial cells. However, it is doubtful whether these colonies arise directly from the circulating $CD34^+$ stem cells. Consistent with previous findings [Tura *et al.*, 2007] and those of George *et al.*, [George *et al.*, 2006] I found no correlation between the number of peripheral blood $CD34^+$ cells and the number of EC-CFU. Studies addressing the origin of endothelial progenitor lineage in adult peripheral blood have demonstrated that monocytes also express endothelial lineage markers such as VEGFR-2 and can differentiate into mature endothelial cells [Schmeisser *et al.*, 2001]. Rehman *et al.* found that the majority of EC-CFU expressed monocyte markers such as CD14, Mac-1, and CD11c, suggesting that they are derived from monocyte-like cells [Rehman *et al.*, 2003]. These findings are confirmed in the elegant studies by Rohde

et al in which they demonstrate formation of colony forming units by mononuclear cells depleted of $CD34^+$ cells and report that the EC-CFU are primarily composed of monocytic and lymphocytic cells [Rohde *et al.*, 2007]. The role of EC-CFU remains unclear and further studies are required to address what *in-vivo* processes they represent.

The concept that functional endothelial cells may originate from a CD14 expressing progenitor is supported by reports that mature endothelial cells isolated from human umbilical vein express CD14 [Jersmann *et al.*, 2001]. Furthermore, Urbich *et al.* demonstrate that isolated $CD14^+$ cells also have the capacity to improve neovascularisation after hind-limb ischaemia [Urbich *et al.*, 2003]. $CD14^+$ monocyte-like cells are more abundant in normal peripheral blood than in bone marrow and are therefore capable of rapidly homing to sites of vascular injury. Whilst $CD34^+VEGFR\text{-}2^+$ cells may ultimately give rise to endothelial cells, they are much less prevalent in peripheral blood than in bone marrow, and are not mobilised following PCI. The findings of this study are consistent with those recently published by Thomas *et al.,* [Thomas *et al.*, 2008a] and together these studies do not suggest an important role for $CD34^+VEGFR\text{-}2^+$ cells in the early response to vascular injury.

Whilst I did not specifically measure $CD14^+$ cells by flow cytometry, I did quantify circulating monocytes, and total leukocyte CD14 mRNA. I identified a significant decrease in both variables immediately after cardiac catheterisation. This fall in circulating monocytes may be explained by immediate localisation of

monocytes to the site of vessel damage: both at the site of arterial puncture and at the site of coronary angioplasty and stenting, although adherence of monocytes to the plastic catheters used during the procedure might explain the rapid but transient fall in circulating monocytes. The number of peripheral blood monocytes returned to pre-procedural levels by 24 hours, and CD14 mRNA levels increased 24 hours after PCI. Mobilisation of $CD14^+$ monocyte-like cells or a change in the function of these cells through up-regulation of surface protein expression may explain the increase in EC-CFU in peripheral blood observed 24 hours after arterial injury. These cells may contribute to vascular repair either through formation of mature endothelial cells and incorporation into the vessel wall, or through the release of angiogenic growth factors at the site of vessel injury.

As it may take up to three months for a complete functional endothelial layer to form following percutaneous coronary intervention [Grewe *et al.*, 2000], I cannot discount a role for CD34 derived progenitors, perhaps released in response to secondary angiogenic factors, in the later stages of vascular repair. It is also possible that other putative EPC populations not measured in the present study, such as those expressing the stem cell marker CD133, may have a role in the response to vascular injury. Further studies are required to explore specifically the role of $CD14^+$ and $CD133^+$ subpopulations, to define the time course of this response to vascular injury, and to assess the impact of EPC mobilisation on restenosis and clinical outcomes.

3.6 CONCLUSIONS

Acute local vascular injury following angioplasty and stenting results in a systemic inflammatory response and increases the number of EC-CFU. Circulating $CD34^{+}VEGFR\text{-}2^{+}$ cells are rare in peripheral blood, and are not mobilised rapidly in the early response to vascular injury. The acute response to injury is mediated by EC-CFU, probably derived from circulating monocytic cells rather than the primitive $CD34^{+}VEGFR\text{-}2^{+}$ cells that have hitherto been considered as the major endothelial progenitor cell source in blood and bone marrow. It is unclear what EC-CFU represent. A better understanding of the cellular response to vascular injury is necessary to allow a more sophisticated approach to reducing the complications of PCI with new strategies designed to enhance vessel repair.

CHAPTER FOUR

THE CONSTITUENTS AND MECHANISMS OF GENERATION OF 'ENDOTHELIAL CELL - COLONY FORMING UNITS'.

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4.1 SUMMARY

The formation of endothelial cell-colony forming units (EC-CFU) is increased by cardiovascular stress, although their function *in-vivo* is unclear. I therefore examined the constituents of EC-CFU and the mechanisms of their generation. The expression of endothelial and haematopoietic antigens on EC-CFU was assessed using immunofluorescent confocal microscopy and flow-cytometry. Colony growth was examined using time-lapse microscopy. In order to interrogate leukocyte/colony interactions, wells containing mature EC-CFU were inoculated with autologous monocytic and lymphocytic cells isolated by fluorescence activated cell sorting. Cellular proliferation within EC-CFU was assessed using the nucleotide analogue bromodeoxyuridine (BrdU) and the inhibitor of cellular proliferation, mitomycin-C. EC-CFU expressed typical endothelial characteristics [CD146, CD105, Tie-2 and eNOS], however several endothelial markers were only weakly expressed or absent [CD31, CD144, von Willebrand factor and vascular endothelial growth factor receptor-2]. EC-CFU strongly expressed the pan-leukocyte marker CD45, the macrophage marker CD68, and also the lymphocyte markers CD4 and CD8. Expression of CD14 on EC-CFU was progressively down-regulated over time from 14.4±3.6% at baseline to 10.9±0.9%, 5.2±0.4%, 3.8±1.2 and 2.5±0.6% on days two to five respectively ($P<0.001$; $N = 6$). Time-lapse microscopy revealed migration of characteristic spindle cells toward mature colonies. Both monocytic and lymphocytic fractions migrated toward mature colonies. Colonies exhibited punctate incorporation of BrdU that progressively increased during colony maturation sequentially from a median of 2.2 (0.0-4.0) $x10^3$cells/μm^3 on day two to 35.2 (18.7-55.6) $x10^3$ cells/μm^3 on day 5 ($p<0.0001$). Mitomycin-C significantly reduced colony

formation.

EC-CFU exhibit endothelial characteristics, but are predominantly $CD14^+$ derived macrophages and are a potent stimulus for lymphocyte migration. Proliferation is necessary for EC-CFU generation, however colony growth also occurs as a result of leukocyte migration. Although confirmatory *in-vivo* studies are required, EC-CFU formation likely reflects leukocyte activation as a reparatory response to vascular denudation or tissue ischaemia.

4.2 INTRODUCTION

Endothelial progenitor cells (EPC) offer promise as a therapeutic target in cardiovascular medicine. Unfortunately, our understanding of EPC biology has been slow to progress, and even a precise definition of an EPC has been lacking. In the absence of a robust phenotypic definition, 'functional' endothelial colony forming assays have been developed, whereby mononuclear cells isolated from the peripheral circulation are cultured under angiogenic conditions in order to generate colonies that exhibit mature endothelial cell characteristics. Formation of 'endothelial' colonies has been interpreted as inferential evidence that circulating endothelial progenitor cells exist in the peripheral circulation. Based on the premise that colonies arise via clonal expansion of a single cell, the enumeration of such colonies has been used to estimate the concentration of endothelial progenitors in the peripheral blood. Such assays have subsequently been used in a huge number of pre-clinical and clinical studies in order to determine the relationship between putative EPC and cardiovascular disorders.

The endothelial cell – colony forming unit (EC-CFU) assay described by Hill *et al*, has been used most extensively [Hill *et al.*, 2003]. In support of their vasculoprotective role, EC-CFU are reduced in patients with overt atherosclerosis [Heeschen *et al.*, 2004], and in cardiovascular disorders such as diabetes mellitus [Loomans *et al.*, 2004], and hypertension [Delva *et al.*, 2007], and an increased capacity to generate EC-CFU is associated with better vascular function as measured by flow mediated arterial dilatation [Hill *et al.*, 2003]. EC-CFU are mobilised in response to cardiovascular stress, such as coronary artery bypass surgery [Roberts *et*

al., 2007], myocardial ischaemia [George *et al.*, 2004] and infarction [Massa *et al.*, 2005; Shintani *et al.*, 2001], and as demonstrated in the previous chapter, discrete endovascular injury caused by percutaneous coronary intervention [Mills *et al.*, 2009]. Several other studies have reported similar findings following angioplasty both in the coronary and peripheral vasculature [Banerjee *et al.*, 2006; Bonello *et al.*, 2006; Garg *et al.*, 2008b; Marboeuf *et al.*, 2008]. These studies were conducted under the specious impression that EC-CFU were a quantitative measure of circulating EPC, however we now appreciate this not to be the case. EC-CFU are related to normal cardiovascular homeostasis, however lack the capacity to form perfusing vessels in animal models of neovascularisation, and widely express hematopoietic markers [Rohde *et al.*, 2007; Yoder *et al.*, 2007]. The pathophysiological significance of EC-CFU therefore remains unclear, although it is plausible that EC-CFU formation reflects leukocyte activation in response to cardiovascular stress. Although presumptively regarded as a proliferative assay this has never been demonstrated, and furthermore the presence of mature leukocytes within EC-CFU indicates cell migration is important in their development. These studies were conducted to confirm the phenotypic characteristics of EC-CFU and test the hypothesis that EC-CFU form by cellular migration rather than proliferation.

4.3 MATERIALS AND METHODS

4.3.1 Immunostaining of endothelial cell-colony forming units

Following informed consent, circulating mononuclear cells isolated by density gradient separation (Histopaque 1.077 g/ml; Sigma-Aldrich, UK) of peripheral blood from healthy volunteers was used to generate EC-CFU as described above (Chapter 2.6.1) Endothelial cells-colony forming units (EC-CFU) (Stem Cell Technologies) were defined as a colony of cells consisting of multiple thin, spindle cells in association with a central cluster of rounded cells [Hill *et al.*, 2003]. The phenotypic characteristics of EC-CFU were determined as described above in chapter 2. Briefly, day five EC-CFU were fixed and permeabilised and blocked with blocked with 10% Goat serum/ 1% bovine serum albumin (BSA), prior to immunostaining with by immunostaining using a battery of surface and intracellular stains including endothelial nitric oxide, CD146, CD34, CD31, CD105, CD144, Tie-2 and VEGFR-2, in addition to the leukocyte markers CD45, CD4, CD8, CD14 and CD68.

Positive and negative controls

Human umbilical vein endothelial cells (HUVEC) served as positive controls for endothelial markers. Having undergone no more than three passages, HUVEC were cultured in EGM-2 (Lonza; UK) on plain glass chamber slides until confluency was acheived. Mononuclear cell (MNC) preparations served as positive controls for haematopoietic markers. 100µl of MNC at a density of 4 x 10^5 cells/mL in 1% BSA solution were cytospun onto glass slides and fixed using methanol for 5 minutes before being stained as above.

The human adenocarcinoma cell-line, HT-29 served as a negative control population. HT-29 cells (courtesy of Dr S. Bader's group, University of Edinburgh) were cultured in RPMI (Gibco/Invitrogen; UK) supplemented with 5% FCS until confluency was achieved. Cells were fixed in methanol and stained as described above.

Confocal microscopy and image analysis

Stained colonies were visualized using a Zeiss 'LSM 510 Meta' confocal fluorescent microscope at x40 with an oil immersion objective lens. Fluorescent intensity was compared qualitatively against the positive and negative controls.

4.3.2 Time-lapse microscopy

For time-lapse analysis developing colonies were identified 24 hours following the replating step. Developing colonies were imaged using an inverted confocal microscope (Leica), situated in an environmentally controlled chamber with an integrated digital camera (Nikon). Colonies were incubated at 37°C at 5% CO_2. Images were captured at a frequency of six per hour for approximately 72 hours.

4.2.3 Fluorescence activated cell sorting.

Cell sorting was performed as described in chapter two. Briefly, mononuclear cells were isolated from the whole blood of healthy volunteers by density gradient centrifugation. Aliquots of mononuclear cells were stained for lymphocytes with anti-CD3 PE-mouse and anti-CD19 APC-mouse pre-and another stained for monocytes using anti-CD14 Cy5-5PE. Cell clumps were removed by agitation followed by

sedimentation and the cell suspension was transferred to fresh culture medium supplemented with 100 U/mL penicillin and 100g/mL streptomycin. Cell sorting was performed by a FACSAria cell sorter (Becton-Dickinson, UK) using CellQuestPro software (Becton Dickinson; UK). Acquisition gates were set using the protocol as described.

4.3.4 Cellular migration

FACS sorted mononuclear cells were stained green ($CD14^+$ monocytes) or red ($CD3^+$ and $CD19^+$ lymphocytes) by incubating sorted fractions for 20 minutes at 37°C at a density of $0.5x10^6$/mL in complete endocult medium with cell-labeling solution (Vybrant™ Di cell-labeling solution; Molecular Probes, UK) at a concentration of 2.5 μL/mL, mixed by gentle pipetting. Cell suspensions were centrifuged at 1500 rpm for five minutes at 37°C to form a cell pellet. The supernatant was gently removed and the cell pellet was resuspended in complete medium. Fluorescently labeled sorted cell fractions containing approximately $5x10^5$ cells were combined with autologous EC-CFU approaching maturity at day four. Cell populations were delivered via an 8μm transwell migration chamber (Co-star;UK) in order to demonstrated active cellular migration and prevent cell clumps contaminating the pre-existing colonies. Colonies were assessed over the following 24 hours using an inverted microscope equipped with an argon laser in order to assess leukocyte/colony interactions.

4.3.5 Colony proliferation

Mononuclear cells from healthy volunteers were used to generate EC-CFU

colonies as described above. At the replating step on day two, four separate wells were prepared and from day two to five an individual well was pulsed with Bromodeoxyuridine (BrdU) (Sigma-Aldrich) at a 100 μmol concentration for six hours at 37°C, 5% CO_2. Following each pulse, unincorporated BrdU was removed by rinsing unattached cells and the well itself twice with fresh medium. Resuspended cells were returned to the well and incubation was allowed to continue thereby providing a 'snapshot' of the amount of proliferation occurring for a six hour period on each day. On day six the supernatant was removed and colonies were fixed and permeablised with 4% paraformaldehyde at room temperature for 30mins. Colonies were then immersed in 0.25% tween-20 for 12hrs before being rinsed with PBS. Permeabilised colonies were incubated with DNAse (Perbio science; United Kingdom) at 500U/mL in 50mMol Tris buffered deionised water; $MgCl_2$10mMol; bovine serum albumin 100μgml^{-1} pH 7.4) at 37°C for 30 minutes. Colonies were then rinsed well with PBS and incubated with anti-BrdU conjugated to alexafluore 488 (Invitrogen; Paisley, UK) at a 1/100 dilution.

Wells were visualised using an inverted fluorescent microscope and integrated digital camera (Nikon Eclipse TS100-F). Proliferation was expressed as the proportion of BrdU positive cells per cubic micron of colony, where colony volume (V) was calculated using the formula $V = (\pi r^2 h)/3$ where r = the radius of the base of the colony and h = the height of the colony was assumed to be equal to r. For control experiments, lymphocytes were obtained by gently decanting the non-adherent population of a mononuclear cell preparation exposed to plastic [Elkord *et al.*, 2005]. Lymphocytes either fresh, or stimulated to proliferate with phytohaemagglutinin (PHA; Wellcome, Kent, UK.) at concentration of 5 μg/mL [Aldhous *et al.*, 2008]

were treated as above in order to obtain negative and positive controls for BrdU incorporation respectively. In separate experiments EC-CFU were cultured in the presence of the anti-proliferative agents, mitomycin C and Actinomycin D, in order to assess the effect of blocking proliferation on colony formation.

Statistical analysis

All experiments were performed at least in triplicate to ensure reproducibility. Continuous variables are reported as mean ± SEM or median and inter-quartile range where appropriate. Statistical analyses were performed with GraphPad Prism (Graph Pad Software, USA) using two-tailed Student's *t*-test, Mann-U Whitney or Wilcoxon paired tests where appropriate. Spearman's test was used to test for correlations between BrdU concentration and colony size. Statistical significance was taken at $P<0.05$.

4.4 RESULTS

4.4.1 Phenotypic characteristics of EC-CFU

EC-CFU expressed a variety of surface antigens considered typical of haematopoietic/leukocytic cells, endothelial cells or both (Figure 4.2). The intensity of immunofluorescence was compared to HUVEC, uncultured MNC and as a universal negative control, the human adenocarcinoma cell line HT29. Of the typical leukocyte markers examined, the common leukocyte antigen CD45 and the macrophage marker CD68 were most intensely expressed. Both were increased compared to MNC and were absent on HUVEC. The lymphocyte markers CD8, and to a lesser extent CD4, were expressed on EC-CFU at a similar level of intensity as uncultured MNC. HUVEC were negative for lymphoid markers. Notably, the monocyte marker CD14 was only very weakly expressed on mature colonies, as compared to uncultured MNC. Consistent with previous reports [Jersmann *et al.*, 2001] very faint CD14 expression was also seen on isolated HUVEC, but otherwise expression of these more typical leukocyte markers was absent on HUVEC and HT-29. Flow-cytometric quantification of CD14 expression on cells retrieved from the EC-CFU assay demonstrated a fall in CD14 expression from 14.4±3.6% at baseline to 10.9±0.9%, 5.2±0.4%, 3.8±1.2 and 2.5±0.6% on days two to five respectively (ANOVA $P<0.001$; $N = 6$) (Figure 4.3). Of the more typical endothelial markers inspected, CD146 and Tie-2 were most intense and were expressed to a comparable degree on both EC-CFU and HUVEC. The vascular endothelial cadherin, CD144 was absent on EC-CFU yet strongly positive on HUVEC. The intensity of expression of eNOS on EC-CFU was weakly expressed though similar to that of HUVEC. Von-Willebrand factor was relatively faintly expressed on both EC-CFU and HUVEC.

VEGFR-2 expression was virtually absent on EC-CFU and was only very faintly expressed on HUVEC. CD31, an endothelial marker that is also expressed on monocytic cells was faintly expressed on EC-CFU, but very brightly on HUVEC. All of the more typical endothelial markers were absent on MNC and HT-29. CD105, a surface protein expressed on both monocytic macrophages and activated endothelial cells, was very intensely expressed. CD105 expression was similarly intense on HUVEC. CD34 was expressed on EC-CFU but to a similar level as MNC and HT-29. There was faint expression of CD34 on HUVEC. Finally Ac-LDL uptake and lectin binding, classical but non-specific characteristics of endothelial cells were present EC-CFU and HUVEC but absent on HT-29.

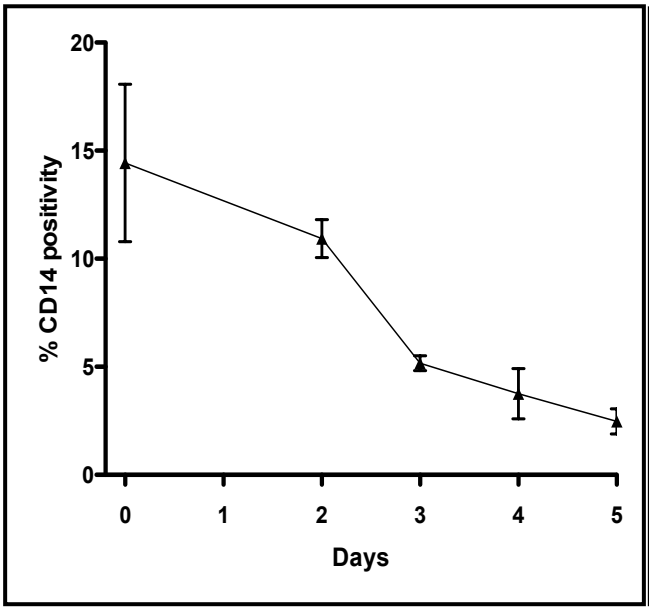
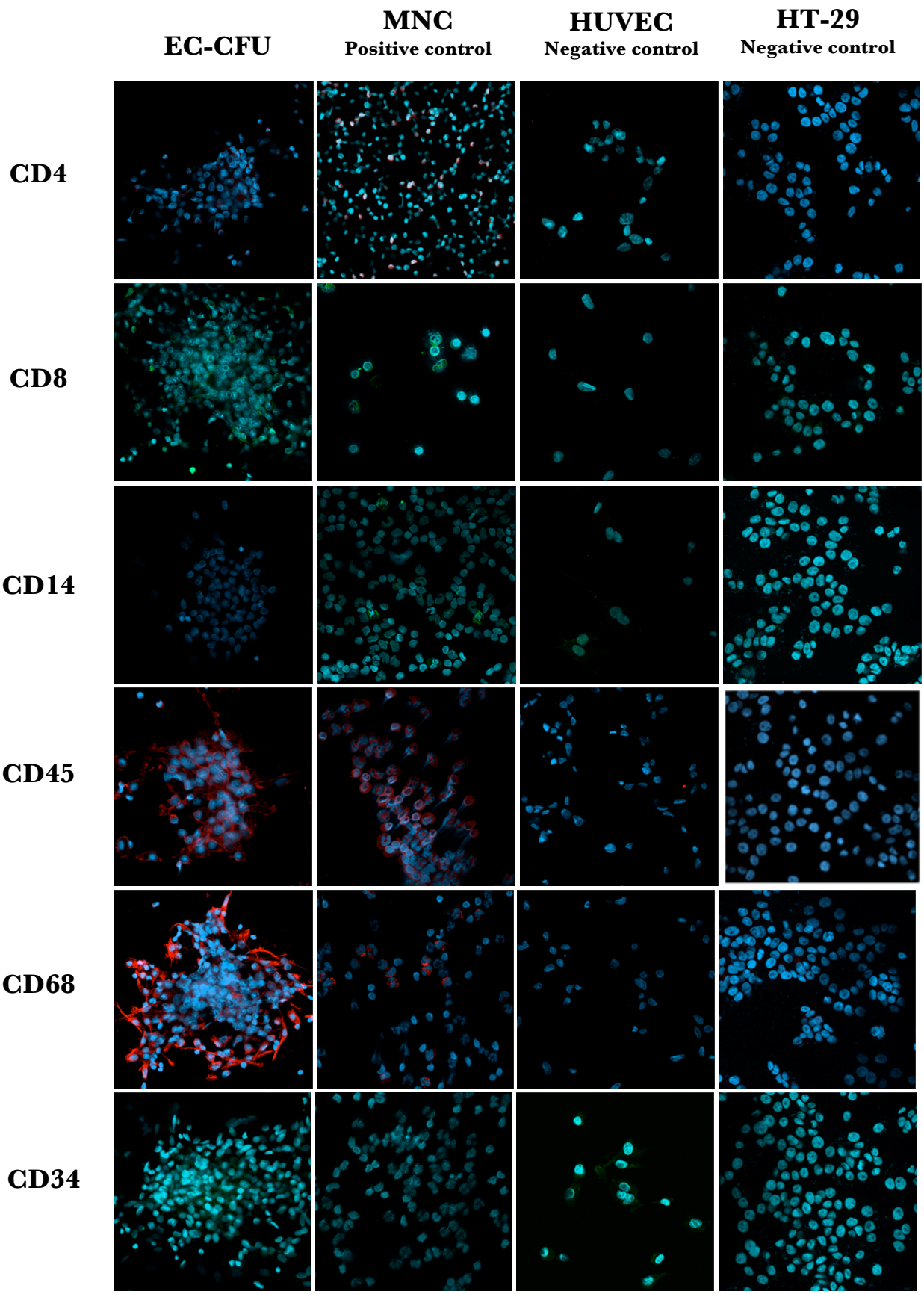
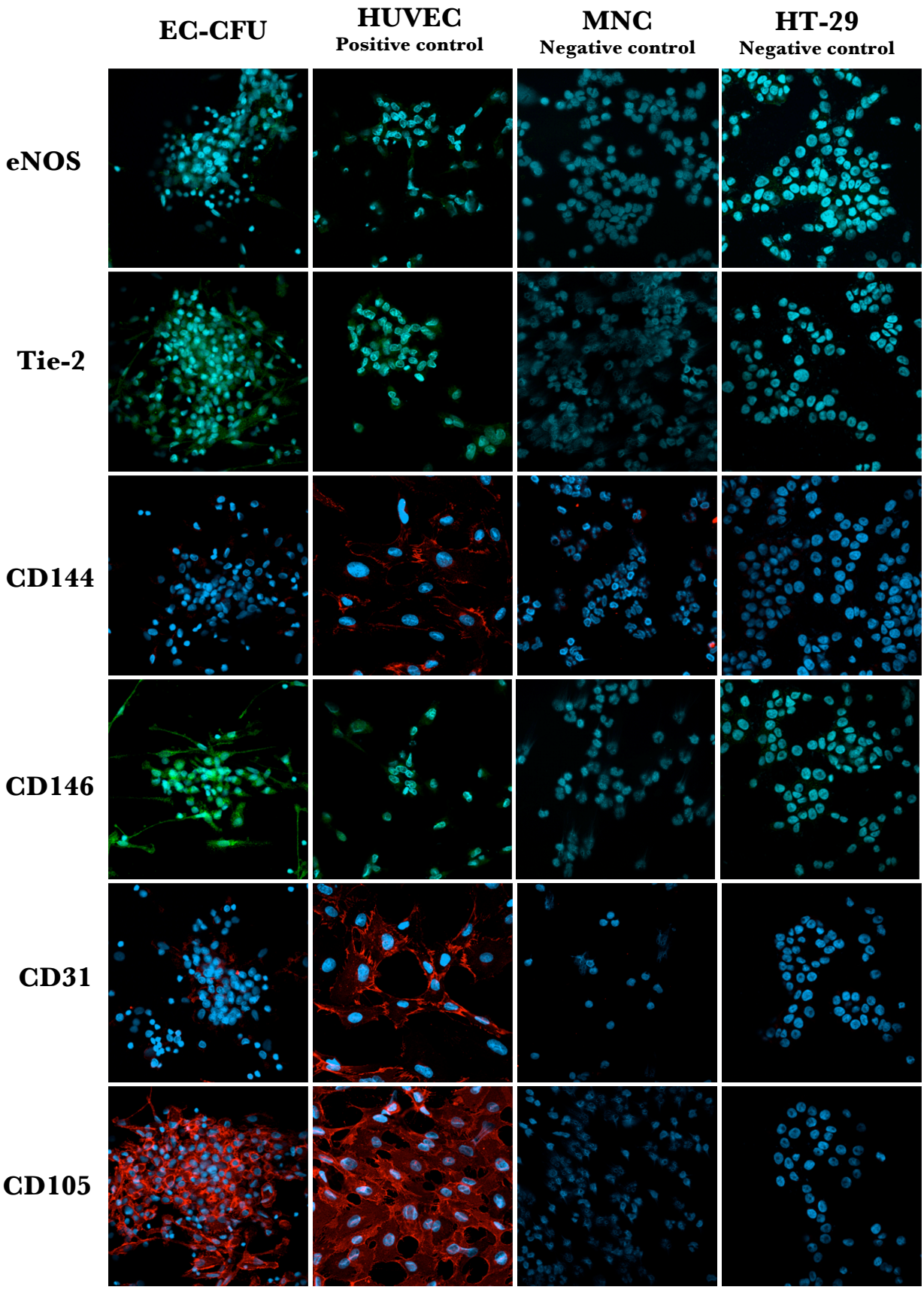


Figure 4.3 Down regulation of CD14 in culture
CD14 expression on cells in the EC-CFU assay was assessed by flow cytometry. There was progressive down regulation of CD14 expression in culture over time from 14.4±3.6% at baseline to 10.9±0.9%, 5.2±0.4%, 3.8±1.2 and 2.5±0.6% on days two to five respectively (ANOVA $P<0.001$; $N = 6$)


EC-CFU
MNC
Positive control
HUVEC
Negative control
HT-29
Negative control
CD4
CD8
CD14
CD45
CD68
CD34

Endothelial markers



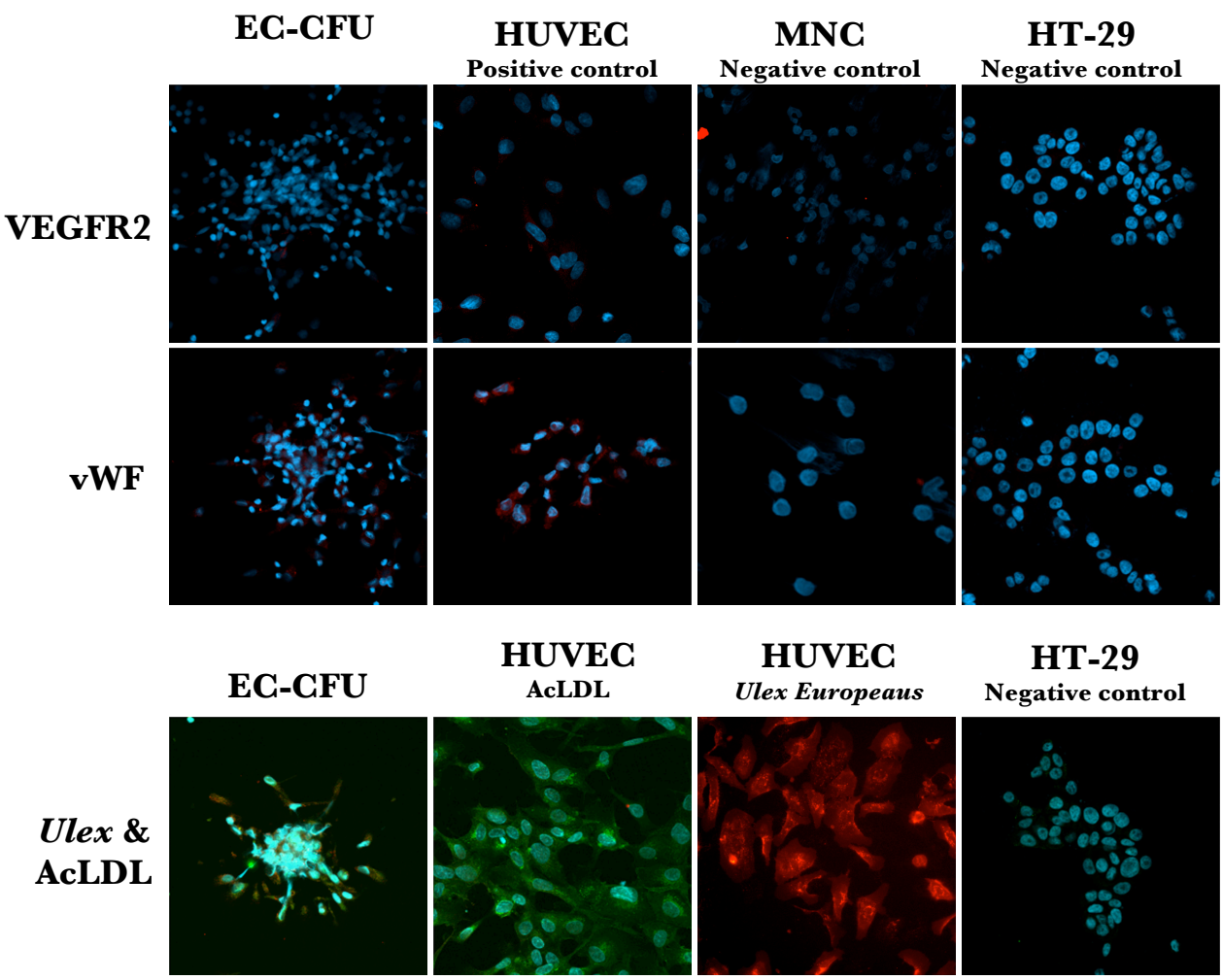


Figure 4.2 EC-CFU phenotypic characterisation: With the exception of Ac-LDL and *Ulex Europeaus*, colonies were stained using primary antibodies followed by a secondary step. Cells are imaged at either x20 or x40 magnification. Mononuclear cells (MNC) and human umbilical vein cells (HUVEC) served as positive and negative controls depending on the antigen in question. The human adenocarcinoma cell line HT-29 served as a universal negative control. Expression of CD45, CD68, and CD105 were most intense. CD4 and CD8 were present throughout EC-CFU. The monocyte marker CD14 was only weakly expressed on EC-CFU. Colonies displayed classical, but non-specific endothelial characteristics; uptake of acetylated low-density lipoprotein and Ulex Uropeaus binding and expressed Tie-2, CD146, CD105 and eNOS. von Willebrand factor, VEGFR-2 and CD31 expression was weak and the vascular endothelial cadherin CD144 was absent. See text for details.

4.4.2 Fluorescence activated cell sorting

Mononuclear cells were sorted into monocytic and lymphocytic fractions on the basis of CD14, CD3 and CD19 expression. Approximately 33±19% of MNC were intact, and approximately 77±11% of these events were singlets (i.e. not cell aggregates). Of the intact singlets, 66±15% fell within the lymphoid gate and 27±8% fell within the myeloid gate. Within the lymphoid and myloid gate cells were further selected on the basis of CD19 or CD3 and CD14 respectively. Such stringent acquisition criteria produced a high purity of cell phenotype. With respect to the T-lymphocyte sort, 80±4% of intact events were $CD3^+$ and 0.3±<0.15% were $CD14^+$. For the B-lymphocyte sort, 87±6% of intact events were $CD19^+$ and 0.4±<0.2% were $CD14^+$. For the monocyte sort, 73±11% of intact events were $CD14^+$ and 0.4%±<0.15% were either $CD3^+$ or $CD19^+$. The absolute quantities of cells recovered from sorts were as follows; 1.0±0.5 $x10^6$, 3.3±0.8 $x10^6$ and 0.8±0.2 $x10^6$ for $CD14^+$, $CD3^+$ and $CD19^+$ cells respectively.

4.4.3 Cellular Migration

Cellular migration time-lapse

Colonies were observed using time-lapse microscopy for approximately 72 hours. Avid migration of both 'round' and 'spindle' cells toward colonies occurred with an associated enlargement of the colony. Cells migrated by rolling or by the projection of cytoplasmic extensions followed in turn by movement of the cell body. Small satellite colonies could be seen to form but these subsequently dissipated. Ultimately colonies began to degrade and disintegrate, adopting an ill-defined and granular appearance (Figure 4.4; see appended movie file).

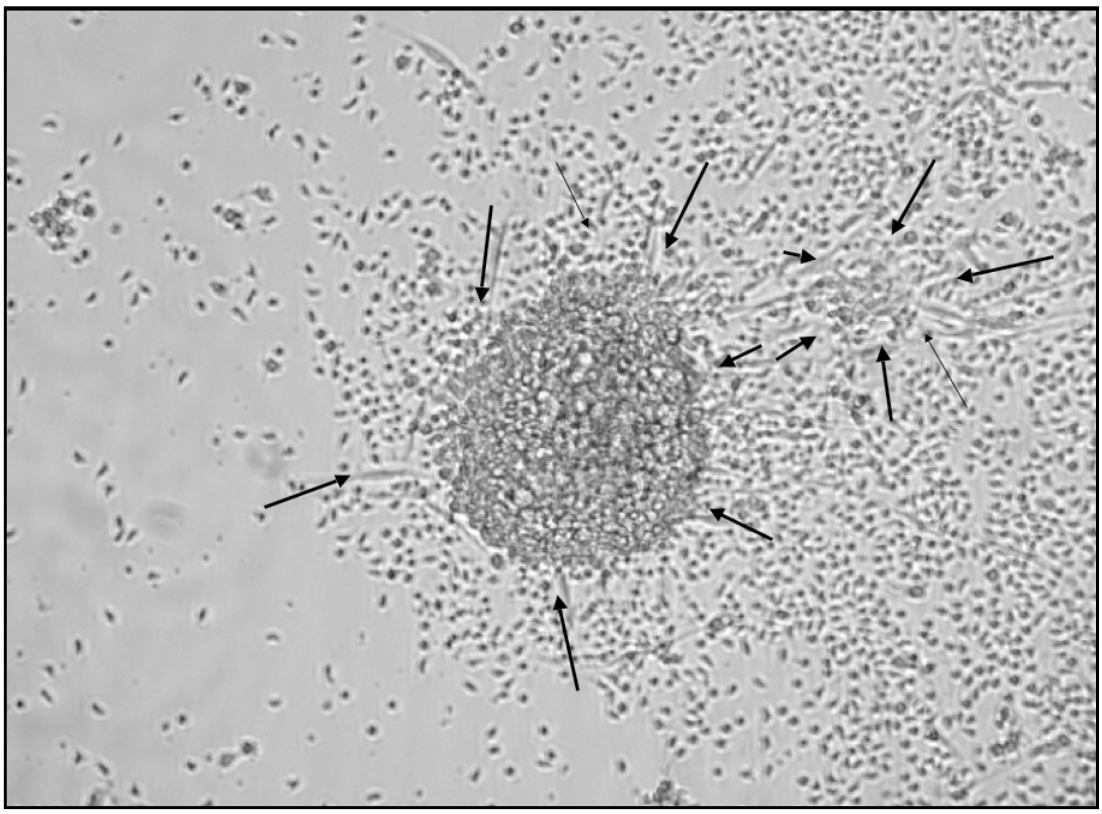


Figure 4.4 Mononuclear cells migrate to and incorporate into EC-CFU: Time-lapse microscopy revealed that both round and spindle shaped cells migrated toward developing EC-CFU. A still image is shown here of a large central colony attracting cells. The direction of cell migration is depicted by the black arrows. A small satellite colony was seen to form at the 2 o'clock position, which also attracted a stream of spindle cells. The number of cells occupying the surface of the well between colonies could clearly be seen to diminish over the period of colony maturation (see time-lapse movies).

Cellular Migration – Inoculation experiments

Via a transwell insert, $5x10^5$ cells from each population were added to a respective well. Within eight hours of inoculation, stained mononuclear cells had migrated through the transwell insert and had settled on the base of the well. Both round and spindle shaped morphology were evident within both monocytic and lymphocytic populations. However, within the first eight hours stained spindle cells were randomly positioned, unlike unstained spindle cells closely associated with colonies that adopted a radial distribution around mature colonies. Definite

incorporation into colonies was evident by 12 hours by both monocytic and lymphocytic cells with dense staining of colonies, predominantly at the periphery of the colonies (Figure 4.5). $CD3^+$ and $CD19^+$ cells behaved similarly. Distinct populations of stained and unstained cells were present throughout, indicating that staining of colonies after 12 hours was not due to contamination of the wells with cellular stain. In order to provide added confirmation of this, EC-CFU were co-incubated with supernatant of washed mononuclear cells following staining. No dye uptake occurred.

4.4.4 EC-CFU proliferation

Freshly adhered MNC on fibronectin showed only very sparse and scattered BrdU uptake (data not shown). EC-CFU exhibited marked BrdU uptake, indicating that cellular proliferation was indeed taking place (Figure 4.6A). As a positive control, MNC stimulated to proliferate with PHA[Inman *et al.*, 1963] formed multiple colonies, all of which exhibited marked BrdU uptake (Figure 4.6B). As a negative control, EC-CFU unexposed to BrdU were stained with anti-BrdU as above. The absence of immunofluorescence confirmed that staining was specific (Figure 4.6C). The proportion of $BrdU^+$ cells in EC-CFU varied directly with the size of a colony ($r=0.66$; 95% confidence intervals (CI) 0.53-0.76, $P<0.0001$; N = five subjects and 109 colonies). The median concentration of $BrdU^+$ cells increased sequentially from a median of 2.2 (0.0-4.0) $x10^3$ cells/μm^3 on day two to 35.2 (18.7-55.6) $x10^3$ cells/μm^3 on day 5 ($P<0.0001$; N=5; Figure 4.7A). After day five, colonies degenerated, and exhibited a diffuse and granular morphology with little definition of cell membranes and nuclei, making the determination of BrdU concentration

unfeasible. BrdU incorporation was also evident in EC-CFU generated form MNC enriched for $CD14^+$ to a purity of >99%. When treated with the cell cycle arrest agent mitomycin C, colony formation was significantly reduced from a median of 21 (5-48) to 2 (0.3-10) colonies per well; P=0.003; N=12: Figure 4.7B). Cells did however remain alive and acquired spindle-shaped morphology, indicating a sub-lethal, anti-proliferative dose of Mitomycin-C. Cells treated with actinomycin D (which ultimately inhibits both cell division and protein synthesis) remained rounded and began to die after one day in culture.

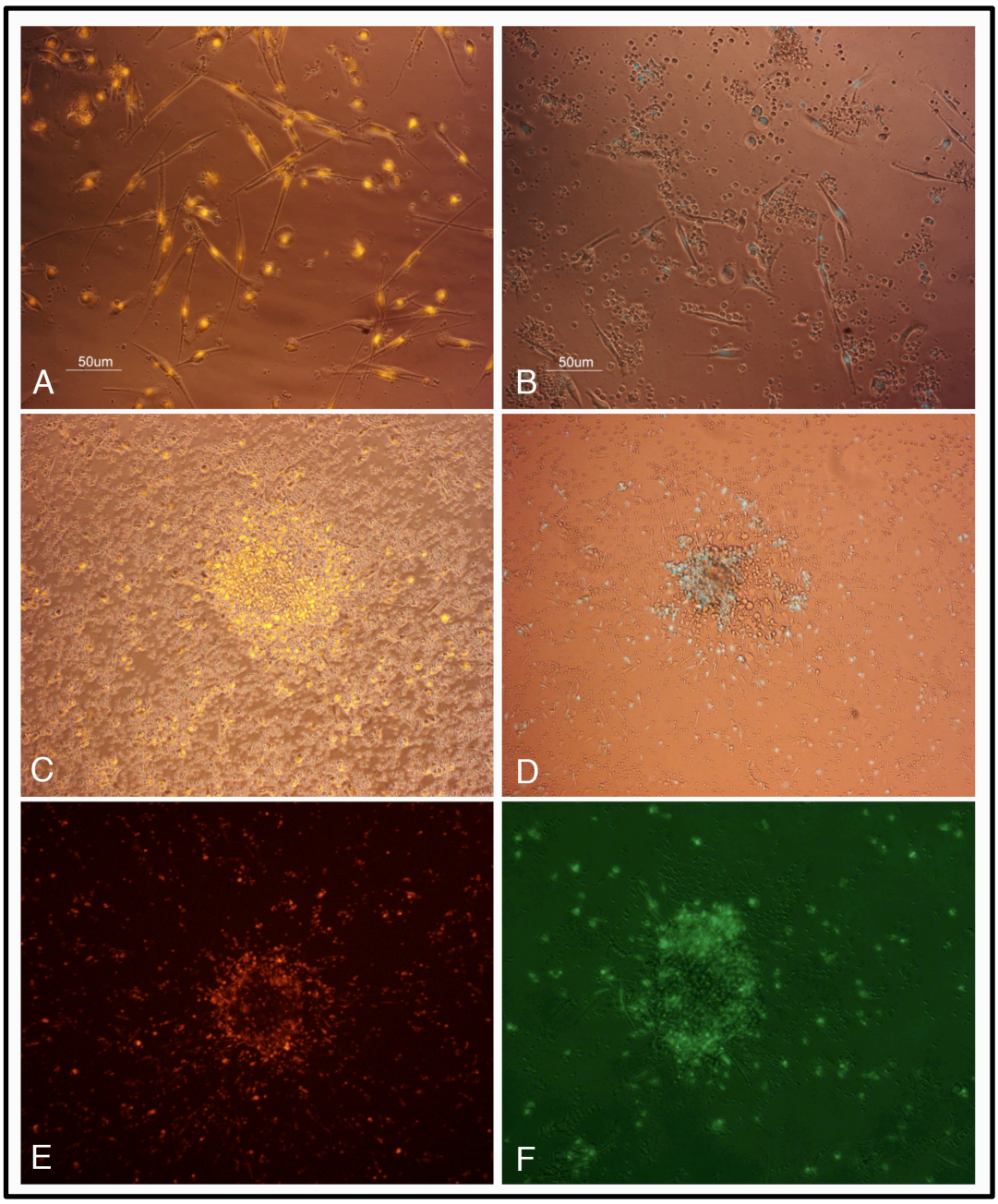


Figure 4.5 Monocytes and lymphocytes migrate and incorporate into EC-CFU: Day 5 EC-CFU cultures inoculated with stained mononuclear cells are displayed. Wells with added $CD14^+$ monocytes stained green are displayed on the right, and $CD3^+$ lymphocytes stained red are displayed on the left. Stained and unstained cells can be readily distinguished from each other. Both cells types adopted a spindle shape morphology but round cells can also be seen **(A and B; x40 magnification)**. Stained mononuclear cells migrated toward and incorporated into EC-CFU **(C-D with phase-contrast; E-F without phase-contrast; x20 magnification**). Photomicrographs representative of 4 experiments.

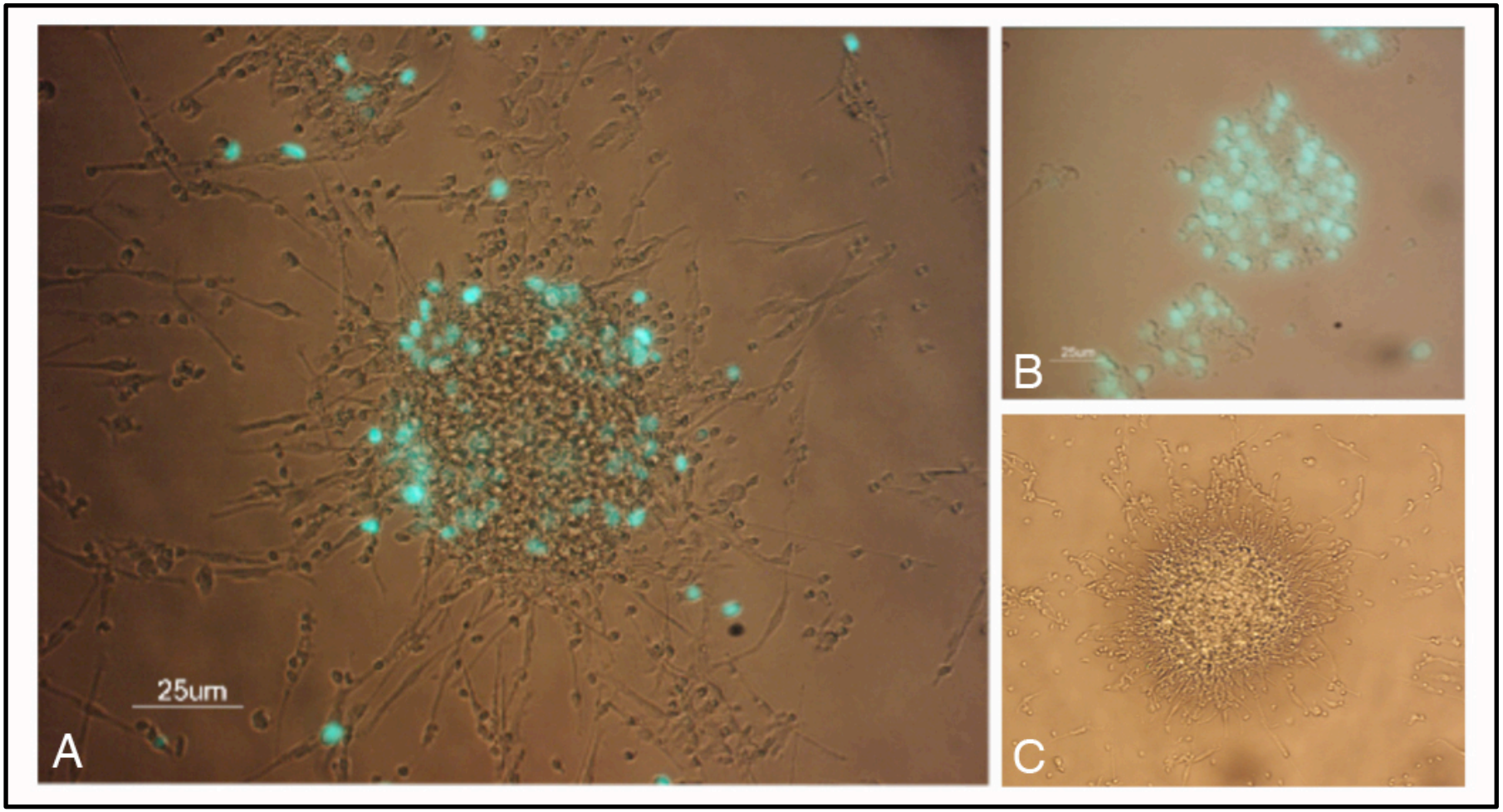


Figure 4.6 Cellular proliferation occurs within EC-CFU: BrdU incorporation into dividing cells within an EC-CFU is shown, indicating that proliferation occurs within EC-CFU (**A**). Lymphocytes stimulated by phytohaemagglutic acid are shown as a positive control (**B**). For a negative control to exclude non-specific binding of anti-BrDU, EC-CFU unexposed to BrdU were stained in the same manner as A&B (**C**).

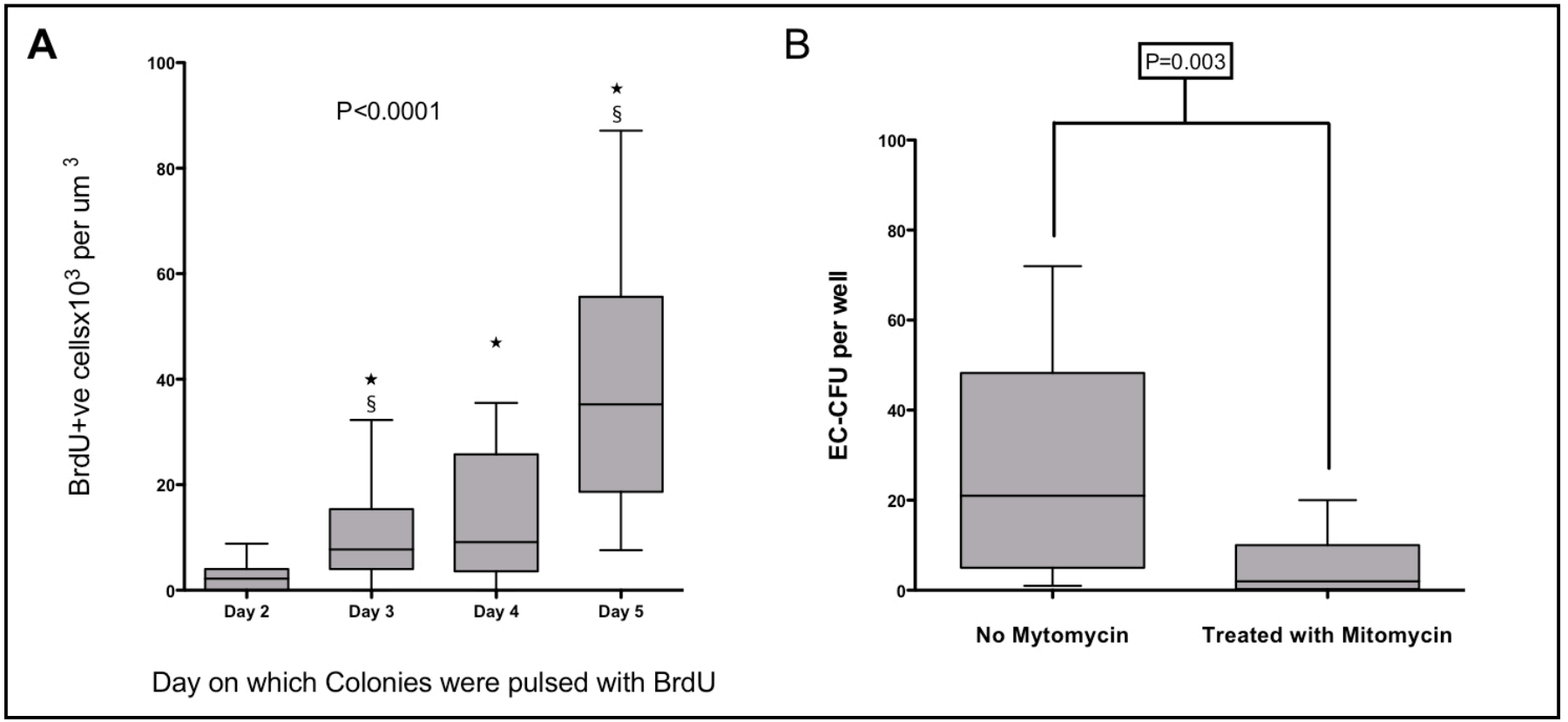


Figure 4.7 Cellular proliferation is required for EC-CFU formation: Sequential pulsing of colonies with BrdU from day 2 – 5 demonstrates that the median concentration of proliferating ($BrdU^+$) cells increased sequentially from a median of 2.2 (0.0-4.0) $x10^3$cells/μm^3 on day two to 35.2 (18.7-55.6) $x10^3$ cells/μm^3 on day 5 (Kruskal-Wallis test P<0.0001; N=5) ★= significantly different from day two; § = significantly different from preceding day (A). Inhibition of proliferation with mitomycin-C at a concentration of 0.25μg/mL dramatically reduced colony formation (21 (5-48) vs 2 (0-10) colonies; P<0.003; N=12) (B). Box and whiskers plots display the median, interquartile range and range. BrdU=Bromodeoxyuridine.

4.5 DISCUSSION

These studies confirm the haematopoietic nature of EC-CFU, and for the first time demonstrate that EC-CFU colonies are a potent stimulus for the migration of monocytes and lymphocytes *in-vitro.* Furthermore, I demonstrate conclusively that cellular proliferation occurs within EC-CFU colonies and that this may be necessary in order for EC-CFU to form.

Consistent with previous reports [Hur *et al.*, 2007; Rohde *et al.*, 2007; Yoder *et al.*, 2007], I found that EC-CFU exhibit a variety of endothelial characteristics. These included the uptake of acetylated low-density lipoprotein and UEA-1 binding, and the expression of Tie-2, CD146, CD31, CD105 and endothelial nitric oxide synthase. Other typical endothelial markers such VEGFR-2 and Von Willebrand factor were only very weakly expressed, or in the case of CD144, not at all. Although often described as classical endothelial characteristics, Ac-LDL uptake, lectin binding and surface expression of CD31, CD144 and CD105 are actually non-specific, and can also a be expressed on cells of myeloid lineage such as monocytes and macrophages [Rohde *et al.*, 2006]. Indeed the most intensely expressed antigens of all were the pan-leukocyte marker CD45, and the macrophage markers CD68 and CD105. The lymphocyte markers CD4 and CD8 were also expressed widely throughout colonies. Of particular note was the fact that the monocyte marker CD14 was expressed only very weakly despite being expressed relatively intensely on uncultured monocytes. Further interrogation of mononuclear cells plated in the EC-CFU assay using flow-cytometric analysis confirmed that CD14 expression is progressively down-regulated in the EC-CFU assay over time. In contrast, the

markers CD68 and CD105 were expressed very weakly on uncultured monocytes but became very intensely expressed on mature EC-CFU. It is recognised that monocytes will up-regulate a variety of endothelial characteristics in culture [Fernandez Pujol *et al.*, 2000; Harraz *et al.*, 2001; Rehman *et al.*, 2003; Schmeisser *et al.*, 2001; Urbich *et al.*, 2003; Zhang *et al.*, 2005; Zhang *et al.*, 2006; Zhao *et al.*, 2003] and will also readily differentiate into macrophages under appropriate environmental cues [Becker *et al.*, 1987]. CD14 expression diminished as CD68 and CD105 expression increased, and it is therefore likely that monocytes differente into macrophages in the EC-CFU assay, consistent with previous studies demonstrating that colonies possess phagocytic function and are unable form perfusing vessels in vivo [Yoder *et al.*, 2007]. It is now recognised that proteins may be transferred between cell types, leading to erroneous conclusions regarding gene expression and phenotype. For instance, platelet derived micro-particles are readily taken up by monocytes leading to apparent 'expression' of such antigens as CD31 and vWF. This may well explain the phenotypic characteristics of EC-CFU [Prokopi *et al.*, 2011].

EC-CFU colonies were thought to be derived from single cells undergoing clonal expansion, and the assay was used as a means of quantifying the concentration of circulating putative EPC [Asahara *et al.*, 1997; Hill *et al.*, 2003]. As a result the spindle cells assumed to be 'emanating' from each colony, were thought to be neo-endothelial cells. Using time-lapse microscopy I have confirmed that, contrary to popular belief, cells do not emerge from EC-CFU, but in fact migrate toward them with a commensurate increase in colony size. Inoculating the EC-CFU assay with immunofluorescently labeled monocytic and lymphocytic fractions, allowed tracking

of specific $CD14^+$, $CD3^+$ and $CD19^+$ cell populations, confirming that both monocytes and lymphocytes may adopt a spindle shape morphology and undergo avid migration toward, and incorporation into, EC-CFU. Having demonstrated that cellular migration was a significant component of colony growth, I wished to determine the significance of cellular proliferation on colony growth, particularly as EC-CFU were originally assumed to be proliferating colonies.

Bromodeoxyuridine (BrdU) is an analogue of the nucleotide thymidine that incorporates into the DNA of actively dividing cells, and serves as a means of identifying the presence of cellular proliferation. Incubation of maturing EC-CFU with immunofluorescently labeled BrdU demonstrated that cellular proliferation occurs throughout EC-CFU, with the concentration of proliferating cells increasing substantially by three, four and 16 fold, on days three four and five respectively. In the presence of mitomycin-C, the number of colonies formed was markedly reduced, indicating that cellular proliferation is necessary to initiate colony formation. Mitomycin-C can have deleterious effects on cell viability, and this may have effected colony formation, however at the low concentrations used in the present study, cytotoxic effects are likely to have been very limited [Sadeghi *et al.*, 1998]. Furthermore, cells were still able to form spindle cells indicating retention of viability, unlike cells treated with actinomycin-D, where cells remained rounded and began to disintegrate after a day in culture. Proliferation therefore appears to be the initiating event in colony formation. The mass of proliferating cells may then serve as a stimulus for further cell migration. Further studies are required to elucidate precisely which cells are proliferating.

In parallel studies (working with Dr Olga Tura-Ceide et al, Scottish Centre for Regenerative Medicine working in the same laboratory as myself) we showed that EC-CFU potential is associated with $CD14^+$ cells and that CD14 cells that are proliferating (see appended manuscript; The constituents and mechanisms of generation of EC-CFU). In these studies enriching for $CD14^+$ cells increased EC-CFU formation. The CD14 depleted fraction did not form colonies. Neither CD133 or CD34 enriched fractions could generate EC-CFU. CD133 and CD34 deplete fractions could generate EC-CFU but to a lesser extent than unfractionated cells. Interestingly $CD34^+$ cells could generate cells if first differentiated into $CD14^+$ cells. CD14 cells therefore appear to be the precursor for EC-CFU however we did not definitively phenotype the $BrdU^+$ cells in the assay.

Leukocytes accumulate at sites of tissue injury as crucial mediators in the inflammatory response necessary for wound healing. This is similarly the case following vascular injury, and the roles of monocyte derived macrophages, and lymphocytes following vascular injury have been well described [Farb *et al.*, 1999; Fujiyama *et al.*, 2003; Inoue *et al.*]. Endothelial injury leads to the release of chemotactic factors such as monocyte chemo-attractive protein-1 (MCP-1) [Ito *et al.*, 1997] and stromal derived factor-1 (SDF-1)[Garg *et al.*, 2008b] and increased expression of various intercellular adhesion molecules on the endothelium. Monocytes attach to sites of vascular injury migrate into the vascular wall, and secrete chemotactic factors that drive further cellular migration to the site of vascular injury and activate endothelial cells in order to enhance re-endothelialisation. Monocytes differentiate into phagocytic macrophages in order to remove noxious

agents and amplify the inflammatory response through antigen presentation to invading lymphocytes attracted by increased SDF-1 production. Activated T-cells similarly regulate the inflammatory response and endothelial cell activation facilitating the restoration of normal vascular homeostasis. EC-CFU and closely aligned populations mobilize to regions of vascular injury in animal models [Asahara *et al.*, 1997; Werner *et al.*, 2003].

In the last chapter I described an increase in EC-CFU following cardiovascular stress in the form of discrete vascular injury (Chapter 3), [Mills *et al.*, 2009]. I shall go on to decribe a similar increase following acute myocardial infarction (Chapter 6) [Padfield *et al.*, 2013], and also provide some evidence to suggest that this is a relatively specific inflammatory response to vascular injury (Chpater 5) [Padfield *et al.*, 2010b]. Although not specifically measured in the present study, EC-CFU are known to secrete a variety of angiogenic cytokines (VEGF, interleukin-8, matrix metalloproteinases, G-CSF, and GM-CSF) [Hur *et al.*, 2007; Rehman *et al.*, 2003] that are capable of inducing leukocyte migration and proliferation, suggesting an active influence by EC-CFU on these processes. In the present study I have observed that the *in-vitro* behaviour of EC-CFU closely mirrors the known *in-vivo* cellular response to vascular injury. I therefore suggest that EC-CFU formation reflects *in-vivo* leukocyte activation, predominantly by monocytes which undergo proliferation and differentiation into macrophages, accompanied by avid cytokine production and the attraction of further monocytes and lymphocytes to amplify a vasculoprotective inflammatory response to vascular injury. The close relationship between EC-CFU and cardiovascular health, combined with their

'endotheloid' phenotype led to the specious conclusion that EC-CFU were vascular progenitors. This study contributes to the growing body of evidence that refutes the hypothesis that EC-CFU are EPC, but does suggest that EC-CFU play a role in vascular inflammation.

4.6 CONCLUSIONS

EC-CFU express endothelial characteristics, but are predominantly $CD14^+$ derived macrophages that act as a potent stimulus for lymphocyte migration. Proliferation is necessary for EC-CFU generation, however colony expansion predominantly occurs as a result of leukocyte migration. EC-CFU formation likely reflects a vasculoprotective inflammatory response to vascular denudation or tissue ischaemia. Further studies of the *in-vivo* behaviour of EC-CFU are needed to confirm whether EC-CFU are a direct measure of vascular inflammation.

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CHAPTER FIVE

CIRCULATING ENDOTHELIAL PROGENITOR CELLS ARE NOT AFFECTED BY ACUTE SYSTEMIC INFLAMMATION

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G Barclay GR, Newby DE, Mills NL, in

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5.1 SUMMARY

Vascular injury causes acute systemic inflammation and mobilises endothelial cell colony forming units (EC-CFU). Whether such mobilisation occurs as part of a non-specific acute phase response or is a phenomenon specific to vascular injury remains unclear. I aimed to determine the effect of acute systemic inflammation on EC-CFU and putative EPC mobilisation in the absence of vascular injury. In a double-blind randomised cross-over study, 12 healthy volunteers received *Salmonella typhus* vaccination or placebo in order to generate an acute systemic inflammatory response. Phenotypic EPC populations enumerated by flow cytometry ($CD34^+VEGFR\text{-}2^+CD133^+$, $CD14^+VEGFR\text{-}2^+Tie2^+$, $CD45^-CD34^+$, as a surrogate for late outgrowth EPC and $CD34^+CXCR\text{-}4^+$), EC-CFU and serum cytokine concentrations [C reactive protein (CRP), interleukin-6 (IL-6), vascular endothelial growth factor (VEGF-A) and stromal derived factor-1 (SDF-1] were quantified during the first 7 days. Vaccination increased circulating leukocyte (9.8 ± 0.6 *versus* 5.1 ± 0.2 $x10^9$/L; P<0.0001), serum IL–6 [0.95 (0–1.7) *versus* 0 (0–0) ng/L; P=0.016] and VEGF-A [60 (45–94) versus 43 (21–64) pg/L; P=0.006] concentrations at 6 hours, and serum CRP at 24 hours [2.7 (1.4–3.6) versus 0.4 (0.2–0.8) mg/L; P=0.037]. Vaccination caused a 56.7 ± 7.6% increase in $CD14^+$ cells at 6 hours (P<0.001) and a 22.4 ± 6.9% increase in $CD34^+$ cells at 7 days (P=0.04). EC-CFU, putative vascular progenitor and serum SDF-1 concentrations were unaffected throughout the study period (P>0.05 for all). Acute systemic inflammation causes non-specific mobilisation of $CD34^+$ cells though does not selectively mobilise putative vascular progenitors. Systemic inflammation *per se* is not the primary stimulus for EC-CFU mobilisation following acute vascular injury.

5.2 INTRODUCTION

A close relationship between cardiovascular disease and systemic inflammation is now well recognised. Elevated C-reactive protein (CRP) concentrations predict the occurrence of adverse cardiovascular events, independently of 'traditional' cardiovascular risk factors, even in apparently healthy individuals with apparently 'normal' CRP concentrations [Ridker *et al.*, 2000]. Acute inflammation such as that occurring in the context of respiratory and urinary tract infections is temporally associated with adverse cardiovascular events, namely myocardial infarction and stroke [Smeeth *et al.*, 2004]. Similarly the incidence of cardiovascular disease in patients with chronic inflammatory conditions, such as rheumatoid arthritis [del Rincon *et al.*, 2001] and systemic lupus erythematosis is disproportionately high, even after adjusting for traditional cardiovascular risk factors [Svenungsson *et al.*, 2001]. These effects may, in part be attributed to a deleterious effect of inflammatory signalling on endogenous vascular repair mechanisms such as EPC function.

Although largely defined by the expression of haematopoietic markers, $CD34^+$ and $CD133^+$, and VEGFR-2 [Asahara *et al.*, 1997; Peichev *et al.*, 2000; Vasa *et al.*, 2001b], Non-haematopoietic, $CD45^-CD34^+$ cells, have also recently been identified as a putative EPC on the basis of their ability to form "late outgrowth" colonies phenotypically and functionally indistinguishable from mature endothelial cell colonies in culture [Case *et al.*, 2007; Timmermans *et al.*, 2007; Yoder *et al.*, 2007]. In addition to $CD34^+$ EPC, other circulating leukocytes have been identified has having regenerative capacity. In particular monocytes expressing VEGFR-2 and

the tyrosine kinase receptor, Tie-2 enhance endothelial regeneration and accelerate restoration of vascular function in animal models of vascular injury [Elsheikh *et al.*, 2005; Nowak *et al.*, 2004]. The EC-CFU assay [Hill *et al.*, 2003], although widely used as a measure of circulating EPC, is now thought not to have capacity to form perfusing vessels [Rohde *et al.*, 2007; Yoder *et al.*, 2007], but rather stimulates vessel growth and repair through the secretion of growth factors [Rehman *et al.*, 2003]. In chapters three and four I demonstrated that EC-CFU, predominantly composed of monocytes and lymphocytes [Padfield *et al.*, 2013], are mobilised in response to vascular injury in the context of an acute systemic inflammatory response [Mills *et al.*, 2009]. Although not true EPC there is compelling clinical evidence that EC-CFU are an important component in the cellular response to vascular injury [Hill *et al.*, 2003; Sobrino *et al.*, 2007; Werner *et al.*, 2005].

The effect of inflammation on the various putative EPC populations is poorly understood with contrasting effects seen during acute and chronic inflammation. Recombinant C-reactive protein (CRP) depresses EC-CFU function *in-vitro* [Verma *et al.*, 2004], and conversely therapy directed against tumour necrosis factor-alpha therapy enhances the mobilisation and function of progenitor cells in patients with rheumatoid arthritis [Ablin *et al.*, 2006]. Chronic inflammatory conditions such as ulcerative colitis, rheumatoid arthritis and systemic lupus erythematosis are also associated with depressed concentrations of circulating EPC [Grisar *et al.*, 2005; Masuda *et al.*, 2007; Moonen *et al.*, 2007; Palange *et al.*, 2006], suggesting that EPC dysfunction is a potential mechanism underlying the accelerated rate of atherosclerosis observed in these conditions. In contrast, acute inflammation may

have the effect of increasing EPC concentrations, for instance following an acute coronary syndrome or tissue ischaemia [Adams *et al.*, 2004; George *et al.*, 2004; Massa *et al.*, 2005], or discrete vascular injury caused by PCI [Banerjee *et al.*, 2006; Mills *et al.*, 2009]. However, it is unclear whether the stimulus for the mobilisation of these populations arises from the systemic inflammatory response or from vascular injury *per se*. It may be that acute systemic inflammation potentiates EPC mobilisation, or indeed acts in a counter regulatory or inhibitory manner so suppress EPC mobilisation.

The aim of this study was therefore to investigate the behaviour of a range of putative EPC populations and EC-CFU, and the factors responsible for their mobilisation in response to an isolated inflammatory stimulus in the absence of denuding endothelial injury. I used an established model of acute systemic inflammation, *Salmonella Typhus* vaccination [Chia *et al.*, 2003], to study the effects of inflammation on the behaviour of EPC in healthy volunteers.

5.3 METHODS AND MATERIALS

The study was performed with the approval of the local research ethics committee, in accordance with the Declaration of Helsinki, and with the written informed consent of all participants. All procedures were performed in accordance with the guidelines of our institution.

5.3.1 Subjects

As described, twelve healthy, non-smoking, non-obese male volunteers were enrolled into the study. All subjects were normotensive without a history of diabetes mellitus, peripheral vascular or coronary artery disease. None of the subjects had undergone typhoid vaccination in the year prior to the study nor had they received vasoactive or non-steroidal anti-inflammatory drugs in the week before the study.

5.3.2 Study design

Using a randomised balanced block double-blind crossover study design, the polysaccharide *Salmonella Typhus* vaccination 0.025 mg (Typhim Vi, Aventis Pasteur MSD, UK) was compared with saline placebo given by intramuscular injection at least 2 weeks apart [Chia *et al.*, 2003]. Subjects attended between 8 and 10 am for venesection and were randomly assigned to vaccine or placebo injection into the deltoid muscle of the non-dominant arm. As previous studies have reported a peak inflammatory response occurring at 6 hours following *Salmonella Typhus* vaccination, subjects were asked to return 6 hours later for repeat blood sampling [Chia *et al.*, 2003]. In order to evaluate the late effects of vaccination on EPC, subjects returned at 24, and 168 hours for further blood sampling. Subjects were advised to maintain

adequate fluid intake of at least 2 L over the first 24 hours and avoid alcohol during the study period. In order to avoid confounding EPC mobilisation, subjects were also asked to abstain from strenuous exertion during the study period [Rehman *et al.*, 2004].

5.3.3 Blood sampling and assays

Venous blood samples (20 mL) were collected into EDTA and serum gel for flow cytometry, mononuclear cell preparations and culture, and separation of plasma and serum. Whole blood was analysed for total cells, differential count and platelets using an autoanalyzer (Sysmex, UK). ELISAs were used to quantify serum interleukin-6 (Invitrogen, UK), VEGF-A (Invitrogen, UK), and stromal derived factor-1 (SDF-1) (R&D systems, UK) concentrations according to the manufacturer's instructions. Serum high sensitivity-CRP was quantified using an immunoturbidimetric method (Dade-Behring Marburg, Germany).

5.3.4 Flow cytometric identification of EPC

Endothelial progenitor cells and angiogenic monocytes were identified using flow cytometry and analysed as described above (section 2.5.2),

5.3.5 Endothelial cell - colony forming units

Endothelial cell-colony forming units (EC-CFU) [Hill *et al.*, 2003] were generated as described above (Section 2.6.1) and counted in a minimum of four wells on day 5.

5.3.6 Assays of EC-CFU function

In order to assess the *in-vitro* effects of an inflammatory milieu on functional aspects of cultured EC-CFU, randomly selected subjects were recalled at the end of the study for repeat venesection. EC-CFU were again generated from peripheral blood, but on this occasion co-incubated with autologous stored serum samples (5% concentration) obtained at either baseline, 6 and 24 hours following exposure to either vaccine or placebo during the initial part of the study. On day five, mature colonies were retrieved using non-enzymatic dissociation solution (Sigma-Aldrich; UK) and underwent functional assays assessing cellular migration and adhesion as described above (Section 2.6.3) and briefly below.

Migration

EPC migratory capacity was assessed using 8μm pore transwell inserts (Corning-Costar; UK). Harvested EC-CFU were resuspended in chemotaxis buffer (EBM-2; Lonza; UK) at a concentration of $2x10^6$ cells/mL. 200μL of cell solution was added to the upper chamber. 600μL of chemotaxis buffer containing 50ng/mL of VEGF (Peprotech; UK) was added to the lower chamber. Cells were incubated at 37°C, 5% CO_2, 95% humidity. At 3 hours the quantity of migrated EPC in the lower chamber was determined using a coulter counter (Multisizer™ 3 Coulter counter®; Beckman-Coulter; UK).

Adhesion

EC-CFU were harvested and re-suspended in EBM2 (Lonza; UK). $5x10^4$ cells were re-plated on Fibronectin and incubated as above. At 30 minutes non-

adherent cells were removed and wells were washed gently with PBS. Adherent cells were visualised under direct focal microscopy. Cellular adhesion was expressed as a function of the density of adherent cells in 10 random fields.

Proliferation

Cellular proliferation within EC-CFU was assessed by the detection of incorporation of the thymidine analogue bromodeoxyuridine (BrdU) (Sigma-Aldrich; UK) as described above (Section 2.6.3). Briefly, mononuclear cells replated on fibronectin at day two were incubated with BrdU. Colonies were fixed and permeabilised on day five and incubated with DNAse (Perbio; UK) for 30 minutes. Colonies were rinsed and incubated with anti-BrdU conjugated to alexafluore-488™ (Invitrogen; UK). Colonies were examined with an inverted microscope at 488nm. Proliferation was expressed as the proportion of BrdU positive cells per unit volume of colony. Colony volume (V) was calculated under the assumption of a conical geometry using the formula: $V = (\pi r^2 h)/3$ where r = the radius of the base of the colony and the height (h) of the colony is assumed to be equal to r.

5.3.7 Data analysis and statistics

Continuous variables are reported as mean ± standard error of the mean or median and interquartile range (IQR) where appropriate. Because of the cross-over design, data were analysed to confirm the absence of a carry over effect prior to comparisons. Statistical analyses were performed with GraphPad Prism (Graph Pad Software, USA) using 2-way analysis of variance (ANOVA) and two-tailed Student's *t*-test or Mann-U Whitney paired tests where appropriate. In previous studies

vascular injury following percutaneous coronary intervention had resulted in an approximately 300% increase in both circulating EC-CFU and serum C-reactive protein concentration [Mills *et al.*, 2009]. Assuming that smaller changes in EC-CFU than those detected following PCI might still be clinically relevant, I aimed to detect at least a 75% increase in EC-CFU. Pilot data from healthy volunteer studies suggested a mean colony count of approximately 17 colonies per well with a standard deviation 16. Therefore given that: $N = (z_{0.5\alpha} + z_{\beta})^2 (\sigma/\delta)^2$. Where $z_{\beta} = 0.84$ and $z_{0.5\alpha} = 1.96$, in order to provide an 80% power at a two-sided significance level of 5%. The approximate number of subjects (N) required to detect a difference of δ given a standard deviation of σ, is 12. Therefore 12 subjects were used in order to detect a 75% increase, providing a wide margin of error in terms of detecting a clinically significant change.

5.4 RESULTS

5.4.1 Study participants

The characteristics of the study population are tabulated below. Total leukocyte concentrations were slightly higher prior to placebo (table 5.1).

Table 5.1. Baseline characteristics of study population

	Placebo	Vaccine	P value
Demographics and basic observations			
Age (years)	26±1.2		-
Male gender (%)	100		-
Temperature (°C)	36±0.2	36±0.1	0.90
Blood Pressure (mmHg) - *Systolic*	136±2	130±4	0.13
- *Diastolic*	79±3	73±3	0.13
Pulse rate (bpm)	76±4	71±9	0.16
Haematological and biochemical measures			
Total Leukocytes $x10^9$/L	5.6±0.3	5.1±0.2	0.02
Neutrophils $x10^9$/L	3.1±0.2	2.7±0.2	0.06
Lymphocytes $x10^9$/L	1.9±0.1	1.7±0.2	0.90
Monocytes $x10^9$/L	0.45±0.02	0.45±0.04	0.90
Interleukin – 6 ng/L	0.3±0.2	0.5±0.4	0.10
VEGF-A pg/L	55.8±10	47.6±10	0.07
SDF-1 ng/L	2040±56	2036±45	0.95
CRP mg/L	0.4 (0.2-0.9)	0.4 (0.2-0.8)	0.40
Angiogenic cell populations			
$CD34^+$	3.38±0.28	2.92±0.24	0.16
$CD34^+VEGFR\text{-}2^+$	0.51±0.09	0.38±0.04	0.17
$CD34^+CD133^+VEGFR\text{-}2^+$	0.14±0.04	0.15±0.06	0.85
$CD45^-CD34^+$	0.48±0.06	0.39±0.04	0.23
$CD34^+CXCR\text{-}4^+$	0.67±0.1	0.97±0.16	0.13
$CD14^+$	390±30	350±30	0.43
$CD14^+VEGFR\text{-}2^+$	50±10	60±10	0.51
$CD14^+Tie\text{-}2^+$	2.0±0.5	1.0±0.2	0.25
$CD14^+Tie\text{-}2^+VEGFR\text{-}2^+$	0.6±0.2	0.7±0.2	0.42
EC-CFU per well	19 (5-46)	14 (9-50)	0.62

Values are median and interquartile range or the mean ± standard error. Statistical comparisons are made using paired a Student's t-test or Wilcoxon test where appropriate. Angiogenic cell populations quantified by flow cytometry are expressed as $x10^6$/L. bpm = beats per minute; CD = cluster of differentiation; CRP = C reactive protein; EC-CFU = endothelial cell – colony forming unit; SDF-1 = stromal derived factor –1; VEGF(R-2) = vascular endothelial growth factor (receptor-2).

5.4.2 *Salmonella Typhus* vaccination induces an inflammatory response

Following vaccination, subjects reported mild flu-like symptoms although these were not associated with any change in temperature, heart rate or blood pressure. No major adverse events occurred. *Salmonella Typhus* vaccination induced a leukocytosis in all subjects that was maximal at 6 hours (Δ 4.6±1.1x10^9/L, P<0.0001) and remained at 24 hours (P=0.037). This was driven predominantly by a neutrophilia (Δ 4.3±1.1x10^9/L, P<0.0001) and to a lesser extent by a monocytosis (Δ 0.19±0.03x10^9/L, P=0.004). Saline placebo injection had no effect on the differential cell counts (table 5.2). Following vaccination, there was an increase in serum IL–6 [0.95(0–1.7) *versus* 0(0–0) ng/L; P=0.016] and VEGF-A [60(45–94) *versus* 43(21–64) pg/L; P=0.006] concentrations at six hours that returned to baseline levels by 24 hours. Serum CRP concentrations increased later, peaking at 24 hours [2.7(1.4–3.6) *versus* 0.4(0.2–0.8) mg/L; P<0.001], returning to baseline at one week. Saline placebo injection had no effect on the cytokine profile (P>0.2; Figure 5.2).

5.4.3 $CD34^+$ Endothelial Progenitor Cell Phenotype

Six hours following saline placebo, $CD34^+$, $CD34^+VEGFR\text{-}2^+$ and $CD34^+CD133^+VEGFR\text{-}2^+$ cell concentrations had fallen by 19.5±8.5% (P=0.033), 42±9.5% (P=0.001) and 53.6±12.5% (P=0.037; figure 5.3) respectively. Concentrations were comparable to baseline at 24 hours and one week. Six hours following vaccination, $CD34^+$ populations did not fall (P>0.2), and by 1 week circulating $CD34^+$ cells had increased by 22.4±6.9% (P=0.04). $CD34^+VEGFR\text{-}2^+$ and $CD34^+CD133^+VEGFR\text{-}2^+$ sub-populations did not change during the study

period (P>0.2; figure 4.4). The late outgrowth precursor $CD45^{-}CD34^{+}$ similarly fell at 6 hours following placebo by 7.4%±11, however this did not reach statistical significance. There were no differences in $CD45^{-}CD34^{+}$ cells following vaccination compared to placebo (P>0.2; figure 5.4). Circulating $CD34^{+}CXCR\text{-}4^{+}$ cell concentrations were similar prior to vaccination and placebo. Whilst $CD34^{+}CXCR\text{-}4^{+}$ cell concentration followed a trend similar to that of $CD34^{+}$ cells, the proportion of $CD34^{+}$ cells expressing CXCR4 was not significantly changed throughout the study period (P=0.78; figure 5.4). Serum SDF-1 concentrations were similarly unaffected by vaccination or placebo (Figure 5.2).

5.4.4 $CD14^{+}$ Angiogenic monocyte

Following vaccination, I observed an increase in circulating $CD14^{+}$ cells with a peak of 54.8±4.8% at 6 hours (P<0.005). A median of 0.18% (IQR, 0.08-0.24%) of $CD14^{+}$ cells expressed Tie-2 and VEGFR-2, although the levels of this population did not change in response to vaccination (P>0.2). Similarly $CD14^{+}Tie2^{+}$ and $CD14^{+}VEGFR\text{-}2^{+}$ populations were unaffected by vaccination (P>0.2; Figure 5.5). $CD14^{+}$ populations were unaffected by placebo (P>0.05 for all).

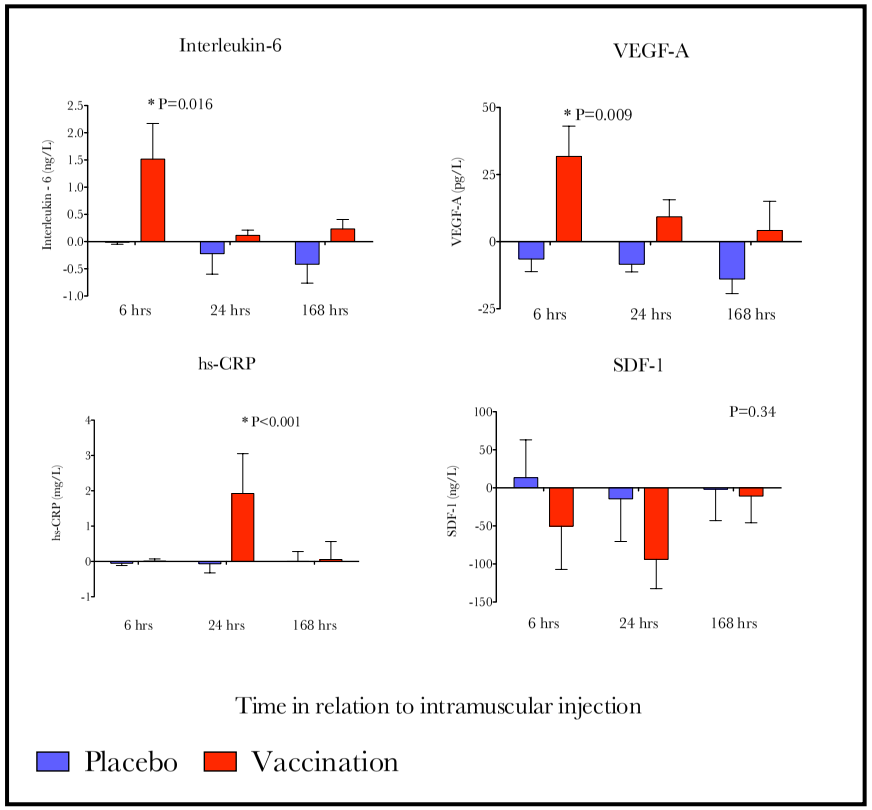

Figure 5.2 Serum cytokines concentration following *Salmonella typhus* vaccination: Vaccination resulted in an increase in serum interleukin-6 (P=0.016) and vascular endothelial growth factor-A (VEGF-A) (P=0.009) concentrations at 6 hours and a subsequent increase in CRP concentrations (P<0.001) at 24 hours. Serum stromal derived factor – 1 (SDF-1) concentrations were unaffected by either placebo or vaccination. Data are presented as the median change from baseline ± the interquartile range (CRP) or the mean and standard error (IL-6, SDF-1 and VEGF-A)

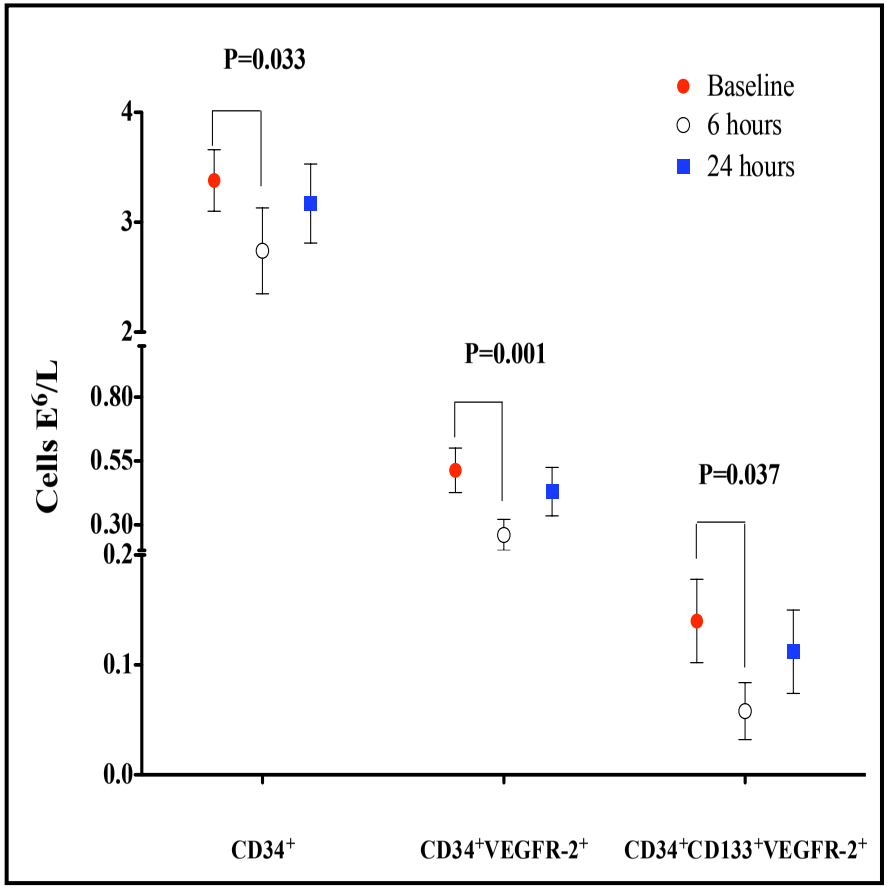

Figure 5.3 Circadian variation of CD34⁺ progenitor cells: Circadian variation of CD34 populations was evident following placebo, with significant reductions in $CD34^+$ (P=0.033), $CD34^+VEGFR\text{-}2^+$ (P=0.001) and $CD34^+CD133^+VEGFR\text{-}2^+$ (P=0.037) cells by mid afternoon.

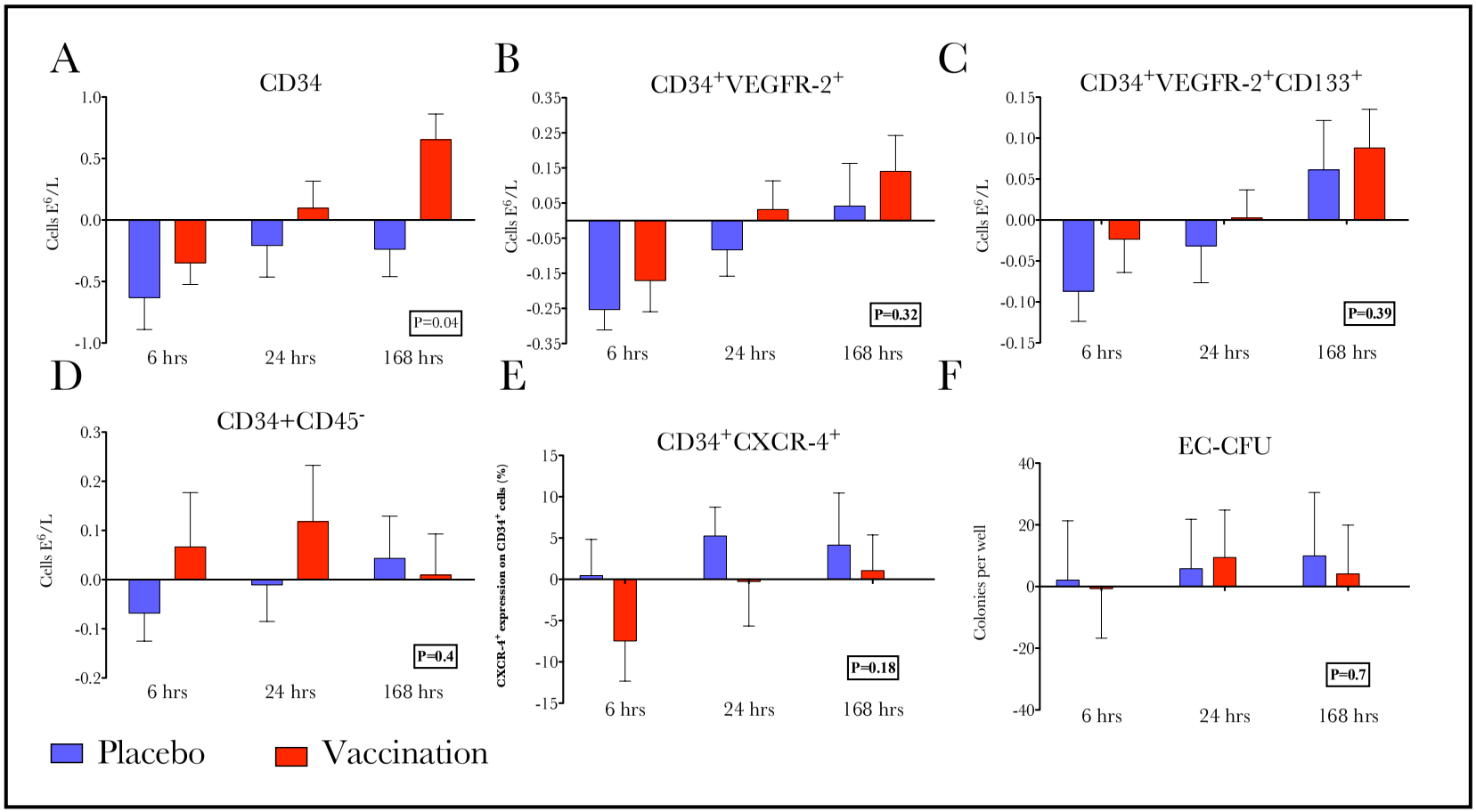

Figure 5.4 Effect of vaccination on circulating CD34⁺ populations and EC-CFU: Following vaccination the fall in $CD34^+$ populations from base line at 6 hours was attenuated **(A-C)** and a significant increase in $CD34^+$ cells occurred at one week (P=0.04) **(A)**. However, $CD45^-CD34^+$, $CD34^+VEGFR\text{-}2^+$ and $CD34^+CD133^+VEGFR\text{-}2^+$ cells were not altered by vaccination (P=0.4, P=0.32 and P=0.39 respectively) **(C-E)**. EC-CFU were similarly unaffected (F) Data are presented as the absolute mean change from baseline ± SEM **(A-E)**, and as the absolute median change from baseline ± IQR **(F)**.

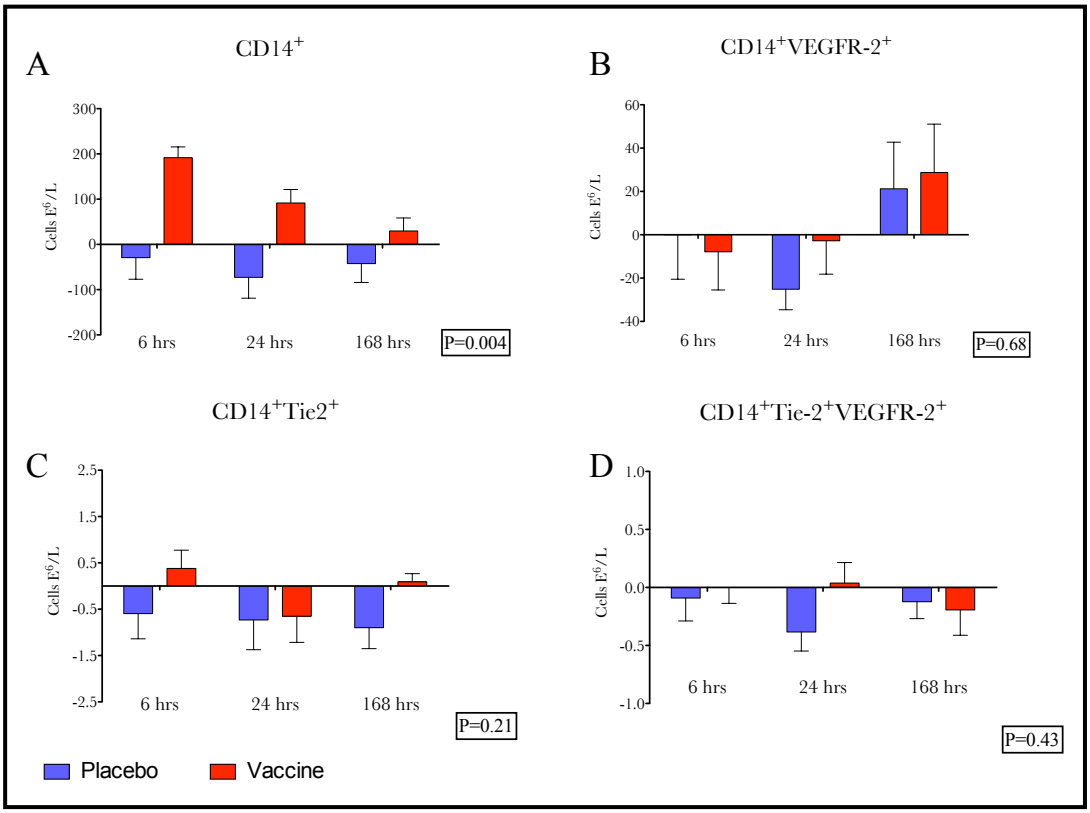

Figure 5.5 Effect of vaccination on circulating CD14+ populations.
Vaccination resulted in a significant mobilisation of CD14+ cells compared to placebo (P<0.001) (A). CD14+ subpopulations bearing Tie-2 and or VEGFR-2 were not significantly different from placebo throughout the study period (P>0.2) (B-D). Data are presented as the absolute median and IQR change from baseline.

5.4.5 Endothelial Cell - Colony Forming Units

Mean baseline numbers of CFU were similar prior to vaccination and saline placebo (33±11 *versus*. 24±6; P=0.6), and remained unchanged throughout the study period (P=0.7) (figure 5.4).

EC-CFU function

Co-incubation with stored serum inhibited EC-CFU generation and almost completely abolished colony growth at a concentration of 40% (figure 5.6).

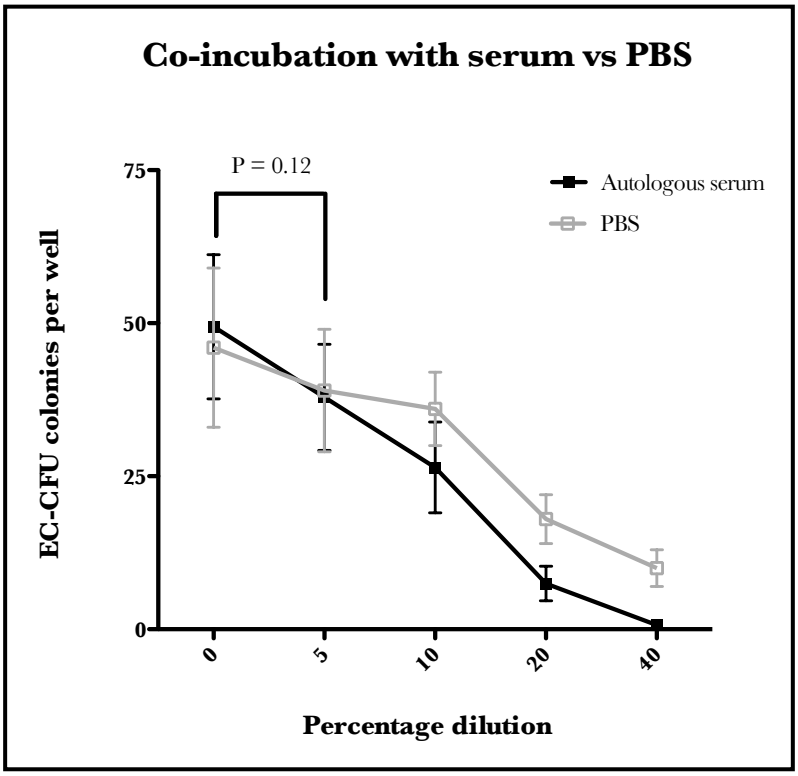

Figure 5.6 Co-incubation of EC-CFU with autologous serum: Serum inhibits EC-CFU generation, beyond any effect purely related to dilution of growth medium. (ANOVA; P<0.001; N=5.) This difference was negligible at a dilution concentration of 5% (P=0.12). Data are medians ± IQR.

In order not to excessively inhibit EC-CFU formation, a 5% dilution was used to compare the effects of serum from vaccinated *versus* non-vaccinated subjects. EC-CFU were generated under normal conditions or in the presence of serum obtained 6 hours following intramuscular injection. Eighteen wells were seeded from six subjects. Consistent with the initial dose ranging study, serum caused a mild but non-significant reduction in the number of EC-CFU that were generated; 13 (9-28) colonies per well with no serum added, *versus* 7 (3.7-29) and 12 (5-19) colonies per well (ANOVA, P=0.49) with serum obtained following saline placebo or vaccination respectively. Migration towards VEGF-A were similar under all three conditions; 2796 (2333-4000) cells migrated with no serum added, 4008 (2890-5738) in the presence of serum obtained following placebo injection, and 2850 (2548-4333) cells with serum obtained following vaccination (ANOVA, P=0.25). Adhesion to fibronectin was similar without the addition

of serum or following the addition of serum obtained following placebo injection or vaccination (17(9-26) *versus* 10(8-38) *versus* 20(9-44) colonies per well; P=0.59). Proliferative capacity of EC-CFU was significantly reduced by the addition of serum, 1.4 (1.02-2.01) *versus* 0.77 (0.42-1.20) and 0.90 (0.52-1.35) BrdU $^{+}$ve cells/μm^3 with serum obtained following saline placebo or vaccination respectively (ANOVA; P=0.0004), however there was no difference between the two types of serum (P>0.23).

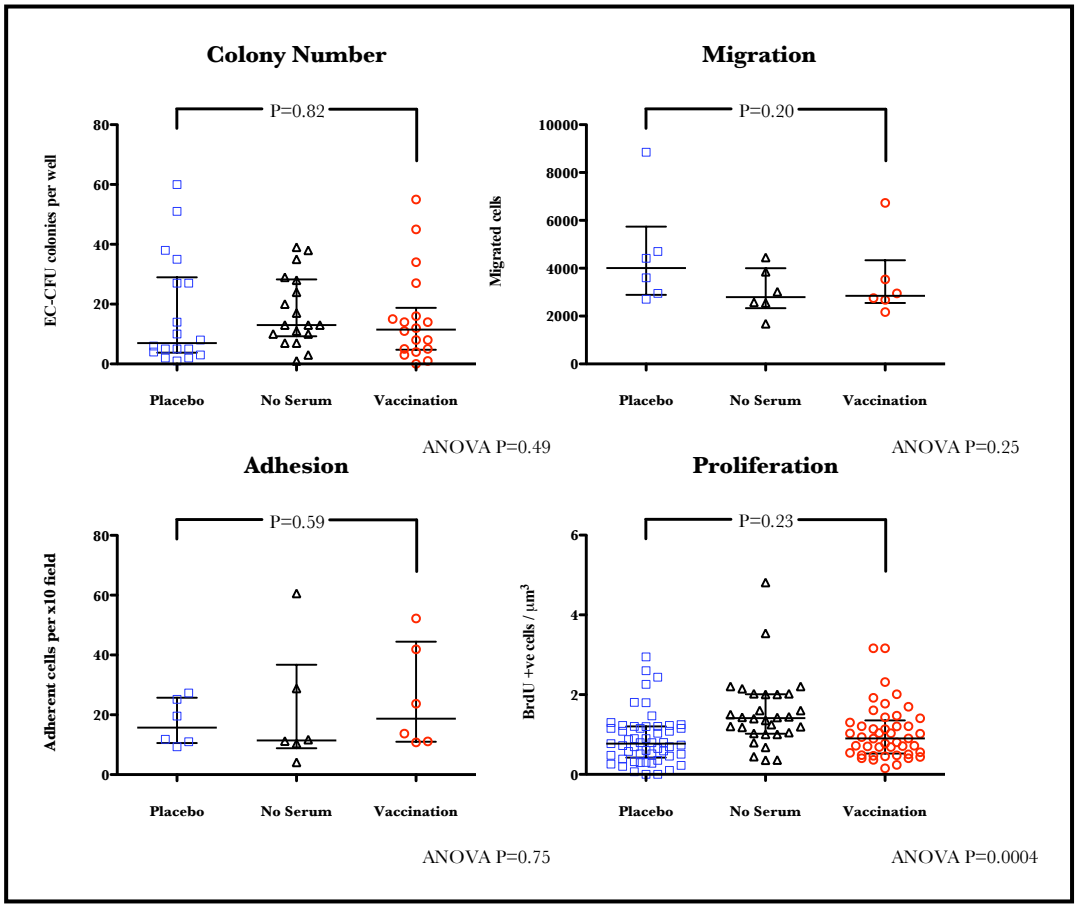

Figure 5.7 Effect of autologous serum on functional aspects of cultured EPC: EC-CFU were generated in the presence of 5% serum obtained 6 hours post intramuscular injection or no serum. Eighteen wells were seeded from six subjects. Consistent with the initial dose ranging study serum caused a mild reduction in EC-CFU quantity. Migration towards VEGF-A and adhesion to fibronectin were similar under all three conditions with no significant differences between the two types of sera. Proliferative capacity of EC-CFU was significantly reduced by the addition of serum however it was there was no difference between serum obtained following vaccination or saline placebo. Data are expressed median (IQR) per well (colony generation), per subject (adhesion and migration) and per colony.

5.5 DISCUSSION

Putative haematopoietic EPC expressing CD34, EC-CFU and late outgrowth colonies are mobilised in response to vascular injury such as that occurring in the context of percutaneous coronary intervention or an acute coronary syndrome [Huang *et al.*, 2007; Massa *et al.*, 2005; Mills *et al.*, 2009; Shintani *et al.*, 2001; Wojakowski *et al.*, 2004]. These clinical events also cause a systemic inflammatory response, however it remains unknown whether inflammation occurring in this context is a physiological event that may potentiate EPC mobilisation, or an inhibitory pathological process detrimental to vascular repair. In this study, I have examined the behaviour of a wide variety of putative vascular progenitors in response to an inflammatory stimulus using a model of acute systemic inflammation in the absence of a discrete vascular injury. The study demonstrates a biphasic response to acute systemic inflammation, with early and late mobilisation of $CD14^+$ and $CD34^+$ cells respectively. However, specific vascular progenitor subpopulations including late outgrowth colony precursors, $CD45^-CD34^+$ progenitor cells, and even leukocyte derived EC-CFU were unaffected by systemic inflammation.

Whilst EC-CFU are not mature endothelial cells, they do have an established association with cardiovascular disease and it is likely that EC-CFU have a vasculoprotective role through the secretion of a variety of angiogenic factors [Rehman *et al.*, 2003]. EC-CFU are mobilised in response to discrete vascular injury such as that occurring during percutaneous coronary intervention in association with an inflammatory response [Mills *et al.*, 2009]. However, neither EC-CFU or putative EPC were affected by the systemic inflammatory environment generated by

Salmonella Typhus vaccination, indicating that EPC and EC-CFU mobilisation are phenomena relatively specific to vascular injury, and that inflammation *per se* does not directly mobilise these populations.

It should be recognised however that acute systemic inflammation induced by *Salmonella Typhus* vaccination does have deleterious effects on endothelial function. The model employed here has been shown to impair endothelium-dependent vasodilatation [Hingorani *et al.*, 2000], and furthermore that anti-inflammatory treatment ameliorates these adverse effects [Kharbanda *et al.*, 2002; Vlachopoulos *et al.*, 2005]. It would therefore appear that whilst the transient inflammatory response to *Salmonella Typhus* vaccination is sufficient to cause endothelial dysfunction, it is not sufficient to cause significant vascular denudation and stimulation of vascular progenitor cell mobilisation.

The interactions between EPC mobilisation and systemic inflammation are obscure. Both EC-CFU and late outgrowth EPC secrete pro-inflammatory cytokines such as tissue factor and monocyte chemo-attractant protein–1, a property potentiated by stimulation by other inflammatory mediators such tumour necrosis factor – alpha. This might suggest that EPC have a possible pro-inflammatory role [Zhang *et al.*, 2009], and indeed many inflammatory states are characterised by neovascularisation and increased capillary density. However several studies support a direct inhibitory effect of inflammation on EPC. Experimental *in-vitro* studies suggest that CRP down-regulates the production of angiogenic chemokines by EC-CFU and impairs EC-CFU migration toward VEGF [Suh *et al.*, 2004]. Mayr *et al* recently

reported a two-thirds reduction in the proportion of circulating EPC ($CD34^+CD133^+VEGFR\text{-}2^+$) and EC-CFU 4-6 hours following intravenous lipopolysaccharide (LPS) infusion [Mayr *et al.*, 2007]. The pro-inflammatory effects of LPS infusion results in a marked monocytopenia and lymphocytopenia, where as the inflammatory response to *Salmonella Typhus* vaccination in the present study involved a monocytosis and had little effect on circulating lymphocytes. Given that EC-CFU are derived from monocytic [Rehman *et al.*, 2003] and lymphocytic subpopulations [Hur *et al.*, 2007], it is quite possible that the reduction in EC-CFU in Mayr's study was determined principally by a profound mononuclear cell depletion. Mayr *et al.,* also reported a reduction in $CD34^+CD133^+VEGFR\text{-}2^+$ following LPS induced inflammation, which appears to contradict the findings of our present study. However, as $CD34^+CD133^+VEGFR\text{-}2^+$ concentrations were reported relative to the total leukocyte count, which was markedly increased due to a neutrophilia [Jilma, 1999] it is entirely possible that the absolute number of circulating progenitors was unchanged. Unfortunately, absolute numbers were not reported preventing definitive conclusions from this study and making it difficult to compare these findings directly with my own.

I interpret the mobilisation of CD34 and CD14 cells as a non-specific response to inflammatory mediators induced by *Salmonella Typhus* polysaccharide exposure. $CD14^+$ is a receptor for endotoxin predominantly expressed on circulating monocytes [Wright *et al.*, 1990]. The mobilisation of monocytes is part of an acute phase response and is common to a wide variety of inflammatory, infective and neoplastic conditions. CD34 functions as a regulator of cellular adhesion and

identifies a naïve population of cells widely regarded as having 'stem cell' capacity. Mobilisation of $CD34^+$ cells is similarly non-specific and occurs in response to a variety of inflammatory stimuli. Concordant with the findings of the present study, a delayed mobilisation of $CD34^+$ cells occurs in healthy volunteers treated with G-CSF [Sato *et al.*, 1994], and in patients following acute myocardial infarction [Shintani *et al.*, 2001].

Numerous factors responsible for EPC mobilisation, homing and recruitment into new or injured vessels have been identified [Tousoulis *et al.*, 2008]. Although current understanding of these processes remains incomplete, two cytokines, SDF-1 and VEGF-A are thought to be of particular importance to EPC mediated re-endothelialisation through stimulation of their cognate receptors: CXCR4 and VEGFR-2 respectively [Adams *et al.*, 2004; Asahara *et al.*, 1999; Grunewald *et al.*, 2006; Kalka *et al.*, 2000a; Seeger *et al.*, 2009; Yla-Herttuala *et al.*, 2007]. In the present study, *Salmonella Typhus* vaccination caused a significant increase in VEGF-A, however this alone was insufficient to mobilise putative EPC or EC-CFU. SDF-1 is thought to act down stream of VEGF in order to enhance the incorporation of EPC into sites of neo-endothelialisation [Grunewald *et al.*, 2006] and is increased in response to vascular injury [Schober *et al.*, 2003]. That SDF-1 and the concentration of those circulating cells expressing its receptor, CXCR-4 were unchanged following vaccination, supports the hypothesis that the necessary pathways for EPC mobilisation and homing are not solely activated by a non specific inflammatory stimulus.

Finally, the concentration of circulating $CD34^+$ populations fell over the six hour period following placebo, confirming a previously reported circadian variation of EPC concentrations [Thomas *et al.*, 2008b]. Interestingly this circadian variation of EPC was attenuated by acute systemic inflammation in our study, presumably through disruption of normal trafficking of cells to and from the bone marrow.

5.6 CONCLUSIONS

Acute systemic inflammation caused by *Salmonella Typhus* vaccination stimulates early and late mobilisation of $CD14^+$ and $CD34^+$ populations respectively. However, despite significant secretion of VEGF, systemic inflammation *per se* does not cause mobilisation of vascular progenitors including a surrogate measure of late outgrowth colonies. This would indicate that humoral factors specific to vascular injury are necessary for EPC mobilisation. It is likely that endothelial denudation or tissue ischaemia is necessary for this to occur.

CHAPTER SIX

ENDOTHELIAL PROGENITOR CELLS, ATHEROMA BURDEN, AND CLINICAL OUTCOME IN PATIENTS WITH CORONARY ARTERY DISEASE

Published by Padfield GJ, Tura O, Freyer E, Barclay GR,

Turner M, Newby DE & Mills NL,

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6.1 SUMMARY

Endothelial progenitor cells (EPC) are considered potential therapeutic targets in the treatment of patients with cardiovascular disease. I evaluated the relationship between putative EPC, atheroma burden, and clinical outcome. EPC populations were determined by flow cytometry and culture of circulating mononuclear cells in 201 patients undergoing coronary angiography for suspected angina or an acute coronary syndrome (ACS). Survival free from revascularisation, recurrent myocardial infarction, and death was determined at 3 years.

Circulating $CD34^+VEGFR\text{-}2^+$ and $CD34^+VEGFR\text{-}2^+CD133^+$ cells were rare (<0.009% of mononuclear cells), were not increased in patients with an ACS, and did not relate to atheroma burden or clinical outcome ($P>0.1$ for all). In contrast $CD34^+CD45^-$ cells were increased in patients with coronary artery disease compared to those with normal coronary arteries ($P=0.008$) and correlated positively with atheroma burden ($r=0.44$, $P<0.001$). Increased concentrations of circulating $CD34^+CD45^-$ cells were associated with a shorter cumulative event-free survival ($P<0.02$). Pro-angiogenic monocytes ($CD14^+VEGFR\text{-}2^+Tie\text{-}2^+$) and endothelial cell-colony forming units (EC-CFU) were increased in patients with an ACS ($P<0.01$ for both), however concentrations only reflected myocardial necrosis, and not the extent of coronary disease or clinical outcome.

Neither traditional EPC or EC-CFU were related to the extent of coronary artery disease or clinical outcome. However, $CD34^+CD45^-$ cells were increased in patients with coronary atheroma and predicted future cardiovascular events with concentrations reflecting the extent of vascular injury.

6.2 INTRODUCTION

Cell therapy offers the potential to accelerate neo-vascularisation and enhance vascular repair through the differentiation and proliferation of progenitors into functionally mature phenotypes. However, clinical trials testing the efficacy of intra-coronary or intra-myocardial delivery of progenitor cells or the implantation of stents designed to capture circulating progenitors have failed to deliver consistent clinical benefits. An incomplete understanding of the progenitor cell populations involved in vascular repair continues to limit progress in this area.

As described above, EPC have traditionally been defined on the basis of the co-expression of VEGFR-2 and the stem cell marker, CD34 [Cheng *et al.*, 1996; Shalaby *et al.*, 1995], and on occasion the co-expression of CD133 has also been used as evidence of cellular naivety [Peichev *et al.*, 2000; Yang *et al.*, 2004]. An inverse relationship between circulating $CD34^+VEGFR\text{-}2^+$ cells and cardiovascular outcome has been described [Schmidt-Lucke *et al.*, 2005; Vasa *et al.*, 2001b; Werner *et al.*, 2005], and $CD34^+$ [Shintani *et al.*, 2001], $CD34^+VEGFR\text{-}2^+$ and $CD133^+VEGFR\text{-}2^+$ [Friedrich *et al.*, 2006; Gill *et al.*, 2001] populations are mobilised following angiogenic stress. However, whilst these populations accelerate re-endothelialisation and attenuate neo-intimal hyperplasia in experimental models of vascular injury [Takamiya *et al.*, 2006; Walter *et al.*, 2002; Werner *et al.*, 2002], it should be recognised that cell preparations enriched for double positive $CD34^+VEGFR\text{-}2^+$, or triple positive $CD34^+VEGFR\text{-}2^+CD133^+$ cells have never been shown to differentiate into endothelial cells. Recent studies have identified non-haematopoietic $CD34^+$ subpopulations, negative for the pan-leukocyte marker CD45

($CD34^+CD45^-$), that are capable of forming late-outgrowth endothelial colonies. These cell types have robust proliferative potential and are morphologically indistinguishable from mature endothelial cells. The $CD34^+CD45^-$ population therefore is thought most likely to represent a true endothelial progenitor [Case *et al.*, 2007; Timmermans *et al.*, 2007; Yoder *et al.*, 2007].

Monocytes, identified by the surface expression of CD14, augment differentiation and proliferation of naïve progenitor cells, secrete pro-angiogenic factors [Rehman *et al.*, 2003], and can also adopt endothelial characteristics *in vitro* [Fernandez Pujol *et al.*, 2000]. In particular, angiogenic monocytes expressing the 'endothelial' surface receptors VEGFR-2 and Tie-2 accelerate re-endothelialisation with demonstrable improvements in endothelial function under experimental conditions of vascular injury [Elsheikh *et al.*, 2005; Nowak *et al.*, 2004], and facilitate neo-angiogenesis in the context of neoplasia [Venneri *et al.*, 2007]. Combined with a relative abundance in the peripheral circulation, such properties have made $CD14^+$ subpopulations attractive therapeutic targets in the field of cardiovascular medicine. Partly composed of monocytic fractions, the traditional early outgrowth EPC colony described by Hill *et al* [Hill *et al.*, 2003], although now recognised to be unable to form mature endothelial cells, is still considered to be relevant to cardiovascular repair via paracrine pro-angiogenic activity [Rehman *et al.*, 2003].

In order for cell therapy to become a reality, our understanding of the role of putative progenitor cells must be further developed. I therefore sought to clarify the relevance of circulating $CD34^+$ and $CD14^+$ sub-populations by examining their

behaviour in response to an acute coronary syndrome, and their relationship to the extent of coronary atherosclerosis and the occurrence of adverse clinical events.

6.3 METHODS

6.3.1 Subjects

The study was performed with the approval of the local research ethics committee in accordance with the Declaration of Helsinki, and the written informed consent of all volunteers. Patients were recruited following referral for coronary angiography for the investigation of suspected stable angina or following an acute coronary syndrome (ACS), defined as an increase in the plasma troponin concentration or evidence of myocardial ischemia (>1 mm ST deviation) on a 12-lead electrocardiogram in the context of anginal chest pain. Patients with significant co-morbid illness, hematological or internal malignancy, hepatic or renal failure or concurrent infection were excluded from the study. Clinical characteristics, cardiovascular risk factors, and medication during admission were documented.

6.3.2 Coronary angiography

All patients underwent diagnostic coronary angiography performed via the femoral or radial artery with 5-6F arterial catheters using standard angiographic projections. Patients with suspected angina were classified as having coronary artery disease based on the presence of at least one ≥ 50% stenosis of a major epicardial arterial segment. Coronary artery disease severity was graded using the Gensini scoring system [Gensini, 1983, Ringqvist *et al.*, 1983]. Patients underwent percutaneous coronary intervention at the discretion of the operator as described.

6.3.3 Blood sampling and assays

Prior to coronary angiography, peripheral venous blood was collected and anti-coagulated with EDTA (Sarstedt-Monovette, Germany) for flow cytometry and isolation of peripheral blood mononuclear cells. Whole blood was analysed for the differential leukocyte count using an autoanalyser (Sysmex, UK). Plasma troponin concentrations were measured in the regional clinical reference laboratory using the Abbott Architect *STAT* troponin I assay (Abbott Laboratories, Abbott Park, IL).

6.3.4 Flow cytometric identification of EPC

Endothelial progenitor cells were characterised phenotypically using flow cytometry as described above in the methods section. Briefly, cells were directly stained and analysed for phenotypic expression of surface proteins using pre-conjugated anti-human monoclonal antibodies. Unstained samples were used to provide negative controls and to establish positive stain boundaries. Flow-cytometric analysis was performed as described above. Approximately 500,000 events were acquired in the leukocyte gate for each sample. The absolute concentration of cells per mL of blood was calculated by equating the total number of events in the leukocyte gate to the total leukocyte count from the full blood count.

6.3.5 Endothelial cell - colony forming units

Endothelial cell-colony forming units (EC-CFU) [Hill *et al.*, 2003] were generated as described above (Section 2.6.1) and counted in a minimum of four wells on day 5.

6.3.6 Clinical outcomes

Clinical outcomes were obtained through review of medical records and using the TrakCare software application (InterSystems Corporation, Cambridge, MA, USA); an electronic patient record system used by the Acute Hospitals Division of Lothian National Health Service (NHS) Health Board, United Kingdom. Myocardial infarction was defined as admission with chest pain or ST-segment deviation of ≥0.5 mm with evidence of myocardial necrosis using plasma troponin concentrations of ≥0.2 ng/mL as the diagnostic threshold [Mills *et al.,* 2011]. Revascularisation included all percutaneous and surgical coronary artery procedures. Hospitalisation for cardiovascular causes included any unplanned hospitalisation for acute coronary syndrome, heart failure, stroke or uncontrolled arrhythmia. Major adverse clinical events were a composite of cardiovascular death, recurrent myocardial infarction, coronary revascularisation or cardiovascular hospitalisation.

6.3.7 Data analysis and statistics

At a significance level of 5% and based on power calculations derived from previous studies [Mills *et al.*, 2009; Padfield *et al.*, 2010b], we estimated that a sample size of n=90 would give 80% power of detecting a putative clinically meaningful 15% difference in phenotypic EPC ($CD34^+VEGFR\text{-}2^+$) between patients with stable angina and ACS. A sample size of n=90 per group will give 95% power, at a 5% significance level, to detect meaningful correlations ($r^2>0.12$) between measures of EPC number and clinical variables including Gensini score. Flow cytometric analyses, EC-CFU enumeration, and Gensini scoring were performed by an observer

blinded to the patient's clinical profiles. Statistical analyses were performed with SPSS version 17 (SPSS Inc, Chicago, USA). Continuous variables are reported as mean ± standard error or median (inter-quartile range) where appropriate. Student's *t*-test, Mann-Whitney tests and Pearson's Chi-Square tests were used for comparisons between groups where appropriate. Spearman's test was used to test for correlation analysis between variables. For partial correlation analyses controlling for covariates, data were normalised where appropriate. Cell populations were categorised into tertiles after natural logarithmic transformation in order to evaluate associations between circulating progenitors and clinical endpoints. Multivariate Cox-regression analysis was performed to determine associations between progenitor cells and event-free survival with adjustment for the diagnosis of ACS on enrollment and cardiovascular risk factors. Hazard ratios represent the predicted change in the hazard between the lowest and the highest tertiles. Statistical significance was taken at a two-sided P value <0.05.

6.4 RESULTS

6.4.1 Study population

I enrolled 201 patients undergoing coronary angiography for the investigation of suspected stable angina (n=90) or ACS (n=111) to the study (Table 6.1).

Table 6.1: Demographic, clinical and angiographic characteristics

	All patients (n=201)	ACS (n=111)	Stable angina (n =90)	P value
Demographics				
Age, years	61 (11)	60 (11)	63 (11)	0.10
Male gender, (%)	168 (83.6)	96 (86.5)	72 (80.0)	0.22
Body mass index, kg/m^2	28.7 (0.4)	28.6 (0.6)	28.9 (0.6)	0.69
Clinical characteristics				
Diabetic, (%)	30 (14.9)	16 (14.4)	14 (15.6)	0.82
Ever smoked, (%)	133 (66.2)	77 (69.4)	56 (62.2)	0.29
Hypertension, (%)	105 (52.2)	46 (41.4)	59 (65.6)	0.001
Family history of CHD, (%)	84 (41.8)	43 (38.7)	40 (44.4)	0.41
Hyperlipidaemia, (%)	151 (75.1)	73 (65.8)	78 (86.7)	0.001
Peripheral vascular disease, (%)	15 (7.5)	6 (5.4)	9 (10.0)	0.22
Prior myocardial infarction, (%)	39 (19.4)	21 (18.9)	18 (20.0)	0.85
Cerebrovascular disease, (%)	10 (5.0)	5 (4.5)	5 (5.6)	0.73
LVSD, (%)	21 (10.4)	11 (9.9)	10 (11.1)	0.78
Prior PCI, (%)	45 (22.4)	21 (18.9)	24 (27.0)	0.18
Prior CABG, (%)	14 (7.0)	6 (5.5)	8 (8.9)	0.34
Biochemistry				
Troponin I, ng/mL	-	0.42 (0.2-3.3)	<0.2	0.001
Creatinine, mg/dL	1.04 (0.02)	1.05 (0.02)	1.04 (0.02)	0.53
Cholesterol, mg/dL	174 (4)	177 (4)	167 (4)	0.10
HbA1c, (%)	5.8 (5.5-6)	5.7 (5.4-6)	5.9 (5.6-6.1)	0.005
Angiographic findings				
Coronary segment score	6.6 (0.3)	6.8 (0.3)	6.2 (0.4)	0.21
Gensini score, units	20 (8.5-42)	21 (11-48)	17 (5-35)	0.052

Continuous variables are expressed as the mean (±standard error) or median (interquartile range) with statistical comparisons performed using Students t-test or Mann-Whitney where appropriate; Categorical variables are expressed as the absolute number of cases (% of group) with statistical comparisons performed using a Chi-square; ACS = acute coronary syndrome; CABG = coronary artery bypass grafting; LVSD = left ventricular systolic dysfunction.

Patients were predominantly male (84%) with a mean age of 61±11 years. Both groups were well matched for age, gender, cigarette smoking, diabetes mellitus, previous revascularisation and renal function (P>0.1 for all). Therapy for cardiovascular risk factors and for the treatment of acute coronary syndrome differed slightly between groups (Table 6.2). The Gensini score was greater in patients with an ACS than those with suspected stable angina (21 (11-48) *versus* 17 (5-35); P=0.052).

Table 6.2. Medical therapy of the study population

Medication	ACS (n=111)	Stable angina (n=90)	P value
Aspirin	107 (97)	86 (96)	0.51
Clopidogrel	100 (91)	54 (60)	0.0001
LMWH	78 (71)	3 (3)	0.0001
Warfarin	2 (2)	1 (1)	0.7
Beta-blocker	81 (74)	71 (79)	0.39
ACE inhibitor	64 (58)	38 (42)	0.03
ARB	7 (6)	6 (7)	0.92
Statin	103 (94)	84 (93)	0.93
Nicorandil	9 (8)	13 (14)	0.16
Long acting nitrate	20 (18)	21 (23)	0.37
Ca-channel antagonist	20 (18)	30 (33)	0.01
Diuretic	12 (11)	25 (28)	0.002
Insulin	0 (0)	1 (1)	0.27
Metformin	6 (5)	5 (6)	0.96
Sulphonylurea	7 (6)	3 (3)	0.57
PPI	25 (23)	25 (28)	0.39

Data are expressed as the absolute number of cases (%) with statistical comparisons performed using a Chi-squared test. LMWH = low-molecular weight heparin; ACE = angiotensin converting enzyme; ARB = angiotensin receptor blocker. PPI = proton pump inhibitor. Statistical significance is taken at a P value of <0.05.

Patients undergoing elective angiography were further classified as having coronary heart disease (n=70) based on the presence of at least 1 ≥ 50% stenosis of a major epicardial arterial segment. Patients with an ACS were further classified as having unstable angina (n=39) or acute myocardial infarction (n=72) based on the plasma

troponin concentration. The median plasma troponin concentration in patients with ACS was 0.42 (0.20-3.3) μg/

6.4.2 Progenitor cell populations

Progenitor populations were determined on the day of admission in elective patients undergoing angiography, and 3 (1-5) days following hospital admission in patients with ACS (Table 6.3).

$CD34^+$ progenitor cells

$CD34^+$ cells were readily detectable in the peripheral circulation and were increased in patients following an ACS compared to those with suspected stable angina (3.44 (2.54-4.85) *versus* 2.84 (2.15-4.18) $x10^6$ cells/L; P=0.04). The majority of circulating $CD34^+$ cells were $CD45^+$, and the proportion of $CD45^+$ cells did not differ between groups. The less abundant $CD34^+CD45^-$ population was increased in patients with ACS compared to patients with stable angina (1.05 (0.70-1.64) *versus* 0.90 (0.55-1.30) $x10^6$ cells/L; P=0.02) (Table 6.3). The concentration of $CD34^+CD45^-$ cells was increased in patients with both stable and unstable coronary disease compared to patients with normal coronary arteries (ANOVA, P=0.008). The presence of myocyte necrosis did not influence $CD45^-CD34^+$ concentrations (P=0.71; Figure 6.1). When patients with an ACS were further categorised according to the time from the onset of symptoms to enrolment in the study, the $CD34^+CD45^-$ concentration remained constant. (P=0.72; Figure 6.2). Regardless of CD45 expression,

$CD34^+VEGFR\text{-}2^+$ and $CD34^+VEGFR\text{-}2^+CD133^+$ cells were rare and frequently undetectable in both

Table 6.3. Selected putative progenitor population frequencies

	ACS	Stable angina	P value
$CD45^\pm$			
$CD34^+$	3.44 (2.54-4.85)	2.84 (2.15-4.18)	0.04
$CD34^+VEGFR\text{-}2^+$	0.13 (0.07-0.27)	0.10 (0.06-0.18)	0.06
$CD34^+VEGFR\text{-}2^+CD133^+$	0.05 (0.02-0.12)	0.05 (0.03-0.09)	0.33
$CD34^+CD133^+$	1.26 (0.85-1.79)	1.08 (0.69-1.81)	0.14
$CD133^+$	1.81 (1.34-2.68)	1.70 (1.19-2.36)	0.17
$CD133^+VEGFR\text{-}2^+$	0.15 (0.08-0.27)	0.12 (0.07-0.22)	0.15
$CD45^+$			
$CD34^+$	2.18 (1.5-2.96)	1.9 (1.34-2.71)	0.16
$CD34^+VEGFR\text{-}2^+$	0.09 (0.04-0.2)	0.07 (0.04-0.14)	0.11
$CD34^+VEGFR\text{-}2^+CD133^+$	0.05 (0.02-0.1)	0.04 (0.02-0.08)	0.37
$CD34^+CD133^+$	1.17 (0.73-1.68)	0.98 (0.65-1.58)	0.25
$CD133^+$	1.74 (1.22-2.58)	1.59 (1.05-2.19)	0.19
$CD133^+VEGFR\text{-}2^+$	0.13 (0.07-0.25)	0.11 (0.06-0.21)	0.18
CD45-			
$CD34^+$	1.05 (0.7-1.64)	0.9 (0.55-1.3) *	0.02
$CD34^+VEGFR\text{-}2^+$	0.03 (0-0.06)	0.02 (0-0.05)	0.29
$CD34^+VEGFR\text{-}2^+CD133^+$	0 (0-0)	0 (0-0)	0.74
$CD34^+CD133^+$	0.06 (0.01-0.12)	0.06 (0.01-0.13)	0.89
$CD133^+$	0.08 (0.03-0.14)	0.07 (0.03-0.15)	0.69
$CD133^+VEGFR\text{-}2^+$	0 (0-0.02)	0 (0-0.02)	0.61
$CD14^+$			
$CD14^+$	474.4 (384.1-591)	457 (356-547.2)	0.29
$CD14^+VEGFR\text{-}2^+$	74.8 (47.1-136.9)	53.9 (30.6-103.1) *	0.02
$CD14^+Tie\ 2^+VEGFR\text{-}2^+$	9 (3.9-20.5)	4.5 (2.1-8.8) **	0.003
EC-CFU	11.5 (3.5-27.0)	6.3 (2.0-16.5) **	0.005

Values are medians and interquartile range. $CD34^+$ and $CD14^+$ populations are cell concentration $x10^6$/L. EC-CFU are expressed as the number of colonies formed per 800,000 mononuclear cells plated. Statistical comparisons are made using a Mann-Whitney test with significance at the 0.05* and 0.01** level (2-tailed). VEGFR-2 = vascular endothelial growth factor receptor – 2

groups and were not increased in patients with an ACS compared to those with undergoing elective angiography (0.13 (0.07-0.27) *versus* 0.10 (0.06-0.18) x10^6 cells/L, P=0.06, and 0.05 (0.02-0.12) *versus* 0.05 (0.03-0.09) x10^6 cells/L, P=0.33 respectively; Table 6.2, Figure 6.1).

$CD14^+$ pro-angiogenic monocytes

$CD14^+$ subpopulations were determined in a subgroup of 119 patients with a similar clinical profile to the main cohort (table 6.4): 50 patients with suspected stable angina and 69 patients with ACS. The concentration of circulating $CD14^+$ cells was not different in patients with ACS compared to elective group (474 (384-591) *versus* 457 (356-547) x10^6 cells/L; P=0.29). However there was an increase in $CD14^+VEGFR\text{-}2^+$ (74.8 (47.1-136.9) *versus* 53.9 (30.6-103.1) x10^6 cells/L; P=0.02) and $CD14^+VEGFR\text{-}2^+Tie\text{-}2^+$ cells (9.0 (3.9-20.5) *versus* 4.5 (2.1-8.8) x10^6 cells/L; P=0.003) in patients with an ACS (Table 6.3). Concentrations of circulating angiogenic monocytes in patients with ACS were higher in patients with myocardial infarction compared to patients with stable angina or normal coronary arteries (ANOVA, P=0.015; Figure 6.1). The $CD14^+VEGFR\text{-}2^+Tie\text{-}2^+$ concentration was highest in patients recruited on the same day as the onset of symptoms, falling progressively to concentrations comparable to patients with stable angina after ~ five days (P=0.025; Figure 6.2).

EC-CFU colonies

EC-CFU colonies expressed a variety of endothelial and haematopoietic characteristics as described above and were increased in patients with ACS

compared to patients with suspected stable angina (12 (4-27) *versus* 6 (2-17); P=0.005; Table 6.3). Differences were determined by higher concentrations in those patients with myocardial infarction compared to patients with stable angina or normal coronary arteries (ANOVA, P=0.018; Figure 6.2).

6.4.3 Clinical correlates

In patients undergoing elective angiography for suspected stable angina, there was a positive correlation between $CD34^+CD45^-$ cells and the Gensini score (R=0.44; P<0.0001; Table 6.5). In contrast, $CD45^+$ cells expressing VEGFR-2^+, $CD133^+$ and VEGFR-2^+CD133^+ did not correlate with coronary artery disease severity (P>0.1 for all). The correlation between $CD34^+CD45^-$ cells and extent of coronary atheroma persisted after controlling for cardiovascular risk factors and the use of cardiac medication (r=0.35; P=0.04). Similarly, when patients were stratified into quartiles according to Gensini score, $CD34^+CD45^-$ concentrations increased in a step-wise fashion with each increment of severity of coronary artery disease (Median $CD34^+CD45^-$ concentrations were 0.75 (0.45-1.13), 0.91 (0.58-1.52), 1.01 (0.71-1.53) and 1.34 (0.76-1.59) $x10^6$ cells/L, given Gensini scores of <9, 9-20, 21-42 and >43 respectively; ANOVA = 0.013; Figure 6.3). Circulating $CD14^+$ cells did not correlate with coronary artery disease severity (r=0.221; P=0.08). $CD14^+$VEGFR-2^+ cells correlated weakly with CAD severity (r=0.29; P=0.03), but not after adjusting for cardiovascular risk factors (r=0.15; P=0.39).

6.4.4 Clinical events

Patients were followed up for approximately three years: median 1,068 (970 – 1145) days with no loss to follow up. During this period 123 patients underwent coronary revascularisation (64%), with 28 patients undergoing coronary artery bypass grafting (15%) and 95 patients percutaneous coronary intervention (49%). In 57 patients (30%) revascularisation was performed ad-hoc following the initial diagnostic angiogram. Six patients died (3%), five from cardiovascular causes and one from lung cancer. Fifty-seven patients were hospitalised for a cardiovascular event (30%), 15 patients had a recurrent myocardial infarction (7%), and a first major adverse cardiovascular event occurred in 74 patients (35%) during the follow up period.

The concentration of $CD34^+CD45^-$ cells was the only population that predicted clinical events. There were no differences in event free survival according to concentrations of circulating $CD45^+CD34^+$, $CD34^+VEGFR\text{-}2^+$, $CD34^+VEGFR\text{-}2^+133^+$, $CD14^+VEGFR\text{-}2^+$ or $CD14^+VEGFR\text{-}2^+Tie\text{-}2^+$ cells ($P > 0.3$ for all). The $CD34^+CD45^-$ cell concentration was significantly higher in patients with recurrent myocardial infarction (1.40 (0.88-1.64) *versus* 0.96 (0.62-1.44) $x10^6$ cells/L; P=0.023), undergoing coronary revascularisation (1.05 (0.75-1.53) *versus* 0.77 (0.47-1.27) $x10^6$ cells/L; P=0.003) or with a first major adverse coronary event (1.04 (0.7-1.53) *versus* 0.87 (0.45-1.27) $x10^6$ cells/L; P=0.010) during the follow up period. There were no differences in $CD34^+CD45^-$ cell concentration with respect to death or hospitalisation (P>0.05 for all).

Patients were stratified into tertiles according to the concentration of $CD34^+CD45^-$ cells (Table 6.6). Baseline characteristics of the patients in each tertile were similar with the exception of higher Gensini score in those patients with the highest $CD34^+CD45^-$ cell concentrations (P=0.001). Event rates were lower in patients in the lowest tertile compared to the highest tertile: for myocardial infarction hazard ratio (HR) 0.20 (95% confidence intervals (CI) 0.06 to 0.67, P=0.009); for revascularisation HR 0.47 (95% CI 0.29 to 0.877, P=0.003); and for major adverse cardiovascular events HR 0.50 (95% CI 0.27 to 0.92, P=0.026). In patients with higher circulating $CD34^+CD45^-$ cell concentrations, the cumulative event-free survival was lower for coronary revascularisation (P=0.01), recurrent myocardial infarction (P=0.02), or any major adverse cardiovascular event (P=0.02). In a cox-regression analysis correcting for covariates, these associations remained significant for all covariates with the exception of the Gensini score (Table 6.7).

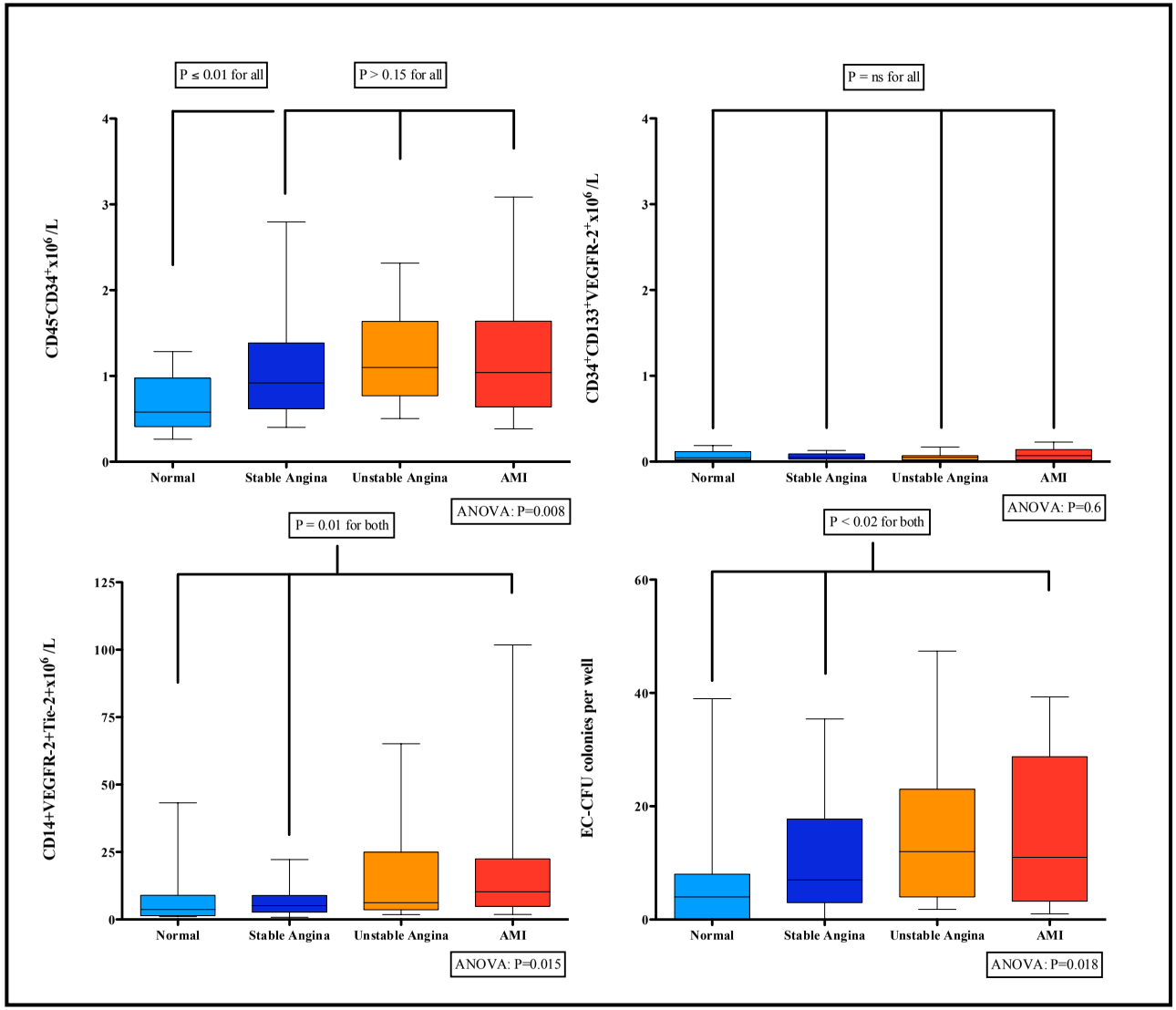

Figure 6.1 Putative progenitor frequencies according to clinical presentation, and the presence of coronary artery disease severity or myocyte necrosis: $CD34^+CD45^-$ concentration was significantly lower only in those patients undergoing elective angiography with normal coronary arteries. In those patients with at least 1 >50% stenosis affecting a major epicardial segment the $CD34^+CD45^-$ concentration was similar regardless of presentation or troponin concentration (A). Triple positive $CD34^+VEGFR\text{-}2^+CD133^+$ cells were extremely rare and were similar in all groups (B). Angiogenic monocytes $CD14^+VEGFR\text{-}2^+Tie\text{-}2^+$ and EC-CFU were increased in unstable patients with evidence of myocyte necrosis (C & D respectively). Data are expressed as the median concentration ± the interquartile distance. Error bars are the 10^{th} - 90^{th} centiles

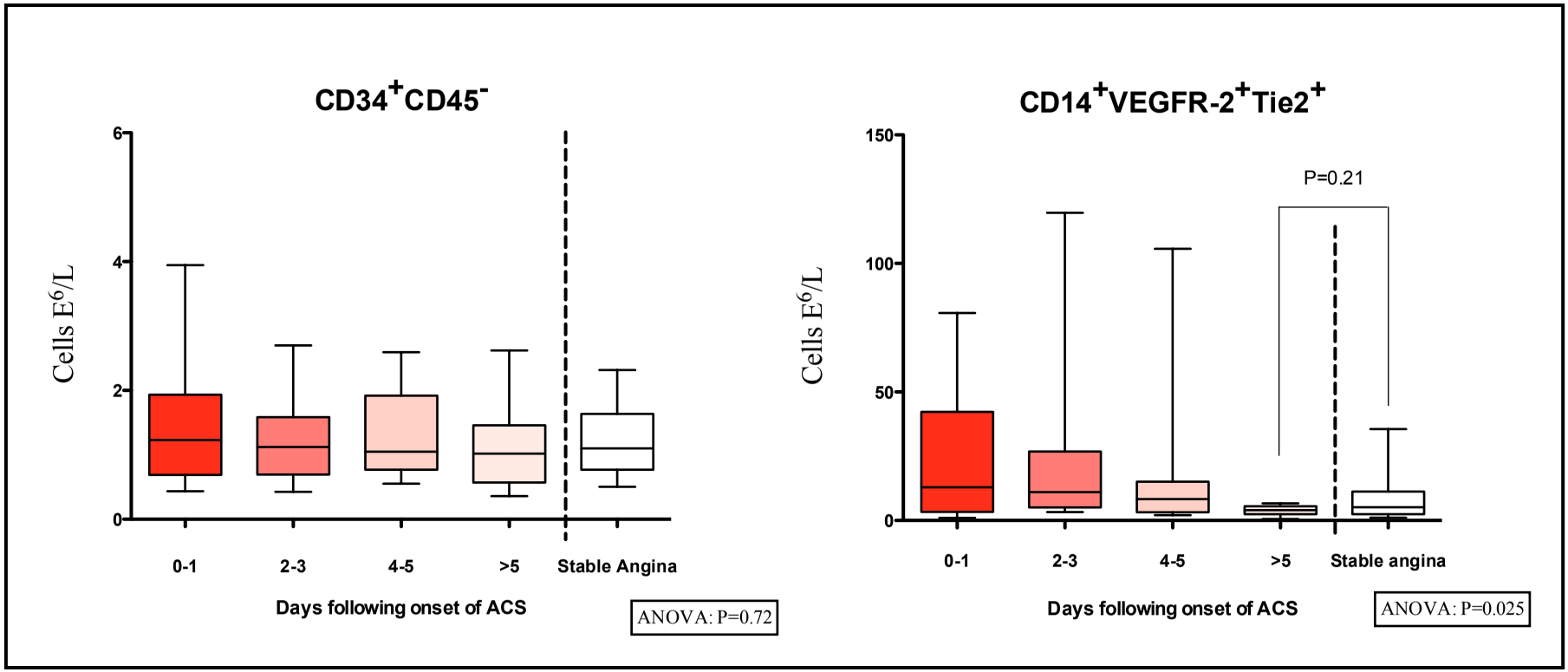

Figure 6.2: Differential behaviour in response to acute vascular stress by $CD34^+CD45^-$ and $CD14^+VEGFR\text{-}2^+Tie\text{-}2^+$ cells.
Patients were stratified according to the time from the onset of symptoms to enrolment into the study. $CD34^+CD45^-$ cell concentrations were not affected by the presence of acute vascular injury as concentrations were similar regardless of the time from the onset of symptoms. Angiogenic monocyte concentrations however were highest in patients in whom cell levels were determined soon after the onset of symptoms, and showed a time dependent decay, falling to levels comparable to those found in patients undergoing elective angiography after day 5. These findings indicate that angiogenic monocytes are mobilized acutely in response to an acute coronary syndrome. Data are expressed as the median concentration ± the interquartile distance with error bars between the 10^{th} and 90^{th} centiles.

Table 6.4. Demographic, clinical and angiographic characteristics of CD14 group

Characteristic	ACS (n=69)	Stable angina (N=50)	P value
Age, years	56 (1)	56 (1)	0.81
Male gender, (%)	59 (86)	34 (68)	0.23
Body mass index, kg/m^2	28 (1)	29 (1)	0.33
Cardiovascular risk factors and medical history			
Diabetic, (%)	10 (14)	8 (16)	0.82
Never smoked, (%)	46 (67)	30 (60)	0.46
Hypertension, (%)	27 (39)	33 (66)	0.004
Family history of CHD, (%)	24 (35)	24 (48)	0.15
Hyperlipidaemia, (%)	45 (65)	42 (84)	0.23
Peripheral vascular disease, (%)	3 (4)	5 (10)	0.22
Prior myocardial infarction, (%)	14 (20)	7 (14)	0.38
Cerebrovascular disease, (%)	3 (4)	2 (8)	0.93
Left ventricular dysfunction, (%)	6 (9)	3 (6)	0.58
Prior PCI, (%)	13 (19)	12 (24)	0.46
Prior CABG, (%)	4 (6)	3 (6)	0.98
Biochemical and angiographic parameters			
Troponin I concentration, ng/mL	0.36 (0.2 - 2.2)	<0.2	<0.0001
Creatinine concentration, mg/dL	1.04 (0.03)	1.01 (0.04)	0.36
Cholesterol, mg/dL	182 (5)	173 (7)	0.36
HbA1c, (%)	5.7 (5.4 - 6)	6 (5.7 - 6.2)	0.01
No. of diseased coronary segments	6.8 (0.4)	5.1 (0.5)	0.01
Gensini Score, units	23 (13.5 - 45)	11.5 (1 - 24.5	0.0002
Medical therapy			
Aspirin	67 (97)	47 (94)	0.41
Clopidogrel	62 (89)	30 (60)	<0.001
LMWH	47 (68)	1 (2)	<0.001
Warfarin	1 (1)	0 (0)	0.39
Beta-blocker	51(74)	39 (78)	0.61
ACE inhibitor	42 (61)	18 (36)	0.01
ARB	2 (3)	5 (10)	0.1
Statin	66 (96)	46 (92)	0.4
Nicorandil	7 (10)	9 (0.18)	0.22
Long acting nitrate	14 (20)	13 (26)	0.46
Ca-channel antagonist	11 (16)	19 (38)	0.01
Diuretic	8 (12)	12 (24)	0.07
Insulin	0 (0)	1 (2)	0.24
Metformin	1 (1)	2 (4)	0.38
Sulphonylurea	3 (4)	1 (2)	0.76
Proton pump inhibitor	16 (23)	18 (36)	0.13

Categorical data are expressed as the absolute number of cases (%) with statistical comparisons performed using a Chi-squared test. Continuous variables expressed as the mean (±standard error) or median (interquartile range) with comparisons performed using Students t-test or Mann-Whitney where appropriate. Statistical significance is taken at a two sided P value of <0.05.

Table 6.5. Bi-variate and partial correlation analysis between $CD34^+CD45^-$ and coronary atheroma burden in patients undergoing elective angiography.

	Correlation R	P value	Adjusted P value
$CD45^+$			
$CD34^+$	0.09	0.40	-
$CD34^+VEGFR\text{-}2^+$	0.16	0.14	-
$CD34^+VEGFR\text{-}2^+CD133^+$	0.08	0.48	-
$CD34^+CD133^+$	0.06	0.57	-
$CD133^+$	0.06	0.60	-
$CD133^+VEGFR\text{-}2^+$	0.05	0.65	-
$CD45^-$			
$CD34^+$	0.44**	0.0002	0.04
$CD34^+VEGFR\text{-}2^+$	0.08	0.45	-
$CD34^+VEGFR\text{-}2^+CD133^+$	0.06	0.55	-
$CD34^+CD133^+$	0.12	0.26	-
$CD133^+$	0.12	0.28	-
$CD133^+VEGFR\text{-}2^+$	0.08	0.44	-
$CD14^+$			
$CD14^+$	0.22	0.09	-
$CD14^+VEGFR\text{-}2^+$	0.29*	0.03	0.39
$CD14^+VEGFR\text{-}2^+Tie^-2^+$	0.23	0.10	-
EC-CFU	0.07	0.53	-

Partial correlations made between normalised putative EPC concentration and the Gensini score corrected for age, gender, BMI, diabetes and smoking status, creatinine, total cholesterol and HbA1c. Correlations are significant at the <0.05* and <0.01** level (2-tailed). VEGFR-2 = vascular endothelial growth factor receptor – 2, EC-CFU = Endothelial cell – colony forming unit.

Table 6.6. Demographic, clinical and angiographic characteristics of the study population stratified by $CD34^+CD45^-$ concentration

	Total n=193	Low n=65	Medium n=64	High n=64	P value
Demographics					
Age, years	61±11	62±10	61±11	62±11	0.89
Male gender, n (%)	162 (84)	55 (85)	51 (80)	56 (88)	0.48
Body mass index, kg/m^2	29±6	29±6	29±6	28±6	0.52
Cardiovascular risk factors and medical history					
Diabetic, n (%)	27 (14)	10 (15)	8 (13)	9 (14)	0.89
Never smoked, n (%)	67 (35)	21 (32)	27 (42)	26 (41)	0.37
Hypertension, n (%)	100 (52)	36 (55)	30 (47)	30 (47)	0.61
Family history of CHD, n (%)	80 (42)	28 (43)	29 (45)	23 (36)	0.53
Hyperlipidaemia, n (%)	145 (75)	52 (80)	44 (69)	49 (77)	0.32
PVD, n (%)	15 (8)	5 (8)	0 (0)	10 (16)	0.004
Prior MI (%)	37 (19)	13 (20)	9 (14)	15 (23)	0.40
AMI, n (%)	107 (55)	30 (46)	36 (56)	41 (64)	0.12
CVD, n (%)	19 (5)	3 (4.6)	1 (2)	5 (8)	0.25
LVSD, n (%)	21 (11)	7 (11)	5 (8)	9 (14)	0.53
Prior PCI, n (%)	43 (22)	12 (19)	15 (24)	16 (25)	0.64
Prior CABG, n (%)	14 (7)	7 (11)	1 (2)	6 (10)	0.09
Biochemical and angiographic parameters					
Troponin I, ng/mL	2.1±6.8	0.2 (0.2-0.5)	0.2(0.2-0.4)	0.2(0.2-1.1)	0.45
Creatinine, mg/dL	1.67±0.36	1.60±0.28	1.61±0.34	1.75±0.37	0.04
Cholesterol, mg/dL	174±43	170±43	170±43	174±4.3	0.95
HbA1c, %	6.0±0.9	6.1±1.1	5.9±1.0	5.9±0.6	0.38
No. of diseased coronary segments	6.6±0.4	6.4±0.5	5.9±0.4	7.4±0.4	0.03
Gensini Score, units	20 (8-43)	11 (4 – 27)	19 (12-32)	30 (13-73)	0.001
Medical therapy					
Aspirin, n (%)	186 (96)	64 (99)	64 (100)	60 (94)	0.35
Clopidogrel, n (%)	150 (78)	44 (68)	56 (88)	50 (78)	0.03
LMWH, n (%)	79 (41)	22 (34)	30 (47)	27 (42)	0.31
Warfarin, n (%)	3 (2)	1 (2)	2 (3)	1 (2)	1.0
Beta-blocker, n (%)	148 (77)	54 (83)	47 (73)	47 (73)	0.33
ACE inhibitor, n (%)	98 (51)	33 (51)	31 (49)	34 (53)	0.87
ARB, n (%)	13 (7)	8 (12)	2 (3)	3 (5)	0.09
Statin, n (%)	172 (89)	61 (94)	58 (91)	61 (95)	0.56
Nicorandil, n (%)	21 (11)	4 (6)	6 (9)	12 (19)	0.05
Long acting nitrate, n (%)	38 (20)	11 (17)	10 (16)	17 (27)	0.24
Ca-channel blocker, n (%)	47 (24)	16 (25)	13 (20)	18 (28)	0.59
Diuretic, n (%)	35 (18)	13 (20)	7 (11)	15 (23)	0.17
Insulin, n (%)	1 (1)	1 (2)	0 (0)	0 (0)	0.37
Metformin, n (%)	11 (6)	4 (6)	3 (5)	4 (6)	0.91
Proton pump inhibitor, n (%)	48 (25)	19 (29)	15 (23)	14 (22)	0.60

Patients are grouped into tertiles according to the $CD34^+CD45^-$ concentration. Continuous variables are expressed as the mean (standard error) or median (interquartile range) with statistical comparisons between groups performed using one-way analysis of variance. Categorical variables are expressed as the absolute number of cases (%) with statistical comparisons performed using a Chi-squared test.

Table 6.7: Survival according $CD34^{+}CD45^{-}$ concentration.

Cardiovascular event	Total n=193 (%)	Low n=65 (%)	Medium n=64 (%)	High n=64 (%)	Log rank (P value)	P adjusted for Gensini
All cause mortality	6 (3.1)	1 (1.5)	3 (4.7)	2 (1.6)	0.57	0.42
Recurrent MI	15 (7.3)	1 (1.5)	4 (6.3)	10 (15.6)	0.02	0.18
Revascularisation excluding index event	66 (34.2)	17 (26.2)	24 (37.5)	25 (39.1)	0.03	0.05
All revascularisation procedures	123 (63.7)	31 (47.7)	45 (70.3)	47 (73.4)	0.01	0.63
Cardiovascular hospitalisation	57 (29.5)	19 (29.2)	20 (31.3)	18 (28.1)	0.87	0.35
MACE	74 (35.2)	23 (35.4)	26 (40.6)	25 (39.1)	0.02	0.07

Adverse event rate at a median follow up of 1068 (970-1145) days. Group 1 includes patients with a natural log number ranging from -8.50 to -7.20, group 2; -7.18 to -6.67 and group 3 -6.67 to -5.31. A multivariate cox-regression analysis was performed to determine the association between cell counts and event-free survival, adjusting for age; sex; a diagnosis of an acute coronary syndrome at the time of enrollment; smoking status; a history of hypertension, diabetes, or hyperlipidemia; known left ventricular dysfunction; prior percutanous coronary intervention; the severity of coronary artery disease determined by the Gensini score; and medical therapy. Hospitalisation was due to cardiovascular events, recurrent MI, revascularisation, uncontrolled arrhythmia or heart failure. MI = myocardial infarction,; MACE = major adverse cardiovascular event.

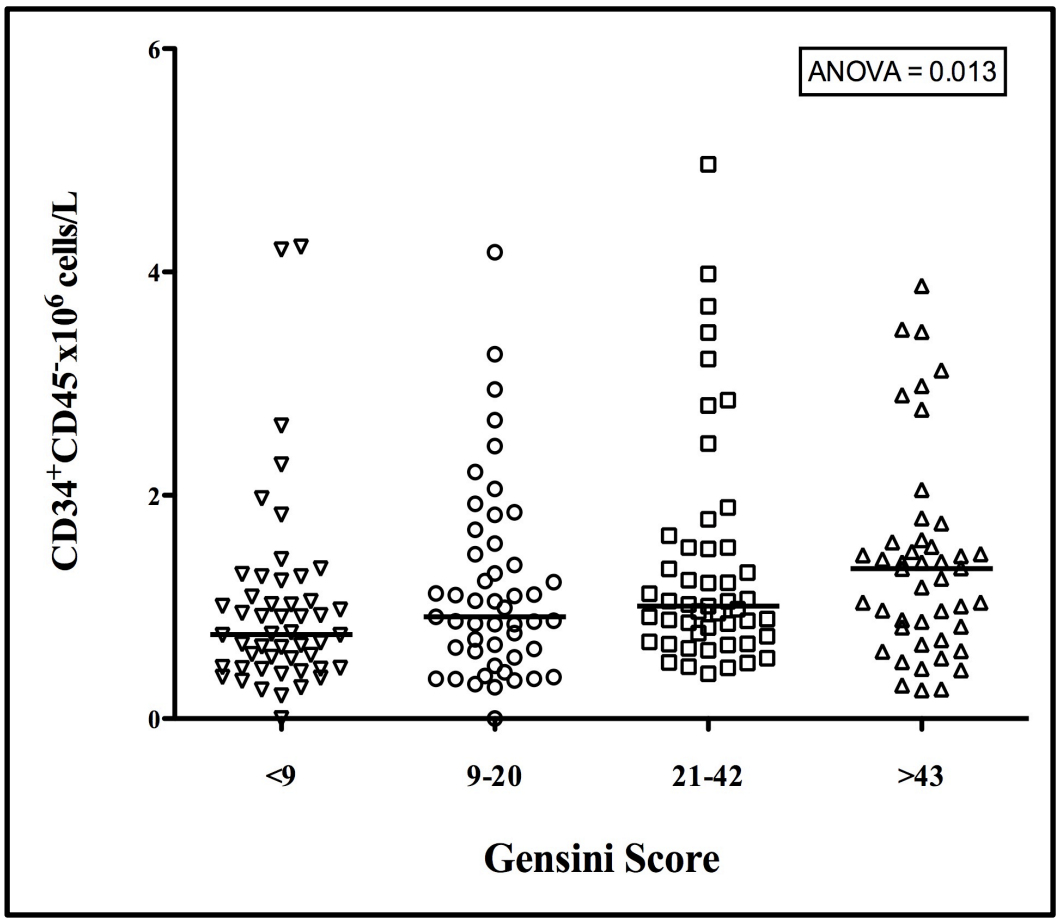

Figure 6.3. $CD34^+CD45^-$ concentration according to Gensini score
Patients were stratified into quartiles according to Gensini score. Median $CD34^+CD45^-$ concentrations fell in a step-wise fashion with each decrement in the severity of coronary artery disease (ANOVA P=0.013).

6.5 DISCUSSION

In 201 patients with coronary artery disease, I have evaluated a comprehensive range of cellular markers that have been used to identify circulating putative EPC. Traditional EPC populations determined by phenotype ($CD34^+$ $VEGFR\text{-}2^+$) or cell culture (EC-CFU) are not related to the extent of coronary artery disease or clinical outcome, but do appear to be increased in patients who have sustained a myocardial infarction. In contrast, it is the $CD34^+CD45^-$ cells that are increased in patients with coronary atheroma and predict future cardiovascular events, suggesting that they represent a measure of on going chronic vascular injury and repair.

Previous studies have demonstrated that $CD34^+$ cells are mobilised following acute myocardial infarction with the peak in concentration varying from 24 hours to a week following the onset of symptoms [Brehm *et al.*, 2009; Massa *et al.*, 2005; Shintani *et al.*, 2001; Spevack *et al.*, 2006; Turan *et al.*, 2007; Wojakowski *et al.*, 2004]. Similarly, I have observed a higher $CD34^+$ count in patients with an acute coronary syndrome, determined wholly by an elevated $CD34^+CD45^-$ concentration in the ACS group. However, in elective patients, where circulating concentrations did not reflect the dynamic response to acute vascular injury or myocardial ischemia, I detected a significant positive correlation between the $CD34^+CD45^-$ concentration and the severity of coronary atheroma. The difference in the $CD34^+CD45^-$ count between the stable and unstable patients was in fact driven by the proportion of

patients in the elective group with normal coronary arteries who had significantly lower circulating $CD34^+CD45^-$ concentrations. Indeed, amongst patients with angiographically proven coronary artery disease, the CD34+CD45- concentration was similar regardless of whether the patient presented with stable or unstable disease. Furthermore there was no evidence of a dynamic response in the $CD34^+CD45^-$ concentration when patients with an ACS were examined with respect to the time from the onset of symptoms. The circulating $CD34^+CD45^-$ concentration therefore provided a measure of vascular injury and atheroma burden but not acute angiogenic stress.

These observations are consistent with those of Guven *et al*, who described a positive correlation between coronary artery disease severity and late outgrowth colonies [Guven *et al.*, 2006]. The source of $CD34^+CD45^-$ cells has not been well established and whether or not their presence in the circulation represents a reparatory response remains unclear. Mature endothelial cells may also express CD34 [Fina *et al.*, 1990], and are also $CD45^-$. $CD34^+CD45^-$ cells may therefore be circulating endothelial cells, and simply represent vascular detritus rather than bone marrow derived progenitor cells. One might predict that if $CD34^+CD45^-$ cells possess regenerative capacity, that increased concentrations of $CD34^+CD45^-$ cells would protect against adverse coronary events. I observed the opposite effect, however it is quite plausible that the reparatory capacity of $CD34^+CD45^-$ cells mobilised in response to vascular injury was insufficient to overcome the multitude of

noxious stimuli to which the endothelium is constantly exposed. The function of $CD34^+CD45^-$ cells is therefore not adequately described here, although their presence in the circulation does serve as a biomarker of atheroma burden and predicts the occurrence of adverse coronary events, namely recurrent myocardial infarction and the need for coronary revascularisation.

$CD34^+$ populations expressing CD133 or VEGFR-2 were neither affected by the presence of acute cardiovascular stress nor related to the extent or severity of coronary artery disease. Mobilisation of $CD133^+$ cells following extensive tissue injury such as coronary artery bypass grafting or burns is recognised [Gill *et al.*, 2001], and Friedrich *et al* describe a $CD133^+CD34^-VEGFR\text{-}2^+$ population thought to be a precursor of $CD34^+$ cells that is up-regulated in unstable atheromatous plaques [Friedrich *et al.*, 2006]. Quirici *et al*, report pluripotency of $CD133^+$ cells with an ability to form endothelial-like cells with rapid population doubling-times in culture [Quirici *et al.*, 2001]. However, Timmermans *et al*, found that $CD133^+$ are devoid of endothelial potential, and that this property lies exclusively in circulating $CD34^+CD45^-CD133^-$ cells [Timmermans *et al.*, 2007]. Although $CD34^+VEGFR\text{-}2^+$ and $CD34^+VEGFR\text{-}2^+CD133^+$ cells have been used previously in clinical studies, I found that both were exceedingly rare and were frequently undetectable. These populations are difficult to quantify accurately, even in carefully conducted, large clinical studies such as this one. I therefore hesitate to draw definitive conclusions regarding the absence of VEGFR-2 or CD133 expression on $CD34^+$ sub-populations

but do not believe that they are likely to play a significant role in the acute response to vascular injury or myocardial infarction.

Consistent with the presence of acute cardiovascular stress, there was an increase in EC-CFU concentrations in those patients presenting with an acute coronary event as compared to those undergoing elective angiography. Although the significance of these early outgrowth populations remains unclear, it is likely that EC-CFU potentiate re-endothelialisation and neo-vascularisation via the secretion of angiogenic cytokines and enhanced proliferation and migration of endothelial cells and EPC [Rehman *et al.*, 2003; Sieveking *et al.*, 2008]. Consistent with this hypothesis, I report an association between angiogenic monocytes co-expressing VEGFR-2 and Tie-2 and acute myocardial infarction. Furthermore in patients with an ACS I observed a time dependent decay in the angiogenic monocyte subpopulation concentration indicating that these cells are mobilised acutely in response to cardiovascular stress. An increase in circulating VEGFR-2 expressing monocytes following myocardial infarction has been reported previously in a very small clinical study, and intra-myocardial injection of this population has improved ventricular function in a murine model of infarction [Bruno *et al.*, 2006]. Contrary to our findings, Hu *et al* found no difference in $CD14^{+}VEGFR\text{-}2^{+}$ cells between patients with stable or unstable coronary artery disease and healthy controls. However, this study included only 25 patients, two weeks following the initial presentation with an acute coronary syndrome, and troponin concentrations were not reported. Comparison with our study is therefore limited [Hu *et al.*, 2008]. I did not detect a robust correlation between coronary artery disease severity and

$CD14^+VEGFR\text{-}2^+Tie\text{-}2^+$ concentration. I believe that unlike $CD34^+CD45^-$ cells, angiogenic monocytes are mobilised acutely in response to cardiovascular stress and are possibly a more sensitive measure of myocardial injury than vascular or endothelial injury.

6.6 CONCLUSIONS

Traditional EPC expressing $CD34^+VEGFR\text{-}2^+$ and $CD133^+$ are impractical to measure and are unlikely to mediate cardiovascular regeneration. $CD34^+CD45^-$ cells are not mobilised acutely following an ACS, but an increased concentration indicates a greater severity of coronary atheroma burden, and predicts the occurrence of adverse coronary events. The $CD34^+CD45^-$ concentration likely reflects underlying cardiovascular injury, but additional studies are required in order to determine whether these populations have a reparatory function or are tissue resident cells liberated from injured tissue. Pro-angiogenic monocytes ($CD14^+VEGFR\text{-}2^+Tie\text{-}2^+$) and endothelial cell-colony forming units (EC-CFU) are increased in patients with an ACS, however concentrations reflect acute myocardial necrosis and do not predict the extent of coronary disease or clinical outcome.

CHAPTER SEVEN

PERCUTANEOUS CORONARY INTERVENTION CAUSES A RAPID BUT TRANSIENT MOBILISATION OF $CD34^{+}CD45^{-}$ CELLS

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7.1 SUMMARY

$CD34^+CD45^-$ cells are increased in patients with coronary artery disease. I wished to determined the behaviour of $CD34^+CD45^-$ cells in response to discrete vascular injury during percutaneous coronary intervention (PCI).

Using flow cytometry I quantified circulating $CD34^+CD45^-$ cells, in addition to traditional $CD34^+VEGFR\text{-}2^+$ EPC, angiogenic monocytes ($CD14^+VEGFR\text{-}2^+Tie\text{-}2^+$) and intercellular adhesion molecule expression (CXCR-4 and CD18) in patients before and during the first week following diagnostic angiography (n=13) or PCI (n=23). Vascular endothelial growth factor-A (VEGF-A) and C-reactive protein (CRP) were quantified by ELISA. PCI, but not angiography alone, increased circulating neutrophil and CRP concentrations, peaking at 24 and 48 hours respectively ($P<0.002$ for both). Serum VEFG-A concentration was unaffected by either procedure ($P>0.2$ for both). $CD34^+CD45^-$ cell concentrations were unchanged following angiography alone ($P>0.4$), but were transiently increased six hours following PCI [median (IQR): 0.93 (0.43-1.49) *versus* 1.51 (0.96-2.15) $x10^6$ cells/L; $P=0.01$], returning to normal by 24 hours. Adhesion molecule expression on $CD34^+$ cells, and traditional EPC, angiogenic monocyte and VEFG-A concentrations were unchanged ($P>0.1$ for all). PCI therefore causes a transient release of $CD34^+CD45^-$ cells into the peripheral circulation, without affecting factors associated with $CD34^+$ mobilisation from the bone marrow. $CD34^+CD45^-$ cells are likely released directly from the vessel wall. Traditional putative EPC and angiogenic monocytes are unlikely to be important in the acute response to vascular injury.

7.2 INTRODUCTION

Percutaneous coronary intervention (PCI) involves the deployment of rigid stent struts at high-pressures that strip the treated vessel of its endothelium and cause laceration of the underlying intima. Vascular injury caused by PCI initiates platelet aggregation and thrombus formation [Mak *et al.*, 1996], and triggers an inflammatory cascade that drives vascular smooth muscle proliferation and neo-intimal hyperplasia [Farb *et al.*, 1999]. The loss of endogenous thrombolytic function and anti-inflammatory signalling normally provided by the endothelium potentiates a maladaptive response to vascular injury that can lead to in-stent restenosis or thrombosis. Re-endothelialisation of the denuded arterial segment is therefore a crucial component of vascular healing, necessary to protect against adverse consequences following PCI.

EPC are mobilised in response to cardiovascular injury such as myocardial infarction [Shintani *et al.*, 2001; Wojakowski *et al.*, 2004], improve organ perfusion and function in animal models of ischaemia [Friedrich *et al.*, 2006; Gill *et al.*, 2001; Kong *et al.*, 2004a; Takamiya *et al.*, 2006; Walter *et al.*, 2002; Werner *et al.*, 2003; Werner *et al.*, 2002; Yoshioka *et al.*, 2006], and are negatively associated with adverse cardiovascular outcomes [Schmidt-Lucke *et al.*, 2005; Vasa *et al.*, 2001b; Werner *et al.*, 2005]. Circulating EPC concentrations are regulated by angiogenic growth factors such as vascular endothelial growth factor and stromal derived factor-1 acting through their cognate receptors, VEGFR-2 and CXCR-4 [Asahara *et al.*, 1999; Dimmeler *et al.*, 2001; Llevadot *et al.*, 2001; Seeger *et al.*, 2009; Yla-Herttuala *et al.*, 2007]. Peripheral homing and incorporation of EPC are dependent on the

expression of surface adhesion molecules, including beta-integrins such as CD18 [Chavakis *et al.*, 2005]. Given their putative vasculoprotective role it is plausible that EPC might be mobilised in order to facilitate vascular repair following injury. However, several investigators have shown previously that acute mobilisation of traditional phenotypic $CD34^+VEGFR\text{-}2^+$ EPC in response to a discrete vascular injury during PCI does not occur [Mills *et al.*, 2009]. Mobilisation of $CD34^+VEGFR\text{-}2^+$ cells following PCI may however be delayed, as measurements have not been performed beyond 24 hours [Mills *et al.*, 2009; Thomas *et al.*, 2008a].

In the last chapter I described that traditional EPC were unaffected by the presence of an acute coronary syndrome (ACS) and were unrelated to the severity of coronary atheroma in a cohort of over 200 patients undergoing coronary angiography [Padfield *et al.*, 2013]. This would indicate that traditional EPC are not clinically relevant to atherosclerosis or acute atherothrombotic events. Indeed EPC defined in the traditional fashion identify populations capable of forming haematopoietic rather than endothelial colonies in culture, that lack significant proliferative potential or the capacity to incorporate into perfusing vessels *in-vivo* [Yoder *et al.*, 2007]. In contrast, non-haematopoietic $CD34^+CD45^-$ cells form endothelial colonies with robust proliferative potential and can incorporate into perfusing vessels [Case *et al.*, 2007; Timmermans *et al.*, 2007; Yoder *et al.*, 2007]. Although not mobilised acutely by an ACS, I found that non-haematopoietic, $CD34^+CD45^-$ concentrations, are directly associated with atheroma burden and predict the occurrence of adverse coronary events. Circulating $CD34^+CD45^-$ cells

therefore serve as a measure of vascular injury, but their origin and pathophysiological significance are unknown. In particular it is unclear whether or not they are bone marrow derived cells with regenerative capacity or are released directly from the vessel wall itself.

Monocytes accumulate at sites of new vessel formation and augment the differentiation and proliferation of endothelial cells via the secretion of pro-angiogenic factors [Fernandez Pujol *et al.*, 2000; Rehman *et al.*, 2003; Schmeisser *et al.*, 2001; Urbich *et al.*, 2003; Zhao *et al.*, 2003]. Vascular injury associated with PCI mobilises monocyte derived angiogenic colony forming units (EC-CFU) within 24 hours, indicating that this population participates in the acute cellular response to vascular injury [Mills *et al.*, 2009]. The specific sub-populations responsible for EC-CFU generation are unknown, however monocytes expressing the endothelial epitopes vascular endothelial growth factor receptor–2 (VEGFR-2) and Tie-2 accelerate re-endothelialisation and improve left ventricular function following experimental cardiovascular injury [Bruno *et al.*, 2006; Elsheikh *et al.*, 2005; Nowak *et al.*, 2004], and as described in chapter five, are mobilised following an acute coronary syndrome. Their behaviour in response to discrete vascular injury however is also unknown.

I hypothesised that re-endothelialisation following PCI may be accelerated by circulating angiogenic monocytes and EPC [Padfield *et al.*, 2010a], and conducted this study in order to determine the behaviour of a variety of putative angiogenic cell populations in response to a discrete vascular injury caused by PCI.

7.3 METHODS AND MATERIALS

7.3.1 Study population

The study was performed with the approval of the local research ethics committee in accordance with the Declaration of Helsinki, and the written informed consent of all volunteers. Patients undergoing elective coronary angiography at Edinburgh Royal Infirmary (ERI), Edinburgh, United Kingdom, for the investigation of suspected angina (Canadian Cardiovascular Society grade 2) or as part of an evaluation prior to surgery for valvular heart disease were identified prospectively and invited to take part in the study. Patients with a recent acute coronary syndrome (<3 months), significant co-morbid illness, haematological or internal malignancy, hepatic or renal failure or concurrent infection were excluded from the study. Clinical characteristics and medical therapy during admission were documented.

7.3.2 Coronary angiography

Coronary angiography was performed via the femoral or radial artery using 5-6F arterial catheters and standard angiographic projections. Stenoses affecting a major epicardial artery of ≥50% were defined as significant, and the overall atheroma burden was graded using the Gensini scoring system [Gensini, 1983, Ringqvist *et al.*, 1983]. Patients underwent percutaneous coronary intervention at the discretion of the operator following pre-treatment with oral Clopidogrel 300 mg and 5000 IU of intravenous unfractionated heparin. Intracoronary stents were implanted following lesion optimisation by balloon pre-dilation and intracoronary glyceryl trinitrate.

7.3.3 Blood sampling and assays

Prior to coronary angiography peripheral venous blood anti-coagulated with ethylene diamine tetra-acetic acid (EDTA) (Sarstedt-Monovette, Germany) was collected for flow cytometry in all subjects. Whole blood was analysed for the differential leukocyte count using an autoanalyzer (Sysmex, UK). Plasma troponin concentrations were measured using the ARCHITECT *STAT* troponin I assay (Abbott Laboratories, Abbott Park, IL). An ELISA was used to quantify serum VEGF-A concentration (Invitrogen, UK) ([Padfield *et al.*, 2010b] and serum high sensitivity-CRP was quantified using an immunonephlometric method (Dade-Behring Marburg, Germany) [Mills *et al.*, 2008] as described above.

7.3.4 Flow cytometric identification of EPC

Endothelial progenitor cells and angiogenic monocytes were phenotyped as described in previous chapters.

7.3.5 Data analysis and statistics

Continuous variables are reported as the mean ± SEM or median and IQR where appropriate. Statistical analyses were performed with SPSS version 17 (SPSS Inc, Chicago, USA). The D'Agostino and Pearson test was used to test for normality of distribution. Analyses were performed using parametric or non-parametric analysis of variance (ANOVA) where appropriate. Two-tailed Student's *t*-test, Mann-Whitney tests and Pearson's Chi-Square tests for between and within group comparisons where appropriate. Statistical significance was taken at a two-sided P value of 0.05.

7.4 RESULTS

7.4.1 Subjects and procedures

Thirteen patients underwent diagnostic coronary angiography alone and 23 underwent PCI using intracoronary stents. Patients were well matched in terms of age and sex and with respect to their cardiovascular risk profile and baseline medications (Table 7.1).

Table 7.1: Demographic, clinical characteristics and medical therapy of the study population

	Angiography (n=13)	PCI (n=23)	P value
Demographics			
Age, years	63 ± 2.7	61 ± 2.3	0.51
Male gender, no. (%)	10 (77)	19 (83)	0.68
Body mass index, kg/m^2	27.5 ± 1.8	28.9 ± 0.9	0.51
Clinical characteristics			
Smoking status, n (%)			
Current smoker	4 (31)	2 (9)	0.11
Ex smoker	6 (46)	11 (48)	0.71
Non smoker	3 (23)	10 (44)	0.22
Hypertensive, n (%)	6 (46)	19 (83)	0.02
Diabetic, n (%)	2 (15)	3 (13)	0.85
Hyperlipidaemia, n (%)	8 (62)	17 (74)	0.32
Family history of IHD, n (%)	5 (39)	16 (70)	0.07
Previous MI, n (%)	5 (39)	13 (57)	0.30
CABG, n (%)	1 (8)	2 (9)	0.92
PCI, n (%)	2 (15)	10 (44)	0.09
PVD, n (%)	2 (15)	3 (13)	0.85
LVSD, n (%)	3 (23)	6 (26)	0.84
Stroke, n (%)	1 (8)	1 (4)	0.67
Creatinine mg/dL	103 ± 4	98 ± 3	0.34
Cholesterol mg/dL	4.3 ± 0.3	4.4 ± 0.2	0.95
	Medical therapy		
Aspirin, n (%)	9 (77)	23 (100)	0.05
Clopidogrel, n (%)	4 (31)	18 (78)	0.01
Beta-blocker, n (%)	7 (54)	17 (74)	0.22
Statin, n (%)	10 (77)	22 (96)	0.09
ACE inhibitor, n (%)	4 (31)	14 (61)	0.08
ARB, n (%)	1 (8)	1 (4)	0.67

Continuous variables are expressed as the mean ± standard error or median (interquartile range) with statistical comparisons between patients with ACS and suspected stable angina performed using Students t-test or Mann-Whitney where appropriate. Categorical variables are expressed as the absolute number of cases and the group proportion (%) with statistical comparisons performed using a Chi-squared test. ACE = angiotensin converting enzyme; ARB = angiotensin receptor blocker; CABG = coronary artery bypass; IHD = ischaemic heart disease; LMWH = low-molecular weight heparin; LVSD = left ventricular systolic dysfunction; MI = myocardial infarction; PCI = percutaneous coronary intervention; PVD = peripheral vascular disease.

Table 7.2: Procedural characteristics of the study population

	Angiography (n=13)	PCI (n=23)	P value
Radial access, n (%)	9 (69)	18 (78)	0.55
Coronary disease severity			
Normal/Minor, n (%)	2 (15)	1 (5)	0.27
1 vessel, n (%)	2 (15)	10 (46)	0.09
2 vessel, n (%)	2 (15)	6 (27)	0.46
3 vessel, n (%)	7 (54)	5 (23)	0.05
3 vessel or LMS, n (%)	9 (69)	5 (23)	0.01
Gensini score, units	34 (26 - 79)	26 (16 - 80)	0.70
Target vessel, n (%)			
LAD	-	11 (48)	-
LCx	-	10 (44)	-
RCA	-	5 (22)	-
SVG	-	2 (9)	-
Multi-vessel	-	7 (30)	-
Balloon inflation time, s	-	44 (30 - 54)	-
Number of stents	-	1 (1-2)	-
Drug eluting stents	-	13 (57)	-
Stent length, mm	-	25.3 ± 3.2	-
Stent diameter, mm	-	3.1 ± 0.1	-
Pre-dilatation, n (%)	-	18 (78)	-
Post-dilatation, n (%)	-	4 (17)	-
Case time, minutes	16 ± 1.6	36 ± 2.2	<0.001

Continuous variables are expressed as the mean ± standard error or median and interquartile range with statistical comparisons between patients with ACS and suspected stable angina performed using Students t-test or Mann-Whitney where appropriate. Categorical variables are expressed as the absolute number of cases and the group proportion(%) with statistical comparisons performed using a Chi-squared test. Statistical comparisons were performed using Student's t-test, Mann-Whitney test or Chi-squared analysis where appropriate. LAD = left anterior descending; LCx = left circumflex; PCI = percutaneous coronary intervention; RCA; right coronary artery; SVG = saphenous vein graft.

Patients undergoing diagnostic coronary angiography alone were more likely to have left main stem or triple vessel disease (P=0.001), although Gensini scores were similar (26 (16-80) versus 34 (26-79) units; P>0.70). Patients undergoing PCI required a longer total procedure time compared to diagnostic angiography alone (36

± 2.2 versus 16 ± 1.6 minutes; P<0.001). Patients received a median of 1 (1-2) stent with a median total balloon inflation time of 44 (30-54) seconds. Drug eluting stents were used in 57% of patients (Table 7.2). No significant complications arose during the course of the study and all patients were discharged within 24 hours of the procedure.

7.4.2 Measures of inflammation and myocyte necrosis

All parameters were similar in both groups at baseline (P>0.05 for all; Table 7.3). The median troponin concentration at 24 hours was undetectable in both groups (P=0.13). Following PCI, the leukocyte count was increased (P=0.04 for ANOVA of repeated measures), peaking at 24 hours (7.21±0.28 versus 6.24±0.20 $x10^6$ cells/L; P=0.002). Serum CRP also increased following PCI (P=0.002 for ANOVA of repeated measures), peaking at 48 hours (3.95 (1.26-8.26) versus 1.02 (0.64-2.33) mg/L; P=0.001). Serum VEGF-A concentrations were unchanged following PCI (P=0.16 for ANOVA of repeated measures). Diagnostic coronary angiography alone had no effect on the total leukocyte count, CRP, or serum VEGF-A concentration throughout the study period (P>0.5 for ANOVA of repeated measures for all).

7.4.3 Progenitor cell populations

All cell concentrations were similar in both groups at baseline (P>0.05 for all; Table 7.4). Following PCI circulating $CD34^+CD45^-$ cell concentrations were increased (P=0.04, for ANOVA of repeated measures), peaking at 6 hours (1.51 (0.96-2.15) *versus* 0.93 (0.43-1.49) $x10^6$ cells/L; P=0.01), returning to baseline

concentrations at 24 hours (Table 7.4 and figure 7.1). The $CD34^+CD45^-$ concentration was unaffected by diagnostic coronary angiography alone (P=0.97 for ANOVA of repeated measures; Table 7.4 and figure 7.1). Traditional EPC phenotypes expressing $CD34^+VEGFR\text{-}2^+$ and those expressing $CD34^+VEGFR\text{-}2^+CD133^+$ and $CD34^+CD45^+$ were unaffected in both groups (P>0.5 for all; Table 7.4). CXCR-4 and CD18 expression on $CD34^+$ cells was 47 ± 7% and 36 ± 5% respectively at baseline and were unchanged throughout the study period in both groups (P>0.2 for all; data not shown). The total CD14 count was not affected by either procedure (P>0.5 for both). Angiogenic monocytes expressing Tie-2 and VEGFR-2 comprised 0.72% (0.28-2.65) of circulating $CD14^+$ cells and were unchanged throughout the study in both groups (P>0.2 for all; Table 7.4).

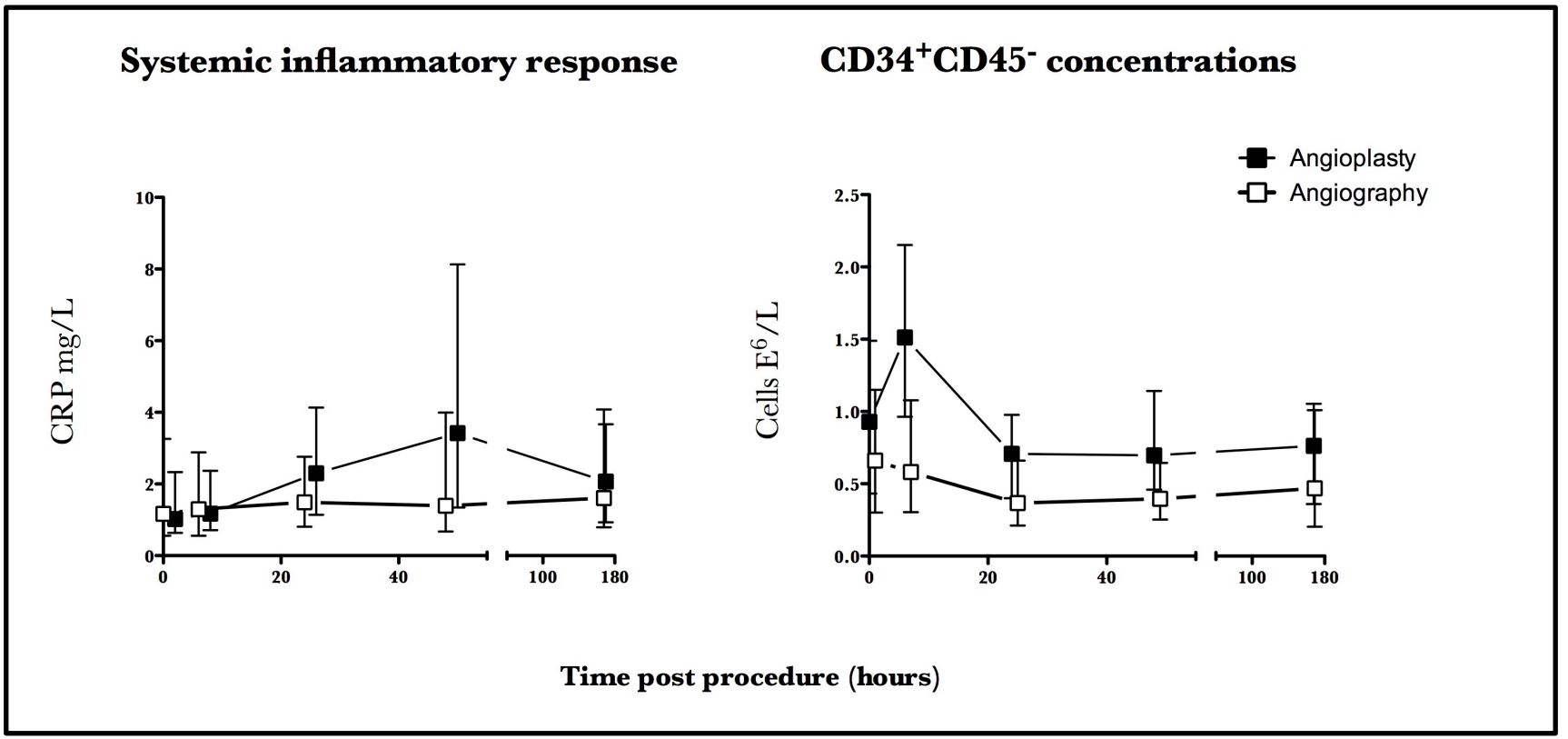

Figure 7.1 Inflammatory response and CD34$^+$CD45$^-$ cell release following percutaneous coronary intervention: PCI rapidly but transiently mobilised CD34$^+$CD45$^-$ cells into the peripheral circulation, peaking at 6 hours; P=0.01. Circulating concentrations had fallen back to baseline by 24 hours. In contrast, the systemic inflammatory response caused by PCI was relatively prolonged, with CRP concentrations continuing to increase for at least 48 hours; P=0.001. Diagnostic coronary angiography alone did not cause an inflammatory response or effect the CD34$^+$CD45$^-$ concentration; P>0.5 for all. Data are median and interquartile range.

Table 7.3. Inflammatory response following diagnostic angiography and PCI

	0 hrs§	6 hrs	24 hrs	48 hrs	72 hrs	168 hrs	ANOVA
Angiography							
Leukocytes ($x10^9$/L)	6.7 ± 0.5	6.9 ± 0.7	7.1 ± 0.5	6.9 ± 0.6	7.2 ± 0.6	6.3 ± 0.7	0.24
Neutrophils ($x10^9$/L)	4.1 ± 0.4	4.1 ± 0.4	4.4 ± 0.4	4.1 ± 0.3	4.4 ± 0.4	4.1 ± 0.4	0.57
Lymphocytes ($x10^9$/L)	1.7 ± 0.2	2.0 ± 0.3	1.8 ± 0.2	1.9 ± 0.3	1.9 ± 0.3	1.7 ± 0.3	0.07
Monocytes ($x10^9$/L)	0.56 ± 0.03	0.59 ± 0.06	0.59 ± 0.05	0.58 ± 0.05	0.58 ± 0.04	0.56 ± 0.05	0.93
CRP (mg/L)	1.2 (0.6 - 3.3)	1.3 (0.6 - 2.9)	1.5 (0.8 - 2.8)	1.4 (0.7 - 4.0)	-	1.6 (0.8 - 4.1)	0.97
VEGF-A (pg/L)	76 ± 13	54 ± 10	69 ± 9	68 ± 8	-	79 ± 13	0.16
PCI							
Leukocytes ($x10^9$/L)	6.3 ± 0.2	6.8 ± 0.3	7.2 ± 0.3*	7.0 ± 0.4*	6.7 ± 0.3	6.5 ± 0.3	0.04
Neutrophils ($x10^9$/L)	3.8 ± 0.2	4.2 ± 0.3	4.7 ± 0.2*	4.3 ± 0.3	4.0 ± 0.2	3.8 ± 0.3	0.04
Lymphocytes ($x10^9$/L)	1.8 ± 0.1	1.9 ± 0.1	1.7 ± 0.2	1.9 ± 0.1	1.9 ± 0.1	1.8 ± 0.1	0.41
Monocytes ($x10^9$/L)	0.49 ± 0.02	0.54 ± 0.04	0.54 ± 0.03	0.54 ± 0.03	0.51 ± 0.03	0.48 ± 0.04	0.14
CRP (mg/L)	1.1 (0.7 - 2.5)	1.3 (0.8 - 2.4)	2.6 (1.0 - 4.5)*	3.4 (1.2 -8.4)**	-	2.0 (0.9 - 3.6)	0.002
VEGF-A (pg/L)	72 ± 8	57 ± 5	57 ± 5	56 ± 5	-	50 ± 5	0.16

Data are expressed as median and IQR or mean ± SEM. Statistical analyses are performed using repeated measures ANOVA (Freidman's test for non-parametric data) and Bonferroni's or Dunn's Multiple comparison test where appropriate. Comparisons between time points are made using a paired Student's t-test or Wilcoxon test where appropriate. *P<0.05 and **P=0.001 for pre-procedure versus time point. §Between group baseline comparison was made for each variable using a Mann-Whitney or unpaired Student's t test where appropriate. CRP = C-reactive protein; PCI = percutaneous coronary intervention; VEGF-A = vascular endothelial growth factor – A.

Table 7.4 Cell frequencies following diagnostic angiography and PCI

	0 hrs*	6 hrs	24 hrs	48 hrs	72 hrs	168 hrs	ANOVA
Angiography							
$CD34^+$	2.45 (1.54-3.21)	2.17 (1.59-2.94)	1.63 (1.37-2.28)	1.74 (1.31-2.75)	2.17 (1.35-3.13)	1.88 (1.66-2.79)	0.14
$CD34^+VEGFR\text{-}2^+$	0.27 (0.09-0.71)	0.36 (0.08-1.50)	0.13 (0.03-0.41)	0.21 (0.09-0.48)	0.06 (0.02-0.40)	0.13 (0.07-0.87)	0.22
$CD34^+VEGFR\text{-}2^+CD133^+$	0.04 (0.01-0.55)	0.10 (0.03-0.44)	0.10 (0.0-0.36)	0.05 (0.01-0.19)	0.03 (0.0-0.49)	0.03 (0.0-0.24)	0.44
$CD34^+CD45^+$	1.77 (0.90-2.48)	1.11 (0.94-1.87)	1.31 (0.97-1.96)	1.36 (1.08-1.69)	1.46 (0.86-1.96)	1.36 (0.81-1.67)	0.73
$CD34^+CD45^-$	0.71 (0.31-1.20)	0.60 (0.31-1.70)	0.39 (0.27-0.82)	0.41 (0.26-0.67)	0.52 (0.21-1.4)	0.61 (0.21-1.1)	0.43
$CD34^+CD45^-VEGFR\text{-}2^+$	0.06 (0.01- 0.31)	0.08 (0.02-0.31)	0.02 (0.002-0.17)	0.06 (0.0-0.13)	0.02 (0.0-0.29)	0.05 (0.01-0.11)	0.33
$CD14^+Tie\,2^+VEGFR\text{-}2^+$†	3.0 (1.8-11.1)	2.8 (1.2-14.3)	2.0 (1.3-10.8)	2.5 (0.70-6.37)	3.2 (2.1-12.6)	2.7 (0.97-8.61)	0.39
PCI							
$CD34^+$	2.58 (1.70-3.36)	2.94 (2.07-4.05)	2.36 (1.74-3.52)	2.42 (1.75-3.29)	2.20 (1.60-2.64)	2.49 (1.36-3.25)	0.08
$CD34^+VEGFR\text{-}2^+$	0.13 (0.02-0.52)	0.18 (0.05-0.85)	0.17 (0.03-0.47)	0.22 (0.02-0.56)	0.12 (0.05-0.41)	0.11 (0.03-0.38)	0.3
$CD34^+VEGFR\text{-}2^+CD133^+$	0.05 (0.0-0.92)	0.14 (0.0-1.3)	0.04 (0.0-0.77)	0.08 (0.0-0.75)	0.03 (0.0-0.74)	0.04 (0.0-0.72)	0.27
$CD34^+CD45^+$	1.41 (0.84-2.14)	1.69 (0.97-2.34)	1.79 (1.12-2.69)	1.64 (1.28-2.11)	1.72 (0.96-2.07)	1.63 (0.96-2.34)	0.6
$CD34^+CD45^-$	0.93 (0.43-1.5)	1.51 (0.96-2.15)§	0.71 (0.40-0.98)	0.70 (0.46-1.14)	0.52 (0.21-0.73)	0.76 (0.36-1.05)	<0.0001
$CD34^+CD45^-VEGFR\text{-}2^+$	0.09 (0.0-0.32)	0.17 (0.02-0.52)	0.09 (0.01-0.37)	0.06 (0.0-0.36)	0.05 (0.0-0.19)	0.04 (0.02-0.16)	0.1
$CD14^+Tie\,2^+VEGFR\text{-}2^+$	3.1 (0.98-7.1)	4.3 (2.4-13)	4.1 (1.8-11)	3.1 (0.30-13)	3.1 (0.55-6.1)	2.1 (1.1-9.3)	0.68

Data are expressed as the median and interquartile range. Statistical analyses are performed using repeated measures ANOVA (Freidman's test) with Dunn's Multiple comparison post test. Comparisons between time points are made using a Wilcoxon test. *Between group comparisons at baseline were made for each variable using a Mann-Whitney test. †n=10. §P=0.01 for baseline compared to time point. ANOVA = analysis of variance; CD = cluster of differentiation; CRP = C-reactive protein; PCI = percutaneous coronary intervention; VEGFR-2 = vascular endothelial growth factor receptor-2.

7.5 DISCUSSION

I have examined the behaviour of a variety of circulating angiogenic cells in response to a discrete vascular injury caused by PCI. I observed a rapid, but transient increase in circulating $CD34^+CD45^-$ cells into the peripheral circulation at six hours following PCI, with circulating concentrations returning to baseline at 24 hours. The increase in $CD34^+CD45^-$ cells preceded the systemic inflammatory response to vascular injury, which peaked at 48 hours. Whilst the number of $CD34^+CD45^-$ cells increased, there was no change in the expression of surface adhesion molecules on $CD34^+$ cells or in VEGF-A concentrations. Taken together, these observations suggest that the increase in $CD34^+CD45^-$ cells following PCI is not mediated by a circulating cytokine or growth factor acting on the bone marrow, but rather results from mechanical injury and the release of CD34+CD45- cells from the coronary artery directly. Traditional phenotypic EPC populations ($CD34^+VEGFR\text{-}2^+$ and $CD34^+VEGFR\text{-}2^+CD133^+$) and, haematopoietic $CD45^+$ subpopulations including angiogenic monocytes were unaffected by discrete vascular injury up to one week following PCI, suggesting these populations are not mobilised in the acute response to vascular injury and are unlikely to play a direct role in vascular repair.

Two small clinical studies have demonstrated a fall in $CD34^+VEGFR\text{-}2^+$ cells within the first few hours of coronary angioplasty [Lee *et al.*, 2009; Thomas *et al.*, 2008a]. Neither of these studies used a control group, and interpretation is therefore limited, but it is likely that this 'dip' in $CD34^+$ concentration is explained by the

diurnal variation that I described in chapter five [Padfield *et al.*, 2010b; Thomas *et al.*, 2008b]. Previous studies have not specifically examined the behaviour of $CD34^+CD45^-$ cells following PCI. Pelliccia et al, found that higher concentrations of $CD34^+VEGFR\text{-}2^+CD45^-$ and $CD133^+VEGFR\text{-}2^+CD45^-$ cells prior to revascularisation identified patients more likely to develop restenosis [Pelliccia *et al.*, 2010]. I described previously that a direct relationship exists between coronary atheroma burden and $CD34^+CD45^-$ cells [Padfield *et al.*, 2013], and both $CD34^+CD45^-$ cell derived late outgrowth colonies [Guven *et al.*, 2006].

$CD34^+CD45^-$ cell derived late outgrowth colonies [Guven *et al.*, 2006] are associated with atheroma burden in the coronary arteries. Therefore, it seems likely that $CD34^+CD45^-$ cells are a measure of vascular injury, however whether or not they are derived from the bone marrow or possess reparatory function remains unclear. Mature endothelial cells also express CD34 [Fina *et al.*, 1990] and are $CD45^-$, and circulating $CD34^+CD45^-$ cells may therefore simply reflect shedding of vascular detritus to the peripheral circulation as a consequence of cell turnover in atherosclerotic arteries. The early and transient appearance of $CD34^+CD45^-$ cells in the circulation following vascular perturbation observed in the current study is consistent with this hypothesis. Furthermore, despite mobilisation of $CD34^+CD45^-$ cells, neither plasma VEGF concentration nor the expression of the surface receptors, CXCR-4 or CD18 on circulating $CD34^+$ cells were affected by PCI. Stem cell mobilisation occurs via activation of the phosphatidylinositol 3-

kinase/Akt/endothelial nitric oxide synthase pathway (PI3K/Akt/eNOS). PI3K/Akt/eNOS is responsible for the regulation of cellular mobilisation, proliferation and migration, and apoptosis in a variety of biological systems, including the cardiovascular system [Shiojima *et al.*, 2002]. Activation of PI3K/Akt/eNOS occurs via angiogenic factors such as VEGF through stimulation of nitric oxide synthesis by bone marrow stromal cells [Dimmeler *et al.*, 2001; Llevadot *et al.*, 2001]. Increased nitric oxide bioavailability leads to cleavage of intracellular adhesions between stem cells and stromal cells of the bone marrow [Heissig *et al.*, 2002], allowing them to mobilise to the peripheral circulation in response to a stromal cell-derived factor-1 gradient generated by bone marrow stromal cells acting through the CXCR-4 receptor [Asahara *et al.*, 1999; Seeger *et al.*, 2009; Yla-Herttuala *et al.*, 2007]. Cell surface adhesion molecules such as CD18 mediate EPC homing and cells adhesion and are required for incorporation of EPC into the vasculature [Chavakis *et al.*, 2005]. Although I did not performed a comprehensive evaluation of the factors involved in the mobilisation of stem cells from the bone marrow, the absence of any change in VEGF concentration or CD18 and CXCR-4 expression further supports the hypothesis that $CD34^{+}CD45^{-}$ cells were not mobilised from the bone marrow, but released directly from the injured coronary artery.

Although I did not observe an increase in circulating $CD34^{+}CD45^{+}$ cells following PCI in the present study, this phenomenon has been described. However previous studies have also included patients with significant myocardial necrosis [Bonello *et al.*, 2006; Inoue *et al.*, 2007; Schober *et al.*, 2005]. Peak mobilisation varied

from 24 hours to a week, and was proportional to the degree of systemic inflammation with $CD34^+CD45^+$ concentrations predicting future development of in-stent restenosis. Whether $CD34^+CD45^+$ cells contribute directly to the development of restenosis is unknown, although it is interesting to note that the use of drug eluting stents was associated with less inflammation and $CD34^+CD45^+$ mobilization. This suggests drug-eluting stents may in part suppress restenosis by attenuating inflammatory signalling to the bone marrow by suppressing local vascular inflammation. $CD34^+CD45^+$ cells may therefore contribute to the development of in-stent restenosis, although the mechanism is unclear. $CD34^+$ cells have the capacity to adopt smooth muscle cell characteristics *in-vitro* and may therefore potentiate the development of in-stent restenosis by acting as circulating smooth muscle progenitor cells (SMPC) [Sata *et al.*, 2002]. Putative SMPC have been reported to express both CD34 and VEGFR-2 [Simper *et al.*, 2002], and both CD34 and CD133 are expressed at higher concentrations in the neo-intima of restenotic lesions compared to de novo lesions [Skowasch *et al.*, 2003]. Inoue, *et al*, demonstrated that circulating mononuclear cells of patients with in-stent restenosis exhibit a propensity to develop a smooth muscle phenotype over that of an endothelial phenotype compared to those without [Inoue *et al.*, 2007]. Therefore plasticity of circulating $CD34^+$ cells may lead to acceleration of neo-intimal hyperplasia if a pro-inflammatory microenvironment in a particular patient dictates such a response.

Monocytes will express a variety of endothelial characteristics *in-vitro* under appropriate angiogenic stimulation [Fernandez Pujol *et al.*, 2000; Rehman *et al.*,

2003; Schmeisser *et al.*, 2001; Urbich *et al.*, 2003; Zhao *et al.*, 2003]. Monocyte chemo-attractant protein-1 (MCP-1) is released by endothelial cells in response to shear stress and tissue ischemia [Ito *et al.*, 1997], causing monocytes to accumulate at sites of new vessel formation, adhere to injured endothelium, and accelerate re-endothelialisation and improve endothelial vasomotor function [Elsheikh *et al.*, 2005; Nowak *et al.*, 2004]. However, the remarkable plasticity of monocytes may result in a diverse response to vascular injury depending on the local microenvironment. For instance there is a strong correlation between circulating monocyte concentration and the development of in-stent restenosis [Fukuda *et al.*, 2009], and MCP-1 is significantly elevated, in both the plasma [Cipollone *et al.*, 2001; Hokimoto *et al.*, 2000] and in the coronary atherectomy specimens of patients who develop in-stent restenosis following PCI [Hokimoto *et al.*, 2002]. Furthermore, treatment with anti-MCP-1 monoclonal antibodies following balloon angioplasty in rats inhibits neo-intimal hyperplasia [Furukawa *et al.*, 1999]. The role of monocytes following vascular injury is therefore diverse and again probably depends on the local microenvironment. PCI mobilises monocyte derived angiogenic colony forming units (EC-CFU) within 24 hours although whether specific sub-populations participate in the acute cellular response to vascular injury is unknown [Mills *et al.*, 2009]. In chapter five I observed that monocytes expressing Tie-2 and VEGFR-2 are mobilised acutely following an ACS, however this appeared to be as a consequence of myocyte necrosis, rather than the presence of atheroma burden or the presence of an ACS *per se*. In this study, $CD14^{+}Tie\text{-}2^{+}VEGFR\text{-}2^{+}$ were not mobilised acutely by PCI, suggesting that mobilisation of this monocytic subpopulation occurs in response to more extensive myocardial injury rather than discrete vascular injury

7.6 CONCLUSIONS

Discrete vascular injury associated with PCI causes a rapid, but transient release of $CD34^+CD45^-$ cells into the peripheral circulation, in the absence of a concurrent increase in $CD34^+$ adhesion molecule expression or VEGF-A secretion. I conclude that $CD34^+CD45^-$ cells are released directly from the vessel wall through mechanical injury to the coronary artery. Traditional EPC and VEGFR-2^+Tie-2^+ monocytes are unaffected by PCI, and are unlikely to be important in the acute response to vascular injury.

CHAPTER EIGHT

CONCLUSIONS AND FUTURE DIRECTIONS

8.1 SUMMARY OF THESIS FINDINGS

The endothelium is critical in maintaining normal vascular homeostasis through the regulation of vascular tone and endogenous fibrinolysis, and the inhibition of vascular inflammation and neo-intimal proliferation. Disruption of endothelial function as a result of denuding vascular injury caused by PCI is of major significance in the development of procedure related complications, namely in-stent restenosis and thrombosis. Devising a means of accelerating re-endothelialisation following PCI is therefore desirable. The discovery of bone marrow derived EPC within the adult circulation has offered the potential to realise this aspiration. Compelling evidence exists that EPC exist, however their identity and significance remain undetermined and cell therapy for cardiovascular disorders remains out of reach. In order to develop effective strategies to enhance vascular repair following PCI, we need a better understanding of the role of EPC following vascular injury.

These studies were conducted in order to clarify the relationship between putative EPC and vascular injury. I have examined the behaviour of specific populations previously implicated in vascular repair, namely $CD34^+$ cells expressing VEGFR-2 and $CD133^+$, and monocytic fractions expressing Tie-2 and VEGFR-2 and in addition to the inaptly named, endothelial cell - colony forming unit assay. Given their presumed vasculoprotective role, I hypothesised that these cell populations would be mobilised in response to discrete vascular injury but would be diminished in the patients with significant atherosclerosis as a result of chronic vascular injury. I further hypothesised that EPC would be mobilised as part of an inflammatory response but that excessive systemic inflammation may be deleterious to EPC function.

8.1.1. Dissociation of phenotypic and functional endothelial progenitor cells in patients undergoing percutaneous coronary intervention.

Circulating concentrations of $CD34^+VEGFR2^+$ cells and their presumed progeny, EC-CFU were determined in patients before, and 24 hours following PCI or diagnostic coronary angiography alone. Vascular injury associated with PCI caused significant systemic inflammation, commensurate with a three-fold increase in EC-CFU. $CD34^+VEGFR\text{-}2^+$ EPC were unaffected and furthermore circulating concentrations of EC-CFU and $CD34^+VEGFR\text{-}2^+$ cells did not correlate with each other. EC-CFU and $CD34^+VEGFR\text{-}2^+$ cells are therefore distinct populations, mobilised via dissociated mechanisms. These findings indicate that EC-CFU are in some way involved in the acute response to vascular injury, unlike $CD34^+VEGFR\text{-}2^+$ cells which are unchanged acutely in response to PCI.

8.1.2. EC-CFU - characterisation and mechanisms of generation.

Phenotypic analysis demonstrated that while EC-CFU display some endothelial antigens, they predominantly express haematopoietic surface antigens, widely expressing the pan leukocyte marker CD45 and most intensely the macrophage markers CD68 and CD105 in addition to the lymphocyte antigens CD4 and CD8. Direct visualisation of developing EC-CFU confirmed that they are a powerful stimulus for the migration of monocytes and both T and B-lymphocytes. In addition, EC-CFU contain proliferating cells which are instrumental to colony formation. Therefore EC-CFU do not appear to represent circulating progenitors, and although

confirmatory *in-vivo* studies are required, it is more likely that EC-CFU serve as an *in-vitro* measure of the cellular response to vascular injury, whereby activated leukocytes form a nidus of cellular proliferation and induce cell migration to the site of vascular injury and differentiation of monocytes into macrophages.

8.1.3. Circulating EPC are not affected by acute systemic inflammation

Mobilisation of EC-CFU occurs in the context of systemic inflammation. In order to determine whether this is a specific response to vascular injury, *Salmonella typhus* vaccination was used to generate acute systemic inflammation in healthy volunteers in the absence of discrete cardiovascular injury. Interestingly despite significant systemic inflammation, neither EC-CFU or EPC changed during the course of the study, suggesting that factors specific to vascular injury are responsible for the mobilisation of EC-CFU following PCI.

8.1.4. EPC, atheroma burden and clinical outcome in patients with coronary artery disease

Dysfunction of endogenous endothelial repair mechanisms would be expected to potentiate atherosclerosis. I therefore evaluated the relationship between putative EPC populations, coronary atheroma burden, and clinical outcomes in 201 patients undergoing coronary angiography for suspected angina or following an acute coronary syndrome (ACS). This larger clinical study highlighted the rarity of circulating $CD34^+VEGFR\text{-}2^+$ and $CD34^+VEGFR\text{-}2^+CD133^+$ cells, and importantly demonstrated that these populations were neither increased in patients with an ACS nor related to atheroma burden or survival free from revascularisation,

recurrent myocardial infarction, or death. Circulating concentrations of the recently identified putative $CD34^+CD45^-$ EPC population were increased in patients with coronary artery disease, and correlated positively with atheroma burden. Increased concentrations of circulating $CD34^+CD45^-$ cells were also associated with a shorter cumulative event-free survival. Pro-angiogenic monocytes ($CD14^+VEGFR\text{-}2^+Tie\text{-}2^+$) and EC-CFU were increased in patients with an ACS, however concentrations only reflected myocardial necrosis, and did not predict the extent of coronary disease or clinical outcome. Unlike circulating angiogenic monocyte concentrations, there was no evidence of a dynamic response by $CD34^+CD45^-$ cells following an ACS, suggesting that $CD34^+CD45^-$ cells may be a measure of atheroma burden and vascular injury, rather than a reparatory response to vascular injury.

8.1.5. Percutaneous coronary intervention causes a rapid but transient mobilisation of $CD34^+CD45^-$ cells.

Although postulated to be involved in vascular repair, I found that circulating $CD34^+VEGFR\text{-}2^+$ concentrations were unrelated to coronary atheroma burden and were unaffected by vascular injury associated with ACS or PCI. $CD34^+CD45^-$ cells were increased in patients presenting with ACS, however this phenomenon was in fact associated with a greater severity of coronary atheroma in these patients. I therefore returned to elective PCI as a model of discrete vascular injury in order to interrogate the significance of the association between atheroma burden and circulating $CD34^+CD45^-$ cell concentration and to determine the presence of any

late mobilisation of $CD34^+VEGFR\text{-}2^+$ cells. Populations were measured before and during the first week following PCI compared to diagnostic coronary angiography alone. PCI caused a significant systemic inflammatory response and traditional phenotypic EPC were unaffected by PCI, and showed no evidence of delayed mobilisation. However, $CD34^+CD45^-$ cell concentrations were transiently increased six hours following PCI, returning to normal by 24 hours. Interestingly this transient mobilisation occurred in the absence of any increase in $CD34^+$ adhesion molecule expression or VEGF-A production, suggesting that $CD34^+CD45^-$ cells are released directly from the vessel wall following mechanical injury.

8.1.6 Summary

Vascular injury caused by PCI is associated with systemic inflammation commensurate with an increase in circulating EC-CFU. EC-CFU formation appears to represent a specific cellular response to vascular injury, whereby proliferating cells serve as a migratory stimulus for monocytes and lymphocytes. $CD34^+CD45^-$ cells are mobilised within 6 hours following PCI and are increased in the circulation relative to the degree of atheroma burden and act as an indication of the extent of vascular injury as increased numbers of CD34+CD45- cells predict poor clinical outcome. Circulating $CD34^+CD45^-$ concentrations are probably constituents of the vessel wall, and rather than having a reparatory function, likely reflect the extent of atheroma burden and vascular injury. 'Traditional' phenotypic $CD34^+VEGFR\text{-}2^+$ putative EPC are unrelated to the extent of vascular injury, are not mobilised following PCI or ACS and probably have little role in the acute response to vascular injury.

8.2 LIMITATIONS OF THE STUDIES

There are a number of limitations of the studies described above. Of particular note, and of relevance to all of the clinical studies, was the extreme rarity of the putative EPC populations I was studying. Such rare event analysis by flow cytometry is prone to large intra-observer variability. To reduce this problem, a large number of total FACS events were acquired (>500,000 per sample), although even with this number of events it was difficult to acquire a satisfactory number of positive events. This was particularly so for triple positive cell fractions which were rare with a large co-efficient of variation. This raises concern that my conclusions that traditional EPC are not mobilised in response to discrete vascular injury or related to coronary disease may be a result of a type 2 statistical error. It is however reassuring that other published series have similarly failed to show such a relationship.

In the registry of patients undergoing angiography I recognise the limitations in drawing conclusions about clinical outcomes in a study of this size. The observation of an increased event rate in association with high circulating $CD34^{+}CD45^{-}$ levels therefore requires confirmation from larger studies, however the finding is consistent with the association between $CD34^{+}CD45^{-}$ cells and the severity of coronary disease I observed in these patients. Further to this I would note that Gensini scoring, whilst gauging severity of coronary disease, is an indirect measure of coronary atheroma burden, and may underestimate it. Nevertheless angiographic severity of coronary disease is well correlated with atheroma burden and for the purposes of the correlation analyses I conducted, Gensini scoring is a reasonable surrogate measure of atheroma. My finding that $CD34^{+}CD45^{-}$ cells are associated with greater degrees

of vascular injury is of most interest as these cells are possible precursors of late outgrowth endothelial colonies or endothelial cell forming colonies (ECFC). It is unfortunate therefore that I was unable to measure ECFC directly, however production of ECFC from adult peripheral blood was most infrequent. More often than not I was unable to grow any colonies at all (even following enrichment for their putative precursor $CD34^+CD45^-$). I therefore did not develop this assay to a stage where it was applicable to the clinical studies I undertook.

The mechanism by which circulating $CD34^+45^-$ cells increased following PCI associated vascular injury was also not robustly determined. Although it is plausible these cells were mobilised from the coronary directly, this remains somewhat speculative and further studies are required to determine this definitively. Furthermore it would also have been useful if I had determined the presence of mature endothelial cell markers on these cells.

In my characterisation of EC-CFU I did not measure directly which cells in the assay were proliferating. However, in parallel experiments (see appended manuscript; The constituents and mechanisms of generation of 'EC-CFU'.) cells enriched for CD14 generated multiple EC-CFU and showed significant Brd-U uptake indicating that $CD14^+$ cells are likely responsible for proliferation however this needs further confirmation.

8.3 FUTURE DIRECTIONS

Several questions have been generated by these studies that are to be addressed in ongoing studies. Primarily we wish to determine whether certain cell types are associated with favourable vascular repair that may be harnessed in order to reduced ISR following PCI.

8.3.1. The STARS trial

Are peri-procedural EPC concentrations related to in-stent restenosis? Does anti-inflammatory treatment affect EPC mobilisation following PCI?

In the absence of an intact endothelium persistent inflammation encourages smooth muscle hypertrophy and in-stent restenosis [Toutouzas *et al.*, 2004]. Elevated pre-procedural serum CRP concentrations predict adverse outcomes after coronary stent implantation and are associated with a greater degree of angiographic restenosis. This would suggest that systemic inflammation inhibits vascular repair and is associated with an adverse proliferative response [Walter *et al.*, 2001a]. Studies addressing the effect of corticosteroids on the development of ISR have brought mixed results, probably as a result of insufficient dosing of glucocorticoids in some of these studies [Lee *et al.*, 1999; Pepine *et al.*, 1990; Sahara *et al.*, 2003; Versaci *et al.*, 2002].

In collaboration with Dr Mark de Belder at James Cook University Hospital Middlesbrough (JCUH), we are participating in the STeroids Against Re-Stenosis (STARS) Trial, a 2x2 randomised controlled trial examining the effect of oral

Prednisolone and two different stent designs on the development of in-stent restenosis following PCI. The study population is undergoing protocol driven repeat coronary angiography and IVUS examination at six months following stent insertion, and therefore provides the ideal vehicle with which to assess the relationship between EPC and adverse vascular remodelling. We are using this cohort of patients to conduct a prospective study testing the hypothesis that increased circulating progenitors and mobilisation following angioplasty will encourage early endothelialisation and prevent persistent vascular inflammation and in-stent restenosis. This study also provides an opportunity to explore the effect of anti-inflammatory treatment on EPC mobilisation and function.

Experimental Design

Patients undergoing coronary angiography with a view to follow on PCI undergo baseline evaluation of clinical, biochemical and putative EPC concentration. Between 8 and 24 hours prior to angiography patients are randomised in a double-blind fashion to receive oral prednisolone 40 mg or placebo in addition to empirical proton pump inhibition with Lansoprazole 30mg. Study medications are prepared at JCUH pharmacy and are identifiable only by a study reference code. Following the decision to treat any de novo lesion affecting a native coronary artery with a reference diameter of > 3mm with a BMS, patients are randomised by sealed envelope in a single blind fashion to receive either Zeta™ or Vision™ bare metal stents (Abbott Vascular; UK). Exclusion criteria are the intended use of drug eluting stent, primary PCI for AMI, re-stenotic lesions, vein graft lesions or any unprotected left main stem PCI. Prior to PCI target lesions are assessed fluoroscopically in two

orthogonal views following the administration of 200ug of intra-coronary GTN. Oral prednisolone 40mg daily, is continued for a total of 28-days, and aspirin 75mg and clopidogrel 75 mg daily, are given to all patients for at least three months following PCI. The prescription of other pharmacological agents is at the discretion of the attending physician. Patients are re-evaluated at six and 24 hours and again at one and four weeks in order to assess clinical status and perform serial assays of haematological and biochemical assays and EPC measures. At six months patients undergo repeat coronary angiography using the same orthogonal views and intravascular ultrasound (IVUS) examination of the stented segment. Patients treated with drug eluting stents (DES), or those not undergoing PCI receive no further study medication, but are invited to return at 24 hours to measure the response of putative EPC. The protocol for the STARS trial is summarised on page 63.

The primary outcome measure will be in-stent neointimal hyperplasia at 6 months. Neointimal hyperplasia within the stented arterial segment will be quantified by IVUS and volume calculated using a well-validated edge-detection algorithm as described previously [von Birgelen *et al.*, 1997]. Briefly, the coronary artery is cannulated with a guiding catheter, and a 3.2F 40-MHz IVUS imaging catheter (Atlantis SR Pro, Boston Scientific) advanced into the artery. IVUS examination is performed at 0.5 mm/s with a motorised pullback device (Boston Scientific). Computerised 3D reconstructions of the stented segments will be performed offline by a single blinded operator using the TomTec computer system (Echoscan, TomTec Imaging Systems) [Newby *et al.*, 2001]. A secondary outcome measure will be angiographic restenosis, defined as a >50% reduction in target lesion minimum

lumen diameter (MLD) as determined by blinded off-line quantitative coronary angiographic (QCA) analysis immediately post-stent deployment and at 6 months (Figure 8.1).

In order to test the hypothesis that reduced EPC numbers prior to and following PCI are associated with in-stent restenosis, we will quantify circulating putative EPC ($CD34^+CD45^-$, $CD34^+VEGFR\text{-}2^+$, $CD34^+VEGFR\text{-}2^+CD133^+$) and EC-CFU concentrations at baseline and at six hours following the procedure at the interval associated with peak mobilisation of $CD34^+CD45^-$ cells as determined in chapter seven. In addition, blood samples will be obtained at 24-hours, at one week and at one month following PCI. Baseline EPC concentrations and the response to PCI may then be related to the extent of neo-intimal hyperplasia. Plasma and serum will be prepared for storage and batch analysis for inflammatory cytokines and CRP. Data from the intervention and placebo arms of the STARS study will be assessed separately.

Expected Outcomes: We anticipate that a robust response by EC-CFU in response to vascular injury will be associated with enhanced vascular healing and a reduction in neo-intimal hyperplasia. However, consistent with previous studies, persistent vascular inflammation is likely to be associated with an increase in neo-intimal hyperplasia. Therefore a sustained increase in EC-CFU may similarly be associated with an increase in in-stent restenosis. As $CD34^+CD45^-$ cells probably

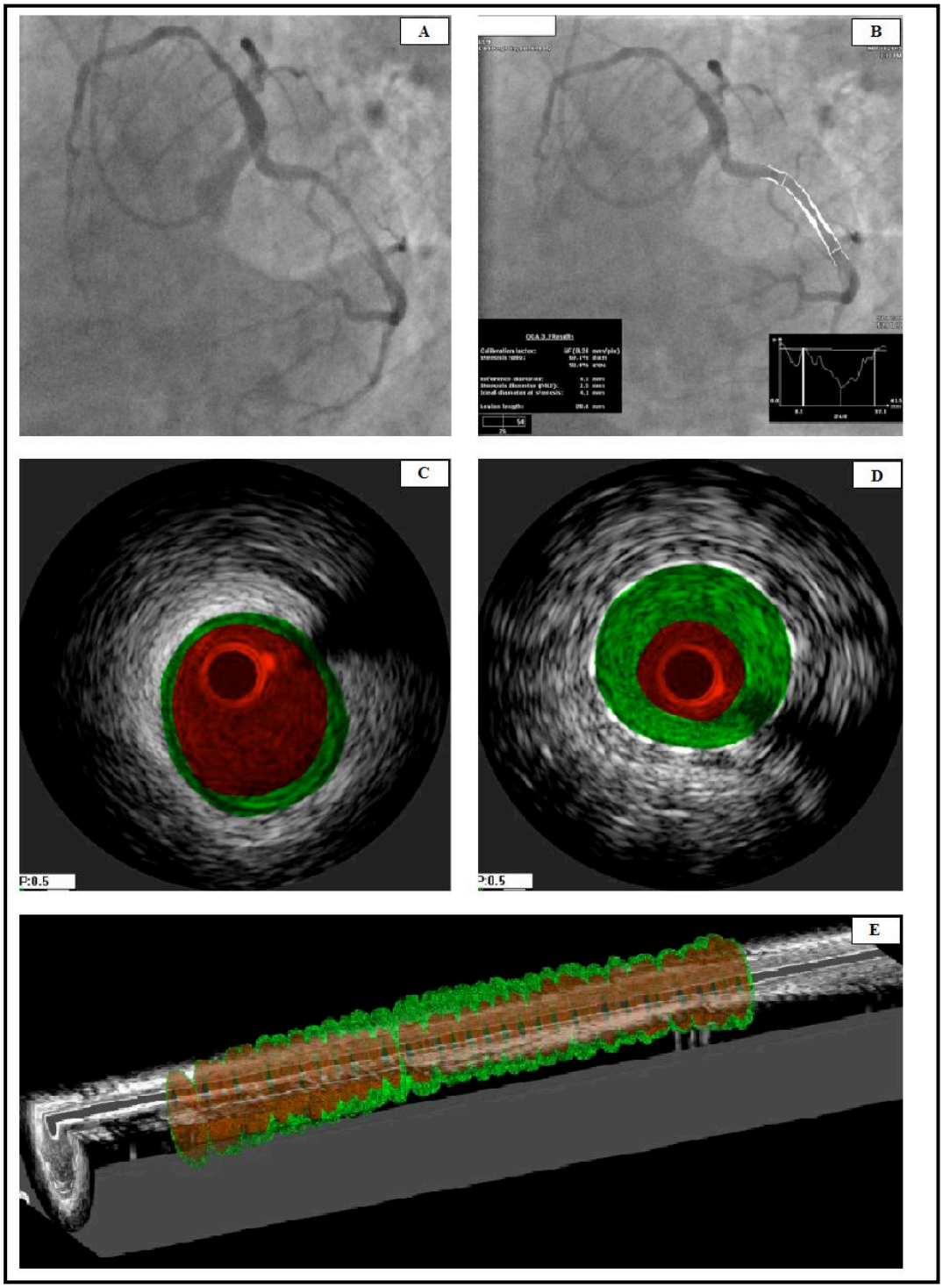

Figure 8.1 Evaluation of proximal circumflex lesion by QCA and IVUS: A: An LAO caudal view of the left coronary artery demonstrating lesions in the proximal left anterior descending artery and a restenotic lesion in the circumflex. **B**: The circumflex (Cx) has been analysed using edge detection software providing a reference vessel diameter and percentage stenosis. **C**: The relatively healthy proximal segment of Cx same lesion as assessed by IVUS. There is mild concentric plaque, mainly between 3 and 6 o'clock. **D**: In stent. There is marked, concentric neointimal hyperplasia shown here in green. **E**: a volume rendering depicting the volume of neo-intima calculated by summation of each segment.

reflect the degree of vascular injury, we anticipate a positive correlation between increased $CD34^+CD45^-$ cells and in-stent neointimal hyperplasia at 6 months. However, if $CD34^+CD45^-$ cells possess reparatory function we will observe a negative correlation. It is likely that traditional putative EPC bearing CD34 and VEGFR-2 will have little bearing on the development of ISR however this has yet to be prospectively assessed in a clinical trial.

8.3.2. Where are $CD34^+CD45^-$ cells mobilised from following PCI?

We wish to determine the regenerative capacity of $CD34^+CD45^-$ cells and their effect on vascular healing. As we have identified $CD34^+CD45^-$ cells as a potential therapeutic target in regenerative medicine, determining their source of origin is of interest. If of bone marrow origin, $CD34^+CD45^-$ cells may be potentially harvested and are likely to have a high regenerative capacity, unlike cells merely sloughed from the vasculature. To determine the source of origin of $CD34^+CD45^-$ cells and their regenerative capacity we propose parallel studies using transgenic mice subjected to vascular injury and a direct comparison of $CD34^+CD45^-$ concentrations in the coronary arterial and venous blood before and following PCI in man.

Animal model experimental design

Transgenic mice expressing green fluorescent protein (GFP) will be bred at the MRC Centre for Regenerative Medicine, Edinburgh University. Non-GFP expressing SCD/NOD mice exposed to a sub-lethal dose of irradiation to induce

bone marrow suppression will undergo haematopoietic reconstitution by intravenous transfusion of haematopoietic stem cells ($2x10^6$ cells) from the GFP expressing strain. Transfected mice will be maintained in sterile conditions until bone marrow full recovery has been achieved. Mice will then be anaesthetised and exposed to endovascular injury by angioplasty using a 2F fogarty catheter introduced into the femoral artery. The presence of $GFP^+CD34^+CD45^-$ cells circulating in the peripheral blood will be determined by flow cytometry in injured mice at baseline and at one, six and 24 hours hours following vascular injury. Animals will be humanely killed at 4 weeks post vascular injury and the presence of GFP expressing endothelial cells at the site of vascular injury will be determined by immunohistochemistry. The role of the $CD34^+CD45^-$ cells on neo-intimal hyperplasia will be further assessed directly using an *ex-vivo* expanded $GFP^+CD34^+CD45^-$ late outgrowth population obtained using FACS of peripheral blood withdrawn from GFP expressing mice. Following vascular injury in non-GFP^+ SCD/NOD mice as described, $GFP^+CD34^+CD45^-$ cells will be transfused via a tail vein. Mice will be killed humanely at 12 weeks and the degree of neo-intimal hyperplasia related to vascular injury will be compared with a control population subjected to vascular injury without treatment with cell infusion.

Clinical study experimental design

We will recruit 40 patients requiring elective PCI for flow limiting coronary stenoses. Patients with a recent acute coronary syndrome or coronary intervention (<3 months), renal or hepatic failure, or a systemic inflammatory disorder or

malignancy will be excluded. Standard baseline data will be collected including, cardiovascular risk factors, past medical history, medical therapy, clinical presentation and angiographic findings. Peripheral venous blood will be collected for enumeration of $CD34^+CD45^-$ cells, in addition to routine clinical tests including lipid profile, HbA1c, fasting glucose, renal and liver function.

Coronary angiography will be performed via the right radial artery with 6F arterial catheters. Prior to PCI a 6F sheath will be placed in the right femoral vein and the coronary sinus will be cannulated using a suitable guide catheter (Amplatz or SO2 sheath) a venous sampling run will be performed involving simultaneous blood sampling from the proximal coronary artery via a guide catheter and vein at t=0 and 30s and at 1, 3, 5, 10 and 15 minutes. The operator will then proceed to perform PCI. At the onset of the first balloon inflation a further sampling run identical to the control run will be performed. Once a satisfactory result has been achieved from PCI, catheters and sheaths will then be removed and vasculotomies closed under manual pressure or the use of a compression device. The patient will then be returned to recovery, and at six hours following PCI a further peripheral venous sample will be taken to confirm a systemically detectable mobilisation of $CD34^+CD45^-$ cells and an inflammatory response (IL-6, differential leukocyte count).

Expected Outcomes

Animal studies: We anticipate that if $CD34^+CD45^-$ expressing cells are derived from the bone marrow we will observe an increase in $GFP^+CD34^+CD45^-$ cells following endovascular injury. If $CD34^+CD45^-$ cells are derived from the vessel wall

$CD34^+CD45^-$ cells will be GFP^-. The regenerative capacity and of $CD34^+CD45^-$ cells will be determined by the proportion of $CD34^+CD45^-GFP^+$ that constitute neo-endothelium following vascular injury and the impact on the development of neo-intima.

Clinical studies: We anticipate that vascular injury will cause an early increase in $CD34^+CD45^-$ cells that will be sustained to six hours. If $CD34^+CD45^-$ cells are liberated from the coronary artery they will necessarily pass through the coronary veins before entering the systemic circulation, and will therefore be detected at higher concentrations in the coronary sinus compared to the coronary artery proximal to the site of vascular injury. If $CD34^+CD45^-$ cells are derived from the bone marrow we will not observe such a rise in the $CD34^+CD45^-$. Indeed if $CD34^+CD45^-$ cells are BM derived and are specifically homing to sites of vascular injury, we expect that $CD34^+CD45^-$ cells will be detected at lower concentrations in the venous blood due to engraftment of cells in the injured coronary artery.

8.3.3. Can intracoronary stent design enhance EPC engraftment?

The modern strategy adopted to avoid ISR is based on the use of drug-eluting stents designed to suppress cellular proliferation [Muldowney *et al.*, 2007]. However, local anti-proliferative therapy may also interfere with stent re-endothelialisation and increase the risk of stent thrombosis. Therefore directly accelerating re-endothelialisation may be a more favourable strategy. Efforts to attract 'EPC' using stents coated with monoclonal antibodies directed against CD34 have reported

acceptable safety data [Aoki *et al.*, 2005; Co *et al.*, 2008; Duckers *et al.*, 2007; Lee *et al.*; Miglionico *et al.*, 2008; Silber *et al.*], however CD34 capture stents may potentiate ISR [Cervinka, 2009] possibly through attracting non-endothelial vascular precursors. This difficulty in identifying the relevant cell type may be obviated by coating stents with pro-angiogenic biopolymers that specifically enhance re-endothelialisation without accelerating smooth muscle hyperplasia [Chan *et al.*, 2008; Mooney *et al.*, 2008].

Experimental design

From a library of polyurethane and polyacrylate polymers [Anderson *et al.*, 2004; Tourniaire *et al.*, 2006], we have generated a polymer microarray on aminoalkylsilane-treated, agarose coated glass slides. Putative late outgrowth cells sourced from umbilical cord blood [Tura *et al.*, 2007], human umbilical vein cells and $CD34^{+}CD45^{-}$ cells will be cultured to confluence and then seeded onto the microarray. Arrays are then stained for endothelial antigens following a period of incubation. Endothelial growth on each polymer member can then be determined by scanning of the polymer spots using automated software Pathfinder™ (IMSTAR S.A., Paris, France) (Figure 8.5).

Sponge experiments

Using an established sponge model of *in-vivo* angiogenesis [Hague *et al.*, 2002] we can assess the in-vivo effect of these polymers on angiogenesis. Polyurethane sponges can be dip-coated in growth factors or polymers of interest and cultured with various cell populations or implanted subcutaneously in severe combined

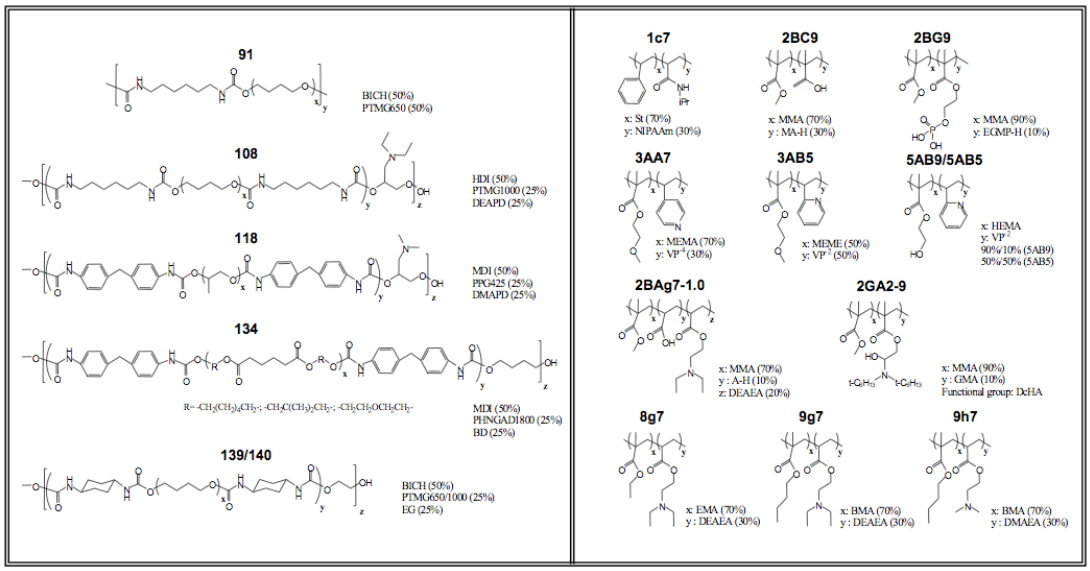

Figure 8.4 Polymer library: Molecular structure of selected polyurethane and polyacrylate polymers used to culture HUVEC and EOC. Chemical compositions of the best eighteen polymers. Abbreviations: St: Styrene; NIPAAm: N-isopropylacrylamide; MMA: methyl methacrylate; MA-H: methacrylic acid; EGMP-H: ethylene glycol methacrylate phosphate; MEMA: 2-methoxyethylmethacrylate; V^{-4}: 4-vinylpyridine; V^{-2}: 2-vinylpyridine; HEMA: 2-hydroxyethylmethacrylat; A-H: acrylic acid; DEAEA: 2-(diethylamino)ethyl acrylat; GMA: glycidyl methacrylat; DnHA: di-n-hexylamine; EMA: ethyl methacrylate; BMA: butyl methacrylate; DMAEA: 2-(diethylamino)ethyl acrylate; BICH: 1,3-bis(isocynanatomethyl)cyclohexane; PTMG: poly(butylene glycol); BD: 1,4-butanediol; HDI: 1,6-diisocyanohexane; DEAPD: 3-(diethylamino)-1,2-propanediol; MDI: 4,4'-methylenebis(phenylisocyanate); PPG: poly(propylene glycol); DMAPD: 3-dimethylamino-1,2-propanediol; PHNGAD: poly[1,6-hexanediol/neopentylglycol/diethylene glycol-alt-(adipic acid)]diol; EG: ethylene glycol. See figure overleaf. See figure 8.5

immunodeficient/non-obese diabetic (SCD/NOD) mice. Sponges are subsequently retrieved and examined for evidence of neoangiogenesis using, scanning electron microscopy and immunostaining. In preliminary experiments certain polymers have shown promise. For instance the polyacrylate, 8G7 was associated with a high density of endothelial coverage using late out-growth and HUVEC on the microarray, and sponges impregnated with 8G7 showed increased formation of vascular networks compared to growth factor reduced matrigel® (GFR-M) treated sponges (Figure 8.6a (1-6)). Furthermore, histological analysis using haematoxylin/eosin immunostaining of excised sponges has demonstrated significantly greater vascularisation in 8G7 coated sponges compared with GRF-M treated control sponges (Figure 8.6 b).

Similarly, the concentration of haemoglobin within explanted sponges (a surrogate measure of perfused blood vessels) was ~3-fold higher in 8G7 coated sponges than in GRF-M treated sponges (Figure. 8.6c). Supplementary analysis using quantitative polymerase chain reaction (qPCR) also indicates that 8G7 coating promotes endogenous neovascularisation with increased relative expression of mouse vWF compared with sponges treated with GFR-M. Expression of human vWF was unchanged (Figure. 8.6d).

Stent experiments

Polymers with angiogenic potential will be applied to intracoronary stents to explore their potential clinical utility. Coated stents are co-incubated with late outgrowth endothelial cells or HUVEC for several days before being inspected using SEM for evidence of increased cell attachment and confluence. Platelet and monocyte adherence to intra-coronary stents are critical components in the development of both stent thrombosis and restenosis, therefore it will be important to determine the propensity for monocytes to bind to polymers identified as having angiogenic potential. We have performed preliminary studies examining the effects of 8G7 on *in-vitro* re-endothelialisation and platelet-monocyte binding of inta-coronary stents by means of scanning electron microscopy. These have demonstrated improved endothelialisation associated with 8G7 coating with encouragingly low levels of platelet monocyte binding (Figure 8.7).

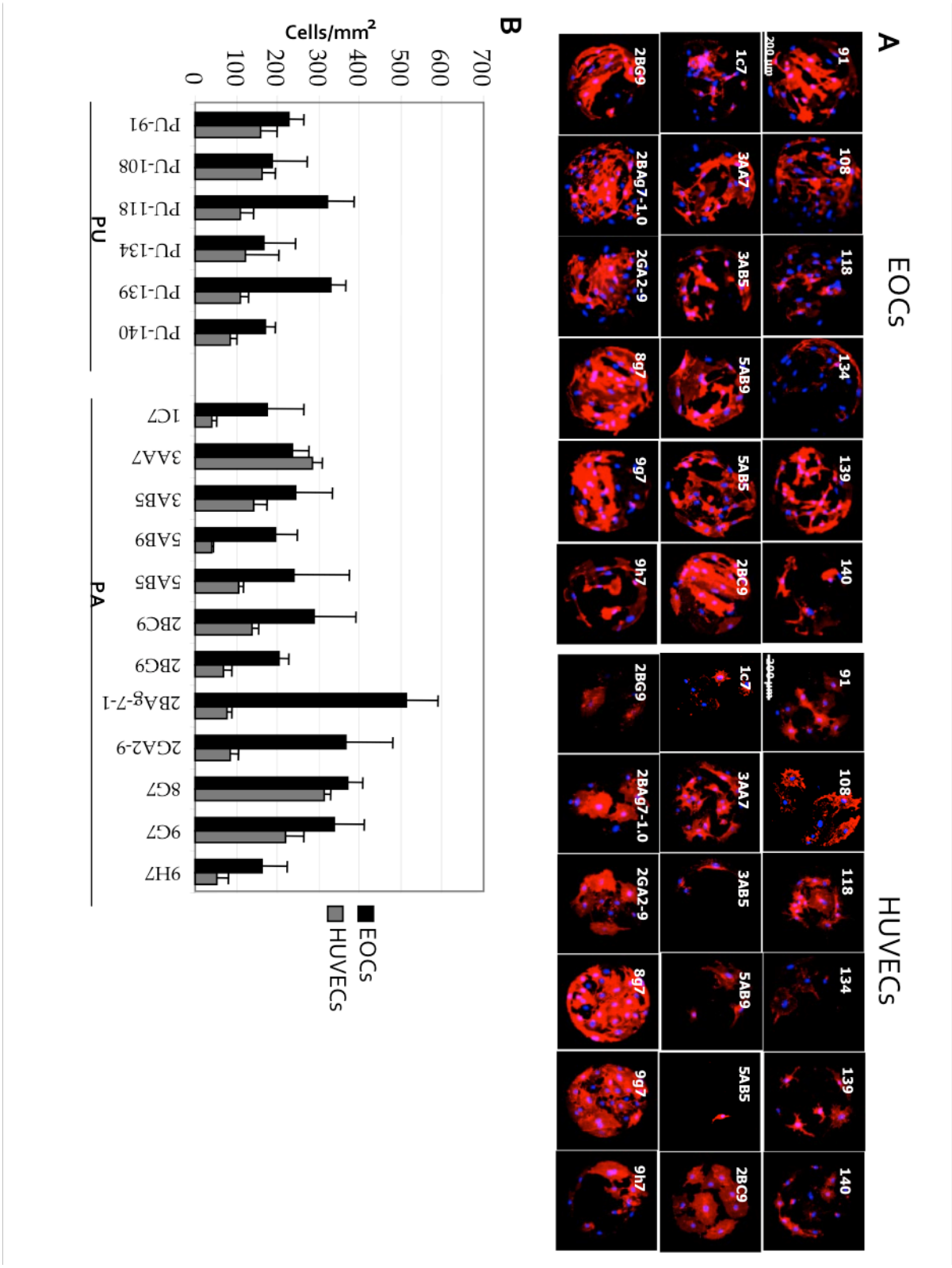

Figure 8.5 Endothelial late outgrowth cells and HUVEC incubated on polymer microarrays for 3 days: Endothelial late outgrowth cells (endothelial colony forming cells) and human umbilical vein endothelial cells were grown on various polyurethane and polyacrylate polymers. The rhodamine channel (red) shows the endothelial phenotype (CD31). Cell nuclei are stained with DAPI (Hoechst 33342, blue).

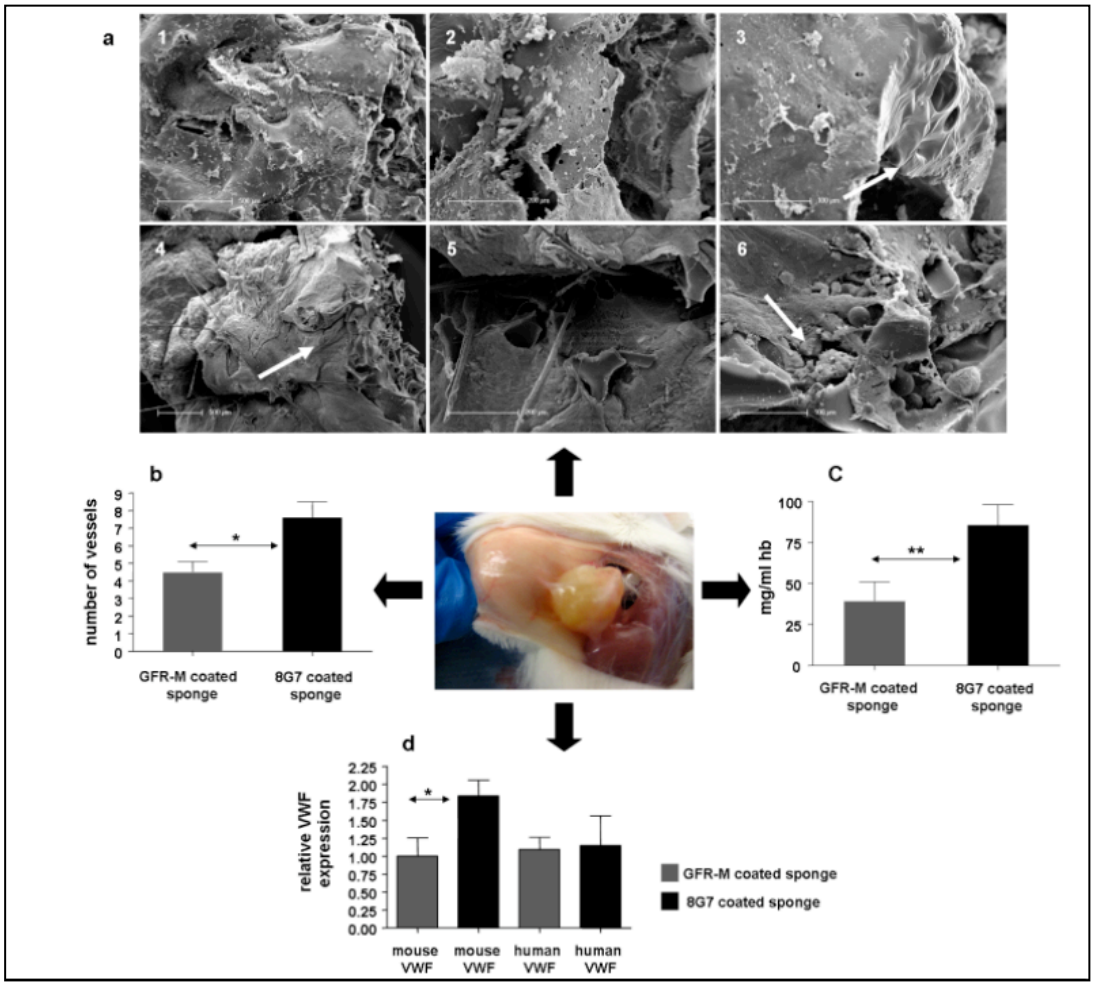

Figure 8.6. Subcutaneous sponge implant assay.
Here sponges excised from male SCID/NOD mice after 20 days following implantation are analysed for evidence of neovascularisation. **a**) Electron microscopy analysis of sponges impregnated with the polyacrylate, 8G7 (**1-3**) and growth factor reduced matrigel (GFR-M) (**4-6**) loaded with endothelial outgrowth cells (EOC) (arrows show vascular networks). **b**) Blood vessel density in sponges, determination by Chalkley counts on two sections per sponge. The results presented are a mean of four animals ± SEM. *P<0.05. **c**) Haemoglobin assay: measure of the amount of haemoglobin (mg/ml) present in the samples (n=3) **P<0.01 **d**) Relative expression of both mouse and human von Willebrand factor measured by qPCR n=3. *P<0.05. Statistical analyses were performed using Mann-Whitney U test.

Figure 8.7 *In-vitro* stent experiments: Late outgrowth EPC , platelets and monocytes were incubated with an uncoated or an 8G7-coated chromium-cobalt alloy coronary stents. After 96 h incubation, in endothelial basal medium, cells were fixed and examined by electron microscopy. Late outgrowth EPC binding is shown on **a,b**) an uncoated stent and **c,d**) an 8G7 coated stent. Platelet binding is shown on **e,f**) uncoated and 8G7 coated stents **g,h**). Monocyte binding on uncoated stents **i,j**) (arrows (**j**) show modest monocyte binding) and 8G7 coated stents **k,l**). Late outgrowth EPC cultured with the uncoated stent showed no cell attachment whereas late outgrowth EPC cultured with the 8G7-coated stent exhibited a consistent adhesion with around 80% confluence. Platelet adhesion on the uncoated stent was relatively increased compared to the 8G7-coated stent. Virtually no monocytes binding to the uncoated stent

a
50 µm
b
50 µm
c
100 µm
d
50 µm
e
100 µm
f
50 µm
g
100 µm
h
50 µm
i
100 µm
j
50 µm
k
100 µm
l
100 µm

Expected results

We anticipate that by using comprehensive assessment of a wide variety of polymers we will identify a platform that will encourage endothelial seeding and proliferation. Polymers such as 8G7 that show potential for improving re-endothelialisation may then be then employed in animal models of PCI to determine whether they affect re-endothelialisation, thrombus formation and neo-intimal hyperplasia.

8.4 CLINICAL PERSPECTIVE

The technological advances in PCI over the last 30 years have been quite remarkable. At its inception, simple balloon angioplasty was considered most adventurous, the use of intra-coronary stents have now become routine practice, and stent design has become increasingly more refined, providing greater flexibility and deliverability of intra-coronary stents. Drug eluting stents are now well established as a means of reducing in-stent restenosis, and as the volume and complexity of percutaneous coronary interventions continues to increase, we move forward into the era of bio-absorbable stents. These developments combined with the development of a wide array of adjunctive equipment for accessing, imaging, and recannalising diseased coronary arteries, has allowed increasingly more complex lesions to be treated effectively with high degrees of safety and a commensurate improvement in patient outcomes. However, endothelial injury associated with PCI has remained a constant feature, with little technological advance specifically addressing this important obstacle. A means of accelerating re-endothelialisation of implanted stents continues to be most attractive. So far attempts to achieve this therapeutic goal have been disappointing, be that through direct transfusion or pharmacological mobilisation of putative progenitor cells, or by the use of innovative EPC 'capture' stents. Perhaps these shortcomings have been largely due to an incomplete understanding of the cell populations involved in neo-endothelialisation and neo-intimal formation.

An improved understanding of endogenous vascular repair via circulating progenitors may allow us to move forward in developing novel treatments for

iatrogenic vascular injury incurred during PCI. The studies contained within this thesis provide some contribution to our understanding of the characteristics and clinical relevance of a variety of putative EPC populations that may be of use in subsequent pre-clinical and clinical studies of EPC. Identifying the optimal cell populations and means of regulating their behaviour remain important research goals in this field.

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APPENDIX

PUBLICATIONS ARISING FROM THESIS

ORIGINAL ARTICLES

Padfield, GJ, et al. The Constituents and Mechanisms of Generation of 'Endothelial Cell - Colony Forming Units'. *Cardiovascular Research* 2013b; (in press). [Appended as PDF]

Padfield GJ, et al. Endothelial progenitor cells, atheroma burden and clinical outcome in patients with coronary artery disease. *Heart.* 2013; 99(11): 791-8. [Appended as PDF]

Padfield GJ et al. Circulating endothelial progenitor cells are not affected by acute systemic inflammation. *AJP: Heart and Circulatory Physiology.* 2010; 298: 2054-61. [Appended as PDF]

NL Mills, et al. Dissociation of phenotypic and functional endothelial progenitor cells in patients undergoing percutaneous coronary intervention. *Heart.* 2009; 95(24): 2003-8. [Appended as PDF]

REVIEW ARTICLE AND BOOK CHAPTER

Padfield GJ. (2012) Endothelial Progenitor Cells in the Treatment of Vascular Disease, in: Willis MS and Homeister JW. (ed) *Molecular and Translational Medicine Part 2*, New York: Springer Science. Pages 283-327.

Padfield, GJ, et al. Understanding the Role of Endothelial Progenitor Cells in Percutaneous Coronary Intervention. *J Am Coll Cardiol* 2010 55: 1553-1565. [Appended as PDF]

SUPPLEMENTARY ELECTRONIC FILES

Appended publications

Time lapse images of EC-CFU formation