CURRENT CONCEPTS AND NEW DEVELOPMENTS FOR AUTOLOGOUS IN VIVO ENDOTHelialISATION OF BIOMATERIALS FOR INTRAVASCULAR APPLICATIONS

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Abstract

Circulating endothelial progenitor cells (EPCs) in the peripheral blood of adults represent an auspicious cell source for tissue engineering of an autologous endothelium on blood-contacting implants. Novel materials biofunctionalised with EPC-specific capture molecules represent an intriguing strategy for induction of selective homing of progenitor cells. The trapped EPCs can differentiate into endothelial cells and generate a non-thrombogenic surface on artificial materials. However, the success of this process mainly depends on the use of optimised capture molecules with a high selectivity and affinity. In recent years, various biomedical engineering strategies have emerged for in situ immobilisation of patient’s own stem cells on blood contacting materials. The realisation of this in vivo tissue engineering concept and generation of an endothelium on artificial surfaces could exceedingly enhance the performance of not only small calibre vascular grafts and stents, but also, in general all blood-contacting medical devices, such as heart valves, artificial lungs, hearts, kidneys, and ventricular assist devices.

Keywords: Tissue engineering, biocompatibility, implants, stem cells, endothelial cells, cardiovascular tissue.

Introduction

Atherosclerosis is a chronic inflammatory disease of the arterial wall, which leads to the asymmetric focal thickening of the intima (the innermost layer of arteries). It is the leading cause of death in the developed countries, present in almost one-third of all deaths. During atherosclerosis, the accumulation of low density lipoproteins within the intima leads to the formation of plaques that consist of lipids, inflammatory and immune cells (mainly macrophages and T cells), collagen fibres, smooth muscle cells (SMCs), debris from dead cells and calcium. Over time, the progressive plaque enlargement can cause narrowing (stenosis) or even occlusion of blood vessels. One of the treatment methods to reopen blockages in the arteries is the percutaneous transluminal angioplasty, which is mostly performed with stent deployment to keep the vessel open as long as possible.

However, if the atherosclerotic lesion in the occluded vessel is extensive and the vessel is no longer reliable, bypass surgery is performed using an autologous vascular graft. For this purpose, the patient’s own internal mammary artery or saphenous vein is used to route blood flow around the obstructions. However, many patients do not have suitable autologous vessels because of previous surgical harvest or pathological vessel alterations due to progressed atherosclerosis. Furthermore, it is often difficult to obtain a sufficient graft length for effective bypass. In such cases, there is a great need for synthetic vascular grafts to replace or bypass diseased vessels. At present, the synthetic vascular grafts of choice are made of expanded-polytetrafluoroethylene (e-PTFE, Gore-Tex®) or polyethylene terephthalate (PET, Dacron®). Although these grafts exhibit long term patency for more than ten years at large graft diameters, the grafts with a less than 6 mm inner diameter are associated with high occlusion rates. The occlusion is mainly caused due to the thrombogenicity of the synthetic materials or the intimal hyperplasia at the anastomotic sites (Burkel, 1988).

Despite the tremendous progress in biomaterial development and surface modifications in recent years, the native non-thrombogenic endothelium still represents the ideal surface for blood contact. Hitherto, numerous research groups seeded synthetic material surfaces with endothelial cells (ECs) (Deutsch et al., 1999; Meinhart et al., 2001) to create an autologous endothelium in vitro (Fig. 1). However, this application necessitates an initial surgery to obtain a vessel biopsy from the patient and to isolate the patient’s own ECs for graft seeding (Villalona et al., 2010). The isolated ECs are grown until the required

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cell number is achieved. Thereafter, grafts are seeded with these cells and cultivated until they reach confluence. Finally, the in vitro endothelialised grafts are implanted into the patient. Due to this extensive procedure, the in vitro endothelialisation of grafts is very time-consuming and expensive, and harbours the risk of bacterial contamination and, therefore, is often unfeasible in clinical practice.

EPCs – A fascinating alternative cell source for endothelialisation of vascular grafts

In 1997, Asahara and colleagues (Asahara et al., 1997) identified bone marrow-derived endothelial progenitor cells (EPCs) in the circulating peripheral blood of adults that are capable of attaining EC characteristics in vitro. In further studies, it was demonstrated that these cells are able to repair injured blood vessels and to regenerate the function of ischaemic organs by vasculogenesis and angiogenesis (Hristov et al., 2003; Szmityko et al., 2006).

The presence of these precious endothelial progenitor cells in adults offers scientists the possibility to generate an autologous endothelium on vascular grafts or stents (Szmityko et al., 2006) without the need of a vessel biopsy for isolation of ECs. However, since the discovery of EPCs, there is a controversy about the identity of true circulating EPCs (Hur et al., 2004; Prater et al., 2007; Yoder et al., 2007). At least 2 fundamentally different EPC populations can be obtained from peripheral blood by in vitro cultivation of mononuclear cells (MNCs), namely the “early” EPCs (also called colony forming unit-endothelial cells (CFU-ECs)) or “late” EPCs (also known as endothelial colony forming cells (ECFCs) or blood outgrowth endothelial cells (BOECs)) (Prater et al., 2007).

Different cultivation methods for isolation of EPCs from MNCs are used by scientists. “Early” EPCs are similar to Asahara’s EPCs and can be obtained by using the commercially available kit EndoCult™ from StemCell Technologies (Grenoble, France). For this purpose, MNCs are isolated from the peripheral blood by density gradient centrifugation and incubated for 48 h on fibronectin-coated plates in EndoCult™ medium to remove adherent monocytes. Nonadherent cells are removed and re-plated on new fibronectin-coated dishes. CFU-EC colonies appear within 4-7 days of culture as discrete colonies comprising round cells in the centre and spindle-shaped cells sprouting at the periphery (Hill et al., 2003). “Late” EPCs can be obtained using the cultivation method reported by Lin and Ingram (Ingram et al., 2004; Lin et al., 2000). MNCs are incubated on collagen I-coated plates in endothelial specific growth medium. Growth medium is gently changed every day to discard non-adherent cells. ECFC colonies appear from the adherent cell population, after 2-3 weeks of culture and display typical endothelial cobblestone morphology. Hur and colleagues also demonstrated that both EPC populations can be obtained by using one culture protocol (Hur et al., 2004). For this purpose, they seeded MNCs on 2% gelatin-coated plates and incubated in endothelial specific growth medium. The first medium change was performed 6 days after plating. Thereafter, the medium was changed every 3 days. The “early” EPCs appeared after 3 to 5 days and their number increased for 2 weeks. Thereafter, they did not replicate.
and gradually disappeared within 4 weeks after plating. The “late” EPCs appeared in 2 to 4 weeks after plating. “Early” EPCs express both endothelial and monocytic markers, such as CD14 and CD115, and exhibit a limited proliferation capacity (Rehman et al., 2003; Rohde et al., 2006; Zhang et al., 2006). In contrast, “late” EPCs are highly proliferative cells, which are found in lower cell densities (0.05-0.2 cells/ml) than “early” EPCs (50-500 cells/ml) in the peripheral blood of adults (Prater et al., 2007). Both EPC populations contribute to angiogenesis, but only “late” EPCs are able to form de-novo functional blood vessels in vivo (Critser and Yoder, 2010). However, Yoon and colleagues demonstrated that the transplantation of mixed EPCs, “late” and “early” EPCs, in athymic nude mice with hindlimb ischaemia results in synergistic augmentation of angiogenesis compared with any single type of EPC transplantation (Yoon et al., 2005). Hitherto, the exact in vivo functions of these different EPC populations are still unclear. In contrast to “early” EPCs, “late” EPCs exhibit high proliferation capacity and seem to be more appropriate for in vitro endothelialisation of large surfaces, such as vascular prosthesis, because of the ability to quickly generate a high number of ECs. Thus, these cells are probably best suited for rapid endothelialisation of prostheses. However, hitherto the in vivo endothelialisation efficiency of “late” and “early” EPCs is not well-studied. The capturing of “early” EPCs on implants could be also effective for in vivo endothelialisation of artificial surfaces. In vivo, “early” EPCs that secrete higher levels of angiogenic cytokines than “late” EPCs, may support endothelialisation by secreting proangiogenic cytokines (Hur et al., 2004; Zampetaki et al., 2008) and increase the homing of circulating EPCs. Furthermore, they may ensure the proliferation of “late” EPCs and the survival of matured ECs. Thus, the immobilisation of both types of EPCs could synergistically enhance the quality of the generated endothelium. Therefore, further in vivo studies are necessary to clarify the precise roles of “early” and “late” EPCs in view of the endothelialisation efficiency on implants.

A major obstacle for understanding, interpretation, and comparison of the previously published reports about EPCs is the lack of standardised methods for characterisation and naming of these cells. Research groups use the term “EPC” for a heterogeneous group of cells, such as for either “early” EPCs or “late” EPCs obtained in vitro. Other groups apply flow cytometry for quantification of EPCs, which are positive for CD34/CD133/VEGFR2 (Massa et al., 2005; Peichev et al., 2000), CD34/CD133 (Allanore et al., 2007), or CD34/VEGFR2 (Su et al., 2010). In turn others isolate CD34 positive cells using MACS (Magnetic Activated Cell Sorting) (Weber et al., 2004) and designate these cells as EPCs. Thus, for better interpretation of the data published in the literature, there is an urgent need for standardised identification and designation methods.

Strategies to increase mobilisation, homing, and incorporation of EPCs
EPCs are present in low numbers in the circulating peripheral blood of adults. Thus, different strategies exist to boost the number of circulating EPCs and to increase homing and incorporation of these cells at sites of vascular injury, ischaemia, or active angiogenesis. One strategy to improve homing and incorporation of EPCs at desired tissues is the in vitro pre-treatment of EPCs. They can be pre-stimulated by incubation with small molecules or genetically modified to activate signalling pathways involved in survival, overexpression of antiapoptotic proteins, or expression of adhesion molecules (Seeger et al., 2007). Another strategy is the delivery of proteins or genes to the target tissue for in situ induction of chemokines and chemoattractant factors to enhance the attraction and recruitment of EPCs from the blood stream. Furthermore, the elevation of the EPC number in the peripheral blood by systemic in vivo application of drugs can accelerate angiogenesis, vasculogenesis, and repair of an injured endothelium. Different strategies to increase vascularisation and homing of EPCs are illustrated in Fig. 2. Since the number and function of EPCs inversely correlates with risk factors for coronary artery disease (Vasa et al., 2001b; Hill et al., 2003; Werner et al., 2007b),

![Figure 2: Different strategies for increased homing and incorporation of EPCs. (A) Systemic in vivo delivery of drugs for mobilisation of EPCs from the bone marrow into the peripheral blood. (B). In vitro pretreatment of EPCs or (C) pretreatment of the target tissue for in situ recruitment of EPCs to the target tissue.](cells-materials.org)
patients suffering from coronary artery disease can be medically treated to increase the number and functional activity of EPCs, thereby further improving the prospects of success for EPC based therapies.

**Increasing the number and function of EPCs**

Numerous cytokines, growth factors, drugs and hormones, such as vascular endothelial growth factor (VEGF) (Asahara et al., 1999), angiopoietin-1, granulocyte colony-stimulating factor (G-CSF), stromal cell-derived factor-1 (SDF-1) (Askari et al., 2003; Ceradini et al., 2004), erythropoietin (Heeschen et al., 2003), hepatocyte growth factor (HGF), oestrogen (Iwakura et al., 2003; Strethlow et al., 2003), and statins (Duckers et al., 2007b; Vasa et al., 2001a) induce the mobilisation of EPCs from the bone marrow into the circulating peripheral blood and can be used in vivo to boost the number of circulating EPCs from the bone marrow into the circulating peripheral blood (Table 1). Furthermore, the ex vivo expansion of EPCs is often necessary for in vitro experiments or for potential clinical cell therapy of

<table>
<thead>
<tr>
<th>Strategies for</th>
<th>Drugs and Cytokines</th>
<th>Condition</th>
<th>Investigators</th>
<th>Effects</th>
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<tbody>
<tr>
<td>increasing the number of EPCs</td>
<td>HMG-CoA reductase inhibitors (statins) e.g. atorvastatin</td>
<td>in vivo</td>
<td>(Vasa et al., 2001a)</td>
<td>Increase in number of EPCs</td>
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<td>ACE inhibitors e.g. ramipril</td>
<td>in vivo</td>
<td>(Min et al., 2004)</td>
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<td>AT II antagonists e.g. olmesartan, irbesartan</td>
<td>in vivo</td>
<td>(Bahlmann et al., 2005)</td>
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<td>Erythropoietin</td>
<td>in vivo</td>
<td>(Heeschen et al., 2003)</td>
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<td>G-CSF</td>
<td>in vivo</td>
<td>(Honold et al., 2006)</td>
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<td>VEGF</td>
<td>in vivo</td>
<td>(Asahara et al., 1999)</td>
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<td>PPAR-γ agonists e.g. rosiglitazone</td>
<td>in vivo</td>
<td>(Pistrosch et al., 2005)</td>
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<td>increasing the function of EPCs</td>
<td>PPAR-γ agonists e.g. pioglitazone</td>
<td>in vitro/in vivo</td>
<td>(Gensch et al., 2007)</td>
<td>Prevents apoptosis of EPCs</td>
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<td>HMG-CoA reductase inhibitors (statins) e.g. atorvastatin, mevastatin simvastatin</td>
<td>in vitro</td>
<td>(Assmus et al., 2003)</td>
<td>Delay of EPC senescence</td>
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<td></td>
<td>ACE inhibitors e.g. ramipril</td>
<td>in vitro</td>
<td>(Min et al., 2004)</td>
<td>Increased functional activity of EPCs</td>
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<td>Erythropoietin</td>
<td>in vitro</td>
<td>(George et al., 2005)</td>
<td>Increased proliferative and adhesive properties of EPCs</td>
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<td></td>
<td>in vitro</td>
<td>(Bahlmann et al., 2004)</td>
<td>Increased number of functionally active EPCs</td>
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<td>Thrombin receptor activating peptide (SFLLRN)</td>
<td>in vitro</td>
<td>(Smadja et al., 2005)</td>
<td>Induced proliferation, migration and increased capillary-like structure formation in Matrigel</td>
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<td>Resveratrol</td>
<td>in vitro</td>
<td>(Xia et al., 2008)</td>
<td>Delayed the onset of senescence in EPCs</td>
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<td>Oestrogen</td>
<td>in vitro</td>
<td>(Imanishi et al., 2005a)</td>
<td>Inhibited EPC senescence</td>
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<td></td>
<td>Puerarin</td>
<td>in vitro</td>
<td>(Zhu et al., 2008)</td>
<td>Delayed the onset of senescence in EPCs and increased proliferation of EPCs</td>
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ischaemic diseases. However, the in vitro cultivation of EPCs leads to cellular aging (senescence) and limits the proliferative capacity of these cells. Moreover, in patients with cardiovascular risk factors (Tepper et al., 2002; Imanishi et al., 2005b) the function of EPCs is impaired. Dysfunction of EPCs may have adverse effects on EPC-based endothelialisation of biomaterials and clinical strategies that are performed to enhance the perfusion of ischaemic tissues in patients with coronary and peripheral artery disease. Fortunately, several in vitro studies with different drugs (Leone et al., 2009), such as erythropoietin (Bahlmann et al., 2004), statins (Assmus et al., 2003), and PAR-γ (Peroxisome Proliferator-Activated Receptor-γ) agonists (Gensch et al., 2007; Werner et al., 2007a), demonstrated the ability to stimulate the function of EPCs (Table 1). Using a thrombin receptor activating peptide, SFLLRN (Serine-Phenylalanine-Leucine-Leucine-Arginine-Asparagine), Smadja and colleagues activated PAR-1 (Protease Activated Receptor-1) on human “late” EPCs, which in turn promoted EPC proliferation and enhanced SDF-1/CXCR4-mediated angiogenesis (Smadja et al., 2005). Other in vitro studies with drugs, such as HMG-CoA reductase inhibitors (Assmus et al., 2003), puerarin (Zhu et al., 2008), resveratrol (Xia et al., 2008), or oestrogen (Imanishi et al., 2005a) led to the inhibition of EPC senescence. Therefore, the application of these drugs could be useful to protect EPCs against dysfunction and to improve proliferative and functional capacity of these cells.

Increasing homing of EPCs

Prestimulation of EPCs

Homing of EPCs to the sites where vascular repair and neovascularisation are needed can be enhanced by up-regulation of adhesion molecules on EPCs. In previous studies, the pre-stimulation of EPCs with HMGB-1 (High-Mobility Group Box-1) (Chavakis et al., 2007), SDF-1 (Zemani et al., 2008), 8-pCPT-2′-O-Me-cAMP (Carmona et al., 2008; Patschan et al., 2010), activating β1-integrin antibody (KIM185) or M2β (Chavakis et al., 2005), leptin (Schroeter et al., 2008) and ephrin-B2-Fc chimera (Foubert et al., 2007) increased the homing of systemically administered EPCs. In vivo, the protein HMGB-1 is passively released from necrotic cells and actively secreted by activated immune cells (Rouhiainen et al., 2004). Thus, it serves as a chemoattractant for inflammatory and stem cells and elicits the migration of these cells to the sites of tissue damage (Palumbo et al., 2009). HMGB-1 transduces cellular signals via binding to RAGE (Receptor for Advanced Glycation End-products), Toll-like receptor 2 (TLR2) or 4 (TLR4) (Lotze and Tracey, 2005). From these receptors, RAGE and TLR2 are expressed on EPCs and the pre-stimulation of EPCs with HMGB-1 activates β1 and β3 integrins on EPCs and induces the adhesion to mature ECs, ICAM-1, and fibronectin. Using this process, Chavakis et al. (2007) observed improved homing and adhesion of EPCs at sites of ischaemia and active angiogenesis. Soluble E-selectin (sE-selectin) is another potent angiogenic mediator (Koch et al., 1995; Kumar et al., 2003), which was determined in elevated concentrations by Oh and colleagues (Oh et al., 2007) 24 h after ischaemia. The stimulation of ECs with sE-selectin induced the expression of adhesion molecules ICAM-1 and VCAM-1, which in turn contributed to EPC recruitment in ischaemic tissues and increased angiogenesis. Since EPCs have (additionally to the E-selectin) functional E-selectin ligands on their surface, the sE-selectin can also modulate EPCs. Therefore, the stimulation of EPCs with sE-selectin augmented interleukin-8 secretion and led to enhanced migration, capillary tube formation, and incorporation of EPCs into ECs.

Pretreatment of the target tissue

The local injection of cytokines or chemokines into the target tissue is another promising approach to attract progenitor cells. SDF-1 is one of the most important chemotactic and pro-migratory factors and binds to the G-protein-coupled receptor CXCR4 (CXC Chemokine Receptor 4), which is expressed on monocytes, macrophages, B and T lymphocytes, platelets, megakaryocytes, and CD34 positive cells, including both EPCs and haematopoietic stem cells (Moore et al., 2001). Several studies demonstrated the ability of locally administered SDF-1 to augment the EPC accumulation in a mouse model of hindlimb ischaemia (Yamaguchi et al., 2003; Yu et al., 2009). In further studies, Ceradini et al. (2004) showed that SDF-1 gene expression in ECs is regulated by the transcription factor HIF-1 (Hypoxia-Inducible Factor-1), which is a key mediator of cellular hypoxia response and regulates the expression of genes involved in angiogenesis, glucose metabolism, vascular tone, and oxygen transport (Frede et al., 2009). The induction of SDF-1 expression via HIF-1 increased the migration, homing, and adhesion of circulating CXCR4-positive cells to areas of injury. Furthermore, the ex vivo transfection of EPCs with an HIF-1α-expressing vector increased revascularisation efficacy in an in vivo mouse model of hindlimb ischaemia (Jiang et al., 2008). In comparison to untransfected EPCs, HIF-1α-overexpressing EPCs showed increased proliferative potential and sensitivity towards angiogenic factors. This is probably due to the recruited HIF-1α-expressing EPCs, which promote SDF-1 expression and recruit more EPCs to the injury site, and thereby lead to increased revascularisation. Furthermore, Hiasa and colleagues demonstrated that the intramuscular injection of SDF-1 coding plasmid-DNA into mice significantly augments the homing of progenitor cells to the ischaemic limb and enhances ischaemia-induced vasculogenesis and angiogenesis (Hiasa et al., 2004).

Biofunctionalisation of implant surfaces to capture EPCs from blood stream

The in vivo endothelialisation of blood contacting prostheses inside the body is a promising alternative to the expensive and time-consuming in vitro generation of an endothelium on synthetic materials. For this purpose, blood contacting materials are coated with capture molecules like peptides, proteins, antibodies, magnetic
molecules, or aptamers to enable the homing of EPCs on implant surfaces (Table 2). After the implantation, EPCs are attracted directly from the blood stream to the implant surface and the endothelialisation process starts immediately. Thus, an optimal surface with high biocompatibility can be created and biomaterial induced immune reactions can be avoided. In Fig. 3, the in vivo endothelialisation concept of biofunctionalised implants is shown.

To ensure the successful homing of EPCs on implants, the surface coating should meet two requirements. The first requirement is the immobilisation of highly specific capture molecules. The second requirement is the prevention of unspecific adhesion of other blood cells and serum proteins, which can rapidly cover the immobilised capture molecules and impede the recognition of EPCs. Furthermore, the coatings can contain covalently immobilised growth factors, such as VEGF and Angiopoietin-1 (Chiu and Radisic, 2010) to support the differentiation of EPCs into ECs and the survival and proliferation of these cells.

**Different Capture Molecules for EPCs**

**Monoclonal antibodies (MAB) recognizing EPC surface receptors**

Circulating EPCs are characterised by expression of the early surface markers CD34 (Asahara et al., 1997), CD133, and VEGFR-2 (Vascular Endothelial Growth Factor Receptor-2; KDR) (Miller-Kasprzak and Jagodzinski, 2007) on their surface. Thus, several research groups are focused on creation of coatings with specific monoclonal antibodies, which recognise these main EPC surface receptors.

**CD34 MAB**

The Genous™ EPC capturing stent (OrbusNeich Medical Technologies (Fort Lauderdale, FL, USA) coated with anti-human CD34 monoclonal antibodies is the first biofunctionalised stent for in vivo endothelialisation. The safety and feasibility of this stent was demonstrated in the single-centre HEALING-FIM (Healthy Endothelial Accelerated Lining Inhibits Neointimal Growth-First In Man) study (Aoki et al., 2005). Later, the efficacy of the Genous™ stent to prevent in-stent restenosis was evaluated (Silber, 2006; Duckers et al., 2007a; Duckers et al., 2007b). In this multicentre HEALING II study, patients on statins had more EPCs and less in-stent late loss than those not on statins. Therefore, patients in the HEALING IIB multicentre study were pretreated with atorvastatin prior percutaneous coronary intervention (PCI) in order to increase EPC levels and to clarify the safety and efficacy of the Genous™ stent in conjunction with statins. The statin therapy resulted in a 459% relative increase in the number of EPCs. However, the titre of circulating CD34⁺ stem cells was elevated by only 12% (Garg et al., 2010), which shows that there is a discrepancy between the cells identified as EPCs and CD34⁺ cells. Probably, due to the failure of the therapy to augment CD34⁺ stem cell levels,
Table 2: Capture molecules for EPCs (part 1).

<table>
<thead>
<tr>
<th>Capture Molecules</th>
<th>Application</th>
<th>Study</th>
<th>Investigators</th>
<th>Outcome</th>
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<tr>
<td><strong>Monoclonal antibodies (MAB)</strong></td>
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<tr>
<td>CD34 MAB coated stent (Genous™ stent)</td>
<td><em>in vivo</em> (human) (HEALING-FIM)</td>
<td>(Aoki et al., 2005)</td>
<td>Within 9 months:  - No stent thrombosis  - MACCE (Major adverse cardiac and cerebrovascular events) occurred in only one patient out of 16 patients</td>
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<td></td>
<td><em>in vivo</em> (human) (HEALING II)</td>
<td>(Duckers et al., 2007a)</td>
<td>Patients on statins had more EPCs and less in-stent late loss than those not on statins</td>
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<td><em>in vivo</em> (human) (HEALING IIB)</td>
<td>(Garg et al., 2010)</td>
<td>The pretreatment of patients with atorvastatin prior PCI had no benefit in terms of angiographic outcomes</td>
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<td><em>in vivo</em> (human) TRIAS</td>
<td>(Beijk et al., 2010)</td>
<td>After one year:  - Higher late lumen loss in the Genous™ stent compared with the Taxus stent  - 4 stent thromboses with Taxus stent  - No stent thrombosis with Genous™ stent</td>
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<td>CD34 MAB immobilised onto a sirolimus-eluting stent (SES)</td>
<td><em>in vivo</em> (pig)</td>
<td>(Nakazawa et al., 2010)</td>
<td>Up to 80% endothelialisation at 14 days</td>
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<td>CD34 MAB coated ePTFE grafts</td>
<td><em>in vivo</em> (pig)</td>
<td>(Rotmans et al., 2005)</td>
<td>Rapid endothelialisation Increased neointimal hyperplasia</td>
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<td>CD34 MAB immobilised on electrodes of a microfluidic system</td>
<td><em>in vitro</em></td>
<td>(Ng et al., 2010)</td>
<td>Trapping and accumulation of CD34⁺ cells from PBMCs</td>
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<td>VEGFR-2 MAB coated solid surface</td>
<td><em>in vitro</em></td>
<td>(Markway et al., 2008)</td>
<td>Ability to selectively capture flowing VEGFR-2⁺ HUVECs</td>
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<tr>
<td>VEGFR-2 MAB coated microfluidic device</td>
<td><em>in vitro</em></td>
<td>(Plouffe et al., 2009)</td>
<td>Captured approximately 17-fold more EPCs than only BSA treated devices</td>
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<td>Bispecific molecule (GPVI-CD133 construct) immobilised on collagen coated surfaces or preincubated with EPCs</td>
<td><em>in vitro/in vivo</em> (mouse)</td>
<td>(Langer et al., 2010)</td>
<td>10-fold enhanced adhesion of human CD34⁺ cells under flow conditions compared to collagen alone 14 days after carotid artery injury: Enhanced reendothelialisation of vascular lesions</td>
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<td><strong>Aptamers</strong></td>
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<td>DNA-aptamers immobilised onto PDMS or PTFE discs</td>
<td><em>in vitro</em></td>
<td>(Hoffmann et al., 2008)</td>
<td>Captured EPCs from whole anticoagulated porcine blood under flow conditions</td>
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Table 2: Capture molecules for EPCs (part 2).

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Capture molecules</th>
<th>Mode</th>
<th>References</th>
<th>Results</th>
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</thead>
</table>
| cRGD peptides coated stent | in vivo (pig) | (Blindt et al., 2006) | After 12 weeks:  
- Significantly reduced mean neointimal area and percent area stenosis compared with BMS |
| Polyurethane vascular grafts with YIGSR peptides, diazeniumdiolate NO donor and PEG | in vitro | (Taite et al., 2008) | Encouraged adhesion of ECs  
Reduced platelet adhesion  
Reduced proliferation of SMCs  
Enhanced proliferation of ECs |
| Nanofibrous matrix with MMP-2 cleavage sites coupled to YIGSR peptides and NO donors | in vitro | (Kushwaha et al., 2010) | Enhanced EC growth  
Inhibition of SMC growth  
Reduced platelet adhesion |
| Metacrylic terpolymer with TPS peptide selected by phage display | in vitro | (Veleva et al., 2008) | in serum free medium specific binding to HBOECs |
| Selectins and their ligands | sLe^3 immobilised into a collagen matrix | in vitro | (Suuronen et al., 2009) | Recruited more CD133^+CD34^+L-selectin^+ cells than collagen only matrix |
|  | in vivo (Sprague-Dawley rats) | | Enhanced neovascularisation and improved tissue perfusion of ischemic hindlimbs |
|  | (Suuronen et al., 2009) | |  |
| P-selectin coated microtubes | in vitro | (Narasipura et al., 2008) | significantly higher number of CD34^+ cells on P-selectin coated devices than on CD34 MAB coated devices |
| P-selectin coated blood-compatible plastic tubes | in vivo (Sprague-Dawley rats) | (Wojciechowski et al., 2008) | After 1 hour of blood perfusion:  
- Up to 7-fold enrichment of the CD34^+ cells than on non-coated control tubes |
| Magnetic molecules | SPIO microspheres-loaded ECs | in vivo (rabbit) | (Consigny et al., 1999) | Circumferential delivery of ECs to the luminal surface of injured arteries |
|  | SPIO microspheres-loaded EPCs | in vivo (pig) | (Pislaru et al., 2006a) | After 1 day:  
- Uniform cell coverage on magnetised knitted Dacron grafts |
|  | in vivo (pig) | (Pislaru et al., 2006b) | After 1 day:  
- Magnetised stents retained 6- to 30-fold more EPCs than non-magnetised stents |
|  | SPIO nanoparticles-loaded EPCs | in vivo (Sprague-Dawley rats) | (Kyrtatos et al., 2009) | After 1 day:  
- With magnet 5-fold enhanced cell localisation compared to cell delivery without magnet |
the angiographic outcomes of Genous™ stent were not improved.

In the randomised single-centre TRIAS (TRI-stent Adjudication Study) trial, the Genous™ stent was compared with a conventional drug eluting stent (DES) (the Taxus Liberté paclitaxel-eluting stent) in patients with de novo coronary lesions and a high-risk of coronary restenosis (Beijk et al., 2010). One year outcomes of the study showed that a subgroup of patients had a significantly higher late lumen loss in the Genous™ stent compared with the Taxus stent. However, compared to four stent thromboses with Taxus stent, no stent thrombosis was observed with Genous™ stent. Prior to the completion of the 1-year follow-up, this single-centre study was transformed into a multi-centre randomised, 2-armed TRIAS study (TRIAS High Risk (HR) and TRIAS Low Risk (LR)) to increase patient recruitment. The TRIAS HR study compares the Genous™ stent with the Taxus stent in patients with a high risk of restenosis. Meanwhile, the TRIAS LR study compares the Genous™ stent with the bare metal stent (BMS) in patients with a low risk of restenosis (Klopp et al., 2009). The monitoring of patients will be conducted during a 5-year follow-up period.

Rotmans and colleagues implemented the coating technique, which is used for Genous™ stent, to coat ePTFE grafts with anti-human CD34 MAB (Rotmans et al., 2005). The implantation of these grafts in an in vivo porcine model resulted in rapid endothelialisation, which was verified by lectin staining (a surface antigen shared by several blood-borne cells, including monocytes). However, the endothelialisation was also associated with significantly increased neointimal hyperplasia at the venous anastomosis. This adverse effect might be caused by differentiation of attracted CD34+ cells into vascular SMCs (Shimizu et al., 2001; Simper et al., 2002). Previous studies demonstrated the ability of CD34+ cells to differentiate into diverse cell types, including ECs, cardiomyocytes, and vascular SMCs (Yeh et al., 2003). Haematopoietic stem cells (HSCs) also express CD34 on their cell surface and can differentiate into SMCs in vitro and in vivo (Sata et al., 2002). These cells can contribute to pathological arterial remodelling and lead to the increased proliferation index in anti-CD34 antibody coated grafts. Furthermore, the attracted EPCs can release growth factors, such as VEGF and HGF (Rehman et al., 2003), which can stimulate migration and proliferation of vascular SMCs (Grosskreutz et al., 1999; Ma et al., 2003; Parenti et al., 2004) and lead to increased intimal hyperplasia. Therefore, a combination of the Genous™ stent and drug eluting stent has been developed. Anti-human CD34 antibodies were immobilised onto the surface of a sirolimus-eluting stent (SES) to suppress the neointimal hyperplasia while promoting EC coverage (Nakazawa et al., 2010). The stent deployment in pig coronary arteries resulted in increased endothelialisation compared with the standard SES alone at 3 and 14 days. The endothelialisation of the Genous™ stent was nearly completed at 14 days (98%). Although the endothelialisation rates of the Genous™ stent were approximately 2-fold greater than the SES-anti-CD34 stent at 3 days, the SES-anti-CD34 stent demonstrated up to 80% endothelialisation at 14 days. However, further studies are needed to evaluate the long term impact of these stents on neointimal proliferation.

The ability of anti-CD34 antibody-coated surfaces to capture CD34+ cells from a cell suspension composed of peripheral blood mononuclear cells (PBMCs) was also demonstrated by Ng and colleagues (Ng et al., 2010). Anti-CD34 antibodies were immobilised on silane-PEG SAM (Polyethylene Glycol Self-Assembled Monolayer) passivated electrodes of a microfluidic system. The integrated negative dielectrophoresis (DEP) (Thomas et al., 2009) enabled the trapping and accumulation of CD34+ cells at the centre of electrodes. Thus, this chip system can be applied for label-free quantification of CD34+ cells in PBMC samples, which were termed as EPCs by Ng et al. (2010).

Despite promising results, the described studies should be viewed critically. CD34 is the most commonly used marker for identification and isolation of circulating EPCs. However, CD34 is not only specific for EPCs, but it is also expressed on HSCs. Peichev et al. demonstrated that circulating non-adherent EPCs, which have the ability to differentiate into mature adherent ECs, co-express additionally to CD34, the endothelial as well as haemangioblastic marker VEGFR-2, and the haematopoietic stem cells antigen CD133 on their surface (Peichev et al., 2000). Only 0.002% of the total PBMCs were CD34 positive, and only 0.44±0.2% of the total CD34+ cells were in fact EPCs (defined by additional expression of VEGFR-2 and CD133). That means that 99.6% of the anti-CD34 antibody attracted cells are not EPCs. A multitude of these cells have the ability to differentiate into proinflammatory cells. Thereby, intimal hyperplasia can be further accelerated, which is associated with deterioration of the graft patency. Furthermore, the precise characterisation of captured cells onto anti-CD34 antibody-coated implants using immunohistochemistry may improve the interpretation and understanding of the clinical outcomes.

**VEGFR-2 MAB**

The major mediator of the angiogenic and mitogenic effects of VEGF is the VEGFR-2, which is, in addition to mature ECs, also present on the circulating EPCs (Ferrara et al., 2003). Therefore, antibodies against VEGFR-2 can be also used to attract EPCs onto artificial grafts. In a model system under low shear rates, Markway and colleagues demonstrated the ability of protein G-oriented anti-VEGFR-2 MB to selectively capture flowing VEGFR-2 human umbilical vein endothelial cells (HUVECs) onto a solid surface (Markway et al., 2008). The protein G-orientation of antibodies enhanced the availability of the antigen-binding domains and resulted in approximately 2.5-fold higher capture efficiency than directly adsorbed antibodies did. After binding, antibody mediated adhesion was not disrupted, even if the shear rate was increased to arterial shear rate. Furthermore, Plouffe et al. conjugated the surface of a microfluidic device with antibodies against CD34 or VEGFR-2 to capture in vitro cultivated ovine peripheral blood-derived EPCs (Plouffe et al., 2009).
Under low shear stress, anti-CD34 antibody-coated microfluidic devices were able to capture approximately 20-fold more EPCs than only BSA treated devices. Also, the coating with anti-VEGFR-2 antibody resulted in approximately 17-fold higher EPC binding versus BSA controls. Thus, the ability of anti-VEGFR-2 antibody coated capture devices to bind EPCs was demonstrated. However, further studies with a mixture of cells, such as PBMCs, could be performed to ensure the specificity and to determine the efficiency of this antibody. Moreover, as an alternative to the anti-VEGFR-2 antibody, the electrostatically or covalently bound ligand of VEGFR-2 (namely, VEGF) could be used to attract EPCs on implant surfaces (Anderson et al., 2009).

CD133 MAB

Recently, a promising new concept for further modification of EPC specific capture molecules was developed by Langer and colleagues to guide the captured EPCs to vascular lesions (Langer et al., 2010). A bispecific molecule (called GPVI-CD133 construct) was designed, consisting of a recombinant form of the soluble platelet collagen receptor glycoprotein VI (GPVI) targeting collagen, which is the main component of the injured vessel wall, and a monoclonal antibody targeting CD133 on EPCs. In vitro, the preincubation of the collagen surface with the GPVI-CD133 construct enhanced the adhesion of human CD34+ cells, which were termed as EPCs, by approximately tenfold under flow conditions compared to collagen alone. In NOD/SCID (Non-Obese Diabetic/Severe Combined Immunodeficiency) mice, 14 days after injury of the carotid artery and application of human EPCs, enhanced reendothelialisation of vascular lesions was observed when EPCs were preincubated with the bispecific construct compared to EPCs treated with the single components of the construct alone.

Despite these promising results, the use of collagen specific ligand to guide the captured EPCs to the injured endothelium should be viewed critically. Platelets have two major receptors that mediate the direct platelet-collagen interaction: the integrin α,β3 and GPVI (Best et al., 2003). These receptors are involved in platelet attachment to the injured arterial wall and play a crucial role in the initiation of haemostasis and thrombosis in vivo. The high number of platelets (150,000-450,000 per μl blood) expressing collagen receptors enables the immediate recognition of vascular injuries. Therefore, the vascular lesions can be covered by platelets before the bispecific construct can arrive at the location in effective numbers. Because of this, it could be necessary to use very high amounts of bispecific construct to compete against platelets. Furthermore, EPCs localised in the bone marrow or immediately after migration into the circulation are CD133 positive; however, circulating EPCs obviously lose CD133 and begin to express typical mature EC surface markers (Hristov et al., 2003). Thus, EPCs in the peripheral blood that lost CD133 and differentiated into more mature ECs cannot be recognised by the bispecific construct. Thereby, a smaller number of EPCs can be captured. Unfortunately, it should be also mentioned that CD133 is also expressed by haematopoietic stem cells. However, the use of bispecific molecules to capture EPCs and to guide them to the desired tissues is a promising approach.

Aptamers

Due to their ability to fold into specific three-dimensional structures, short single-stranded oligonucleotides, called aptamers, can bind to a given target with high affinity and specificity. They are generally 70-90 nucleotides long and can be selected using the SELEX (Systematic Evolution of Ligands by EXponential enrichment) combinatorial chemistry process (Ellington and Szostak, 1990; Tuerk and Gold, 1990), which mimics the Darwinian evolution in test tubes (Tavitian and Haberkorn, 2009). Iterative cycles of in vitro selection and amplification allow the enrichment of target specific aptamers from a large chemically synthesised random sequence library composed of up to 10^15 different sequences. Typically, the selected aptamers bind their targets with dissociation constants (K_d) in the low picomolar (10^-12 M) to low nanomolar (10^-9 M) range (Nimjee et al., 2005). Since the discovery of SELEX technology in 1990, aptamers have received tremendous attention in the fields of targeted drug delivery (Chu et al., 2006b; Chu et al., 2006a; Farokhzad et al., 2006; Huang et al., 2009; McNamara et al., 2006), diagnosis (Shangguan et al., 2007; Tang et al., 2007), imaging (Blank et al., 2001; Schäfer et al., 2007), target validation (Blank and Blind, 2005; Cerchia and de Franciscis, 2007), therapeutics (Bock et al., 1992; Cosmi, 2009), biosensing (Bing et al., 2010; Liu et al., 2009), and cell fishing (Guo et al., 2006; Schäfer et al., 2007; Phillips et al., 2009; Pan et al., 2010). After 20 years of effort, aptamers have been generated against a broad range of targets, including small molecules, peptides, proteins, drugs, and even whole cells (Daniels et al., 2003; Cerchia et al., 2005; Ohuchi et al., 2006; Mallikaratchy et al., 2007; Raddatz et al., 2008) or organisms (Ulrich et al., 2002; Chen et al., 2007).

Using the SELEX technology, Hoffmann and colleagues were able to enrich DNA-aptamers against peripheral blood derived porcine CD31 positive EPCs (Hoffmann et al., 2008). The selected aptamer candidates were immobilised onto polydimethylsiloxan (PDMS) or polytetrafluoroethylene (PTFE) discs, using a haemocompatible reactive six-arm star-shaped polyethylene glycol (starPEG) coating (Hoffmann et al., 2006). The antithrombogenic starPEG coating prevents the undesired protein adsorption. Thereby, the covering of immobilised capture molecules by serum proteins can be avoided and the specific binding of circulating EPCs onto blood contacting implants can be significantly increased. In a modified Chandler-loop model (Chandler, 1958), discs with immobilised aptamers or control discs with nonsense aptamers were incubated with whole anticoagulated porcine blood. One of the generated aptamers was able to capture CD31 (PECAM-1) and CD144 (VE-cadherin) positive EPCs under flow conditions. These cells were cultivated for 10 days in vitro and exhibited properties of endothelial cells. Thus, the ability of aptamers to capture EPCs from peripheral blood was demonstrated in an in vitro closed-loop model under flow conditions.
Further cell-SELEX experiments are currently being performed to generate human EPC specific aptamers. Because cell-SELEX technology uses whole living cells as the target to generate cell specific aptamers (Guo et al., 2008), it is not necessary to know the molecular composition of the cell surface. However, it is important to perform SELEX experiments with defined and well characterised EPC populations to prevent the loss of enriched aptamers by incubation with altered cells in the next selection rounds. Furthermore, since cell-SELEX has the potential to develop aptamers for unknown molecules, the selected aptamers can then be used to purify and identify the target for aptamer recognition. In this way, new EPC specific biomarkers can be discovered.

Peptides

Extracellular matrix derived peptides

In recent years, specific peptide motifs of the extracellular matrix (ECM) components that interact with cell integrins inspired scientists to use these peptides for biofunctionalisation of biomaterial surfaces to enable accelerated in situ endothelialisation (de Mel et al., 2008).

Cyclic RGD (cRGD) peptides

Several ECM proteins, including fibronectin, vitronectin, and laminin, contain the RGD (Arg-Gly-Asp) sequence, which binds to integrins receptors on the cell surface and enables the adhesion of cells to ECM. Since the conformation of cRGD peptide mimics more closely the native ligand, this peptide is preferred for surface immobilisation. Several groups demonstrated in their studies the expression of integrins with cRGD binding motifs on EPCs (Peled et al., 2000; Walter et al., 2002). Therefore, Blindt and colleagues coated stents with a new polymer that contained cRGD peptide and examined the capability of these stents to bind EPCs and to reduce neoointimal hyperplasia. These stents were implanted in porcine coronary arteries. Histomorphometric analyses after 12 weeks demonstrated that the mean neoointimal area and percent area stenosis were significantly reduced in cRGD-loaded polymer stents by accelerated endothelialisation compared with the unloaded polymer or bare metal stents (Blindt et al., 2006). However, despite these first positive results, it should be mentioned that integrins with RGD binding motifs are not only present on EPCs. Above all, platelets exhibit glycoprotein GPIIIa, the most abundant platelet receptor, on their surface. It recognises the RGD sequence (Sanchez-Cortes and Mrksich, 2009) in several adhesive proteins, such as fibrinogen, fibronectin, vitronectin, von Willebrand factor, and thrombospondins. Since platelets are present in a much higher number in the peripheral blood than EPCs, they can quickly bind to cRGD peptides and cover the implants within the shortest time. Therefore, capture molecules cannot bind to EPCs anymore. Moreover, the additional promotion of the platelet adhesion to the artificial grafts may lead to an enormous acceleration of the thrombus formation.

YIGSR peptides

Another peptide, which has also been used to promote endothelialisation on biomaterials, is the laminin-derived (Tyr-Ile-Gly-Ser-Arg) YIGSR peptide (Jun and West, 2004; Jun and West, 2005a; Jun and West, 2005b). Taite and colleagues incorporated this peptide, a diazeniumdiolate nitric oxide (NO) donor, and polyethylene glycol (PEG) into the backbone of polyurethane with the aim to improve the long-term patency of polyurethane vascular grafts (Taite et al., 2008). The modification encouraged the adhesion of ECs to the material. Simultaneously, both the incorporation of diazeniumdiolate NO donor and PEG reduced the platelet adhesion to the polyurethane surface. Additionally, the incorporated NO donor reduced proliferation of SMCs, but enhanced proliferation of ECs.

Recent tissue engineering applications are focused on mimicking biological, chemical, and nanostructural properties of natural ECM by self-assembly of peptide amphiphiles (PAs) into nanofibre networks. The viscoelastic properties, degradability, and bioactivity of such networks might be tuned by altering the length and chemical composition of PAs (Jun et al., 2008). Kushwaha and colleagues developed a native endothelial ECM mimicking nanofibrous matrix, which could be used as a self-assembled coating on vascular implants (Kushwaha et al., 2010). The nanofibrous matrix is formed by self-assembly of two different PAs that consist of hydrophobic tails coupled to hydrophilic functional peptide sequences. One PA (C16-GTAGLIGQYIGSR) contained a matrix metalloproteinase-2 (MMP-2) cleavage site (GTAGLIGQ) coupled to the endothelial cell-adhesive ligand (YIGSR). The other PA (C16-GTAGLIGQKKKKK) contained a matrix metalloproteinase-2 (MMP-2) cleavage site (GTAGLIGQ) that was coupled to a polylysine (KKKKK) group to form NO-donating residues. The release of NO from the nanofibrous matrix significantly enhanced EC growth and reduced SMC growth. Furthermore, a 150-fold decrease in platelet adhesion was determined on the NO-releasing nanofibrous matrix compared to the collagen-I coated surface. Such a nanofibrous matrix with multiple components can synergistically enhance the generation of an endothelium on biomaterials by supporting EC proliferation, inhibition of SMC growth, and platelet adhesion. Thus, endothelialisation is improved and the thrombosis and restenosis are prevented. However, the described studies were performed with ECs. Therefore, similar experiments might be performed with EPCs to examine whether EPCs behave similarly to ECs. Nevertheless, the modification of biomaterials by coatings with NO-releasing self-assembled PAs or by incorporation of NO donors and PEG are auspicious strategies that can be used in combination with each EPC capture molecule. Such a biomaterial could provide the optimal conditions for rapid in vivo endothelialisation of small-diameter vascular grafts.

Peptides selected by phage display

Phage display technology (Smith, 1985), which uses a combinatorial peptide library to identify novel cell specific...
peptide ligands, has the advantage that the selection can be performed without prior knowledge of cell-surface molecules, such as in the cell-SELEX technology. Thus, it is a promising strategy to select new EPC specific peptide ligands. Using this technique, Veleva et al. (2007) isolated a peptide ligand (TPSLEORTVYKAK, called TPS) that binds to human blood outgrowth endothelial cells (HBOECs). This peptide was covalently coupled to a methacrylic terpolymer matrix (Veleva et al., 2008). In serum free medium, TPS peptide retained the binding affinity towards HBOECs and demonstrated limited binding to HUVECs. However, in the presence of serum proteins, cell-specific binding was inhibited. It is possible that the unspecific binding of serum proteins to the methacrylic terpolymer matrix led to the covering of EPC capture molecules and prevented the specific recognition of cells. Thus, protein-resistant terpolymers should be developed to ensure selective binding of EPCs. Especially for in vivo applications, the inhibition of unspecific serum protein adsorption to the implant surfaces plays an important role. Moreover, the identification of cell surface molecules that are recognised by TPS peptide could help to improve the prospects of success of this EPC capture molecule.

Selectins and their ligands
The selectins, E-, L-, and P-selectin, are important cell adhesion molecules that play a key role in tethering and rolling of leukocytes on inflamed endothelium (Kansas, 1996). Several groups demonstrated that EPCs exhibit the P-selectin glycoprotein ligand-1 (PSGL-1) on their cell surface, which mediates the recruitment of EPCs on activated endothelium by binding to P- and E-selectin (Vajkoczy et al., 2003; Foubert et al., 2007; Pfosser et al., 2009). Additional studies also demonstrated the expression of E-selectin as well as its ligand (Oh et al., 2007) and L-selectin (Biancone et al., 2004) on EPCs.

Since several studies had verified the ability of the oligosaccharide sialyl Lewis^i (sLe^i) to bind E-selectin (Brunk and Hammer, 1997), P-selectin (Rodgers et al., 2000), and L-selectin (Greenberg et al., 2000). Suuronen and colleagues (Suuronen et al., 2009) immobilised sLe^i into a collagen matrix and demonstrated enhanced adhesion of EPCs. Ischaemic hindlimbs receiving this matrix revealed increased neovascularisation and improved tissue perfusion. Charles et al. (2007) coated surfaces with P-selectin-, E-selectin-, or L-selectin-IgG and investigated the differences in rolling velocities of CD34 expressing and non-expressing cells. For this purpose, MNCs were isolated from human adult bone marrow and separated in CD34+ fraction using Dynabeads and in CD34− fraction by depletion of CD34+ cells. Thereafter, cells were perfused over immobilised selectins. CD34+ cells exhibited slower rolling and stronger adhesion than CD34+ cells when interacting with selectins in flow microchannels. Due to distinguishable rolling behaviour of CD34+ cells from that of CD34+ cells on selectin coated surfaces, selectin coated stents or grafts might be developed for the selective capturing of CD34+ cells from blood flow. Therefore, Narasipura and colleagues performed additional studies to determine whether CD34+ cells from human bone marrow-derived MNCs could be specifically captured on P-selectin functionalised microtubes (Narasipura et al., 2008). Thus, flow devices were coated with recombinant human P-selectin/F, chimera or anti-CD34 antibody to capture and enrich CD34+ cells. The total number of captured CD34+ cells on P-selectin coated devices was significantly higher than on antibody coated devices. It is possible that the superior binding of cells to P-selectin is induced by the preferred flow-mediated nature of selectin binding. Later, the same research group implanted blood-compatible plastic tubes coated with P-selectin into the femoral artery of rats to determine whether CD34+ cells could be captured directly from circulating blood (Wojciechowski et al., 2008). After 1 h of blood perfusion, the percentage of captured CD34+ cells to total captured MNCs on P-selectin coated tubes was enriched up to 7-fold over the CD34+ population on non-coated control tubes. However, only 28% of the captured MNCs were CD34+ cells.

PSGL-1 is expressed on all white blood cells and plays an important role in recruitment of white blood cells into inflamed tissue. In contrast to low EPC amounts in the peripheral blood, the number of white blood cells in 1 ml blood is approximately 4 × 10^6 - 1.1 × 10^7 cells. Because of this high cell amount, white blood cells can bind rapidly to the capture molecules and block them. Thereby, efficient capturing of EPCs cannot be ensured. Also, sLe^i is constitutively expressed on granulocytes and monocytes.

Magnetic Molecules
Labelling of EPCs with superparamagnetic iron oxide (SPIO) particles offers another attractive possibility to capture EPCs on implants. Using an external magnetic device, SPIO-loaded EPCs can be attracted to the desired place in vivo. The first attempts in this area were performed with SPIO microspheres-loaded ECs. By applying a magnet over an injured femoral artery in combination with animal rotation, Consigny and colleagues demonstrated nearly circumferential delivery of SPIO-loaded ECs to the luminal surface of injured arteries (Consiny et al., 1999). Later, Pislaru et al. (2006a) annealed a flexible magnetic sheet to external surface of a knitted Dacron graft and filled them with SPIO microspheres-loaded porcine EPCs for 10 min before placement. Grafts precoated with EPCs, were implanted in porcine carotid arteries. After one day, the grafts were evaluated and uniform cell coverage was observed on the magnetised surfaces. In order to enable the application of this technology for stents, Pislaru and colleagues generated magnetisable stents by coating commercially available stainless-steel stents with a 10 μm thick layer of nickel (Pislaru et al., 2006b). Stents were magnetised and implanted for one day in porcine coronary and femoral arteries. SPIO microspheres-loaded EPCs were delivered locally during transient occlusion of blood flow. Magnetised stents retained 6- to 30-fold more labelled EPCs than non-magnetised stents. In addition, in a recent study, Kyratas et al. (2009) magnetically tagged human EPCs with a FDA-approved superparamagnetic contrast agent (Endorem®), which is clinically used for magnetic
resonance imaging of the liver and spleen. SPIO nanoparticles-loaded EPCs were administered into the injured rat carotid arteries with and without the presence of an external magnetic device for 12 min. Magnet actuator was placed at a 5 mm vertical distance from the target tissue. After one day, 5-fold enhanced cell localisation was detected at the site of carotid artery injury compared to cell delivery without the magnet. Although this application is very promising, the capturing of superparamagnetic-labelled EPCs on magnetic surfaces is time consuming and expensive. EPCs have to be isolated and cultivated for approximately 2 weeks under GMP conditions and an additional incubation for 16 h is necessary for labelling of these cells in vitro. However, these obstacles could be overcome by functionalisation of SPIO nanoparticles (McCarthy and Weissleder, 2008) with EPC-specific peptides, antibodies, aptamers, or proteins. Thus, the isolation of EPCs is no longer necessary. However, the success of this approach depends on the availability of highly selective EPC-specific ligands. Moreover, further studies are needed to determine the long-term influence of iron particles on proliferation and function of captured EPCs. Furthermore, it should be demonstrated that the release of iron particles from EPCs does not lead to a local inflammation, which can trigger the intimal hyperplasia. Additionally, the development of biocompatible magnetisable graft materials is required to perform long-term in vivo studies.

Conclusions

Rapid in vivo endothelialisation of intravascular implants by capturing circulating autologous EPCs directly from the blood stream via biofunctionalised implant materials is a fascinating concept to enhance the haemocompatibility of blood contacting materials. However, the success of this strategy is highly influenced by the specificity and affinity of EPC capture molecules. Although numerous approaches have already been designed to immobilise EPCs on synthetic materials, further studies, more specific capture molecules, and improvements are necessary to upgrade the in vivo self-endothelialisation and for it to reach clinical maturity.

References


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Discussion with Reviewers

Reviewer I: What is in your opinion the most specific approach for the in vivo vascularisation of biomaterials in terms of EPC?
Authors: The success of the in vivo endothelialisation depends on the haemocompatibility of the coating technology and the specificity of the used EPC capture molecules. Any class of capture molecules such as peptides, antibodies or aptamers, with high biocompatibility, which meet the specificity requirement, is appropriate for the biofunctionalisation of biomaterials for in vivo tissue engineering of an endothelium. Despite the previous developments in this area, more specific capture molecules are necessary that exclusively target EPCs in the circulating peripheral blood. In addition, standardised definitions for EPC subpopulations and characterisation methods are necessary for an accurate assessment of the in vivo endothelialisation on implants.

Reviewer I: In extreme injuries cases, other tissues also required tissue-engineered constructs to be repaired (e.g. bone). In these cases, one of the unsolved problems is the promotion of a micro-vascular network within a tissue-engineered implant that also involves other cell types. Do you think that the methods you described could be transposed to other applications and promote the formation of such a capillary-like network within porous biomaterials without ending with a full endothelialisation of the material?
Authors: The major obstacle in generating artificial three dimensional (3D) large structures, such as engineered tissues or organs, is the efficient oxygen and nutrient supply to cells within 3D constructs. Because of the thickness, the diffusion is limited. Thus, a microvascular network is necessary for sufficient perfusion of the tissue. Furthermore, the repopulation of the construct with different types of cells is another challenge for tissue engineers. There are different approaches to improve the success of engineered tissue constructs. Heike Mertsching and colleagues demonstrated that the preserved vessel network of decellularised biological vascularised matrixes (BioVaM) from porcine small intestine segments can be reseeded with EPCs to line the graft vessel bed with a functional endothelium. Harald Ott and Doris Taylor have even managed to create a beating heart in the laboratory. Using cells from newborn rats, they were able to repopulate acellularised rat hearts. For the repopulation, a mix of heart- and endothelial cells as well as stem- and progenitor cells was prepared by mincing hearts from newborn rats. Four days after injection of these cells into the left ventricles of the decellularised hearts and pumping a solution of oxygen and nutrients through the network of preserved blood vessels, the previously nonliving heart-matrix began to beat. These fascinating results demonstrate the feasibility to recellularise even whole organ matrixes with different types of cells. The results also show that the generation of artificial tissues requires well-defined scaffold geometries within a tissue engineered matrix. Furthermore, for targeted cell-homing in three dimensional constructs, cell specific capture molecules can be immobilised at defined positions. For example, by using the cell-SELEX technology, cell type specific aptamers can be selected. These capture molecules can be immobilised in 3D constructs to catch the cells at desired places. Additionally, the incorporation of growth factors and degradable polymers, which prevent during the repopulation the migration of cells to undesired locations, can be used to control cell growth and migration.

Reviewer I: In terms of the different possibilities to achieve an in vivo vascularisation, which of these approaches might have the potential to result in a clinical application.
Authors: Hitherto, the most progressed approach to capture circulating EPCs from the peripheral blood is the use of monoclonal antibodies (MABs), namely antibodies targeting CD34. Stents coated with anti-CD34 MABs are already in clinical use for in vivo endothelialisation. However, CD34 is not only expressed on EPCs but also on haematopoietic stem cells. Thus, anti-CD34 MABs are not selective for only EPCs and therefore, in our opinion, this approach is not convincing. In our very own ranking for capture molecules that have the potential to result in a clinical application, aptamers take the first place, peptides are on the second position, subsequently come MABs and finally magnetic beads. However, it should be mentioned that the best in vivo endothelialisation outcome and long-term biocompatibility depends on the specificity of EPC capture molecules that are used for biofunctionalisation. Despite the current developments in this area, several research groups are still searching for new more specific EPC markers and capture molecules that exclusively target EPCs in the circulating peripheral blood.

Reviewer II: Taking into account the low frequency of EPC within the circulation and the unsolved question of specific markers to identify these cells, how would the authors rank the individual methods they summarise in this review in terms of specificity and efficacy or outcome in vivo?
Authors: The best in vivo endothelialisation outcome and long-term safety stands or falls with the specificity of the used EPC capture molecules. Hitherto, the major limitation of the used capture molecules that are reviewed in this manuscript is that they are not highly selective for EPCs. In this regard, cell-SELEX and phage display technologies are auspicious methods to select highly specific EPC capture molecules. Using these methods, new up to now undiscovered markers on circulating EPCs can be
discovered. Especially SELEX technology, which contains approximately $10^{15}$ different oligonucleotides in the start library as possible target binding ligands, has the ability to select EPC specific capture molecules by using EPCs as target cells. Furthermore, the use of anti-VEGFR2 antibodies could be more selective for circulating EPCs than anti-CD34 or anti-CD133 antibodies, because CD34 is also expressed by haematopoietic stem cells, and CD133 expression decreases after mobilisation of the EPCs from bone marrow into the peripheral blood. Also the magnetic labelling of EPCs is a promising approach, however it needs the previous isolation of EPCs and the effect of iron particles on survival of EPCs is not well studied. Also the incorporation of additional molecules, such as NO donors, PEG, or growth factors, into the backbone of antithrombogenic biomaterials is a promising approach to enhance the adhesion of EPCs and the proliferation of captured EPCs on biomaterials. Using these modifications, the adhesion of platelets and serum proteins can be also reduced, which in turn can enhance the capturing efficacy of capture molecules. Despite the present developments in this area, more specific capture molecules are necessary that exclusively target EPCs in the circulating peripheral blood. In addition, standardised definitions for EPC subpopulations and characterisation methods are required for an accurate evaluation and comparability of the used EPC capturing methods in regard to specificity and in vivo endothelialisation efficiency on implants.

Reviewer II: The procedure for endothelialisation of grafts described (Introduction) is indeed time consuming, expensive and possibly leading to contamination, but what also about the viability of the endothelial cells when the implant is introduced in vivo, especially at long term? Was this ever tested? Couldn’t this be an additional possibility of drawback for this ex vivo approach which even further validates the presented approach of EPC recruitment?

Authors: Thank you for this interesting comment. A detailed study of the long-term viability of endothelial cells (ECs) on in vitro endothelialised and implanted grafts is so far unknown to us. But, a study, which determines the in vivo viability of these ECs and their ability to maintain a confluent EC-layer after implantation, would be very interesting. It is known that after a finite number of divisions in vitro, mature ECs undergo senescence, like most human somatic cells. The EC senescence in long-term in vitro cell cultures may limit initially the ability to produce sufficient number of cells to seed the implants. Due to aging of the vasculature or pathological changes of the ECs caused by cardiovascular diseases, the population doublings can differ from one patient to another patient. On the other hand, the in vitro generated endothelium on grafts can contain after a long time of cultivation several senescent ECs. After implantation, the ECs are exposed to enormous continuous sheer stress of blood flow, which can cause the partial loss of ECs. The proliferation of neighbouring ECs is necessary to compensate the cell loss and to fill the gaps. Therefore, the use of cells with high-proliferative potential and low senescence is required to enable the long-term patency and biocompatibility of grafts. Thus, the direct capturing of highly proliferative EPCs without the need of isolation procedure and long in vitro cultivation periods has a huge potential to maintain a confluent endothelium over a long time. Thereby, the long-term safety of implants could be ensured.