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Endothelial Outgrowth Cells: Function and Performance in Vascular Grafts

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The clinical need for vascular grafts continues to grow. Tissue engineering strategies have been employed to develop vascular grafts for patients lacking sufficient autologous vessels for grafting. Restoring a functional endothelium on the graft lumen has been shown to greatly improve the long-term patency of small-diameter grafts. However, obtaining an autologous source of endothelial cells for *in vitro* endothelialization is invasive and often not a viable option. Endothelial outgrowth cells (EOCs), derived from circulating progenitor cells in peripheral blood, provide an alternative cell source for engineering an autologous endothelium. This review aims at highlighting the role of EOCs in the regulation of processes that are central to vascular graft performance. To characterize EOC performance in vascular grafts, this review identifies the characteristics of EOCs, defines functional performance criteria for EOCs in vascular grafts, and summarizes the existing work in developing vascular grafts with EOCs.

Introduction

Cardiovascular disease is a major health burden worldwide. With more than 7.5 million cardiovascular operations performed in the United States in 2010, there is high demand for novel solutions to treat vascular disease.[1] A key limitation in the field is the inability of the current materials, such as expanded polytetrafluoroethylene (ePTFE) and Dacron® (polyethylene terephthalate fibers), to achieve long-term patency in vascular grafts <5 mm in diameter.[2–4] Current vascular grafts predominantly fail by either thrombus formation or intimal hyperplasia, leading to occlusion.[5,6] Treatment options for vascular disease would be significantly improved with the development of functional, small-diameter blood vessels to replace diseased tissue.

In vivo, endothelial cells (ECs) line the luminal surface of blood vessels and are responsible for regulating numerous processes, including hemostasis and thrombosis, the transport of blood constituents into tissues, smooth muscle cell proliferation, and vascular tone.[7] Recognizing the capacity for ECs to properly coordinate these processes *in vivo*, much effort has been placed into developing vascular grafts with an endothelialized lumen that can dynamically interact with the local environment.[8–12] In patients lacking a suitable saphenous vein to use as a bypass graft, endothelialized ePTFE grafts demonstrated improved patency rates at a 9-year follow up.[9] A review of EC isolation and graft seeding techniques highlighted the utility of vein ECs in maintaining vascular graft patency, though isolations from veins typically have low yields and are invasive.[13] Thus, despite promising results in clinical trials, the limited availability of autologous ECs and the donor site morbidity resulting from EC harvest have limited the clinical potential of EC-seeded vascular grafts.

The discovery of circulating endothelial progenitor cells (EPCs) that could be isolated from peripheral blood and differentiated to an endothelial-like phenotype provided a promising new autologous cell source for endothelialization.[14] Since this discovery, research has sought to characterize the various subtypes of cells that differentiate from circulating progenitors,[15–18] develop protocols for EPC characterization and quantification,[19–22] and establish the clinical relevance of EPC count.[23,24] Among the phenotypically and functionally diverse progeny of EPCs, a population termed endothelial outgrowth cells (EOCs; also known as late outgrowth, blood outgrowth endothelial, or endothelial colony-forming cells) are particularly promising for vascular tissue engineering applications.[25] The features of EOCs that make them well suited for tissue engineering include (1) robust proliferation rate, (2) high expansion potential, and (3) simple sourcing via venipuncture.[16,25–27] The high proliferation rate and expansion potential of EOCs allows very few colonies to be quickly expanded to the high cell number required for tissue engineering applications, and venipuncture is considerably less invasive than harvesting ECs from excised tissue.

In this review, the isolation and validation procedures for EOCs will be briefly described, followed by physiological methods to characterize EOC performance in vascular grafts, and concluding with current vascular tissue engineering strategies utilizing EOCs. Although cellular adhesion and proliferation have been used to characterize the performance of EOCs on vascular grafts,[28] this review aims to define

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Table 1. Defining Properties of Endothelial Outgrowth Cells

	Properties of EOCs
Morphology	Cobblestone under static conditions, will elongate with unidirectional fluid shear stress[26,46,60,75,78]
Surface marker expression	$CD31^+$, vWF^+, $CD146^+$, $CD309^+$, $CD34^+$, $CD133^-$, $CD14^-$, $CD45^-$[17,19,25,26,33]
Functional verification	Bind UEA-1 lectin and uptake AcLDL, angiogenic behavior in Matrigel™[17,25,26]
Source	Progenitor isolated from peripheral blood, colonies appear after >7 days in culture[25]
Population doubling time	1–3 days[18,26,33,35]
Expansion potential	$>10^9$ cells[18,25–27]
Extracellular matrix production	Robust production of collagen IV and fibronectin[73]

Verification of a number of these properties can be performed to validate true EOC phenotype.
EOCs, endothelial outgrowth cells; vWF, von Willebrand factor; UEA, ulex europaeus agglutinin; AcLDL, acetylated low-density lipoprotein.

EOCs' ability to regulate the biological processes required of a vascular endothelium. Since the improper regulation of thrombosis, intimal proliferation, and inflammation typically underlies vascular graft failure,[6] these processes will be presented as performance criteria for a successful vascular graft. This review summarizes the results of multiple studies of EOCs regarding these processes to provide a broad report on EOC function with the understanding that subtle inter-lab variations in culturing conditions may produce individual variations in findings. Other derivatives of EPCs, as well as therapies utilizing undifferentiated EPCs, will not be discussed here; however, the authors direct interested readers to other reviews[15,29,30] for discussions on other EPC-derived cell types.

Endothelial Outgrowth Cells

As first demonstrated by Asahara *et al.*, EC-like cells can be derived by culturing the adherent, $CD34^+$ fraction of mononuclear cells from a peripheral blood draw in a pro-EC growth medium.[14] These cells differentiate 3–7 days post-plating, express numerous EC genes and surface antigens, and enhance angiogenesis in a mouse hind limb ischemia model. However, subsequent studies with these cells indicated that the cells were unable to achieve a cobblestone-like EC morphology or form capillary-like tubes in Matrigel™, and the cells expressed monocytic surface antigens.[31,32] In parallel with these developments, Lin *et al.* cultured peripheral blood mononuclear cells and noted occasional colonies of cells with cobblestone morphology and a high expansion capacity that appeared to originate from bone marrow.[25] The cells in these colonies became known as EOCs, and in contrast to the cells characterized by Asahara *et al.*, these cells appeared after >7 days in culture, expressed CD146 (MCAM, clone P1H12), had angiogenic behavior in Matrigel, and lacked CD14 expression. Due to a lack of a unique marker defining either the progenitor population giving rise to EOCs or EOCs themselves, a combination of surface antigen expression and functional assessment is currently required to distinguish the EOC population from other EPC-derived cell types.[15] A summary of defining properties exhibited by EOCs is listed in Table 1.

The multifaceted and still-evolving definition of EPCs and their derivatives has consequently led to significant inter-lab variability in the terminology of EPCs and EOCs. However, multiple groups have shown definitively that the highly proliferative, non-monocytic, cobblestone cells discussed henceforth in this review are derived (in humans) from a rare $CD34^+CD133^-CD146^+$ circulating progenitor.[19,33,34] The scarcity of these progenitors in peripheral blood has been the greatest challenge in their characterization. From a translational standpoint, the low frequency in blood reduces the probability of successful outgrowth from any given blood draw. Fortunately, the procedure of drawing blood is minimally invasive and could be repeated for patients if the initial attempt is unsuccessful. In addition, multiple cell characterization methods should be utilized to confirm the presence of EC markers (CD31, VEGFR-2, CD34, CD146, and von Willebrand factor), the absence of leukocyte markers (CD14, CD45), *in vitro* tube formation in Matrigel™, and acetylated low-density lipoprotein uptake. Thorough cell characterization is necessary to confirm the EOC phenotype due to the multiple cell types that may be generated using the current standard derivation protocols.[15,16,19,35,36] Further, our group and others have noted that colonies of outgrowth cells occasionally assume a large, irregular shape with slow proliferation and limited expansion potential.[33,35,37] In the absence of any definitive markers that distinguish these various EPC-derived populations, screening each colony is currently necessary to validate the EOC phenotype.

Performance Criteria for EOCs in Vascular Grafts

There are two general strategies for engineering vascular grafts using EOCs: covering existing clinically-used synthetic materials with EOCs to limit blood-material contact and improve patency, or incorporating vascular cell types in addition to EOCs (e.g., smooth muscle cells) with a biodegradable or naturally derived scaffold to recreate native blood vessel structure and function. For both approaches, EOCs should function as a healthy, native endothelium to facilitate long-term graft success. Chiefly, these functions include (1) serving as an antithrombotic interface with the blood, (2) limiting leukocyte invasion and other inflammatory processes, and (3) inhibiting intimal hyperplasia, particularly at the graft anastomoses. These biological processes, methods to characterize them, and recent research elucidating EOCs' performance in them will be further described individually.

Thromboprotection

Native ECs possess a variety of surface-bound and secreted molecules that influence the local hemostatic environment.[38] Thrombomodulin and the endothelial Protein C

receptor, expressed on the EC surface, work in conjunction to activate Protein C, which, in turn, inactivates the coagulation Factors Va (FVa) and VIIIa (FVIIIa) to limit coagulation. Prostacyclin and nitric oxide (NO) secreted by ECs locally inhibit platelet adhesion and activation. EC secretion of tissue plasminogen activator (tPA) catalyzes the production of plasmin, which breaks down fibrin clots. Conversely, up-regulation of tissue factor expression on the surface of ECs after vascular injury facilitates the activation of FX to FXa, which then converts the zymogen prothrombin to the active enzyme thrombin to generate a fibrin thrombus. ECs also facilitate coagulation by releasing von Willebrand factor and P-selectin from Weibel–Palade bodies to initiate platelet aggregation and activation. In addition, ECs basally deposit von Willebrand factor to promote platelet aggregation in the event that ECs are detached from their underlying matrix. Platelet aggregation and activation initiates the coagulation cascade and the production of a fibrin thrombus. Developing a luminal surface that can actively regulate and respond to thrombotic pathways is a significant motivation for endothelializing vascular grafts.

The thrombogenicity of EOCs on vascular grafts can be characterized by a number of systems of varying complexity that are reviewed by McGuigan and Sefton in an in-depth discussion of evaluating biomaterial thrombogenicity.[39] Purified systems that measure the activity of a limited number of players in coagulation (e.g., FX and Protein C activation, NO production) provide quick and straightforward information on cell thrombogenicity and are highly useful for studying individual signaling pathways. However, these results may not translate to *in vivo* performance due to the highly interconnected thrombotic regulatory mechanisms. Methods that study graft performance using whole blood, particularly at physiological flow rates, provide the best indication to *in vivo* thrombogenicity, though these systems can confound the roles of individual pathways.[40,41]

EOCs express the fundamental proteins used by ECs to regulate thrombosis, including tissue factor, thrombomodulin, endothelial Protein C receptor, tPA, and von Willebrand factor.[26,27,35,36,42–48] Regulation of thrombus formation by EOCs has been suggested *in vitro* by studies demonstrating reduced platelet adhesion on EOC-lined materials and the generation of the anticoagulant activated Protein C.[45,46,48] In addition, in response to pro-thrombotic stimuli, EOCs increase their expression of tissue factor, decrease plasma clotting time, and increase tPA expression.[36]

A number of strategies have been used in an attempt to improve EOCs' thromboprotective function before graft implantation. Similar to ECs, the most common method to reduce EOCs' thrombogenicity is to precondition the cells with fluid shear stress (described later). Instead of promoting the anti-thrombotic behavior of cells through external stimulation, EOCs can also be genetically modified for improved performance.[43,47,49,50] Thrombomodulin-overexpressing EOCs have demonstrated increased Protein C activation, decreased platelet adhesion under static conditions, and prolonged clotting times compared with native EOCs.[47] In addition, EOCs overexpressing prostacyclin had similar proliferation, decreased apoptosis after hydrogen peroxide treatment, and increased angiogenic properties compared with native EOCs.[51] Together, these studies have shown that EOCs have the potential to regulate thrombosis by similar mechanisms as ECs and can provide a thromboresistant lining on graft materials.

Inflammatory response

Inflammation is characterized by an increase in vascular endothelial permeability, fluid, and leukocyte transit across the endothelium into the tissue, leukocyte degradation of foreign objects, and cytokine secretion.[52] While acute inflammation is beneficial to the healing process, chronic inflammation can result in detrimental remodeling by processes such as fibrosis and calcification, which alter the local mechanical properties and reduce graft functionality.[53] When activated by injury or inflammatory cytokines, ECs facilitate inflammation by (1) releasing chemotactic factors to attract circulating leukocytes, (2) expressing selectins to allow leukocyte rolling adhesion along the vessel wall, (3) loosening intercellular junctions with adjacent ECs, and (4) expressing integrin ligands to permit leukocyte diapedesis into the tissue. Although this review will highlight the role of only a few endothelial inflammatory markers, more thorough reviews of leukocyte adhesion,[54] transmigration,[55] and atherogenesis[56] have been previously published. Circulating leukocytes can be transiently bound by E- and P-selectins on cytokine-stimulated ECs to facilitate rolling adhesion along the vessel wall. Slowing the leukocytes permits the formation of stronger intercellular bonds to the endothelium via ICAM-1 (intercellular adhesion molecule 1; CD54) and VCAM-1 (vascular cell adhesion molecule 1; CD106). Leukocyte diapedesis across the endothelium is facilitated by PECAM (CD31) and ICAM-1.[57]

The typical *in vitro* characterization of ECs' response to inflammatory cytokines is the measurement of surface expression of ICAM-1, VCAM-1, or E- and P-selectin. As with native ECs, EOCs significantly up-regulate these adhesion proteins compared with their basal state in response to inflammatory cytokines or aberrant flow conditions. The inflammatory response of EOCs can be induced by treating the cells with the cytokine tumor necrosis factor-α (TNF-α), which results in an increase in both ICAM-1 expression[58] and prothrombotic tissue factor expression.[59] In addition, oscillatory fluid shear has a pro-inflammatory effect on EOCs, resulting in increased monocyte adhesion.[60] The processes of inflammation and thrombosis are highly interdependent; in both ECs and EOCs, there is an increase in thrombogenicity after inflammatory cytokine stimulation. TNF-α stimulation of EOCs results in tissue factor protein up-regulation similar to ECs and shortens the time to tissue factor/FVII-dependent thrombin generation and plasma clotting.[36,59] In summary, EOCs demonstrate an inflammatory response *in vitro* to TNF-α and oscillatory shear stress similar to ECs.

Inhibiting intimal hyperplasia

In vivo, ECs limit vascular smooth muscle cell migration and proliferation predominantly through the release of NO generated by endothelial nitric oxide synthase (eNOS). Commonly, eNOS gene or protein expression levels are used as an indicator of NO production. Alternatively, NO can be measured in an *in vitro* assay quantifying NO metabolites,[58,61] a fluorescent NO indicator probe,[17] or a function-based NO assay that measures smooth muscle cell relaxation after controlled activation of eNOS.[27] The majority of groups

have shown EOCs to have a lower expression of eNOS than mature ECs,[17,26,35,46,58] though some groups have found no difference between cell types.[36] However, eNOS expression does not directly correlate with NO production, as evidenced by studies demonstrating that EOCs have lower eNOS expression but similar NO production as aortic ECs when stimulated by calcium[17] or fluid flow.[58]

Intimal hyperplasia after vascular injury can also be reduced by activated Protein C.[62,63] Activated Protein C acts to inhibit inflammation-induced EC apoptosis and permeability, thereby maintaining functional EC regulation of underlying smooth muscle cells.[64] Similar to ECs, EOCs can activate Protein C.[46,65] To enhance Protein C activation, EOCs were engineered to overexpress thrombomodulin via adenoviral transfection. Although *in vitro* Protein C activation was increased,[47] thrombomodulin overexpression by EOCs seeded onto an ePTFE graft did not significantly reduce intimal hyperplasia or thrombus formation compared with non-transfected EOCs in a rat model.[66] The lack of improvement may be due to the reduced activity of human thrombomodulin with rat Protein C, the limited duration of thrombomodulin overexpression, or suppression of transcription factors downstream of thrombomodulin due to disturbed blood flow at the proximal anastomosis that dampened thrombomodulin-mediated effects.

The capacity of EOCs to limit intimal hyperplasia has been demonstrated in multiple animal models. EOCs transplanted in a balloon-injured rabbit carotid artery reduced injury-induced intimal hyperplasia; this reduction was enhanced by transplanting EOCs that overexpressed eNOS.[50] In a canine interpositional carotid implant model, EOC-seeded decellularized arteries reduced intimal thickness 3 months post-implantation throughout the length of the graft compared with grafts without EOCs.[67] Grafts wrapped with EOC-seeded scaffolds showed a significantly reduced intima-to-media ratio at 14 days post-implant, suggesting the EOCs released paracrine factors, such as NO, to inhibit intimal proliferation.[68] Together, these studies show the potential for EOC-seeded vascular devices to limit intimal hyperplasia via NO release and Protein C activation.

Response to fluid shear stress

For vascular graft applications, EOCs need to remain adherent to the graft under physiologic shear stresses, averaging 15–20 dyn/cm^2 in arteries to 1–6 dyn/cm^2 in veins.[69] The EC response to fluid shear stress is well established. As reviewed in,[69,70] ECs subjected to arterial levels of fluid shear stress align in the direction of flow, increase production of NO, decrease expression of inflammatory adhesion molecules, and increase thrombomodulin expression.

Since fluid shear stress has a significant effect on EC phenotype, characterizing EOCs' response to fluid shear stress has been an area of active study. When subjected to fluid shear stresses of 10–15 dyn/cm^2, EOCs elongate in the direction of flow.[26,37] EC and EOC alignment, independent of fluid shear stress, alters immunogenicity and matrix protein deposition.[71–73] Both EOCs and aortic ECs exhibited decreased VCAM-1 mRNA expression when subjected to a fluid shear of 25 dyn/cm^2.[74] There are conflicting results on the relative gene expression of von Willebrand factor after fluid shear, as one group found no difference in von Willebrand factor gene expression at fluid shears of 5 or 25 dyn/cm^2,[74] but another group found that 15 dyn/cm^2 increased von Willebrand factor gene expression.[46] Compared with ECs, EOCs have an increased production of thrombomodulin and corresponding Protein C activation, but do not increase tissue factor expression after steady fluid shear stress, indicating a superior thromboprotective phenotype in arterial flow conditions.[46,60] Steady shear causes EOCs to increase eNOS gene expression[37,42,60] and NO production, suggesting an improved capacity to limit intimal hyperplasia.[37,58,61,75] Models that incorporate both fluid shear stress and inflammatory cytokines enable a better characterization of how these factors interact to influence EOC behavior. After 24 h of 6 dyn/cm^2 shear stress preconditioning, TNF-α-induced tissue factor activity of EOCs was reduced compared with non-preconditioned EOCs.[76] Fluid shear stress preconditioning shows promise for improving vascular graft performance. However, the additional time and equipment necessary for such treatment may not be practical for some applications.

In vivo, vascular branch points and bifurcations can result in disturbed blood flow conditions.[70] ECs exposed to disturbed flow exhibit a dysfunctional phenotype with increased expression of tissue factor, von Willebrand factor, ICAM-1, VCAM-1, E-selectin, and reduced NO secretion.[69] This altered expression profile results in local susceptibility to thrombosis and atherosclerotic plaques.[69,77] Bidirectional fluid flow with peak shear stresses of ± 0.3 dyn/cm^2 was used to simulate regions of disturbed flow and induced a similar up-regulation of tissue factor gene expression in EOCs as in ECs, indicating a similar prothrombotic response to oscillatory shear stresses.[78] In addition, oscillatory shear stress (0 ± 10 dyn/cm^2, 1 Hz) significantly increased monocyte adhesion on EOCs relative to EOCs under a steady shear.[60] Since vascular regions with disturbed and oscillatory blood flow are prone to EC dysfunction, there is motivation to develop a better understanding of EOC phenotype under similar disturbed flow conditions. A summary of the existing research characterizing EOCs' response to fluid shear stress is presented in Table 2.

Vascular Tissue Engineering Applications Using EOCs

Vascular tissue engineering strategies have typically sought to use EOCs either to cover current synthetic graft materials or to integrate EOCs into a natural or biodegradable synthetic scaffold, potentially with other cell types, to mimic native vessel structure and protein composition. To ensure complete coverage of the tubular graft lumen, seeding is generally performed using multiple inoculations with manual or automated rotation, or by perfusing a cell suspension through the graft wall.[35,36,42,66,79–81]

Synthetic scaffolding

Current clinical vascular grafts are constructed of synthetic materials, most commonly ePTFE and Dacron. Since both of these are hydrophobic materials lacking natural adhesion peptides, surface modification of these materials is required before lining the lumen with EOCs. In addition, a period of cell culture after seeding is required to ensure EOC

Table 2. A Summary of Studies Characterizing How Fluid Shear Stress Affects Endothelial Outgrowth Cell Function and Response to Tumor Necrosis Factor α Treatment

Flow conditions	*Flow system*	*Substrate*	*Shear stress*	*TNF-α treatment*	*Results (described as flow treated EOCs compared to static unless stated otherwise)*	*Reference*
Steady flow	Parallel plate	Fibronectin-coated Teflon A films spun-cast on glass	15 dyn/cm^2 for 24 or 28 h	N/A	Increased eNOS, TM, ICAM-1 gene expression No significant difference in VCAM-1 or E-selectin gene expression	(58)
		Fibrin-based scaffold	15 dyn/cm^2 for 24 h	10 U/mL for 24 h	Increased NO production Decreased platelet adhesion Attenuated TNF-α-induced upregulation of VCAM-1 and ICAM-1	(48)
		Gelatin-coated plastic	5 or 25 dyn/cm^2 for 5 h	N/A	Decrease in VCAM-1 gene expression at 25 dyn/cm^2 No difference in vWF gene expression with either shear stress	(74)
		Collagen I-coated glass	15 dyn/cm^2 for 24 h	N/A	Increased TM, eNOS, TFPI gene expression Increased TM protein Increased APC activity No differences in TF gene or protein	(46)
	Orbital rotator	Collagen-coated TCPS	6.0 dyn/cm^2 for 6 or 24 h	110 U/mL for 2 h	Decreased TF and eNOS gene expression at 6 and 24 h Increased eNOS gene expression at 6 and 24 h following TNF-α treatment Attenuated TNF-α induced TF activity at 6 and 24 h	(76)
Steady and oscillatory flow	Cone and plate	Collagen I-coated TCPS	24 h treatment with steady shear of 10 dyn/cm^2, or oscillatory shear of 0 ± 10 dyn/cm^2	N/A	Oscillatory shear reduced eNOS and P-selectin genes vs. steady shear Steady shear decreased monocyte adhesion vs. oscillatory Steady shear increased TM, ePCR and CD39 genes vs. oscillatory shear No significant difference in the gene or protein expression of E-selectin or VCAM-1 in oscillatory shear vs. steady shear	(60)
Steady, pulsatile, and oscillatory flow	Perfusion bioreactor	Fibronectin-coated Sylgard tubes	24 h treatment with either low unidirectional shear of 0.3 ± 0.1, pulsatile shear of 6 ± 3 dyn/cm^2; or oscillatory shear of 0.3 ± 3 dyn/cm^2	N/A	Unidirectional increased TF gene expression Pulsatile decreased tPA gene expression Bidirectional increased TF, decreased tPA gene expression No condition had an effect on VCAM-1 or E-selectin gene expression	(78)

TNF-α, tumor necrosis factor α; N/A, not available; eNOS, endothelial nitric oxide synthase; TM, thrombomodulin; ICAM-1, intercellular adhesion molecule 1; VCAM-1, vascular cell adhesion molecule 1; vWF, von Willebrand factor; TFPI, tissue factor pathway inhibitor; APC, activated protein; TF, tissue factor; TCPS, tissue culture polystyrene; tPA, tissue plasminogen activator.

adhesion, spreading, and complete coverage of the luminal surface. This culture period also allows for *in vitro* pre-conditioning of the graft, for example, by fluid shear stress or growth factors. Thus, when developing EOC-seeded synthetic grafts, there are, in general, three variables: (1) synthetic scaffold material, (2) biofunctional coating, and (3) culture time and conditioning.

Since the current standard material for large-diameter vascular grafts is ePTFE, a few groups have sought to modify this material for improved cell adhesion and use it as a scaffold for EOCs. ePTFE modified with the biodegradable, polyester elastomer poly(1,8-octanediol-*co*-citrate) and coated with fibronectin facilitated the adhesion of EOCs through 24 h of 10 dyn/cm^2 shear stress.[36] Studies of EOCs seeded onto ePTFE coated with either collagen I or fibronectin indicated that EOCs had a more robust adhesion and proliferation potential on either of these proteins than on α-elastin or collagen IV.[65] With either collagen I or fibronectin coatings, EOCs formed a confluent monolayer and were able to remain adherent at supraphysiological shear stresses.[65]

The mechanical properties, particularly the elasticity, of polyurethanes have stimulated investigation into their utility as vascular graft materials. The harmful degradation products of polyurethanes have eliminated their use in long-term implants. However, they are still used as arteriovenous grafts to permit vascular access for dialysis patients due to polyurethane's ability to reseal after puncture. Segmented polyurethane coated with photo-cured gelatin was seeded with EOCs and subjected to a 12-h shear stress of 30 dyn/cm^2.[35] The EOCs remained adhered to the graft and elongated in the direction of flow. Segmented polyurethane films coated with collagen I, resulting in grafts with internal diameters of ~4.5 mm and 6 cm in length, were also seeded with autologous canine EOCs and evaluated as an interpositional carotid artery graft.[79] After 3 months, all six grafts were patent and showed a smooth luminal surface lacking thrombi. Smooth muscle cells were identified in the graft media, though it is unclear whether the smooth muscle cells had migrated into the graft from adjacent tissue, or whether the EOCs had transdifferentiated into a smooth muscle cell phenotype.

Several groups use electrospinning to create polymeric micro- to nano-sized fibers in a matrix that mimics the native extracellular matrix architecture. In comparison to thin films of the same composition, EOCs grown on an electrospun fibrous scaffold composed of hexylmethacrylate, methylmethacrylate, and methacrylic acid had increased proliferation.[44] Whether proliferation is enhanced by aligned fibers rather than by random fiber orientation is controversial, and there may be a necessary degree of fiber alignment that is needed to increase proliferation compared with random fibers.[44,82] These studies that highlight variable EOC behavior on different substrates demonstrate the potential to modulate EOC function through scaffold design.

Synthetic scaffolds possess many advantages for vascular tissue engineering, including easy transportation and storage, inter-graft consistency, and well-defined physical and chemical properties. Seeding the luminal surface of synthetic grafts with ECs has been shown to improve long-term synthetic graft patency for a number of materials.[8–12] However, compared with the body of research studying the effect of EC behavior on synthetic scaffolds, very little work has investigated EOCs' function with regard to these materials. Adhesion to synthetic materials under a fluid shear is often used as the major metric of synthetic scaffold performance; however, a more thorough characterization of the scaffolds' influence on EOCs' biological regulatory function is needed.

Naturally derived and biodegradable scaffolding

Although synthetic materials possess consistent physical and chemical properties and are often less expensive, they lack the instructional biological cues of the native extracellular matrix. Examples of these cues include the fibrous topography of the typical vascular extracellular matrix which aids in cell alignment and adhesion, as well as biochemical signals, such as growth factors, that aid in healing and tissue integration.[83] Naturally derived materials, or biodegradable materials designed to be replaced by cell-deposited matrix proteins, have, therefore, been utilized in an attempt to construct vascular grafts that possess these native signals.

A current technique for generating natural scaffolding for vascular tissue engineering is decellularizing preexisting blood vessels and repopulating the vessels with cells cultured *in vitro*. Decellularized blood vessels provide a scaffold with the complex structure of the native tissue and are highly supportive of EC adhesion.[84–87] Ovine EOCs seeded onto decellularized porcine iliac arteries were pre-conditioned with a fluid shear stress of 25 dyn/cm^2 and produced NO upon stimulation with a thromboxane analog.[27] When implanted into sheep, the EOC-seeded grafts remained patent for approximately 130 days. After explant at 130 days, smooth muscle cells were present in the media of the grafts and conferred vasomotor activity in response to the neurotransmitters norepinephrine, serotonin, and acetylcholine. In another study, tissue-engineered grafts were constructed by selectively seeding bone marrow-derived EOCs onto the lumen and vascular smooth muscle cells onto the exterior of decellularized vessels.[81] These constructs were cultured in a bioreactor with pulsatile flow for 7 days, then implanted as interpositional carotid grafts in canines. At 14 weeks, 9 out of the 10 seeded grafts were patent. No characterization of intimal hyperplasia was performed, though the authors note the number of smooth muscle cells in the graft matrix increased during the implant period. Decellularized arteries have also been modified by covalently binding heparin to the surface before EOC seeding.[67] These grafts were cultured for 7 days under a fluid shear stress of 30 dyn/cm^2 before implantation. In a canine interpositional carotid implant model, the heparin-modified grafts seeded with EOCs demonstrated a higher 3-month patency and reduced intimal thickness throughout the length of the graft compared with unseeded heparin-modified grafts.

Rather than decellularizing an explanted vessel, smooth muscle cells or fibroblasts cultured on a biodegradable scaffold can be used to generate *de novo* a vascular matrix scaffold. In this case, the degradable scaffold serves as a temporary support or pattern to facilitate extracellular matrix protein production by the smooth muscle cells. In one example, smooth muscle cells were cultured on a biodegradable polyglycolic acid rod for 10 weeks in a bioreactor, allowing the smooth muscle cells to generate a tubular extracellular matrix.[42] After decellularizing the scaffold, the mechanical properties were comparable to a human

saphenous vein. The decellularized scaffolds were then seeded with autologous EOCs, preconditioned with 15 dyn/cm^2 shear stress for 24 h, and implanted in porcine carotid arteries as end-to-side grafts to mimic clinical bypass grafts. All three EOC-seeded grafts remained patent at 30 days and trended toward increased luminal area and decreased intimal area compared with autologous venous grafts, some of which occluded by intimal thickening and thrombosis. In another study, smooth muscle cells were seeded on porous, biodegradable poly(glycerol sebacate) scaffolding and cultured for 1 week in a pulsatile flow bioreactor to stimulate smooth muscle cell proliferation and the production of elastin and collagen.[88] EOCs were then seeded onto the graft where they formed a confluent monolayer on the luminal surface. Coculture of EOCs and smooth muscle cells in the grafts increased the ultimate tensile stress and the strain at failure compared with EOC-only seeded grafts; these results were attributed to the matrix proteins deposited by the smooth muscle cells.

A vascular graft composed entirely of autologous tissue is advantageous, as it eliminates the potential for immune rejection. However, such autologous constructs traditionally require blood vessel harvesting to generate a scaffold and isolate vascular cells. In contrast, Aper *et al.*[89] were able to construct a vascular graft using cells and proteins contained within 100 mL of peripheral porcine blood. EOCs and fibrinogen were combined and polymerized by the addition of thrombin to generate a cell-seeded, tubular fibrin graft. The graft was subsequently cultured in a perfusion bioreactor for 5 days. Although the mechanical properties remained insufficient for grafting (burst strength of only 90 mmHg), the incorporation of vascular smooth muscle cells in future designs may improve these shortcomings.

From a biological perspective, natural materials have many potential benefits, including preserving native extracellular matrix architecture, presentation of physical and biochemical cues to support cell seeding and post-implantation integration, and reduced concern of toxic degradation byproducts. However, from a translational perspective, natural materials are often much more expensive and challenging to source (typically including extensive processing to eliminate potential allergens), and have inferior mechanical properties that make them more difficult to surgically manipulate. New methods that shorten *in vitro* graft preparation time, improve mechanical robustness and the ability to withstand surgical handling and suturing would improve the clinical utility of natural scaffolds for EOC-seeded grafts.

Future Research Topics

Research utilizing EOCs in vascular tissue engineering has rapidly grown as investigators employ EOCs to perform endothelial functions. Still, much work needs to be done to translate this research to clinical devices. In concert with the applied research using EOCs in devices, basic research that identifies an EPC-specific marker from which EOCs are derived would enable more efficient EOC quantification and isolation from patients. The dogma that the progenitors of EOCs originate in bone marrow has recently been challenged,[34] and presents another topic for investigation. Although statins[90] and erythropoietin[91] increase the quantity of circulating EPCs, determining the specific factors that induce EPCs in culture to differentiate into EOCs would further enhance protocols for efficient EOC isolation and expansion.

Many vascular graft materials are being developed with the sole performance criterion of supporting the proliferation and adhesion of EOCs under flow. When developing EOC-seeded vascular grafts, a more thorough characterization of how the underlying material impacts the function of EOCs with regard to thrombosis, intimal hyperplasia, and inflammation should be performed. Novel modifications to existing scaffold materials may improve EOCs' functionality in these processes.

In vivo, ECs reside in a complex environment of mechanical cues (fluid shear stress, vascular tone) and biochemical cues (cytokines, growth factors) that ECs integrate to regulate the local vascular environment. Therefore, characterizing how the interplay between biomechanical and biochemical cues cooperatively affect EOC regulation of thrombosis, intimal hyperplasia, and inflammation may more fully define *in vivo* EOC behavior. A mechanistic understanding of the signaling pathways activated by these complex environments may also identify future targets for preconditioning EOCs for improved function. Genetically engineering EOCs to have improved thromboprotection or pro-healing qualities may further improve graft function. Although the increased culture time detracts from the clinical applicability of genetically engineering EOCs, this method would be beneficial for patients with genetic vascular disorders or poor intrinsic EOC function.

Conclusion

EOCs derived from circulating EPCs are an attractive source for engineering a functional, autologous endothelium in vascular grafts. Work characterizing EOCs' role in the processes of thrombosis, intimal hyperplasia, and inflammation has demonstrated EOCs' capacity to function as an autologous endothelium on vascular grafts. Current tissue-engineered constructs that utilize EOCs have exhibited significant improvements in patency compared with non-seeded devices. Basic scientific studies that determine how biomechanical and biochemical cues interact to affect EOC behavior may identify methods to precondition or engineer EOCs for specific performance enhancements. With continued research, EOCs show great potential to advance the field of vascular tissue engineering and improve the long-term function of vascular grafts.

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