Progenitor cells and vascular disease

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Abstract. Vascular progenitor cells have been the focus of much attention in recent years; both from the point of view of their pathophysiological roles and their potential as therapeutic agents. However, there is as yet no definitive description of either endothelial or vascular smooth muscle progenitor cells. Cells with the ability to differentiate into mature endothelial and vascular smooth muscle reportedly reside within a number of different tissues, including bone marrow, spleen, cardiac muscle, skeletal muscle and adipose tissue. Within these niches, vascular progenitor cells remain quiescent, until mobilized in response to injury or disease. Once mobilized, these progenitor cells enter the circulation and migrate to sites of damage, where they contribute to the remodelling process. It is generally perceived that endothelial progenitors are reparative, acting to restore vascular homeostasis, while smooth muscle progenitors contribute to pathological changes. Indeed, the number of circulating endothelial progenitor cells inversely correlates with exposure to cardiovascular risk factors and numbers of animal models and human studies have demonstrated therapeutic roles for endothelial progenitor cells, which can be enhanced by manipulating them to overexpress vasculo-protective genes. It remains to be determined whether smooth muscle progenitor cells, which are less well studied than their endothelial counterparts, can likewise be manipulated to achieve therapeutic benefit. This review outlines our current understanding of endothelial and smooth muscle progenitor cell biology, their roles in vascular disease and their potential as therapeutic agents.

INTRODUCTION

In recent years, there has been huge interest in stem and progenitor cell biology, the pathophysiological roles and potential to exploit these cells for regenerative therapy. Atherosclerosis, a leading worldwide cause of morbidity and mortality, is compounded by the fact that surgical interventions aimed at unblocking or bypassing these lesions are themselves prone to developing atherosclerotic occlusion. Coronary artery bypass grafting, for example, commonly employs saphenous vein portions as conduits. However, the failure rate for these grafts is as much as 20%

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after 1 year and 40% after 10 years (van Brussel *et al.* 1997; Goldman *et al.* 2004). Thus, in order to serve patients better, there is a need to improve the patency of such grafts. Much work has been undertaken on the role of endothelial progenitor cells (EPC) in vascular biology and disease since they were first described in peripheral blood a decade ago (Asahara *et al.* 1997). More recently, haematopoietic cells with the potential to differentiate into smooth muscle cells have been identified and have, likewise, been dubbed smooth muscle progenitor cells (SMPC) (Han *et al.* 2001; Hillebrands *et al.* 2001; Shimizu *et al.* 2001; Sata *et al.* 2002; Simper *et al.* 2002). This review outlines our current understanding of these vascular progenitor cells and discusses their possible roles in disease progression and therapy.

LINEAGE OF VASCULAR PROGENITOR CELLS

Cells from a multitude of sources are reportedly able to differentiate into endothelial and/or smooth muscle cells, including bone marrow, peripheral blood and spleen, as well as cardiac muscle, skeletal muscle, adipose tissue and embryonic stem cells (for review, see Caplice & Doyle 2005). However, there is as yet no definitive description of the origin and differentiation of EPC or SMPC.

Endothelial progenitor cells were initially described as being a subset of CD34⁺ haematopoietic stem cells (HSC) co-expressing vascular endothelial growth factor receptor-2 (VEGFR-2/Flk-1/KDR). These cells were able to differentiate into cells expressing a range of endothelial markers (including CD31, PECAM-1, and the endothelial receptor tyrosine kinase, tie-2), to incorporate acetylated low density lipoprotein (LDL), form tube-like structures *in vitro* and produce nitric oxide in response to vascular endothelial growth factor (VEGF) (Asahara *et al.* 1997; Shi *et al.* 1998). However, CD34 is not exclusively expressed on HSC and can be found at low levels on mature endothelial cells. Therefore, the more immature progenitor cell markers CD133 (prominin/AC133), c-kit (CD117) and stem cell antigen-1 (Sca-1) have also been used (Caplice & Doyle 2005). Some data also indicate that EPCs may exist in populations of CD34⁺ and CD133⁺ mononuclear cells. Indeed, a population of CD34⁺/CD133⁺/VEGFR-2⁺ progenitors, recently identified in peripheral blood, are proposed to be precursors of CD34⁺/CD133⁺/VEGFR-2⁺ EPCs (Friedrich *et al.* 2006) and CD133⁺/CD14⁺ cells isolated from human umbilical cord blood are able to differentiate into mature endothelial cells (Kim *et al.* 2005). CD14⁺ peripheral blood mononuclear cells (PBMC) have been reported to express a range of pro-angiogenic factors, including VEGF, hepatocyte growth factor and granulocyte colony stimulating factor (G-CSF) (Fernandez Pujol *et al.* 2000; Moldovan *et al.* 2000; Harraz *et al.* 2001; Schmeisser *et al.* 2001; Rehman *et al.* 2003), to integrate into ischaemic tissue and to contribute to neo-angiogenesis (Moldovan *et al.* 2000; Harraz *et al.* 2001; Urbich *et al.* 2003). So-called side population cells, characterized by efflux of Hoechst 33342, from skeletal muscle are also reportedly able to engraft into damaged endothelium (Majka *et al.* 2003).

Endothelial progenitor cells contribute to re-endothelialization of transplanted tissue. Indeed, models have shown that the endothelium of aortic allografts consists entirely of recipient-derived cells (Hillebrands *et al.* 2001). In contrast, in cardiac transplant models very few endothelial cells of recipient origin have been observed. It is possible that these differences relate to the use of immunosuppressants in the cardiac but not aortic graft models (Hillebrands *et al.* 2005). In human studies, it has been demonstrated that the number of circulating EPCs is reduced in cardiac allograft patients with established transplant vasculopathy (Simper *et al.* 2003) and this may indicate sequestration of these cells to the graft site. Indeed, up to a third of endothelial cells may be recipient-derived (Minami *et al.* 2005).

The source of smooth muscle cells within neo-intimal lesions is still under debate, as different models have provided different answers. For example, animal models of post-angioplasty restenosis and atherosclerosis have shown that the majority of smooth muscle cells of the intima are of bone marrow origin (Saiura *et al.* 2001; Sata *et al.* 2002). However, while it has been shown in rodents that smooth muscle cells of recipient origin are present in the intima of aortic grafts and a proportion of these cells are of bone marrow origin (Hillebrands *et al.* 2001; Shimizu *et al.* 2001), it has also been reported that bone marrow-derived cells do not contribute to the intimal smooth muscle cell population in such grafts (Li *et al.* 2001; Hu *et al.* 2002a). Furthermore, animal models of cardiac and aortic transplant vasculopathy suggest that the majority of smooth muscle cells of neo-intimal lesions are of recipient origin (Han *et al.* 2001; Hillebrands *et al.* 2001; Religa *et al.* 2002; Sata *et al.* 2002). It appears that no, or very few, smooth muscle cells within cardiac grafts in humans are of recipient origin (Glaser *et al.* 2002; Atkinson *et al.* 2004; Minami *et al.* 2005). In contrast, smooth muscle cells of donor origin have been observed in coronary atherosclerosis plaques in patients having received sex mismatched bone marrow transplantation (Caplice *et al.* 2003).

The adventitia has also been cited as a possible source of SMPC. Indeed, large numbers of Sca-1⁺ cells were observed in the adventitia of aortic roots in apolipoprotein E (ApoE)^{-/-} mice and also in ligated arteries in endothelial nitric oxide synthase (eNOS)^{-/-} mice (Hu *et al.* 2004; Zhang *et al.* 2006). However, it is conceivable that bone marrow-derived SMPCs might first migrate to the adventitia and, subsequently, translocate to the intima. Indeed, non-side population cells from skeletal muscle engraft into the smooth muscle of damaged vessels. Evidence suggests that these cells originate in the bone marrow but migrate to the muscle and subsequently lose their bone marrow markers, although they do retain haematopoietic potential (Jackson *et al.* 1999; Issarachai *et al.* 2002; McKinney-Freeman *et al.* 2002; Majka *et al.* 2003). Some *in vitro* studies even suggest that mature endothelial cells can give rise to smooth muscle cells (Frid *et al.* 2002; Ishisaki *et al.* 2003).

DIFFERENTIATION OF VASCULAR PROGENITOR CELLS

While VEGF is known to play key roles in the differentiation of endothelial cells during embryonic development (Fong *et al.* 1995; Shalaby *et al.* 1995; Ferrara *et al.* 1996) and is required for the differentiation of EPC into mature endothelial cells *in vitro* (Asahara *et al.* 1997; Gehling *et al.* 2000; Zhao *et al.* 2003), there are likely to be other factors involved. For example, macrophage colony stimulating factor and transforming growth factor (TGF)- β 1 both play important roles in endothelial cell maturation during embryogenesis (Risau 1997; Minehata *et al.* 2002), as does vascular endothelial cadherin-mediated cell adhesion (Hirashima *et al.* 1999). Smooth muscle cell differentiation from putative vascular progenitor cells is likewise poorly understood, although that TGF- β 1, TGF- β 3 and platelet-derived growth factor (PDGF)-BB have roles in embryonic development point to their possible involvement (Nakajima *et al.* 1997; Hirschi *et al.* 1998; Hellstrom *et al.* 1999). Indeed, cells expressing a number of smooth muscle specific markers, including α -smooth muscle actin (α SMA), smooth muscle myosin heavy chain and calponin, as well as CD34, VEGFR-1 and VEGFR-2 can be cultured from adult PBMC and VEGFR-2⁺ embryonic stem cells in PDGF-BB-enriched media (Gehling *et al.* 2000; Yamashita *et al.* 2000; Simper *et al.* 2002). In addition, PDGF- β receptor^{-/-} mice show reduced investment of arterioles with smooth muscle cells (Lindahl *et al.* 1997).

MOBILIZATION, HOMING AND RECRUITMENT OF VASCULAR PROGENITOR CELLS

It is believed that under steady-state conditions vascular progenitor cells are maintained in an undifferentiated and quiescent state but are mobilized following physiological stress and subsequently home to sites of vascular damage (Takahashi *et al.* 1999; Cheng *et al.* 2000; Shintani *et al.* 2001; Laufs *et al.* 2004). Indeed, there is a significant and rapid increase in the number of circulating EPC following traumatic vascular injury (Gill *et al.* 2001). The homing ability of vascular progenitor cells was first alluded to when transplanted bone marrow cells were observed lining Dacron grafts in dogs (Shi *et al.* 1998) and a multitude of factors have since been identified as being important for the mobilization and recruitment of vascular progenitor cells, including growth factors, cytokines/chemokines and integrins (Takahashi *et al.* 1999; Hattori *et al.* 2001, 2002; Heeschen *et al.* 2003; Bahlmann *et al.* 2004). Some of the key factors in these processes are highlighted in Fig. 1.

Mobilization of vascular progenitor cells

The mobilization of vascular progenitor cells has largely been studied in the context of EPC and tissue ischaemia. Both VEGF and stromal cell-derived factor-1 (SDF-1) have emerged as key mediators of EPC mobilization (Asahara *et al.* 1999; Kalka *et al.* 2000; Pillarisetti & Gupta 2001; Shintani *et al.* 2001; Massa *et al.* 2005), functioning in a matrix metalloproteinase (MMP)-9-dependent manner (Gu *et al.* 2002; Heissig *et al.* 2002). Quiescent c-kit⁺ cells are retained in the bone marrow niche by interaction with stromal cells. This interaction is supported by membrane-bound Kit ligand (mKitL). However, nitric oxide-mediated activation of MMP-9 leads to cleavage of mKitL and results in release of soluble (s)KitL and the subsequent translocation of vascular progenitor cells into the circulation (Gu *et al.* 2002; Heissig *et al.* 2002). Indeed, both MMP-9 expression and VEGF-induced EPC mobilization are reduced in eNOS^{-/-} mice and this correlates with reduced neo-vascularization following ischaemia (Aicher *et al.* 2004). eNOS-mediated nitric oxide production is dependent on the abundance of caveolin (Cav) (Feron & Kelly 2001) and Cav^{-/-} mice demonstrate defective neo-vascularization following ischaemic injury. In addition, adhesion of Cav^{-/-} EPC to bone marrow stromal cells is resistant to SDF-1-mediated mobilization as a result of defective internalization of the chemokine receptor CXCR4, to which SDF-1 binds exclusively (Lapidot 2001; Sbaa *et al.* 2006). Conversely, attachment of Cav^{-/-} EPC to SDF-1-presenting endothelial cells is significantly increased (Sbaa *et al.* 2006). However, while eNOS appears to be important for EPC mobilization, it does not seem to directly influence EPC function, as assessed by colony formation and migration *in vitro* (Hoetzer *et al.* 2005). In contrast, phosphatidylinositol 3-kinase (PI3K)/Akt-mediated signalling does appear to be important for both EPC mobilization and function. For example, in Akt1^{-/-} mice, the functional activity of EPC, as well as their mobilization in response to ischaemia, is impaired (Ackah *et al.* 2005) and wortmannin, a PI3K inhibitor, has been shown to impair exogenous EPC-mediated reduction of infarct size in a porcine ischaemia-reperfusion model (Kupatt *et al.* 2005).

Erythropoietin (EPO), an endogenous protein produced by the kidney that regulates red blood cell production, has been shown to increase the mobilization of Sca-1⁺/VEGFR-2⁺ cells in mice (Urao *et al.* 2006) and EPC from patients with congestive heart failure that have been treated with EPO exhibit enhanced proliferation and greater adhesion to endothelial cells and fibronectin than EPC from untreated patients. Similar effects were noted when EPO was applied to EPC from healthy individuals *in vitro* and these effects were dose-dependent (George *et al.* 2005b). EPO appears to exert its effects in a nitric oxide-dependent manner (George *et al.* 2005b; Urao *et al.* 2006).

Figure 1. Mobilization, homing and recruitment of vascular progenitor cells. Vascular damage, such as that following vein grafting or stenting, involves endothelial denudation and platelet accumulation. Platelets, along with neighbouring endothelial and smooth muscle cells, secrete various growth factors and cytokines/chemokines, including platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and stromal cell-derived factor-1 (SDF-1). PDGF is important for smooth muscle cell migration and proliferation. VEGF and SDF-1 are important for the mobilization of vascular progenitor cells; circulating in the blood and promoting progenitor cell translocation from the quiescent vascular bone marrow niche. Both endothelial progenitor cells (EPCs) and smooth muscle progenitor cells (SMPCs) of bone marrow origin have been observed in neo-intimal lesions. Once mobilized, vascular progenitor cells are believed to migrate through the circulation to the site of damage where chemokines and integrins mediate their recruitment and there is evidence for both luminal and adventitial routes of entry. For illustrative purposes, vascular progenitor cells are shown as a population of c-kit⁺ cells within the bone marrow, and as EPC and SMPC populations thereafter.

In coronary artery bypass grafting and burns patients, there is an increase (almost 50-fold) in the number of CD133⁺/VEGFR-2⁺ cells in the peripheral blood within 6–12 h after injury and a return to basal levels within 48–72 h, which is mirrored by plasma VEGF levels (Gill *et al.* 2001). However, patients with coronary artery disease (CAD) appear to have reduced numbers

of circulating CD133⁺/VEGFR-2⁺ EPC, although numbers can be increased by taking exercise, or with statin (hydroxymethylglutaryl coenzyme A reductase inhibitor) or GCSF treatment (Vasa *et al.* 2001a; Adams *et al.* 2004; Laufs *et al.* 2004; Powell *et al.* 2005). Statins reportedly increase the number and functional activity of EPC (Dimmeler *et al.* 2001; Llevadot *et al.* 2001; Vasa *et al.* 2001a). However, while EPC numbers are increased in the short term (up to 1 month), it has recently been shown that long-term (3 months) statin treatment actually causes a decrease (almost half that seen prior to treatment) in the number of circulating EPC (Hristov *et al.* 2006). Use of GCSF to mobilize EPC is supported by recent evidence that serum concentrations of endogenous GCSF, in patients with acute myocardial infarction, correlate with the mobilization of CD34⁺ cells and improved left ventricular function (Leone *et al.* 2005), although there is a suggestion that GCSF treatment may increase the risk of vascular events (Hill *et al.* 2005; Zbinden *et al.* 2005). As with EPC, there is evidence that SDF-1 is involved in SMPC mobilization. Indeed, carotid artery ligation in mice leads to an increase in the number of circulating SMPC, which correlates positively with SDF-1 expression, although there appears to be no link between VEGF expression and SMPC mobilization (Zhang *et al.* 2006).

There is also compelling evidence that GCSF can mobilize SMPC. Indeed, exogenous application of GCSF mobilized putative SMPC in a rabbit model of vascular injury and this was associated with increased neo-intimal hyperplasia (Cho *et al.* 2006). Furthermore, data from the recent MAGIC cell (Myocardial Regeneration and Angiogenesis in Myocardial Infarction with GCSF and Intra-coronary Stem Cell Infusion) trial show that GCSF-mediated recruitment of bone marrow-derived cells is associated with an increased rate of restenosis (Kang *et al.* 2004). It has also been demonstrated that plasma GCSF levels are increased in patients following coronary stenting and that this is associated with an increase in the number of circulating CD34⁺ cells. In these patients, late lumen loss positively correlated with the percentage increase in CD34⁺ cells (Inoue *et al.* 2007). While the effects of statin treatment on SMPC mobilization *in vivo* remain to be elucidated, it has recently been reported that the number of α SMA⁺ cells cultured from PBMC is reduced in the presence of pravastatin, which suggests possible inhibitory effects of statins on SMPC (Kusuyama *et al.* 2006a).

Recruitment and integration

It has been proposed that the recruitment of vascular progenitor cells is a multistep process akin to leucocyte recruitment during inflammation, involving chemo-attraction, adhesion and transmigration, although the mechanisms involved still remain to be fully elucidated. Serum levels of SDF-1 are selectively elevated during periods of ischaemia and the SDF-1/CXCR4 axis has been shown to be important for the recruitment of EPC to ischaemic tissue (De Falco *et al.* 2004; Ceradini & Gurtner 2005). Indeed, in rodent models of ischaemia increased EPC recruitment is observed in response to increased local SDF-1 concentrations (Askari *et al.* 2003; Yamaguchi *et al.* 2003) and activation of the thrombin receptor PAR-1 on isolated EPC promotes proliferation, induces CXCR4 expression and enhances migration towards SDF-1 (Smadja *et al.* 2005). SDF-1 expression is regulated by the transcription factor hypoxia-inducible factor-1 in a manner proportional to tissue oxygen tension (Ceradini *et al.* 2004) and adenoviral-mediated over-expression of kinase-deficient integrin-linked kinase, a serine/threonine kinase, which lies upstream of hypoxia-inducible factor-1, in ischaemic limbs leads to reduced expression of SDF-1 with an associated decrease in EPC recruitment (Lee *et al.* 2006). Recent data suggest that CXCR2 may also be important for EPC recruitment. *In vitro*, under flow conditions, a greater number of EPC arrested on immobilized CXCR2 ligands than on immobilized SDF-1 and following wire-induced injury in nude mice, infusion of human PBMC-derived EPC-enhanced endothelial recovery; an effect abrogated when EPCs were pre-treated with an anti-CXCR2 blocking antibody (Hristov

et al. 2007). Based on their observations, the authors suggested that CXCR2 may be important for EPC arrest and CXCR4 for EPC transmigration.

Several studies have also indicated a role for integrins in EPC recruitment. EPCs express high levels of $\beta 1$ -integrins (Deb *et al.* 2004), although they appear to preferentially bind to $\beta 2$ -integrin ligands (Hristov *et al.* 2007). Indeed, while EPC binding to fibronectin is unaffected by the presence of neutralizing antibodies against $\beta 1$ -integrin, antibodies against $\beta 2$ -integrin inhibit EPC adhesion to and migration through endothelial monolayers (Deb *et al.* 2004; Chavakis *et al.* 2005) and compared to wild-type mice, $\beta 2^{-/-}$ mice show significantly impaired recovery after ischaemic injury (Chavakis *et al.* 2005). $\alpha \nu \beta 3$ - and $\alpha \nu \beta 5$ -integrins are also highly expressed by EPC and have been shown to be important for re-endothelialization in a rabbit balloon injury model (Walter *et al.* 2002; Deb *et al.* 2004). Furthermore, implanting stents coated with the $\alpha \nu \beta 3$ ligand cyclic Arg-Gly-Asp resulted in accelerated re-endothelialization and significantly reduced in-stent restenosis in porcine carotid arteries, compared to uncoated stents. This was associated with a significant increase in the early recruitment of either infused EPCs or endogenous CD34⁺ cells (Blindt *et al.* 2006).

Platelets express several adhesion molecules and secrete potent chemokines at sites of vascular injury. *In vitro*, adherent platelets simulate chemotaxis and migration of embryonic EPC under flow conditions, which is inhibited in the presence of neutralizing antibodies against either P-selectin glycoprotein ligand-1 or very late antigen-4 (a $\beta 1$ -integrin). In addition, immobilized platelets exposed to thrombin stimulate chemotaxis and migration of embryonic EPC, as well as their differentiation into mature endothelial cells (Langer *et al.* 2006).

Other factors that may influence EPC recruitment include VEGF. Indeed, overexpression of VEGF-C by fibroblasts embedded in a three-dimensional collagen matrix lead to enhanced EPC invasion of and migration through the matrix (Bauer *et al.* 2005). Evidence also points to a possible role for β -carotene; EPC chemotaxis is up-regulated in response to β -carotene stimulation and several genes involved in EPC homing and adhesion are likewise up-regulated (Kiec-Wilk *et al.* 2005).

As with EPC, the SDF-1/CXCR4 axis appears to be important for the recruitment of SMPC and subsequent neo-intima formation. In a mouse wire injury model, it was shown that apoptosis triggers SDF-1 expression by medial smooth muscle cells and that SDF-1 binds to platelets at the site of injury and mediates CXCR4-dependent SMPC arrest (Zernecke *et al.* 2005). In addition, SDF-1 up-regulates expression of MMP-2, which has been implicated in smooth muscle cell migration and proliferation (Kodali *et al.* 2006). Like EPC, SMPCs express high levels of $\beta 1$ -integrin and in the presence of antibodies against $\beta 1$ -integrin the binding of SMPC to fibrin is significantly reduced (Deb *et al.* 2004). In addition, given its importance in the recruitment of smooth muscle cells during embryonic development and its involvement in the migration of smooth muscle cells from the media to the intima following arterial injury, it is probable that PDGF-BB also plays a role in SMPC recruitment (Hellstrom *et al.* 1999; Buetow *et al.* 2003).

VASCULAR PROGENITOR CELLS AND CARDIOVASCULAR RISK FACTORS

Endothelial cells help to maintain vessel homeostasis and it is believed that EPCs have a role in maintaining the integrity of the endothelium. Increased numbers of CD34⁺/VEFR-2⁺ or CD133⁺ circulating EPCs are associated with a reduced risk of death from cardiovascular causes, and it has been suggested that the number of circulating EPCs may be a useful prognostic tool for

determining the risk of cardiovascular events (Schmidt-Lucke *et al.* 2005; Werner *et al.* 2005). Indeed, there is an inverse relationship between the number of circulating EPC and severity of CAD, independent of traditional risk factors (Kunz *et al.* 2006). In addition, it has recently been demonstrated that patients with acute coronary syndrome have an increased number of apoptotic circulating CD34⁺ cells, which is associated with the extent of coronary stenosis (Schwartzberg *et al.* 2007).

While the risk of developing atherosclerotic lesions increases with age, it has been reported that the number of circulating CD34⁺/VEGFR-2⁺ or CD133⁺/VEGFR-2⁺ EPC are comparable between old and young individuals without major cardiovascular risk factors, although EPC isolated from older individuals do have a lower survival rate than those from younger people and exhibit reduced migration and proliferation (Heiss *et al.* 2005). This may be important in terms of a person's ability to maintain homeostasis and repair damage in ageing vessels. In addition, circulating EPC numbers are reduced in healthy individuals with risk factors for cardiovascular disease (CVD) and it is evident that a reduced number of circulating EPC correlates with endothelial dysfunction (Hill *et al.* 2003). There is, likewise, an inverse correlation between the number of circulating EPC and the number of risk factors in patients with known CAD (Vasa *et al.* 2001b; George *et al.* 2003, 2004), as well as in patients with disorders known to present a higher risk of developing CVD (Tepper *et al.* 2002; Chen *et al.* 2004; Choi *et al.* 2004; Kondo *et al.* 2004; Ghani *et al.* 2005; Grisar *et al.* 2005; Michaud *et al.* 2006).

Rheumatoid arthritis, for example, is associated with increased morbidity and mortality attributable to accelerated atherosclerosis and cardiovascular events (Wolfe *et al.* 1994; Raza *et al.* 2000). In patients with active disease, the number of circulating EPC is significantly reduced and isolated EPC from these people exhibit reduced migratory activity (Grisar *et al.* 2005; Herbrig *et al.* 2006). Other major risk factors for developing CVD, such as smoking and hypercholesterolaemia are also associated with reduced numbers of circulating EPC (Chen *et al.* 2004; Kondo *et al.* 2004; Michaud *et al.* 2006). In people who smoke, the number of circulating EPCs inversely correlates with the number of cigarettes smoked and rapidly increases following cessation of smoking (Kondo *et al.* 2004; Michaud *et al.* 2006). Furthermore, EPCs isolated from smokers exhibit impaired functional activity (Chen *et al.* 2004; Michaud *et al.* 2006) and, *in vitro*, benzo(α)pyrene, a polycyclic aromatic hydrocarbon present in tobacco smoke, dose dependently inhibits EPC colony formation (van Grevenynghe *et al.* 2006). EPCs isolated from hypercholesterolaemic individuals also demonstrate impaired functional activity and display increased apoptosis/senescence in response to oxidized LDL. These observations may underlie the reduced number of EPC seen in these individuals (Imanishi *et al.* 2004).

Incidence of hypertension increases with age, particularly in post-menopausal women, and it is known that oestrogen augments EPC mobilization and recruitment following vascular injury (Iwakura *et al.* 2003; Strehlow *et al.* 2003). It has recently been demonstrated, *in vitro*, that EPC VEGF production and network formation is increased in the presence of 17 β -oestradiol and that 17 β -oestradiol dose dependently inhibits the onset of EPC senescence. Evidence suggests that this may occur through down-regulation of angiotensin II type 1 receptor (AT₁R) expression, which is known to be involved in the regulation of blood pressure (Imanishi *et al.* 2005b,c). Indeed, exposure to angiotensin II accelerates the onset of EPC senescence *via* mechanisms involving increased oxidative stress, and oestrogen down-regulates AT₁R in mature endothelial cells (Nickenig *et al.* 1998; Gragasin *et al.* 2003; Imanishi *et al.* 2005a,b).

Circulating EPCs are also reduced in patients with diabetes mellitus in accordance with disease severity, although numbers can be increased by improving glycaemic control (Kusuyama *et al.* 2006b). Patients with end stage renal disease (ESRD) also have reduced numbers of circulating EPCs, which are further reduced following dialysis. In addition, *in vitro*,

serum from ESRD patients is able to impair the growth of EPC isolated from healthy volunteers (Westerweel *et al.* 2007).

Despite accumulating evidence that SMPCs contribute to alleviating vascular pathological changes, very few studies have looked at how cardiovascular risk factors influence SMPC. However, while cardiovascular risk factors invariably appear to negatively impact on EPC, the limited data available for SMPC are not so clear. For example, circulating SMPCs, like EPCs, are reduced in patients with diabetes mellitus in accordance with disease severity and increase in response to treatments that improve glycaemic control (Kusuyama *et al.* 2006b). In contrast, in patients with ESRD there is no difference in the number of circulating SMPCs compared to healthy controls. Circulating SMPC numbers are likewise unaffected by dialysis in ESRD patients and serum from ESRD patients has no effect on the growth of SMPC *in vitro* (Westerweel *et al.* 2007). A recent investigation of AT₁R blockade as means to prevent in-stent restenosis revealed that significantly more SMPC could be cultured from PBMC isolated from hypercholesterolaemic rabbits than from normal controls. This phenomenon was enhanced in animals that had also undergone carotid artery stenting but could be attenuated with AT₁R blockade (Ohtani *et al.* 2006).

VASCULAR PROGENITOR CELLS AND PERCUTANEOUS REVASCULARIZATION

Percutaneous coronary intervention (PCI) with stent implantation is the most commonly used revascularization technique and is routinely used in the management of patients with symptomatic CAD. However, 10–30% of these cases will go on to develop in-stent restenosis (ISR), despite the development of drug-eluting stents aimed at reducing such pathology (Froeschl *et al.* 2004). Indeed, while sirolimus-eluting stents are often employed to prevent ISR, *in vitro* evidence suggests that sirolimus enhances EPC senescence (Imanishi *et al.* 2006).

While circulating EPCs are increased post-PCI in patients with CAD (Bonello *et al.* 2006), evidence suggests that patients who develop ISR have fewer circulating EPC and that these exhibit reduced functional activity. Furthermore, when compared to patients who do not develop ISR, an increased proportion of these EPC are senescent (Matsuo *et al.* 2006). In addition, in patients with acute coronary syndrome, despite a significant increase in serum VEGF levels following PCI, EPC are not mobilized. However, the opposite is seen in patients presenting for elective PCI (Banerjee *et al.* 2006).

Diabetics have a higher incidence of ISR following PCI. Indeed, in diabetic mice re-endothelialization is significantly reduced following wire injury and is likewise reduced in wild-type mice transplanted with bone marrow from diabetic mice. Both of these phenomena are associated with reduced EPC recruitment. Indeed, it was recently demonstrated that EPCs derived from diabetic mice display reduced migration and reduced adhesion to extracellular matrix proteins *in vitro* (Ii *et al.* 2006). In mice, G-CSF treatment has been shown to reduce neo-intima formation and to increase re-endothelialization after balloon injury, an observation associated with increase in the number of circulating cells expressing CD31, CD34, VEGFR-2 or c-kit (Kong *et al.* 2004a). However, the recent MAGIC cell trial revealed a high rate of ISR in patients receiving G-CSF treatment prior to stent implantation, which may indicate mobilization of SMPC (Kang *et al.* 2006).

Circulating SMPCs may play a major role in the development of ISR. Indeed, in a recent study, while the number of circulating CD34⁺ cells in patients with atherosclerotic CAD varied

following PCI, the frequency and severity of restenosis was the greatest in those patients with increased numbers of circulating CD34⁺ cells. In addition, the pre- and post-PCI change in circulating CD34⁺ cells was independently predictive of late lumen loss, suggesting a possible role for these cells in the development of intimal hyperplasia (Schober *et al.* 2005). Furthermore, c-kit⁺ cells co-expressing α SMA have been identified in ISR lesions (Hibbert *et al.* 2004). Drug-eluting stents have been deployed to try to reduce neo-intimal hyperplasia and, despite its negative impact on EPC, sirolimus has been shown to reduce the formation of neo-intimal hyperplasia following vascular injury. *In vitro* evidence suggests this may be achieved by inhibiting SMPC growth (Fukuda *et al.* 2005).

VASCULAR PROGENITOR CELLS AND VEIN GRAFT INTIMAL HYPERPLASIA

Following grafting of a vein into an artery, a process of remodelling occurs as the vein adapts to the arterial environment; this is characterized by thickening of the intima (intimal hyperplasia), resulting from smooth muscle cell accumulation and extracellular matrix deposition. This intimal hyperplasia acts as a substrate for development of accelerated atherosclerosis and subsequent graft occlusion (Mitra *et al.* 2006). The source of smooth muscle cells within neo-intimal lesions of vein grafts is unclear and it has been reported that they may be of both donor and recipient origin (Hu *et al.* 2002b). By better understanding how intimal hyperplasia and subsequent atherosclerotic lesions develop within these vein grafts, improved post-operative treatment strategies will hopefully be designed that are able to reduce or prevent the occurrence of such pathology.

Using a mouse model of vein graft atherosclerosis, it has been shown that one of the earliest events to occur following the grafting of a vein into an artery, in response to the increased physical stress, is endothelial cell death (Mayr *et al.* 2000). However, re-endothelialization does occur and evidence suggests that circulating EPCs contribute to this process, with about one third of the regenerated endothelium being made up of bone marrow-derived cells. When performed in ApoE^{-/-} mice, vein grafts show reduced endothelial regeneration and enhanced atherosclerosis, which is associated with reduced numbers of circulating EPC. There is a suggestion that adventitia-derived progenitor cells may contribute to the development of atherosclerotic lesions and may be a source of smooth muscle cells within the neo-intima (Xu *et al.* 2003; Torsney *et al.* 2005). Nitric oxide-induced VEGF plays a key role in neo-intimal lesion development in vein grafts, as evidenced that inducible (i)NOS^{-/-} mice, in which VEGF production is abrogated, exhibit increased neo-intimal hyperplasia and reduced EPC homing. However, this can be rescued by local overexpression of VEGF (Mayr *et al.* 2006). Tumour necrosis factor (TNF)- α signalling appears to be important for the progression of vein graft neo-intimal hyperplasia and is involved in the regulation of chemokine and adhesion molecule expression by graft intrinsic cells (Zhang *et al.* 2004; Peppel *et al.* 2005). However, whether TNF- α signalling directly influences recruitment of vascular progenitor cells to the graft remains to be determined.

VASCULAR PROGENITOR CELLS AND ATHEROSCLEROSIS

It has been suggested that reduced numbers of circulating EPCs are associated with the development of atherosclerosis and recent studies show that the number of CD34⁺/VEGFR-2⁺ cells

inversely correlates with the occurrence of atherosclerosis in asymptomatic individuals (Chironi *et al.* 2006; Fadini *et al.* 2006), although when measured at sites other than the carotid artery, this association was not significant after adjustment for Framingham risk score (Chironi *et al.* 2006). It is known that high-density lipoprotein (HDL) protects against atherosclerosis and recent data show that increased serum levels of HDL correlates with an increase in EPC colony formation *in vitro*, but not in the number of circulating CD34⁺/CD133⁺ cells (Pellegatta *et al.* 2006). In ApoE^{-/-} mice, administration of HDL leads to an increased number of Sca-1⁺ endothelial cells in the aortic root following inflammatory insult with lipopolysaccharide (Tso *et al.* 2006) and transfusion of bone marrow from young, but not from old, ApoE^{-/-} mice to high fat diet fed ApoE^{-/-} mice prevents the development of atherosclerosis. Bone marrow from older mice was found to contain fewer cells expressing vascular progenitor cell markers. There was no change in the number of cells with haematopoietic stem cell markers or generalized murine stem cell markers, when compared to bone marrow from young mice. It was concluded that old ApoE^{-/-} mice suffer from exhaustive consumption of their EPC and that this contributed to the development of atherosclerosis (Rauscher *et al.* 2003).

Bone marrow-derived cells form a significant proportion of the smooth muscle cells within atherosclerotic plaques (Sata *et al.* 2002) and recent data indicate that transfer of bone marrow cells or spleen-derived EPCs from ApoE^{-/-} mice to age-matched ApoE^{-/-} mice significantly increases the size of atherosclerotic lesions occurring. Furthermore, atherosclerotic plaques in EPC-transfused mice contained smaller fibrous caps and larger lipid cores than those in untreated controls, as well as a larger number of CD3⁺ cells (George *et al.* 2005a). Cells expressing progenitor cell markers, including CD34, c-kit and VEGFR-2, have recently been identified in human atherosclerotic lesions, and there is an increase in the number of these progenitors in the adventitia of atherosclerotic vessels. These cells may be a source of EPCs and/or SMPCs that contribute to the remodelling process (Torsney *et al.* 2007).

GENETIC MANIPULATION OF VASCULAR PROGENITOR CELLS

From the data discussed herein, it is apparent that progenitor cells contribute to vascular remodelling, and furthermore, that population of these cells may be isolated and deployed therapeutically. For clinical application, it is likely that autologous progenitor cells would be used, in order to avoid possible immunological complications, and the most readily available source is peripheral blood. As detailed earlier, vascular progenitor cells may be isolated from the mononuclear cell fraction of peripheral blood either by positive selection (e.g. CD34⁺, CD133⁺ and/or VEGFR-2⁺ cells) or by selective culture in the presence of VEGF (EPC) or PDGF-BB (SMPC). However, vascular progenitor cells are lineage committed and do not possess the same capacity for self-renewal as pluripotent stem cells. Patients that are likely to undergo such therapy are also likely to exhibit one or more cardiovascular risk factors; there is a relationship between these and progenitor cell dysfunction. Indeed, both hypertension and exposure to oxidized LDL have been shown to accelerate EPC senescence (Imanishi *et al.* 2004, 2005d). Methods to improve the functional capacity of progenitor cells isolated from patients with cardiovascular risk factors therefore need to be devised. One way in which this might be achieved is by genetically manipulating these cells to overexpress vasculo-protective genes. It has already been demonstrated, in a rabbit model of vascular injury, that infusion of EPC overexpressing eNOS leads to reduced neo-intima formation and increased re-endothelialization; significantly, more so than that observed in animals treated with vector-transfected EPCs (Kong *et al.* 2004b). In

addition, our group has recently demonstrated, in a mouse wire injury model, that infusion of CD34⁺ expressing the anticoagulation proteins tissue factor pathway inhibitor or hirudin under an α SMA promoter, co-localized with smooth muscle cells within lesions, and in those areas infiltrated by these modified CD34⁺ cells, neo-intimal formation was abolished (Chen *et al.* 2006).

CONCLUSIONS

While a great deal has been learned about the nature of progenitor cells in recent years, much of this work has focused on the role of EPC in angiogenesis or their therapeutic use following ischaemia (Asahara & Kawamoto 2004; Werner & Nickenig 2006). However, one of the biggest problems facing cardiovascular medicine today is the failure of bypass grafts, through thrombotic (early) or atherosclerotic (late) occlusion. There are limited data on the roles of vascular progenitor cells in the progression of vein graft intimal hyperplasia, and it has yet to be determined whether infusion of EPC, genetically manipulated or not, would be therapeutic in this context. In addition, one avenue that appears to have been overlooked thus far is the therapeutic use of SMPC. If these could be manipulated to overexpress vasculo-protective genes, they might prove more effective in reducing intimal hyperplasia than EPC, being able to enter the lesion before deploying their payload; acting as a 'Trojan Horse'. This notion is supported by the observation that, following wire injury, CD34⁺ expressing anticoagulation proteins under an α SMA promoter, co-localized with smooth muscle cells and abolished neo-intimal formation (Chen *et al.* 2006). While there is evidence supporting the therapeutic application of vascular progenitor cells, it is likely that the most effective progenitor-based therapy will involve some genetic manipulation, exploiting inherent homing and engraftment characteristics of vascular progenitor cells to deliver therapeutic genes specifically to the target tissue.

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