Endothelial progenitor cell-coated stents under scrutiny

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The vascular endothelium protects the vessel wall from inflammatory cell infiltrates, thrombus formation and smooth muscle cell proliferation, in part by synthesizing and secreting various protective substances such as nitric oxide. In the presence of cardiovascular risk factors, the natural defenses of the endothelium are stressed, setting the stage for endothelial dysfunction and initiating atherogenesis (1). Similarly, during vascular interventions, such as percutaneous coronary intervention or bypass grafting, the integrity of the vascular endothelium is violated, setting in motion accelerated endothelial dysfunction, atherosclerosis and restenosis. The lack of an endothelial layer in prosthetic vascular grafts is a strong stimulus for intimal hyperplasia, which may lead to premature graft failure. Efforts to construct more biologically friendly grafts have been pursued, and in the current issue of the *Journal* (pages 1113-1116), Rotmans et al provide an overview of the applicability and the potential risks of using endothelial progenitor cells (EPCs) to seed prosthetic vascular grafts.

EPCs, a subset of circulating bone marrow-derived stem cells, possess the ability to differentiate into functional, mature endothelial cells (2). EPCs are characterized by the coexpression of stem cell markers (clusters of differentiation [CD]133, CD34) and endothelial cell markers (vascular endothelial growth factor receptor-1, vascular endothelial growth factor receptor-2/kinase insert domain receptor, CD31, vascular endothelial-cadherin, von Willebrand factor, E-selectin), and by their ability to form colony-forming units. The accurate characterization of EPCs, however, remains elusive because many of the markers used in phenotyping these cells are nonspecific, and are shared by both hematopoietic stem cells (CD34+) and mature endothelial cells. Thus, the exact identification of EPCs remains tentative, and similar subsets of EPCs have been defined differently in both *in vivo* and *in vitro* studies. For example, some researchers use bone marrow-derived mononuclear cells as the source of EPCs, but they have the potential to differentiate into several different cell types. Some groups use early stem cells (five to seven days of culture) in an endothelial-specific medium that expresses CD34 and one of the endothelial cell markers, while others use late outgrowth colonies (more than two weeks in culture) of stem cells after plating in an endothelium-specific medium. It is this latter group of cells that may truly represent the EPC – the cell that is committed solely to becoming a mature and functional endothelial cell; however, the search continues.

Circulating EPCs migrate to sites of vascular or tissue injury, contributing significantly to re-endothelialization,

thereby limiting neointimal formation and neovascularization (3,4). Traditional risk factors for coronary atherosclerosis are associated with lower levels of EPCs and impaired functioning. The number of EPCs correlates inversely with the Framingham risk score (5), as well as to the outcome of patients with coronary artery disease (6) (circulating CD34+KDR+ cells). Paradoxically, it is those patients who undergo vascular interventions, either cardiac or peripheral, that have both decreased EPC numbers and reduced EPC function, despite having the greatest need for these cells to repair the injured native vessels or to endothelialize prosthetic grafts.

The numbers and function of endogenous EPCs can be mobilized with exercise, statins, peroxisome proliferator-activated receptor- γ agonists, estrogen, erythropoietin and the infusion of granulocyte colony-stimulating factor (7). Alternatively, the infusion of bone marrow-derived mononuclear cells capable of differentiating into endothelial cells, as well as other cell lines in humans, has shown a clinical benefit in the setting of myocardial infarction and peripheral limb ischemia (8,9). Likewise, the injection of stem cells in animal models enhances prosthetic graft endothelialization and decreases intimal hyperplasia (4).

The harvesting of autologous EPCs, and their *in vitro* expansion before reinjection, has proven to be labour intensive, and more importantly, may alter the phenotype of these cells, inducing cellular senescence and impairing their regenerative function. The focus for re-endothelialization has therefore shifted to strategies aimed at maximizing the recruitment and adherence of endogenous circulating EPCs to the sites of endothelial damage and prosthetic conduits. One technique includes impregnating prosthetic grafts with antibodies that are directed toward proteins on the cell surface of EPCs, thereby attracting the EPCs to migrate to the surface of the graft. Anti-CD34 antibody-coated polytetrafluoroethylene prosthetic grafts were evaluated in an *in vivo* porcine model of prosthetic arteriovenous graft failure (10). These antibody-coated grafts were implanted between the carotid artery and the internal jugular vein on one side of the animal, with a noncoated graft implanted on the contralateral side. Histological sections of the excised grafts demonstrated that the anti-CD34 antibody-impregnated grafts resulted in early endothelialization over 72 h, and by 28 days, 85% of the coated surface was covered by endothelial cells, compared with only 32% coverage of the noncoated grafts ($P=0.04$). An unexpected finding for the anti-CD34 coated grafts was the observed increase in intimal hyperplasia isolated to the venous outflow tract. The authors acknowledged that the

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CD34+ progenitor cells, by virtue of their capacity to differentiate into a variety of cell types that include vascular smooth muscle cells, could actively participate in the process of intimal hyperplasia. Furthermore, hematopoietic stem cells, which are also CD34+, have the potential to differentiate into vascular cells that could participate in atherosclerotic plaque formation (11). CD34 binding has the potential to induce intimal hyperplasia by interfering with subsequent EPC proliferation, differentiation and function. Unfortunately, the adherent cells on the grafts were not fully characterized, and the extent to which CD34+ captured cells, and presumably recruited CD34- cells, contributed to the intimal hyperplasia was not quantified. Furthermore, the extent of captured cell function, as illustrated by endothelial nitric oxide synthase expression or nitric oxide generation, was not addressed.

The restriction of intimal hyperplasia to the venous anastomosis site suggests that the porcine arteriovenous shunt model may not be ideal to assess the potential of prosthetic graft material to capture EPCs, because there is turbulent flow, major pressure differential and a mismatch of compliance among the artery, graft and vein. These characteristics, or perhaps the surgical techniques, may have facilitated vascular inflammation and damage that ultimately led to a hyperproliferative cellular response in the EPC capture grafts. However, it is also possible that the increased intimal hyperplasia observed reveals some detrimental and/or nonspecific effect of the anti-CD34 antibody-impregnated grafts not previously appreciated. Of note, three of the nine study animals had bilateral graft thrombosis, which may suggest that a mechanism independent of intimal hyperplasia was involved. It may well be that the rapid proliferation and maturation of the captured CD34+ cells at the anastomotic site may stimulate smooth muscle proliferation, resulting in the formation of an immature neointima that may either later regress or mature further. Only long-term follow-up of these studies will be able to decipher this.

Identical EPC capture technology has been applied to promote the rapid endothelialization of stainless steel stents to assist in the prevention of neointimal proliferation and thrombus formation (12). EPC capture stents were developed using anti-CD34 antibodies immobilized on stainless steel stents. In the first human clinical trial using this technology (the Healthy Endothelial Accelerated Lining Inhibits Neointimal Growth [HEALING]-I clinical trial), 16 patients with de novo coronary artery disease were successfully treated with implantation of an EPC capture stent in a single primary target lesion in a native coronary artery. No subacute thrombosis was observed in the first month of implantation, despite only one month of antiplatelet therapy with clopidogrel. Only one adverse cardiovascular event was observed in nine months of follow-up, with a single patient presenting with a non-ST segment elevation myocardial infarction. This patient underwent revascularization for focal in-stent restenosis. Furthermore, coronary angiography and intravascular ultrasound were performed in 15 patients after six months, and revealed late luminal loss of 0.63 ± 0.52 mm and mean ($\pm$ SD) per cent stent volume obstruction of $27.2 \pm 20.9\%$. Thus, in the short term, the EPC-capture coronary stent has been shown to be safe and feasible for the treatment of de novo coronary artery disease, with its benefit presumably by promoting early re-endothelialization of the stent and repair of the endothelium after disruption with balloon inflation. Furthermore, early endothelialization of the stent negates the need for long-term antiplatelet therapy with

clopidogrel, with its attendant risk for increased major bleeding. Despite the obvious enthusiasm for this technology, there is still the concern, as with the anti-CD34 antibody-coated grafts, that these stents may be hindered by the capacity of CD34+ progenitor cells to differentiate into various cell types, including vascular smooth muscle cells, with the risk of ultimately developing intimal hyperplasia. The risk of intimal hyperplasia can only be defined by long-term follow-up in large, randomized controlled trials, as well as by ongoing surveillance. It is absolutely imperative to understand that the long-term results of these stents are not available, and that until these data become available, no conclusions about long-term safety can be made. It was only in this manner that the long-term risks of the use of drug-eluting stents for late thrombosis and aneurysmal formation became apparent, as well as the deleterious effects of drug-eluting stents in promoting coronary endothelial dysfunction (13,14).

The first EPC capture stent in a Canadian patient was recently implanted at St Michael's Hospital, Toronto, Ontario (MJB Kutryk, personal communication). The efficacy of EPC capture stent for the prevention of in-stent restenosis is being evaluated in the HEALING II clinical trial. Preliminary results have demonstrated, thus far, a 20% greater reduction in neointima covering of the stent at 18 months follow-up compared with six months as assessed by intravascular ultrasound (MJB Kutryk, personal communication). Long-term follow-up is awaited.

Recently, a stent impregnated with an integrin-binding cyclic Arg-Gly-Asp (cRGD) peptide was analyzed in *in vitro* and *in vivo* porcine models for its potential to recruit and bind EPCs and limit coronary neointimal formation (15). EPCs express integrins with integrin-binding cRGD peptide-binding motifs on their surface. It is hypothesized that the homing and differentiation of EPCs depend largely on their adhesion to integrins and the resultant signalling cascade that occurs on cell binding. *In vitro*, the cRGD peptide supported the outgrowth, recruitment and migration of EPCs. *In vivo*, after deployment into the coronary arteries of pigs, there were no significant differences in mean neointimal area and per cent area stenosis at four weeks between bare metal stents and cRGD peptide-loaded and -unloaded polymer stents. However, at 12 weeks, both mean neointimal area (2.2 ± 0.3 mm²) and mean per cent area stenosis ($33 \pm 5\%$) were significantly reduced in cRGD peptide-loaded polymer stents compared with the unloaded (3.8 ± 0.4 mm², $54 \pm 6\%$, $P=0.01$) or bare metal stents (3.8 ± 0.3 mm², $53 \pm 3\%$, $P<0.001$), in part by accelerating stent endothelialization. Thus, using a cRGD peptide goes beyond simply binding and recruiting EPCs, as occurs with anti-CD34 antibody-coated stents or grafts, and actually appears to influence cell proliferation and function. Whether this added biological influence on EPCs translates into a further reduction in restenosis and in stent thrombosis postpercutaneous primary intervention remains to be seen. Furthermore, the specificity of cell binding to cRGD peptides has yet to be determined. Nevertheless, using integrin-binding peptides represents a promising target to accelerate healing after vascular injury. Hopefully, human trials are not too far away.

As Rotmans et al allude to in their overview, the processes of progenitor cell homing, recruitment and differentiation remain poorly understood. As the molecular pathways behind these processes are uncovered, new targets will undoubtedly emerge that may be used to promote re-endothelialization and also to restore endothelial function, which, in turn, will limit atherogenesis and intimal hyperplasia. Furthermore, multiple

interventions may be combined for added success. For example, recently developed bioactive, nitric oxide-eluting polyurethane may form the basis for future prosthetic vascular grafts (16). Endothelial cells exposed to this nitric oxide-eluting polyurethane have shown enhanced rates of proliferation, increased rates of migration and enhanced coverage of the synthetic surface, while the rate of vascular smooth muscle cell proliferation and the level of platelet adhesion have been significantly reduced. Thus, it forms a surface that more closely mirrors a healthy vascular endothelial phenotype. Perhaps the combination of a nitric-oxide synthesizing graft coated with a peptide that not only binds, but also activates EPCs specifically to enhance their function will serve as the ideal synthetic vascular conduit in the future. Furthermore, recruitment of endogenous EPCs, in addition to the therapeutic infusion of genetically modified EPCs with enhanced proliferative or recruiting capabilities such as cells overexpressing endothelial nitric oxide synthase, may serve as a two-pronged approach to promoting vascular repair and maintaining graft patency.

However, despite the eloquent science for these 'magic bullets', clinical efforts should continue to be directed toward cardiovascular risk reduction and disease prevention. The number and function of native EPCs may be highly dependent on smoking cessation, exercise, improved glycemic and blood pressure control, and treatment with 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, all of which maintain a healthy and functional endothelium. Our group has shown that treatment with the peroxisome proliferator-activated receptor- γ agonist rosiglitazone may enhance therapeutic strategies for early re-endothelialization and limit restenosis because it increased EPC proliferation and promoted mononuclear cell differentiation toward the endothelial lineage and away from the smooth muscle cell lineage in vitro (17). Furthermore,

rosiglitazone improved the rate of endothelial restoration in a mouse model of vascular injury and limited neointimal formation by simultaneously increasing the extent of bone marrow-derived endothelial cells and decreasing the number of bone marrow-derived smooth muscle cells in the areas of vascular damage. Thus, the key to overcoming and limiting restenosis and intimal hyperplasia may be a combination of lifestyle changes, pharmacological interventions and EPC-assisted therapy.

EPCs may potentially be an ideal cellular source for restoring endothelial function and seeding prosthetic grafts, but for now, it remains simply an attractive hypothesis pending clinical proof of safety. As our understanding of EPC biology expands, further interventions to exploit and maximize their inherent regenerative properties will emerge. Identifying EPC-specific markers to better target therapies by limiting recruitment of nonendothelial progenitors may be the key to greater success in the future. For now, the investigation of EPC-coated grafts in well-designed, randomized controlled trials is appropriate, and if proven safe and efficacious, may become an important cardiovascular tool. However, continuing long-term surveillance, which is appropriate for any medical therapy, be it a drug or a technical device, will be needed. It is important for clinicians and scientists to remember that the interaction of bone marrow and the vascular wall is highly complex, rapidly changing and poorly understood. We have recently demonstrated (18) that stem cell factor, which is a critical signalling molecule for progenitors, can actually promote neointimal formation and adverse vascular remodelling, again emphasizing the need for ongoing research efforts and care while interpreting the data concerning EPC-coated stents. The technology is sound, and it is our contention that further refinements in the specificity of the antibody coating may help bridge the bench to the bedside.

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