

Review

Pathophysiology of stem cells in restenosis

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Summary. Recent evidence has shown that vascular function depends not only on cells within the vessels, but is also significantly modulated by circulating cells derived from the bone marrow. A number of studies indicate that an early reendothelialization by circulating endothelial precursors after vascular injury prevents excessive cell proliferation and restenosis. Conversely, other studies concluded that the homing of other cell fractions, consisting mainly of smooth muscle precursors, cause pathological remodelling. Different cell types have been identified and characterized so far as circulating precursors able to participate in vascular repair by homing and differentiating towards endothelial cells or smooth muscle cells. Among these, endothelial precursor cells, smooth muscle progenitor cells, mesenchymal stem cells and others have been described. The origins, the hierarchy, the role and the markers of these different cell populations are still controversial. Nevertheless, different strategies have been developed so far in animal models to induce the mobilization and the recruitment of stem cells to the injury site, based on physical training, hormone injection and application of stem cell-capturing coated stents.

It should also be mentioned that the limited data currently available derived from clinical trials provide contrasting results about the effective role of vascular cell precursors in restenosis prevention, thus indicating that conclusions derived from studies in animal models cannot always be directly applied to humans and that caution should be used in the manipulation of circulating progenitor cells for therapeutic strategies.

Key words: Restenosis, Mesenchymal stem cells, Endothelial progenitor cells, Monocyte lineage cells, Clinical trials

Arterial (re)stenosis

Arterial (re)stenosis is a pathophysiological phenomenon that can follow angioplasty, arteriotomy or by-pass creation in humans, and experimental vascular injury in animal models, causing an occlusion of arterial lumen of variable extension that often requires a new revascularization procedure. The vessel reactions to surgery or angioplasty can depend on the genetic background of the organism and from diseases that affect the cardiovascular system, which represent a risk factor for restenosis, such as hypertension, diabetes and hypercholesterolemia. Vascular injury, with cell loss in intima and media tunicae, elastic lamina fragmentation and damage of tissue architecture, leads to excessive pathological repair and remodeling that involve vascular smooth muscle cell (SMC) migration and proliferation, resulting in neointimal hyperplasia. Some kinds of vessel trauma (e.g. induced by external cuff placement, arterial graft or arteriotomy) (Forte et al., 2001; Xu et al., 2004) induce lumen narrowing through a marked neoadventitia rather than by intimal hyperplasia. A large number of studies have focused on the characterization of arterial stenosis and on strategies designed to limit the negative remodeling effect of trauma. Most studies concern balloon angioplasty applied in different models (Bennett et al., 1994; Shi et al., 1994), while others applied vein-to-artery graft models to analyse venous wall adaptation to arterial blood flow (Mannion et al., 1998).

Surgical vessel injury and standard balloon angioplasty can be considered the starting events of processes such as cell proliferation, migration, apoptosis, deposition of extracellular matrix, inflammatory reactions, all leading to vessel remodeling. SMCs within adult blood vessels retain remarkable plasticity and can undergo profound and reversible changes in phenotype in response to environmental influences, such as vascular injury. The switch from contractile differentiated phenotype to synthetic proliferative phenotype in reaction to external stimuli probably represents a survival advantage in higher organisms

since it allows an efficient vascular repair. However, the high degree of SMC plasticity can predispose to an abnormal reaction to external stimuli, contributing to the development or progression of vascular disease (Owens et al., 2004). Vascular injury models have highlighted the activation not only of SMCs and endothelial cells (ECs), but also of other cell populations (Shi et al., 1996). Furthermore, the contribution of bone marrow-derived stem cells to neointima may open up new perspectives (Tanaka et al., 2003).

Programmed vascular cell death or apoptosis plays a key role not only in the normal physiology of tissues, but also in remodeling of injured vasculature. It usually occurs within a few hours or days after injury, mainly in the media and/or in the neointima, depending on the kind of injury and on the presence of atherosclerotic lesions. Vasoactive substances, such as nitric oxide, Angiotensin II and Endothelin-1 that are often altered in injured vessels, are some of the regulators of apoptosis (Pollman et al., 1996).

The pathophysiology of stem cells in injury-induced vascular repair

Recent evidence has shown that vascular function depends not only on cells within the vessels, but is also significantly modulated by circulating cells derived from the bone marrow (Asahara et al., 1997). Stem cells hold a great potential for the regeneration of damaged tissues in cardiovascular diseases. In particular, in the past, it was believed that the regeneration of injured endothelium and media in arteries was due to migration and proliferation of neighboring ECs and SMCs. Recent studies clearly indicated that different stem cell populations, derived from bone marrow and characterized by different markers and with different behaviours, contribute to vascular remodeling after injury. Moreover, different studies indicate that the contribution of bone marrow-derived cells to (re)stenosis depends on the type of model of injury (Tanaka et al., 2003).

Finally, we would like to mention that it has been demonstrated that pluripotent embryonic stem cells are also able to differentiate into vascular ECs in primates, thus revealing primate-specific vascular developmental mechanisms (Yamashita, 2004). In this context, Yamamoto et al. demonstrated that shear stress can induce mouse embryonic stem cell proliferation and the expression of EC specific markers, such as Flk-1, Flt-1, cadherin, but not SMC marker alpha-actin (Yamamoto et al., 2005).

Bone marrow-derived cells

Many studies recently demonstrated that bone marrow is the home of heterogeneous populations of stem cells (Kucia et al., 2005), named hematopoietic and nonhematopoietic stromal cells. The hematopoietic stem cells (HSCs) are able to generate all the hematopoietic

mature lineages, while the stromal (also named mesenchymal) cells provide a suitable environment for self-renewal and differentiation of HSCs. In addition to such a supportive role, mesenchymal stem cells (MSCs) can differentiate toward diverse lineages, as described below. Classically, hematopoietic and nonhematopoietic stem cells have been believed to be easily distinguishable on the basis of their markers, as only HSCs were considered as CD34+. Nevertheless, it should be mentioned that human CD34- HSCs have also been identified (Osawa et al., 1996). In this context, Gallacher identified a rare population of CD34- cells, which were also lineage negative (Lin-) and expressing AC133, which probably represent highly primitive human precursors of CD34+ cells, since they can differentiate and become able to express CD34 (Gallacher et al., 2000). Recent studies by Kucia also identified a population of nonhematopoietic cells that are CD34+, AC133+, Lin-, CD45- in human (Kucia et al., 2005). An important implication from these studies is that antigens that were considered for years as classical markers for HSCs, such as CD34 and AC133, are also expressed on nonhematopoietic cell populations, and vice versa.

Both hematopoietic and nonhematopoietic bone-marrow-derived stem cells have been demonstrated to participate in vascular repair after injury. Nevertheless, it should be mentioned that other tissue-specific niches, as well, distinct from bone marrow, are probably a source of vascular progenitor cells, whose circulation in blood is triggered by vascular damage. In particular, Tintut et al. (2003) demonstrated that MSCs with self-renewal, lineage plasticity and a unique differentiation repertoire are also contained in the artery wall. These vascular MSCs apparently lack adipogenic lineage in their differentiation repertoire, and the Authors suggest that this vascular cell population could represent a stage of commitment one generation below the MSCs in the mesengenic lineage hierarchy.

Recruitment and incorporation of vascular cell precursors at the injury site require a coordinated sequence of events, including chemoattraction, cell adhesion and transmigration, and finally differentiation to ECs or SMCs. All these steps involve a large variety of molecules, including integrins, chemokines, metalloproteases, kinases.

The following paragraphs will provide an overview of the current evidence of the role played by different bone marrow-derived vascular cell precursors in different animal models as well as in different models of vascular injury. In particular, we will overview the data available for MSCs, endothelial progenitor cells (EPCs), smooth muscle precursors, mesoangioblasts (Mabs), hemangioblasts and Monocyte Lineage Cells (MLCs). Such division can be considered arbitrary and is based mainly on the descriptions and definitions provided by the papers we analysed.

Further studies aimed at defining univocal markers and hierarchy of bone marrow-derived stem cells would

be required, as well as a well defined nomenclature to be adopted by all researchers.

Mesenchymal stem cells

MSCs have been first identified in adult bone marrow (Friedenstein et al., 1976). Subsequent studies demonstrated that MSCs are widely distributed in vivo since they have been isolated also from other tissues, including lung, adipose tissue, skeletal muscle, trabecular bone, synovium, and the human umbilical cord perivascular cells derived from the Wharton's jelly (Tuan et al., 2003; Noth et al., 2005; Sarugaser et al., 2005). In particular, in human long-term bone marrow culture, a subset of adherent cells resembles immature SMC in cytoskeletal features such as alpha-smooth muscle actin and vimentin filaments (Charbord et al., 1990). A clone of mouse bone-marrow smooth muscle-like cells has also been isolated (Peled et al., 1991).

MSCs can be distinguished from HSCs on the basis of their cell surface antigens and can be separated from HSCs by their propensity to adhere to cell culture plastics. In fact, MSCs do not express CD34, which conversely is a marker of hematopoietic cells. MSCs in bone marrow have been described as a very rare population; in particular, Wexler estimated the frequency of MSCs in bone marrow nucleated cell populations as 1 in 3.4×10^4 cells (Wexler et al., 2003).

Studies demonstrated that MSCs extensively proliferate *in vitro* while preserving a normal karyotype and telomerase activity on several passages (Pittenger et al., 1999).

Bone marrow-derived MSCs can potentially differentiate along different mesenchymal lineages including those forming bone, cartilage, fat, ligament/tendon, muscle, neurons, astrocytes and bone marrow stroma that supports hematopoiesis (Gao et al., 2001). MSC potentiality has been first described by Prockop in a study showing that transplanted marrow cells engraft nonhematopoietic connective tissues such as spleen and liver (Prockop, 1997, 1998).

Many studies have focused so far on MSCs due to their intrinsic ability to differentiate into functional cell types able to repair the diseased or injured tissue in which they are localised. This trend to adopt the local identity may be correlated to local cytokines and matrix factors, as well as to adequate contact with host cells.

MSCs injected in blood flow 15 minutes before injury in a model of rat carotid arteriotomy (Forte et al., 2001) are able to home at the injury site, as demonstrated by the presence of labeled MSCs in the area around adventitial vasa vasorum detected one week after injury (Forte et al., unpublished data).

Han et al. (2001) demonstrated that bone-marrow-derived MSCs contribute to neointima formation only in a model of severe injury of iliac artery in chimeric mice and not in arteries submitted to minimal damage. In particular, these authors demonstrated that about 56% of alpha-actin-positive cells detectable in a large neointima

induced by scratch injury were derived from bone marrow. These cells resemble fetal/immature vascular SMCs. These data are in agreement with findings published by Tanaka et al. (2003), suggesting that bone marrow cells substantially contribute to lesion formation when arteries are subjected to severe injuries and that these cells expressed alpha-smooth muscle actin but not markers for highly differentiated SMCs.

Endothelial progenitor cells

EPCs are a specific subset of circulating bone marrow-derived cell population and are characterized by coexpression of Sca-1 and vascular endothelial growth factor receptor 2 (VEGFR2 or Flk-1). It is now known that EPCs are a heterogeneous population, derived chiefly from HSCs, and consist of cells at different stages of maturation, ranging from early CD133+ VEGFR2+ to more mature CD34+ VEGFR2+ phenotypes. An exhaustive description of EPC markers is in Sata (Sata et al., 2005). Despite the differences, these cells have the defining characteristic of being able to differentiate into more mature forms, and being able to line the internal elastic membrane of blood vessels they thus play a key role in neovascularization. Asahara and collaborators (Asahara et al., 1997) were the first group to demonstrate that CD34+ hematopoietic cells purified from adults were able to differentiate to an endothelial phenotype (expressing various specific markers, such as vWF) and were named EPCs. Also, nonhematopoietic cells are able to differentiate to EPCs, expressing CD34 and specific endothelial markers.

Smooth muscle progenitor cells

In comparison to EPCs, only a few papers are fully focused on putative smooth muscle progenitor cells and on their markers. Simper D demonstrated for the first time in human that SMCs can derive from blood smooth progenitor cells cultured in endothelial growth medium supplemented with platelet-derived growth factor BB (Simper et al., 2002). These cells were positive for alpha-actin, myosin heavy chain, calponin, CD34, Flt-1 and Flk-1 VEGF receptors, as well as for $\alpha_5\beta_1$ integrin. It should be underlined that CD34 is a surface marker known to be absent from adult human smooth muscle cells. Moreover, smooth progenitor cells do not express Tie-2 receptor, a receptor tyrosine kinase, consistent with an angioblastic lineage distinct from endothelial cells that has been described as Tie-2 receptor positive (Sata et al., 2002).

Deb described the integrin profile of this cell population, underlying that smooth muscle precursors are characterized by a high expression of beta 1 integrin but do not express other integrins, which are conversely typical of the EPCs (Deb et al., 2004). The first evidence that circulating smooth muscle precursors were able to participate in neointima formation in coronaries in a model of heterotopic cardiac transplantation between

wild-type mice and LacZ mice came from Saiura (Saiura et al., 2001). Similarly, Shimizu demonstrated in a model of graft arterial disease that intimal smooth-muscle-like cells derived from bone marrow and were positive to alpha-actin, calponin and SM1 (Shimizu et al., 2001).

Interestingly, Kobayashi demonstrated that multipotent MSCs differentiated toward SMC phenotype under the stimulus represented by shear and compressive stress induced by blood flow in a model *in vitro* (Kobayashi et al., 2004).

Mesoangioblasts

Recently, a novel type of vessel-associated stem cell named mesoangioblast (Mab), that can differentiate into different mesoderm cell types has been described (Minasi et al., 2002).

Mabs are physically associated with the embryonic dorsal aorta in avian and mammalian species and express the key marker of angiopoietic progenitors, such as Sca-1, Kit, Flk-1 and CD34, as well as genes typical of mesoderm, including receptors and signaling molecules for classical mesoderm inducers, such as BMP, Wnt and Notch (Palumbo et al., 2004). Presumably, they derive from a primitive angioblast (Cossu and Bianco, 2003) and are able to efficiently differentiate into endothelium, smooth, and cardiac muscle *in vitro* (Tagliafico et al., 2004) and when transplanted *in vivo*. In particular, it has been demonstrated that the differentiation of Mabs into smooth muscle is dependent upon expression of *msx2* and *necln*, two transcription factors able to induce, in turn, a number of smooth muscle markers (Brunelli et al., 2004; Brunelli and Cossu, 2005). *In vivo* experiments demonstrated that Mabs are as effective as bone marrow progenitor cells in reducing postinfarction left ventricular dysfunction (Galli et al., 2005). These authors also demonstrated that Mabs in this model differentiate into smooth muscle, whereas the production of endothelium is extremely rare. Finally, Mabs are able to produce in the heart several growth factors that stimulate the proliferation of SMCs, but not of ECs.

Mabs are also able to home inside damaged muscle through the general circulation and proliferate there to reconstruct the tissue (Sampaolesi et al., 2003). High mobility group box 1 protein (HMGB1) has been recently identified as a chemoattractant of Mabs at the injury site (Palumbo et al., 2004).

Nevertheless, a positive or negative role of Mabs in vascular injury-induced remodeling has not been clearly established yet.

Hemangioblasts

Hematopoietic cells and ECs develop from mesoderm via a transitional progenitor named hemangioblast. Gene-targeting studies using embryonic stem cells have identified Flk-1 and Scl as important regulatory molecules that specify both hematopoietic and vascular outcome (Bailey and Fleming, 2003). Flk-1

is the VEGF receptor 1 and acts as a receptor tyrosine kinase. Scl is a basic helix-loop-helix transcription factor.

Hemangioblast is present not only during the embryonic development, but its activity persists into adult life. For example, human AC133+ cells from granulocyte-CSF mobilized peripheral blood can differentiate both into hematopoietic and ECs in culture. Moreover, these AC133+ cells can form new blood vessels *in vivo* (Gehling et al., 2000). As a result of this recent finding, questions are currently arising about the roles and the factors influencing the hemangioblast activity in adults and which could be the power of this cell population in therapeutic strategies involving neovascularization. No studies specifically targeting the analysis of the role of adult hemangioblasts in restenosis and to their potential applications are currently available in literature.

Bone marrow monocyte lineage cells

Recent findings suggest that peripheral blood-derived mature CD34- CD14+ monocytes are able to transdifferentiate into ECs under angiogenic conditions (Rehman et al., 2003) and play a role in neovascularization via leukocyte-leukocyte interaction via CD34+ cells (Harraz et al., 2001). Others demonstrated that bone marrow-derived monocyte mononuclear cells (MLCs) differentiate into neocapillaries in ischemic limb or myocardium (Kamihata et al., 2001; Iba et al., 2002). On this basis, Fujiyama demonstrated the effectiveness of bone-derived MLCs in stenosis prevention in a model of carotid angioplasty in immunodeficient nude rats (Fujiyama et al., 2003) (see subsequent paragraph for details).

Another study by Ohtani and colleagues demonstrated that the blockade of VEGF by soluble Flt-1 in a model of intraluminal injury in rabbits, mice and rats, inhibits the recruitment of bone marrow-MLCs with a reduction of neointima formation after injury (Ohtani et al., 2004). Consequently, the exact role of monocytes, as well as of VEGF, in stenosis progression remains unclear.

Transdifferentiation, cell fusion or epigenetic changes?

Recent studies indicate that bone marrow-derived stem cells have a high degree of plasticity, being able to differentiate into different kinds of cells, including ECs and SMCs. Nevertheless, further studies suggest that in some cases bone marrow-derived cells can fuse spontaneously with other somatic cells and subsequently adopt the phenotype of the recipient cells (Terada et al., 2002; Ying et al., 2002). Anyway, it should be mentioned that the study by Ying and Colleagues reported a very low frequency of spontaneous cell fusion, while some *in vivo* transplantation studies reported high levels of transdifferentiation. In these

cases, it is unlikely that the results are due to cell fusion events. However, cell fusion could be a dominant phenomenon in particular conditions.

It should also be mentioned that stem cell plasticity could be related to epigenetic changes in cells exposed to external stimuli (e.g. organ damage, stress). This phenomenon, like cell fusion, is also very rare, but should be taken into account in particular cases.

Endothelium early repair reduces stenosis in animal models

The disruption of ECs in the intima, detected in a number of models of vascular injury, such as angioplasty, by-pass and arteriotomy, is of particular importance in stenosis progression, since it is the cause of a concomitant reduction of vasculoprotective mediators, such as nitric oxide (NO) and prostacyclin. Endothelium disruption also triggers a number of signaling cascades that lead to inflammation, platelet adhesion and cell proliferation.

The injury itself can stimulate the repair of damaged tunicae, including the intima. For example, it has been demonstrated that coronary artery bypass grafting (CABG) acutely increases the number of circulating EPCs (Gill et al., 2001). This increase of circulating EPCs has been supposed to be related to the release of chemokines, such as VEGF. Another report demonstrated that the number of circulating EPCs, as well as the level of plasmatic VEGF, decrease with increasing age of patients, thus requiring further studies to improve the strategies for mobilization, ex vivo expansion, and re-transplantation of EPCs in aging patients (Scheubel et al., 2003).

Also, various risk factors for coronary artery disease, such as smoking, hypertension, diabetes and hypercholesterolemia, affect the number and the functionality of circulating EPCs both in healthy volunteers (Hill et al., 2003) and in patients (Vasa et al., 2001).

Nevertheless, the time required for the native process of endothelium repair appears too long to prevent the early critical events leading to inflammation and neointimal hyperplasia. Since the rapid repair of endothelium and the recovery of its integrity are of prime importance to reduce the consequences of vascular injury occurring during surgical procedures or angioplasty, a number of therapeutic strategies promoting early reendothelialization are currently tested in animal models. A number of these studies are based on the mobilization of stem cells from bone marrow by cytokines, statins and exercise.

Physical activity has been demonstrated to increase the number of EPCs in the peripheral blood flow, bone marrow and spleen, and to decrease the rate of their apoptosis in mice subjected to a physically active lifestyle. Circulating EPCs were found to increase also in patients with stable coronary artery disease who were undergoing physical training (Laufs et al., 2004).

It is well established that cytokines also efficiently mobilize hematopoietic precursor cells from bone marrow (Lapidot and Petit, 2002). Consequently, this approach has been applied in a number of studies aimed at a rapid reendothelialization in injured arteries through the mobilization of circulating EPCs stimulated by the injection of the cytokine granulocyte-colony stimulating factor (G-CSF).

Kong demonstrated in a model of angioplasty in rat carotids that the injection of G-CSF for five days before angioplasty induced a rapid repair of endothelium, a parallel decrease of inflammation and a 60% decrease of neointima thickness in treated animals compared with control animals, thus demonstrating that G-CSF is able to mobilize EPCs to angioplasty-injured arteries (Kong et al., 2004).

The G-CSF plus macrophage-CSF (GM-CSF) has been used for the EPC mobilization also by Cho in a model of rabbit iliac artery submitted to angioplasty and intravascular radiation (Cho et al., 2003). This model is of particular interest since intravascular radiations are applied to reduce neointimal proliferation after coronary intervention (Ahmed et al., 2001) but they also delay endothelial regeneration in the vascular healing process, increase the infiltration of inflammatory cells and induce thrombosis (Salame et al., 2000), thus requiring a prolonged administration of antiplatelet agents. The Authors demonstrated that the daily injection of GM-CSF for one week before angioplasty and for the week following the vessel injury accelerated reendothelialization and reduced monocytes infiltration after intravascular radiation therapy. In this case, the EPC mobilization through GM-CSF potentiated the efficacy of radiation therapy to suppress neointimal proliferation in the injured arteries.

The cell types of bone marrow-derived cells contributing to G-CSF-mediated endothelial regeneration in a vascular repair model have been defined in the study published by Takamiya (Takamiya et al., 2006). In this case, recombinant human G-CSF was injected daily for 14 days starting 3 days before balloon injury in the rat carotid artery. This treatment significantly reduced the neointimal formation in comparison with controls. Moreover, the regenerated endothelium was functional, since it exerted an NO-mediated vasorelaxation response. These cells contributing to endothelium regeneration derived from bone marrow and have been identified as c-Kit+/Flk-1+. All the studies above mentioned suggest that the treatment with G-CSF might be a good approach for the prevention of restenosis after revascularization procedures. Anyway, it should also be considered that G-CSF could have proatherogenic effects, such as the induction of angiogenesis within atherosclerotic lesions and the aggregation of mobilized inflammatory cells within the atheromatous plaque. Consequently, limitations to the therapeutic application of G-CSF could be possible, and further basic and clinical studies focusing on these issues are required.

A similar therapeutic strategy, based on the mobilization of EPCs not by cytokines, but by the statin simvastatin in a model of angioplasty-injured rat carotids, has been proposed by Walter (Walter et al., 2002). The authors demonstrated that a simvastatin daily injection was effective in mobilizing EPCs from rat bone marrow, increasing the number of circulating EPCs and increasing their adhesiveness by upregulating the expression of integrin subunits. Similar conclusions have been established by Werner in mice transplanted with bone marrow expressing enhanced green fluorescent protein, treated with rosuvastatin and finally submitted to carotid injury (Werner et al., 2002). These studies thus established an additional role for statins, independent of cholesterol reduction.

The reports above mentioned are based on the mobilization of unmodified EPCs by different strategies. Kong tested the efficacy of genetically modified rabbit EPCs overexpressing endothelial NO synthase (eNOS) in inhibiting neointimal hyperplasia and thrombosis in balloon injured carotid arteries (Kong et al., 2004). The authors verified that the overexpression of eNOS further enhances the vasculoprotective properties of EPCs, with a significantly reduced size of neointima and the virtual absence of thrombosis in comparison to unmodified EPC-treated rabbits.

Alternatively to EPC mobilization or genetic modification, other groups proposed to prevent restenosis by applying stents coated with EPCs. In this context, it should be mentioned that an ambitious pilot study was proposed by Shirota, in which the Authors tested the preparation and the application *in vitro* of an EPC-inoculated hybrid tissue-covered stent, loaded on a balloon catheter, to be applied during angioplasty at the site of atherosclerotic lesions (Shirota et al., 2003). After 7-day culture, the insertion of this EPC-coated stent into tubular hybrid vascular media tissue inoculated with SMCs resulted in a complete endothelialization of the luminal surface by the EPCs which migrated from the stent and proliferated *in situ*. The EPC-inoculated hybrid tissue-covered stent could represent a novel therapeutic device for restenosis prevention, alternative to the EPC injection protocols described above, aimed at obtaining a rapid reendothelialization after vascular injury. Nevertheless, further studies would be required to assess the effectiveness also in animal models and the long-term behaviour of coating EPCs.

Alternatively to EPC-coated stents, drug-eluting stents coated with various substances able to capture circulating EPCs at the vascular injury site are considered the most current approach to obtain a rapid recovery of damaged endothelium and prevent excessive cell proliferation. Among these, Blindt demonstrated that the application of a stent coated with a polymer containing an integrin-binding cyclic Arg-Gly-Asp peptide was able to significantly reduce neointimal area 12 weeks after its application in porcine coronary arteries (Blindt et al., 2006). This peptide, in fact, was able to attract EPCs at the injury site, thus accelerating

endothelialization. Alternatively, Aoki successfully tested an EPC-capture stent coated with immobilized antibodies against CD34 (Aoki et al., 2005).

Endothelialization may be compromised after intracoronary brachytherapy and after use of antiproliferative stent coatings. In this context, some authors (Virmani et al., 2004b) observed, at autopsy or in atherectomy specimens at 6 and 12 months, an incomplete healing with poor coverage of the stent struts by endothelial cells. Sirolimus-eluting stent (SES) is commonly used to prevent in-stent restenosis but is not infrequently complicated by late angiographic stent thrombosis. Fukuda demonstrated that SES may negatively affect the reendothelialization (Fukuda et al., 2005). Sirolimus is a hydrophobic macrolide with potent immunosuppressive activity. Authors demonstrated that this molecule exerts a potent inhibitory effect on differentiation, proliferation and incorporation at the injury site of both smooth muscle progenitor cells and EPCs. In addition, Imanishi demonstrated that sirolimus is able to accelerate the onset of EPC senescence, one of the functional characteristics of EPCs (Imanishi et al., 2006). The potential association of increased senescence of EPCs with the development of in-stent restenosis has been demonstrated by Matsuo in 46 patients examined 6 months after successful coronary stenting (Matsuo et al., 2006). These observations could explain why sirolimus has an inhibitory effect on neointima hyperplasia but can possibly have a negative delaying effect on the reendothelialization process after severe vascular injury, which may lead to fatal, late thrombosis (Farb et al., 2003; Virmani et al., 2004a). In conclusion, drug-eluting stents have been able to reduce the frequency of in-stent restenosis. Nevertheless, the discovery of the role of circulating EPCs in restenosis sheds new light on the application of drug-eluting stents and suggests caution about the use of sirolimus and of other drugs with potentially deleterious effects on artery reendothelialization after eluting stent implantation.

All the above mentioned studies clearly demonstrate that rapid reendothelialization obtained through the infusion or the mobilization of EPCs leads to the reduction of neointima hyperplasia induced by vascular injury. Anyway, it should be mentioned that other studies (Griese et al., 2003a) failed to reveal a vasculoprotective effect of locally infused EPCs in rat balloon-injured carotid arteries, since the authors did not detect any attenuation in neointima formation. The same protocol was effective in rabbit injured carotid arteries and in bioprosthetic grafts (Griese et al., 2003b), thus revealing that different animal models can originate very different outcomes of the same experimental protocol.

All the reports mentioned above are focused on EPC role and recruitment strategies for a rapid vascular repair after injury. Fujiyama demonstrated in a model of carotid angioplasty in immunodeficient nude rats that locally transfused bone marrow-derived CD34- CD14+ MLCs can also adhere to the injured endothelium when attracted by the monocyte chemoattractant protein-1

(MCP-1) and induce a rapid reendothelialization like EPCs (Fujiyama et al., 2003). These authors also demonstrated that such CD14⁺ MLC are more potent than bone-marrow-derived CD34⁺ cells in the inhibition of neointimal hyperplasia. The transdifferentiation of bone-marrow-derived MLCs to ECs is probably related to the increased expression of VEGF in injured carotids, as supported by *in vitro* studies (Fujiyama et al., 2003). Interestingly, the report by Fujiyama et al. revealed striking differences of behaviour between peripheral blood-derived monocytes and bone-derived-MLCs when interacting with MCP-1, since the first ones migrate in the media and accelerate restenosis after angioplasty, while the second ones only enhance reendothelialization and decrease neointima hyperplasia, thus demonstrating that only bone marrow-derived MLCs have a specific endothelial cell-committed property.

Other therapeutic applications of EPC for (re)stenosis prevention

Autologous EPCs have been used not only for early reendothelialization, but also to coat synthetic vascular prostheses to avoid thrombus deposition. Synthetic prostheses, in fact, as foreign bodies, can induce blood coagulation on their luminal surface, causing graft occlusion, especially in prostheses of small diameter. Noishiki successfully prevented this inconvenience in a model of dog abdominal aorta graft insertion, by infiltrating bone marrow-derived cells into the walls of long-fibril expanded polytetrafluoroethylene vascular grafts (Noishiki et al., 1996). Transplanted cells induced capillary growth and a complete endothelialization of the graft, thus preventing thrombus deposition.

Smooth muscle precursors are responsible for pathological remodelling in (re)stenosis

Almost all the above mentioned studies highlight a positive role for EPCs in vascular repair, since a rapid reendothelialization mediated by these cells reduces hyperplasia and, consequently, the (re)stenosis rate in the examined models. On this basis, a number of therapeutic strategies, aiming at increasing the number of circulating EPCs and their early homing at the injury site have been set up.

These exciting results are counterbalanced by other observations, proposed mainly by the group of M. Sata and supported by other studies that highlight a negative effect of circulating smooth muscle precursors on neointima hyperplasia in different kinds of models that mimic cardiovascular diseases in mice. Sata were the first group demonstrating that mice models of angioplasty-induced stenosis, graft vasculopathy and hyperlipidemia-induced atherosclerosis developed a marked neointima containing a relevant number of bone marrow-derived SMCs (Sata et al., 2002). In particular, this group demonstrated that purified HSCs (c-Kit⁺, Sca-1⁺, Lin⁻) differentiate to SMCs both *in vitro* and *in vivo*.

In fact, these cells were able to express alpha-actin, calponin, SM-myosin heavy chain and h-caldesmon. HSCs were also able to differentiate to ECs.

Surprisingly, successive studies by the same group (Sahara et al., 2005) clarified that highly purified HSCs did not give the same results as previously published, and consequently the transdifferentiation to SMCs and ECs they observed was due to the contribution of other cell types in the c-Kit⁺, Sca-1⁺, Lin⁻ fraction. This fraction, in fact, is considered enriched in HSCs, but MSCs or multipotent stem cells more primitive than HSCs can be included in this fraction.

The negative effect on vascular remodelling by smooth muscle precursors derived from bone marrow observed for the first time by Sata M et al. is also supported by other observations in different models. For example, Miller demonstrated that transient myelosuppression inhibits neointima in rabbit balloon-injured coronary arteries (Miller et al., 2001). Similar effects were obtained by Furukawa in rat carotids through the antibody-mediated inhibition of chemokines (Furukawa et al., 1999), and by Hayashi through the inhibition of the adhesion molecule P-selectin, which may play an important role in the homing of smooth muscle progenitors (Hayashi et al., 2000). Finally, Xu highlighted a negative critical role for bone marrow-derived cells in a model of cuff-induced vascular injury in mice femoral artery (Xu et al., 2004).

These studies suggest that bone marrow-derived pluripotent stem cells can potentially differentiate to unfavourable cell types causing adverse effects in remote damaged tissues where they home. As a consequence, therapeutic strategies should include the pre-selection of the appropriate fraction of precursor stem cells. The negative role played by the smooth muscle precursors in vascular remodelling suggests that these cells could also be viewed as possible target for (re)stenosis prevention. In this context, possible strategies to inhibit selectively the recruitment and homing of this cell fraction have already been proposed (Sata et al., 2005).

Some negative results of clinical trials based on the recruitment of vascular stem cells for restenosis prevention (see below for details) could be related to the above described observations.

Stem cells and restenosis: the clinical trials

While stem cells are currently the object of a number of clinical trials for recovery after acute myocardial infarction (Zohnhofer et al., 2006), human clinical investigations for restenosis prevention through stem cell-mediated early injury repair are currently quite limited.

Of particular interest is the HEALING-FIM (Healthy Endothelial Accelerated Lining Inhibits Neointimal Growth-First In Man) Registry. HEALING I was a single-center, prospective, non-randomized registry trial. It was conducted by Aoki by applying in patients the GenousTM Bio-engineered R stent (OrbusNeich

Company), the first stent designed to accelerate the natural healing response by capturing a patient's own EPCs from the blood stream (Aoki et al., 2005). Once captured, EPCs rapidly form a protective endothelial layer over the stent, providing protection against thrombus and minimizing restenosis. This stainless steel stent is coated with murine monoclonal antibody against human CD34. The first published results of this clinical study were obtained on 16 patients and reveal that this coated stent was safe and feasible. On this basis, the group started with the HEALING II study, which included 63 patients at 10 centers in Europe. Whole blood samples were analyzed to quantify the number of EPCs in each patient. Data showed that the EPC titer directly correlated with angiographic outcomes. There were no target lesion revascularizations in patients with normal numbers of circulating EPCs, while patients with low EPCs were affected by restenotic and cardiac events. It should be mentioned that the large majority of patients with normal EPC levels were on statin therapy, while most in the low EPC group were not. As already mentioned, studies revealed that statin injection is effective in EPC mobilization (Walter et al., 2002). On the basis of these encouraging results, the HEALING III study has been designed to verify and substantiate these findings and will be conducted in 2006 (Silber, 2006). The HEALING III study will also assess the effect of the combination between statin therapy and EPC capturing stents.

The connection between a normal level of circulating EPCs and restenosis incidence reported by the HEALING II trial is at least in part in contrast with findings reported by Schober (Schober et al., 2005). This study focused on the analysis of blood samples drawn from patients with atherosclerotic coronary artery disease before and 1 day after stenting. The percentage of CD34+ cells in blood samples was correlated with the rate of restenosis and the extent of diameter stenosis at follow-up. The analysis clearly revealed that the restenosis rate was higher in patients with a postprocedural increase of CD34+ cells, while patients with a postprocedural decrease of CD34+ cells showed a reduced restenosis incidence.

This discrepancy indicates that further studies are required to clarify the effect of the percentage of circulating EPCs on restenosis, or using the level of EPCs as a risk factor predicting in-stent restenosis.

Another trial, named MAGIC Cell, examined the feasibility and efficacy of G-CSF injections before angioplasty to obtain mobilized peripheral blood cells in patients with acute and old myocardial infarction who underwent coronary stenting for the culprit lesion of infarction (Kang et al., 2004). Results revealed that cell infusion improved cardiac function and promoted angiogenesis. However, the Authors noted an unexpectedly high rate of in-stent restenosis at culprit lesion in patients who received G-CSF, and therefore they prematurely stopped patient enrolment. These results suggest caution when testing stem cell therapy for

restenosis prevention in humans, as proposed also in previously mentioned studies conducted in animal models (Cho et al., 2003; Kong et al., 2004; Takamiya et al., 2006).

Conclusions

The exact role of vascular cell precursors in restenosis pathophysiology both in animal models and in human is not yet well defined, as heterogeneous and contrasting data are currently available. Of course, there is a huge amount of data demonstrating in animal models of vessel injury and in clinical trials that EPC infusion or mobilization favour a positive outcome of revascularization procedures through an early reendothelialization. Nevertheless, other data demonstrate that smooth muscle precursors and other bone marrow-derived stem cells can negatively affect the remodeling phenomena induced by vascular injury.

These contrasting data could be related to different kind of injuries, to the animal models, to the heterogeneity of stem cell populations examined and consequently to the selection of different fractions of these cell populations. Moreover, the overview of published studies highlights the necessity of well defined rules for a marker-based classification and nomenclature of stem cells and the exact definition of their hierarchy.

Local application of well defined vascular cell precursors at the injury site seems promising as a good approach for limiting restenosis in humans, but caution should be used to avoid collateral negative effects.

Acknowledgements. We are grateful to Ms. Maria Rosaria Cipollaro for assistance. This work has been partially supported by MIUR-PRIN 2004 "Regolazione del remodeling vascolare tramite ODN e siRNA in modelli animali di ipertensione" to U.G.

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Accepted October 23, 2006