

## Review

# Endothelial progenitor cells in health and disease

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**Summary.** There is currently great excitement and expectation in the stem cell community following the discovery that multipotent stem cells can be cultured from human fetal tissue and retain their ability to give rise to a variety of differentiated cell types found in all three embryonic germ layers.

Although the earliest sites of hematopoietic cell and endothelial cell differentiation in the yolk sac blood islands were identified about 100 years ago, cells with hemangioblast properties have not yet been identified *in vivo*. Endothelial cells differentiate from angioblasts in the embryo and from endothelial progenitor cells, mesoangioblasts and multipotent adult progenitor cells in the adult bone marrow. Circulating endothelial progenitor cells (EPC) have been detected in the circulation after vascular injury and during tumor growth. The molecular and cellular mechanisms underlying EPC recruitment and differentiation are not yet understood, and remain as one of the central issues in stem cell biology.

For many years, the prevailing dogma stated that the vessels in the embryo develop from endothelial progenitors, whereas sprouting of vessels in the adult results only from division of differentiated endothelial cells. Recent evidence, however, indicates that EPC contribute to vessel growth in the embryo and in ischemic, malignant or inflamed tissues in the adult, and can even be therapeutically used to stimulate vessel growth in ischemic tissues.

**Key words:** Angiogenesis, Endothelial cells, Endothelial precursor cells, Tumour

### The hemangioblast

The close relationship between hematopoietic cells (HC) and endothelial cells (EC) has been seen as an indication that they descend from a common progenitor,

the hemangioblast in the yolk sac, the initial site of hematopoiesis and blood vessel formation during mammalian development (Murray, 1932).

The existence of hemangioblasts has been inferred from the expression of a number of genes in developing HC and EC, such as vascular endothelial growth factor receptor-1 and -2 (VEGFR-1, VEGFR-2) CD34, SCL/Tal-1, Cbfa2/Runx1/AML-1 and PECAM1. The most compelling evidence in support of a common progenitor comes from studies of differentiating embryonic stem (ES) cell cultures, in which “blast colonies” giving rise to both lineages form transiently (Choi et al., 1998).

Single ES cells in culture express VEGFR-2 and give rise to clones containing hematopoietic progenitors and/or committed angioblasts (Yancopoulos et al., 2000). VEGFR-2 knockout mice lack yolk sac blood vessels and organized blood vessels, and have very few hematopoietic progenitors, while VEGFR-2+ mesodermal cells isolated from the posterior area of presomitic chick embryos differentiate into VEGFR-2+ EC or VEGFR-2- HC when cultured in the presence or absence of VEGF respectively (Yancopoulos et al., 2000).

### The blood islands and the angioblasts

Blood islands are aggregations of mesodermal cells that colonize the presumptive yolk sac. Peripheral cells (angioblasts) become EC, whereas central cells become HC. Angioblasts are defined as cells with some, but not all, the markers characteristic of an EC (Risau and Flamme, 1995).

EC derived from the yolk sac induce the amplification of mature myeloid, erythroid, and lymphoid cells and their progenitors (Yoder et al., 1994; Fennie et al., 1995; Lu et al., 1996).

SCL/Tal-1 and VEGFR-2 are coexpressed in isolated mesodermal cells that give rise to EC. Conversely, HC seem to express SCL/Tal-1 only. Blood vessel maturation implies a sequential pattern in which SCL/Tal-1 and VEGFR-2 are expressed first, followed by PECAM-1, CD34, VE-cadherin and later Tie-2.

Finally, SCL/Tal-1 expression is downregulated in mature EC.

### **The para-aortic splanchnopleural (SP) and the aorta-gonad-mesonephros (AGM) regions**

In the mouse embryo, stem cells for the definitive lineages are also produced in the SP region (8.5-9.5 days postcoitum) (Cumano et al., 1996), and thereafter (10.5-11.5 days postcoitum) in the AGM region containing the dorsal aorta, genital ridge/gonads and pro-mesonephros (Wood et al., 1997; Delassus et al., 1999). EC from this region mediate both the differentiation of hematopoietic precursor cells along myeloid, erythroid, and lymphoid pathways, and enable hematopoietic stem cells (HSC) to repopulate lethally irradiated adult bone marrow (Ohneda et al., 1998).

Assuming that yolk sac derived HSC can circulate and seed intraembryonic tissues (including large arteries), their maturation within these two regions and/or fetal liver may first be required before they can contribute to definitive hematopoiesis. The ultimate source of definitive HSC may thus lie in the yolk sac. Alternatively, the SP/AGM regions may generate a second wave of definitive HSC for the fetal liver.

### **Circulating EC and endothelial precursor cells (EPC)**

The presence of circulating EC was first indicated in 1963 using Dacron grafts by Stump et al. (1963) which suggested that new endothelium on the flow surface of the grafts derived from blood-borne cells. EC lining the coronary arteries of a transplanted human heart were then shown to be derived from the recipient and not the donor (Kennedy and Weissmann, 1971). EC have since been shown to line a ventricular assist device (Frazier et al., 1993). In this context it is interesting that circulating EC are detectable in diseases marked by vascular injury, namely sickle cell anemia, acute myocardial infarction, thrombotic thrombocytopenic purpura, and active cytomegalovirus infection (Hladovec et al., 1978; Grefte et al., 1993; Lefevre et al., 1993; Solovey et al., 1995). Normal adults have  $2.6 \pm 1.6$  circulating EC per ml of peripheral blood (Solovey et al., 1995). Most of these cells are quiescent, and at least half are microvascular as defined by CD36 positivity (Solovey et al., 1997). These results are all consistent with the hypothesis that circulating EPC form a monolayer on a graft surface.

### **Specific and nonspecific markers of circulating EPC**

It is impossible to differentiate immature EPC from primitive HSC as those cells share common surface, i.e. AC-133, CD34 or VEGFR-2. There is no exclusive EPC marker. Both EPC and mature EC express similar endothelial-specific markers, including VEGFR-2, Tie-1, Tie-2 and VE-cadherin (Sato et al., 1993; Schnurch and Risau, 1993; Vittet et al., 1996; Eichmann et al., 1997). Identification of differences is further complicated by the

fact that HC subsets express markers similar to those of EC, such as CD34, PECAM, Tie-1, Tie-2, Eph and VEGFR-1, transcription factors such as SCL/tal-1 and AML1, von Willebrand factor (vWF) (Suda et al., 2000; Lyden et al., 2001).

Asahara et al. (1997) used a polyclonal antibody to the intracellular domain of VEGFR-2 to show that CD34<sup>+</sup>, VEGFR-2<sup>+</sup> circulating EPC form colonies that take up acetylated LDL. When CD34<sup>+</sup>, VEGFR2<sup>+</sup>, CD34<sup>-</sup> or VEGFR-2<sup>-</sup> cells were injected into mice, rats, and rabbits undergoing neovascularization due to hindlimb ischemia, CD34<sup>+</sup> and VEGFR-2<sup>+</sup> cells, but rarely CD34<sup>-</sup> or VEGFR-2<sup>-</sup> cells, incorporate into the vasculature in a manner consistent with their being EC (Asahara et al., 1997).

A novel hematopoietic stem cell marker, AC 133, whose function is unknown, is also expressed on EPC subsets, but not on mature EC (Yin et al., 1997). Its expression is rapidly downregulated as hematopoietic progenitors and EPC differentiate (Miraglia et al., 1997; Yin et al., 1997). Furthermore, Peichev et al. (2000) have demonstrated that a small subset of CD34<sup>+</sup> cells from different hematopoietic sources express both AC 133 and VEGFR-2. Incubation of this subset with VEGF, FGF-2 and collagen results in their proliferation and differentiation into AC 133- VEGFR-2<sup>+</sup> mature EC. Maturation and *in vitro* differentiation of these cells abolish AC 133 expression, suggesting that EPC with angioblast potential may be marked selectively with AC 133. Gehling et al. (2000) have demonstrated that AC133<sup>+</sup> cells from granulocyte colony stimulating factor-mobilized peripheral blood differentiate into EC when cultured in the presence of VEGF and stem cell growth factor. Phenotypic analysis revealed that most of these cells display endothelial features, including the expression of VEGFR-2, Tie-2 and vWF. All these data indicate that AC 133 is currently the best selective marker for identifying EPC and that circulating CD34<sup>+</sup>, VEGFR-2<sup>+</sup> and AC 133<sup>+</sup> cells constitute a phenotypically and functionally distinct population of circulating EC that may play a role in postnatal vasculogenesis.

AML1 is expressed in EC in sites where early hematopoietic stem cells emerge, such as the yolk sac (North et al., 1999). AML1-deficient embryos, which lack definitive hematopoiesis, also display defective angiogenesis in the head and pericardium (Takakura et al., 1998).

### **The source of circulating EPC**

Most circulating EPC reside in the bone marrow in close association with hematopoietic stem cells and the stroma, the local proliferation and transmigration of EPC across the bone marrow/blood barrier may be promoted by these stem cells and stroma cells.

When EPC are mobilized from adult bone marrow, they first migrate from a quiescent niche within the bone marrow into a permissive, proliferative

microenvironment, the so-called vascular zone of marrow. The matrix metalloproteinase-9 (MMP-9) as well as the survival/mitogenic activity of the stem cell cytokine SkitL appears critical during this process. Release of EPC from bone marrow into circulation can be induced by granulocyte macrophage colony stimulating factor (GM-CSF) or VEGF and is critically dependent on the activity of endothelial nitric oxide synthase (eNOS) expressed by stromal cells in bone marrow (Aicher et al., 2003). The positive effects of statins on EPC include increasing the number of circulating EPC, reducing senescence, enhancing proliferation rate and differentiation from CD34<sup>+</sup> cells (Dimmeler et al., 2001; Vasa et al., 2001/Circulation). Erythropoietin and estrogen also positively influence EPC number (Heeschen et al., 2003; Strehlow et al., 2003).

EPC in the peripheral blood may derive from the bone marrow and be not yet incorporated into the vessel wall. The following lines of evidence suggest that they constitute the preponderance of circulating bone marrow-derived endothelial lineage cells.

Asahara et al. (1999), employed a bone marrow transplant model in mice sublethally irradiated to demonstrate the incorporation of bone marrow EPC into foci of neovascularization. Four weeks after transplants, when the bone marrow had been reconstituted, a variety of surgical experiments to provoke neovascularization were performed: a) Cutaneous wounds examined 4 and 7 days after skin removal by punch biopsy disclosed a high frequency of incorporated EPC. b) One week after the onset of hindlimb ischemia, EPC were incorporated in capillaries among skeletal myocytes. c) After permanent ligation of a coronary artery, myocardial infarction sites demonstrated incorporation of EPC in foci of neovascularization at the border of the infarct. All these findings clearly indicate that postnatal neovascularization does not rely on angiogenesis alone and that bone marrow EPC contribute to postnatal vasculogenesis.

Shi et al. (1998) implanted an impermeable Dacron graft made in the canine thoracic aorta and demonstrated scattered islands of EC without any evidence of transmural angiogenesis. Later, they used a transplantation model in which marrow cells from donors and recipients were distinguishable to determine whether EC lining a vascular prosthesis are derived from the bone marrow (Shi et al., 1998). After 12 weeks, the graft was retrieved, and cells with endothelial morphology were identified; only donor alleles were detected in DNA from positively stained cells on the graft. These results suggested that a subset of CD34<sup>+</sup> cells located in the bone marrow mobilize to the peripheral circulation and colonize the endothelial flow surface of vascular prostheses. In those studies, grafts were prepared such that their flow surface was accessible to cells from blood, but shielded from external pannus and perigraft endothelial ingrowth.

Lin et al. (2000) found a distinction between vessel wall and bone marrow-derived EC in blood samples

from subjects who had received gender-mismatched bone marrow transplants 5-20 months earlier. They showed that 95% of circulating EC had recipient genotype and 5% had donor genotype. After 9 days of culture, EC derived predominantly from the recipient vessel wall, expanded only 6-fold, compared with 98-fold after 27 days by EC, mostly originated from donor bone marrow cells. These data suggest that most circulating EC in fresh blood originate from vessel walls and have limited growth capability, and that outgrowth of EC is mostly derived from transplantable marrow-derived cells. In animal models, EPC home in on sites of active neovascularization and mobilization of bone marrow-derived EPC, and differentiate into EC in response to tissue ischemia (Takakura et al., 1998). This finding is consistent with postnatal vasculogenesis. Identification of the chemokines that induce mobilization of bone marrow EPC to the peripheral circulation may provide a novel mechanism for their recruitment to sites of vascular trauma to accelerate vascular healing (Rafii, 2000). Moreover, expansion and mobilization of EPC may augment the resident population of EC competent to respond to exogenous angiogenic cytokines (Isner and Asahara, 1999). Kalka et al. (2000) have demonstrated that transplantation of EPC to athymic mice with hindlimb ischemia markedly improves blood flow recovery and capillary density in the ischemic limb and significantly reduces the rate of limb loss.

### **Multipotent adult progenitor cells (MAPC)**

Reyes et al. (2002) have identified a single cell in human and rodent postnatal marrow that they term the MAPC. MAPC were selected by depleting adult bone marrow of HC expressing CD45 and glycophorin-A, followed by long-term culture on fibronectin with epidermal growth factor (EGF) and platelet derived growth factor under low serum conditions. A cell population expressing AC133 and low levels of VEGFR-1 as well as VEGFR-2 and the embryonic stem cell marker Oct-4 emerged. Its culture with VEGF induced differentiation into CD34<sup>+</sup>, vascular endothelial-cadherin<sup>+</sup>, VEGFR-2<sup>+</sup> cells, a phenotype consistent with angioblasts. Subsequently these cells express vWF and markers of mature endothelium, such as CD31, CD36 and CD62-P. They form vascular tubes when plated on matrigel and upregulate angiogenic receptors and VEGF in response to hypoxia. In immunocompetent mice, intravenously injected EC contribute to neovascularization of transplanted tumors and participate in wound healing. Despite extensive efforts, hematopoietic differentiation of human MAPC has not been observed, indicating a significant difference from the pathway by which both HC and EC are generated from hemangioblasts.

### **Do EPC derive from monocytes?**

Peripheral blood mononuclear cells from adult

humans can be enriched in EPC by addition of VEGF, FGF-2, insulin-like growth factor and EGF to the culture medium for 7-10 days. After local injection *in vivo*, these cells contribute to the formation of new vessels in the ischemic limb (Kalka et al., 2000). Harraz et al. (2001) suggested that CD34<sup>+</sup> angioblasts are a subset of CD14<sup>+</sup> monocytic cells and can transdifferentiate into EC, dendritic cells or macrophages. Rehman et al. (2003) demonstrated that EPC isolated from the monocyte/macrophage fraction of peripheral blood did not proliferate, but secreted angiogenic growth factors. Monocytic EPC may enhance the angiogenic process via release of inflammatory mediators that stimulate granulation tissue formation.

### Differences between EPC isolated from bone marrow, peripheral blood and different organs

There are differences between EPC isolated from bone marrow or from peripheral blood, because multipotent progenitor cells from the bone marrow may be a more undifferentiated cell type with higher plasticity.

Differentiation of human bone marrow-derived multipotent progenitor cells towards the endothelial lineage was only induced by seeding them at a high density in serum-free medium with the addition of VEGF, whereas culture in medium with fetal calf serum directed the differentiation into osteoblasts, chondroblasts, and adipocytes (Ryes et al., 2002). Under certain condition, EPC may differentiate into cardiomyocytes (Badorff et al., 2003).

The parenchyma of the systemic vasculature or certain organs may harbor endogenous EPC-like cells. Exposure to organ-specific angiogenic and matrix factors may be necessary to program EPC to home in on and incorporate in a particular tissue. Incubation of EPC with organ-specific growth factors may confer specific instructions for recruitment to a particular organ.

### EPC in vascular diseases

In certain vascular diseases, impaired mobilization of EPC may contribute to the underlying vascularity. The number and migratory activity of circulating EPC are inversely correlated in patients with risk factors for ischemic cardiovascular diseases, such as genetic predisposition, hypertension or smoking (Vasa et al., 2001).

EPC from patients with diabetes mellitus type-2 are characterized by a decreased proliferation capacity, reduced adhesiveness and ability to form capillary tubes *in vitro* (Tepper et al., 2002). Acute myocardial infarction is associated with a rapid increase of EPC in the circulation (Shintani et al., 2001). Vascular trauma, such as coronary bypass grafting or burn injury, induces a rapid but transient mobilization of EPC (Gill et al., 2001). Within 6-12 h after injury, the percentage of circulating EPC increased almost 50-fold before

returning to basal levels within 48-72 h. Plasma VEGF levels were found to be similarly up-regulated, suggesting VEGF as a key regulator signal of EPC mobilization and differentiation.

Vascular trauma and organ regeneration result in the release of chemokines that recruit EPC to the neoangiogenic site. Rapid incorporation of EPC accelerates vascular healing and prevents potential vascular complications secondary to thrombosis and hypoxia. When the endothelium is injured, circulating progenitor cells may adhere to the underlying smooth muscle cells and contribute to neointima formation (Sata et al., 2002).

*Ex vivo* expanded EPC from peripheral blood transplanted into animal models of ischemic hindlimb and acute myocardial infarction successfully augmented neovascularization and resulted in physiological recovery documented as limb salvage and improvement in myocardial function (Kalka et al., 2000; Kawamoto et al., 2001).

EPC seem to be involved in the regeneration of ischemic myocardium by modulation of both angiogenesis and myogenesis in the ischemic cardiac muscle (Jackson et al., 2001; Kawamoto et al., 2001; Kocher et al., 2001). Tissue ischemia results in upregulation of VEGF-A, which through interaction with its receptors VEGFR-1 and VEGFR-2 expressed in EPC, promotes migration of these cells to the vascular site of injury. VEGF-A induces MMP-9 expression, and activation of MMP-9 results in the release of stem cell-active soluble kit ligand and forces the translocation of quiescent VEGFR2<sup>+</sup> c-kit<sup>+</sup> EPC to a permissive zone that is conducive to proliferation and mobilization to circulation. VEGF gene transfer *in vivo* has been shown to mobilize EPC in patients with ischemic coronary disease (Kalka et al., 2000).

A significant improvement of the function of ischemic limbs was maintained during a 6-month follow-up after autologous implantation of bone marrow-derived mononuclear cells (Tateishi-Yuyam et al., 2002). It was suggested to be due to both CD34<sup>+</sup> EPC and angiogenic factors or cytokines released from the CD34<sup>+</sup> bone marrow fraction.

Clinical studies described the ability of autologous bone marrow-derived cells or *ex vivo* expanded autologous EPC to repair infarcted myocardium in humans (Assmus et al., 2002; Stamm et al., 2003; Strauer et al., 2002).

EPC widely express CXCR4, the receptor for stromal cell-derived factor-1 (SDF-1) and a member of the chemokine CXC subfamily (Mohle et al., 1998). Local administration of SDF-1 enhanced vasculogenesis and subsequently contributed to neovascularization *in vivo*, inducing *in situ* recruitment of transplanted EPC in ischemic tissues (Yamaguchi et al., 2003).

One therapeutic use of autologous EPC is to generate nonthrombogenic vascular cells to coat decellularized or biodegradable surfaces. Noishiki et al. (1996) embedded bone marrow cells in synthetic

prostheses before grafting into the aorta of dogs, resulting in generation of a nonthrombogenic endothelial surface. These data suggest that precoating a graft with endothelium derived from EPC facilitates *in vivo* remodeling.

EPC participate *in vivo* in cerebral neovascularization after ischemic stroke (Zhang et al., 2002). A significant increase of EPC was demonstrated after *ex vivo* transfection with adenovirus encoding VEGF (Iwaguro et al., 2002).

### The role of EPC in tumor angiogenesis

Bone marrow-derived stem cells may be a source of EPC recruited for tumour-induced neovascularization. It has been assumed that the additional endothelial cells required to construct new tumour vessels come from the division and proliferation of local endothelial cells. Endothelial cells incorporated into sites of neovascularization, including tumour-induced new blood vessels, may be derived from these precursor cells.

The recruitment of EPC to tumour angiogenesis is a multistep process, including: a) active arrest and homing of the circulating cells within the angiogenic microvasculature; b) transendothelial extravasation into the interstitial space; c) extravascular formation of cellular clusters; d) creation of vascular sprouts and cellular networks; e) incorporation into a functional microvasculature.

As the first step, certain values in the blood (e.g. high VEGF levels) favour and enhance differentiation of hematopoietic stem cells toward the EPC lineage (Takahashi et al., 1999; Gill et al., 2001). High levels of VEGF produced by tumours may result in the mobilization of bone marrow-derived stem cells in the peripheral circulation and enhance their recruitment into the tumour vasculature (Asahara et al., 1999; Hattori et al., 2000). Moreover, Hattori et al. (2000) showed that combined elevation of VEGF and angiopoietin-1 (Ang-1) result in remodeling of the bone architecture, with depletion of the sinusoidal spaces of hematopoietic cells and a parallel increase in bone marrow vascularization and splenomegaly.

Hypoxia can also mobilize EPC from the bone marrow in the same way as hematopoietic cytokines, such as GM-CSF (Takahashi et al., 1999). Malignant tumour growth results in neoplastic tissue hypoxia, and may mobilize bone marrow-derived endothelial cells in a paracrine fashion and thus contribute to the sprouting of new tumour vessels.

Lyden et al. (2001) demonstrated that transplantation and engraftment of  $\beta$ -galactosidase-positive wild-type bone marrow or VEGF-mobilized stem cells into lethally irradiated Id-mutant mice is sufficient to reconstitute tumour angiogenesis. In contrast to wild type mice, Id-mutants fail to support the growth of tumours because of impaired angiogenesis. Tumour analysis demonstrates the uptake of bone marrow-derived VEGFR-2<sup>+</sup> EPC into vessels surrounded by VEGFR-1<sup>+</sup> myeloid cells.

Defective angiogenesis in Id-mutant mice is associated with impaired VEGF-induced mobilization and proliferation of the bone marrow precursor cells. Inhibition of both VEGFR-1 and VEGFR-2 signalling is needed to block tumour angiogenesis and induce necrosis.

Reyes et al. (2002) found that MAPC respond to angiogenic stimuli by migrating to tumour sites and contributing to tumour vascularization. They also found that *in vivo* angiogenic stimuli in a tumour microenvironment are sufficient to recruit MAPC to the tumor bed and induce their differentiation into endothelial cells that contribute to the tumour vasculature.

Vajkoczy et al. (2003) propose the term “angiomorphosis” as the process of enhancing tissue vascularization through: a) active recruitment of EPC from the circulation by endothelial cells and b) the action of EPC as organizers of the angiogenic process.

### Concluding remarks and prospects

Adult, healthy vasculature is thought to be relatively stable and to exhibit a very low endothelial cell turn over rate (Schwartz and Benditt, 1976). Under steady-state conditions, circulating EPC represent only 0.01% of cells in the circulation.

The term “plasticity” is used to indicate that cells which have undergone lineage-specific differentiation can in some way transdifferentiate, or dedifferentiate to a more primitive stage and be reprogrammed to differentiate into a different pathway. Emerging data on the plasticity of stem cells in the bone marrow suggest a new relationship between HC and EC. Further studies on the molecular pathways involved in the regulation of hematopoiesis and angiogenesis are needed to secure the comprehensive understanding of the common events involved and elucidate the molecular basis of this plasticity. Transplanted bone marrow-derived cells possess broad plasticity, capable of transdifferentiation into new cell types, such as liver cells, cardiomyocytes or neurons *in vivo*. More recently, an alternative possibility has been suggested, that spontaneous cell fusion of bone marrow cells with somatic cells of the recipient tissue, rather than transdifferentiation contributes to the development of these new cells (Terada et al., 2002; Ying et al., 2002). Such a spontaneous cell fusion mechanism might also contribute to the conversion of bone marrow-derived EPC into endothelial cells or cardiomyocytes.

Moreover, the molecular and cellular mechanisms underlying EPC recruitment and differentiation are not yet understood, and remain as one of the central issues in stem cell biology. For instance, it is unclear how circulating EPC specifically home in on angiogenic sites, which critical steps underlie their incorporation into new blood vessels, and how effectively these processes compare with the activation of local, preexisting EC.

*Acknowledgements.* Supported by Associazione Italiana per la Ricerca sul Cancro AIRC, Milan, Ministry for Health – Regione Puglia (grant BS2), Rome, Fondazione Italiana per la Lotta al Neuroblastoma, Genoa, The members of the CARSO Consortium (University of Bari, Lega Italiana per La Lotta Contro i Tumori and Regione Puglia), Ministero della Salute (Ricerca Finalizzata FSN 2002), Rome, Italy.

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Accepted July 4, 2005