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Endothelial cell delivery for cardiovascular therapy

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Abstract

Obstructive atherosclerotic vascular disease stands as one of the greatest public health threats in the world. While a number of therapies have been developed to combat vascular disease, endothelial cell delivery has emerged as a distinct therapeutic modality. In this article, we will review the anatomy of the normal blood vessel and the biology of the intact endothelium, focusing upon its centrality in vascular biology and control over the components of the vascular response to injury so as to understand better the motivation for a cell-based form of therapy. Our discussion of cell delivery for cardiovascular therapy will be divided into surgical and interventional approaches. We will briefly recount the development of artificial grafts for surgical vascular bypass before turning our attention towards endothelial cell seeded vascular grafts, in which endothelial cells effectively provide local delivery of endogenous endothelial secretory products to maintain prosthetic integrity after surgical implantation. New techniques in tissue and genetic engineering of vascular grafts and whole blood vessels will be presented. Methods for percutaneous interventions will be examined as well. We will evaluate results of endoluminal endothelial cell seeding for treatment of restenosis and gene therapy approaches to enhance endogenous re-endothelialization. Finally, we will examine some innovations in endothelial cell delivery that may lead to the development of endothelial cell implants as a novel therapy for controlling proliferative vascular arteriopathy. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Blood vessel; Drug delivery; Endothelial cell; Tissue engineering; Vascular injury

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1. Introduction

Obstructive atherosclerotic vascular disease remains one of the greatest public health threats in the world, accounting for an estimated 12.5 million deaths per year from cardiovascular and cerebrovascular disease annually [1]. Countless more suffer loss of limb from peripheral vascular disease. Risk factor modification, novel pharmacotherapies, and innovative technological interventions have markedly reduced the incidence and improved the outcome of atherosclerotic vascular disease [2]. Yet, there is an increasing need for mechanical revascularization of atherosclerotic arteries, such that surgical vascular bypass grafting and percutaneous transluminal coronary angioplasty (PTCA) and endovascular stenting rank amongst the most common procedures performed in the US annually [2]. In 1996, coronary artery bypass grafting (CABG) was performed nearly 600 000 times, and nearly 666 000 patients received PTCA and 177 000 received stenting in the USA alone [3]. While these interventions have enjoyed great clinical success, only 40% of venous bypass grafts remain patent 10 years after surgery [4], PTCA exhibits a 30–40% restenosis rate [5], and stents cannot be placed in every patient or every artery with atherosclerotic disease.

The tissue, cellular, and molecular events that govern the vascular response to injury accompanying these mechanical interventions are becoming increasingly clear [6]. Exogenous analogues of endogenous

vasoregulatory compounds are being characterized, isolated, and cloned, yet no pharmaceutical approach yet has successfully limited the proliferative complications of vascular interventions [7]. In most cases, disappointing clinical results have followed promising tissue culture and pre-clinical animal experimentation [7]. In major part this reflects the complexity of the human disease. The number of cell types involved, the spatial gradients in reaction across involved arteries and adjacent tissues, and the broad period of time over which these events occur make it unlikely that administration of an isolated agent at one point in time will control all vascular reparative events [8]. Thus, tissue and cell-based therapies are increasingly viewed as the logical alternative [8]. The blood vessel does an excellent job of healing itself, and it is hoped that replacing the cellular components of the blood vessel will restore this reparative potential even when individual vascular cell derived agents have failed to generate an effect [8,9].

In this article, we will review the anatomy of the normal blood vessel and the biology of the intact endothelium, focusing upon its centrality in vascular biology and control over the components of the vascular response to injury so as to understand better the motivation for a cell-based form of therapy. Our discussion of cell delivery for cardiovascular therapy will be divided into surgical and interventional approaches. We will briefly recount the development of artificial vascular grafts for surgical bypass before

turning our attention towards endothelial cell seeded vascular grafts, in which endothelial cells effectively provide local delivery of endogenous endothelial secretory products to maintain prosthetic integrity after surgical implantation. New techniques in tissue engineering of vascular grafts and whole blood vessels will be presented. Methods for percutaneous interventions will be examined as well. We will evaluate results of endothelial cell seeding for treatment of restenosis and gene therapy approaches to enhance endogenous re-endothelialization. Finally, we will assess some innovations in endothelial cell delivery that may lead to the development of endothelial cell implants as a novel therapy for controlling proliferative vascular arteriopathy.

2. The normal anatomy of the blood vessel

The normal blood vessel is a complex tubular structure comprised of three concentric tubes or tunics [10]. The *tunica intima*, most intimately positioned at the lumen that supports blood flow, is lined by a monolayer of endothelial cells and is supported by a basement membrane below which resides a sparse layer of vascular smooth muscle cells [10]. The endothelial cells provide a mechanical barrier to the solutes and solvents in plasma, sense alterations in blood flow, plasma constituents and noxious elements, and secrete a variety of powerful chemical mediators that regulate blood cell trafficking, vasomotor tone, growth and vascular remodeling [11]. The smooth muscle cells principally serve as responsive elements that set vascular tone, though they can assume many of the other vasoregulatory biochemical processes in a far less efficient manner than endothelial cells [12]. The bulk of the smooth muscle cells reside in the middle tube, the *tunica media*, in packets or lamellar units bound by elastic bands or lamina. The contraction and relaxation of these units allow the artery to constrict or dilate, thereby regulating blood flow [10]. The outer tube, the *tunica adventitia*, is a loose fibrous network of fibroblasts through which course the vessels that nourish the blood vessel wall, the *vasa vasorum*, and the nerves that supply neural control, the *vasa nervosum* [10].

3. Endothelial cells: controlling the vascular response to injury

The artery is an intensely sensitive organ. The vascular endothelium, covering nearly 700 m² in the average person, lines all of the blood vessels in the body and detects minute changes in the local environment [11]. In the usual quiescent state, endothelial cell turnover rates are on the order of months to years, but these cells are easily damaged by immunologic, oxidant, and mechanical stressors [11]. The endothelial injury that is imposed by mechanical interventions is not subtle [6]. One of the very first events that occurs with vascular manipulation is loss of the integrity of the endothelium as an intact monolayer resulting in significant endothelial denudation and drop-out of endothelial cells in local areas [13]. This event removes the biochemical regulation of many vascular responses and provides the signals for the initiation and propagation of atherosclerosis and accelerated arteriopathy. Over a century ago, Virchow recognized endothelial dysfunction as one of the triad of essential elements needed to alter vascular homeostasis and create thrombotic occlusions [14]. Since then, the vascular response to injury has been well described and consists of temporal phases of thrombosis, inflammation, cellular proliferation, and vascular remodeling [2]. As an example, the vascular response to stent-induced injury is depicted schematically in Fig. 1 [15,16]. It is now evident that each phase of the vascular response to injury is influenced if not controlled by the endothelium. In the sections that immediately follow, we briefly review some of the data that support the centrality of the intact endothelial cell in controlling each individual phase of vascular response to injury to further make the case that restoration of the endothelium may be the most logical approach to treating obstructive vascular disease.

3.1. Thrombosis

Platelet adherence and thrombus formation is one of the initial events following exposure of de-endothelialized vascular structures to blood flow [6,17,18]. In the case of the synthetic vascular graft material, the bare graft is highly thrombogenic with serum protein adherence and platelet deposition

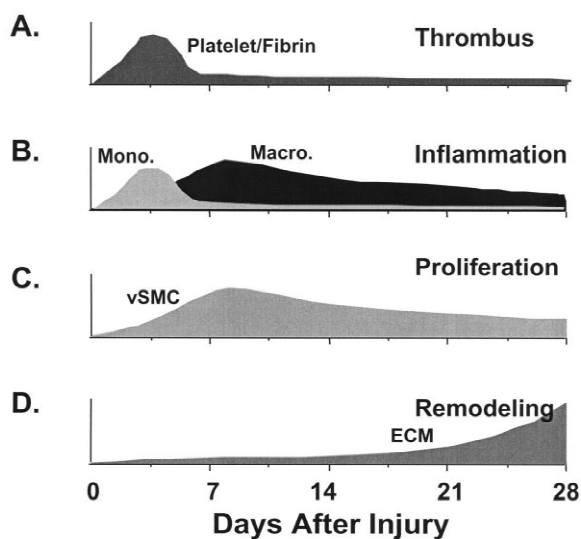


Fig. 1. Schematic representation of the four phases of the vascular response to stent-induced injury. (a) Early after stent implantation, platelet and fibrin rich thrombus accumulates at sites of deep stent strut injury. Thrombus burden peaks 3–4 days after stent deployment and causes the majority of early luminal loss. Within 10 days of stent placement, the occurrence of stent thrombosis and thrombus burden have decreased considerably. Coincident with early thrombus deposition, inflammatory cells (b), in particular surface adherent monocytes (Mono.), are recruited to the arterial lumen. Between 3 and 7 days after stenting, these cells migrate into the neointima as tissue infiltrating macrophages (Macro.). Large numbers of these cells persist within the neointima weeks after stent deployment. Proliferation of vascular smooth muscle cells (vSMC) and monocyte/macrophages within the neointima (c) peaks 7 days after stent implantation, and continues above baseline levels for weeks thereafter. In the final phase of vascular repair after stent-induced injury (d), extracellular matrix (ECM) deposition in the adventitia and throughout the *tunica media* and neointima leads to arterial remodeling. This causes the artery to be compressed upon the struts of the stent from without.

preceding frank clot formation [19–21]. In the case of percutaneous interventions, reviews of the data suggests that the interventions themselves result in significant endothelial denudation, and exposure of bare sub-endothelial structures such as basement membrane causes high levels of protein adherence, platelet deposition and activation, and active thrombus formation [5,15,16]. The intact endothelial cell acts to prevent thrombosis via a number of mechanisms. It resists deposition of proteins and pro-

coagulants by supplying a negatively-charged, physical barrier over the underlying structures of the blood vessel [11,22]. Moreover, the intact endothelium secretes a number of compounds including prostacyclin [23] and nitric oxide [11], which inhibit platelet adherence and degranulation. Endothelial cells also generate tissue plasminogen activator (t-PA) [11,22], which inhibits progression of thrombus formation and promotes fibrinolysis. Thus, these functions amongst others, demonstrate that an intact endothelial cell monolayer potently opposes thrombosis.

3.2. Inflammation

The role of inflammation in vascular graft failure is now well appreciated. A number of studies in animal models with implanted prosthetic vascular grafts have demonstrated that early and chronic adherence of leukocytes corresponds to late graft failure [24–28]. Similar data in various modes of percutaneous intervention exist demonstrating that monocyte and macrophage components drive the process of restenosis via heretofore poorly understood mechanisms resulting in smooth muscle cell proliferation and neointimal hyperplasia [29–31]. It has been hypothesized that increased levels of inflammatory cytokines from mechanical vessel injury induces increased binding of circulating inflammatory cells such as monocytes to remaining endothelial cells which may upregulate adhesion molecules such as L-selectin and others, thereby potentiating an inflammatory cell response resulting in vessel restenosis [18,32,33]. Data in animal models suggest that delivery of endothelial derived compounds inhibits inflammatory cell adherence to the vascular wall and late restenosis [29,34,35], suggesting that intact endothelial cells may also inhibit the inflammatory process induced by vascular injury. In addition, local microbial infection and associated inflammation of synthetic vascular grafts, a disastrous complication of any chronically indwelling foreign body, may be resisted by an intact endothelium [36]. Thus, it appears that the intact endothelium limits the inflammatory cell mediated response to vascular injury.

3.3. Smooth muscle cell proliferation

Late neointimal hyperplasia compromises patency of both synthetic vascular grafts and native vessels after percutaneous interventions. In bypass grafting, the anastomotic sites demonstrate the highest propensity for vascular smooth muscle cell infiltration and proliferation which contributes to late lumen diameter loss [37,38]. Similarly, in mechanically dilated arterial segments, vascular smooth muscle cell proliferation and migration apparently contributes significantly to late restenosis [6,16,39]. The endothelial cell inhibits vascular smooth muscle cell proliferation and migration via a number of paracrine mechanisms [40–42]. Current data suggest that endothelial derived compounds such as heparin [43–46] or nitric oxide [47–50] inhibit vascular smooth muscle cell proliferation in both in vitro assays and in vivo models of restenosis. Consequently, these data suggest that the intact endothelial monolayer inhibits vascular smooth muscle cell proliferation and migration.

3.4. Remodeling

The process of vascular remodeling after surgical vascular graft placement or percutaneous intervention remains incompletely described. Late vascular remodeling appears to be secondary to elaboration of extracellular matrix by vascular smooth muscle cells and the circumferential collagenous contraction of this matrix [51]. For example, the inhibition of extracellular matrix elaboration and enzymes known to promote degradation of extracellular matrix have been shown to decrease late restenosis [52]. And, animal studies show that vascular injury scores increase over time while stent diameter remains constant in experimentally stented arterial segments suggesting vessel contraction [6,17,53,54]. Recent studies in experimental restenosis models have shown that endothelial dysfunction correlates with higher levels of collagen deposition in restenotic vessels, suggesting that the endothelium may exert some level of control over the vascular remodeling process [55]. While further study is required, these preliminary data suggest that the intact endothelial cell may also inhibit or at least blunt late vascular

remodeling and resultant loss of vessel lumen diameter.

4. Endothelial cell delivery – surgical vascular bypass grafts

4.1. Artificial grafts – introduction

Voorhees et al., are likely to have developed the first artificial vascular graft when in 1952 they constructed vascular prostheses out of the fabric Vinyon N [56]. Since that time, scores of researchers have focused upon the production of an ideal synthetic vascular graft. Over the ensuing five decades, many of the complications which threaten long-term graft viability have been investigated, and it has become clear that many complications are related to the artificial graft's inability to combat one or more of the phases of the vascular response to injury. Thus, the concept of the ideal artificial vascular graft has emerged in which the graft would resist thrombosis, inflammation, neointimal proliferation, and late remodeling. It is believed that only such a graft would maintain long-term patency in vivo.

4.2. Synthetic artificial grafts

While many materials have been considered as the scaffolding for grafts, Dacron (polyethylene terephthalate, PET) and Teflon (expanded polytetrafluoroethylene, ePTFE) grafts have emerged as the primary synthetic materials [57]. The high flow rates of large diameter grafts provide long-term patency rates of 85–95% with the need for minimal adjunctive pharmacologic therapy [58]. Smaller diameter grafts (<5 mm), however, have met with early thrombotic complications and late myointimal hyperplasia often leading to total graft occlusion [59]. In fact, less than 50% of small diameter femoropopliteal grafts remain patent 5 years post-implantation [60].

Several material modifications have been employed to improve synthetic graft patency. Prostheses covered with protein coatings that include albumin [61–63], gelatin [64], and various types of collagen [65] have been demonstrated to decrease graft throm-

bogenicity and diminish the need for whole blood pre-clotting which is required for bare polymer grafts. Similarly, the immobilization of antithrombogenic molecules including heparin [66,67] and hirudin [68–70] to the graft's luminal surface have also decreased graft thrombogenicity. In addition, grafts impregnated with antibiotics such as vancomycin [71,72], cephalosporins [73], rifampin [74–79] or others [80,81] manifest a reduced risk of infection. Nonetheless, there are limited clinical data available to facilitate assessment of the clinical efficacy of any one of these bioadditives as a marked improvement over conventional synthetic vascular grafts.

4.3. Endothelial cell seeding of vascular grafts

In the face of the many limitations of synthetic vascular grafts, investigators have turned their attention towards seeding vascular grafts with endothelial cells as a means to improve long-term patency. By employing the endothelial cell's ability to inhibit the full range of the vascular response to injury, investigators hoped to develop the first true cellular delivery therapy for vascular disease. Herring et al. made the first report of such a seeded graft in 1978 [82]. Suspensions of isolated canine venous endothelial cells were used to pre-clot 6 mm Dacron grafts prior to infrarenal implantation in a canine model [82]. At 4 weeks post-implantation, these investigators noted 76% patency rates for seeded grafts vs. 22% for unseeded grafts [37,83]. Explanted grafts possessed an intact Factor VIII-positive staining endothelial lining supported by smooth muscle cells along with penetrating *vasa vasorum* [37,83]. In the few years after Herring's initial reports, work by other groups confirmed the possibility of seeding Dacron grafts with endothelial cells prior to implantation into animal circulation and the recovery of persistent endoluminal, endothelial cells upon these grafts after explantation [84–88]. Many investigators have since contributed to the evolution of this technology, and we will attempt to address some of the critical steps in the seeding process, evaluate some of the determinants of seeded graft performance, review the histopathology and functional assessment of seeded grafts, review the clinical experience with these and like devices, and assess the

use of genetically modified endothelial cells for graft seeding.

4.3.1. Seeding techniques

Initial attempts at seeding vascular grafts showed widely variable efficiency. Endothelial cells were mechanically or enzymatically harvested from autologous venous structures and mixed with whole blood or tissue culture media prior to incubation with the synthetic graft material [82,84,89]. Subsequently, other techniques for endothelial cell seeding have been reported and are discussed below. The primary determinants of seeding success appear to include the source of the endothelial cell, the duration of incubation prior to implantation or exposure to fluid flow, the precise seeding mode, and the substrates upon which the cells are seeded.

4.3.1.1. Source of endothelial cells

One must balance considerations of immune rejection, vascular bed idiosyncrasies and cell health in determining where to obtain cells for graft seeding. Though autografts are tolerated better than allografts and xenografts, one must remember that the hallmark of vascular disease is endothelial dysfunction. Patients with systemic disease that predisposes them to obstructive vascular disease likely have abnormal endothelial cells throughout and the use of these cells may be suboptimal to seed bypass segments. Moreover, the structure and biochemical environments of venous and arterial, microvascular and macrovascular beds, and vessels of different tissues are all unique. Placement of an endothelial cell from one bed in another may result in dysfunctional cell performance. Nevertheless, early experiments employed primary autologous venous endothelial cells and reported limited cell yields, as little as 3×10^4 endothelial cells per harvested vein segment [90], and low levels of cell seeding efficiency onto the synthetic grafts [90]. Alternative cell sources have included crude whole vein homogenates [91,92] and microvascular endothelial cells from adipose tissue [93,94]. The latter cells can be derived from omental fat in animals or humans, and have been isolated from clinical liposuction specimens indicating the possibility for intraoperative autologous harvesting and seeding [95]. Finally, xenograft cells, porcine endothelial cells implanted in a canine thoracoab-

dominal aortic bypass model, have also been shown to inhibit graft thrombosis similar to synthetic vascular grafts seeded with autologous canine endothelial cells. These experiments raised the possibility of using xenograft cells for endothelial cell seeding of vascular grafts [96]. Thus, a variety of cell sources have been identified for endothelial cell seeding of vascular grafts.

4.3.1.2. Duration of cell incubation and seeding mode

The potential use of autologous endothelial cells produces a unique set of problems. Vascular autografts must be created in time for surgery, and often there is little warning between contemplation and initiation of surgery. One must allow for adequate time for cell harvest, isolation, identification and selective growth, and then graft preparation, seeding, incubation, characterization and implantation [59]. Not surprisingly, the incubation time is critical to ultimate seeding efficiency. The data suggest that though shorter incubation times may suffice, longer times are better. For example, one study demonstrated that endothelial cells adhered to grafts with 20-min incubations, but far more so if the time was extended 4.5-fold [97]. Similar reports have confirmed that incubation times of > 60 min and up to 8 h result in higher endothelial cell retention rates after implantation and/or exposure to flow conditions [98]. Therefore, it appears that longer incubations for cell seeding result in enhanced cell retention upon the graft.

Although some would prefer harvesting and seeding of grafts and then placing the graft during the same procedure, this allows no time for error or optimal seeding. Other investigators advocate using completely endothelialized grafts for implantation employing a multi-staged procedure that separates cell harvest and graft implantation after appropriate incubation of the seeded grafts [99]. As in vitro tissue culture and organ culture systems have improved, complete graft endothelialization after in vitro incubation has become readily achievable. Several groups have demonstrated the feasibility of pre-incubating seeded vascular grafts to generate a confluent, endoluminal endothelial cell monolayer prior to graft implantation [100–106]. The time required to achieve a confluent endothelial lining

after in vitro graft culture ranges from 3 days to 2 weeks [100–106]. In both animals and humans, these grafts perform as well or better than single procedure harvest/seeding grafts [101,103–106]. While these pre-incubated grafts require multiple procedures for harvest of endothelial cells and subsequent graft placement, they may offer the added benefit of having a confluent endothelial lining in place prior to exposure to blood flow.

Along with duration of cell seeding prior to exposure to fluid flow, the method of cellular application has proven to be an important factor. Initially, investigators relied upon gravity to seed graft material resulting in eccentric deposition of endothelial cells along the dependent portion of the vascular graft [82]. Rotating the graft material about cell-rich seeding suspensions [107–109], application of an external vacuum [110], or use of surface electrocharging all seem to improve seeding efficiency [111,112], adhesion strength and circumferential uniformity. Such novel approaches to cell seeding have also enhanced cell retention.

4.3.1.3. Graft surface: pre-coating with proteins and growth factors

While cell source and seeding technique are important to ultimate seeding efficiency, early experiments indicated that the substrate upon which the cells were seeded might be the primary determinant of optimal graft endothelialization and subsequent success in vivo. Multiple alternative graft materials have been considered since Herring's initial reports of endothelial cell seeding on Dacron grafts, but considerations other than biocompatibility have come to the fore. Vascular grafts made of ePTFE are used as well as Dacron, but while the latter was easily seeded, only with alteration of porosity of early designs was endothelial cell seeding upon ePTFE possible [113,114]. Moreover, though different bare polymer substrates affect cell adherence, the most significant advances in vascular graft endothelial seeding have resulted from coating the synthetic compounds with biocompatible proteins. Herring's studies utilized pre-clotting of Dacron with blood, which presumably laid down a fibrin-rich substrate upon the Dacron polymer. Specific application of fibronectin protein greatly increased graft surface retention of endothelial cells, even after exposure to

fluid flow conditions [115–117]. Fibronectin not only enhanced cellular attachment, but also accelerated endothelial cell proliferation [116]. However, fibronectin coatings also mildly enhanced platelet deposition, although without generally compromising patency [116]. As a result, a host of other protein-based coatings have been studied as shown in Table 1, each reportedly enhancing endothelial cell adhesion. As increasing understanding of endothelial cell adhesion emerges, more precise, peptide-based protein coatings for vascular grafts are likely to evolve which will at once enhance endothelial cell adherence while also optimizing endothelial secretion of protective vasoactive compounds.

In addition to coating grafts with proteins intended to increase endothelial cell adhesion, several investigators have attempted to enhance endothelial cell proliferation and inhibit smooth muscle cell proliferation by impregnating grafts with endothelial cell growth factors. Greisler et al. showed that pre-coating ePTFE with a fibrin glue containing fibroblast growth factor-1 (FGF-1) prior to implantation into the infrarenal position of New Zealand White rabbits increased capillary ingrowth and endothelial proliferation [118,119]. Since FGF-1 also stimulates smooth muscle cell proliferation, additional studies by this group found that adding an optimal ratio of FGF-1 to heparin along with the fibrin glue limited smooth muscle cell infiltration and proliferation [120,121], and that such pre-treatment prior to endothelial cell seeding enhanced endothelial cell adherence under flow conditions [122]. Moreover, grafts treated with this combination of fibrin glue, FGF-1, and heparin prior to endothelial cell seeding demonstrated de-

creased platelet deposition along the length of the graft [123,124]. Studies in grafts coated with fibrin glue which releases vascular endothelial growth factor (vEGF) along with heparin have shown stimulation of endothelial cell proliferation and suppression of vascular smooth muscle cell proliferation in vitro [125]. Similarly, endothelial cells seeded upon albumin-heparin coated grafts with immobilized basic FGF (bFGF) have been shown to proliferate more rapidly [126]. Such carefully formulated endothelial growth factor laden grafts may accelerate re-endothelialization of seeded grafts while limiting other maladaptive cellular responses. The development of growth factor impregnated grafts remains a rapidly evolving area which holds promise for accelerating endothelialization of seeded grafts.

4.3.1.4. *Effects of flow and shear stress*

While large diameter synthetic vascular grafts have demonstrated high patency rates, smaller diameter synthetic grafts manifest higher early failure rates due to thrombosis. Several studies have examined the effect of endothelial cell seeding upon synthetic graft patency under low flow conditions. In such conditions, endothelial cell seeding decreased accumulation of thrombus in 4 mm Dacron grafts [127]. And, patency was 3-fold higher in endothelial-seeded 4 mm canine carotid arterial bypass grafts in which flow had been reduced to 30% of baseline [87]. Thus, endothelial cell seeding appears to decrease the likelihood of thrombosis under low flow conditions.

While investigators were aware that flow conditions would reduce the yield of endothelial cell

Table 1
Protein coatings of synthetic vascular grafts which increase endothelial cell adherence to synthetic vascular graft material

Protein coating	References
Albumin	[114,126,202]
Collagen (Type I, IV)	[114,117,130,143,203]
Endothelial cell specific monoclonal antibodies	[204]
Extracellular matrix	[205–207]
Fibrin glue	[102,118,119,208]
Fibronectin	[130,143,203,209,210]
Gelatin/Gelseal	[114,117,130,210–213]
Laminin	[143,209,211,214–216]
REDV peptide	[215]
RGD peptide	[217]

seeding, few quantitative assessments were made of the impact of flow upon ultimate endothelial cell density in seeded grafts until nearly 10 years after the initial report by Herring et al. The introduction of fluid flow and concomitant shear stress to a freshly seeded graft decreased endothelial cell retention by nearly one-third from static flow to high flow states when cells were seeded upon ePTFE grafts by a pre-clot technique [128]. Similar findings noted that endothelial cell retention improved when seeded grafts were not immediately exposed to pulsatile shear stress [129]. Additional work reported a predictable trend that when grafts were coated with substrates such as gelatin, upon which endothelial cells are known to adhere more avidly, seeded cells better resisted shear stress and remained attached to the graft material in the face of physiologic ranges of shear stress [130]. Similar findings have been noted by others who showed improved endothelial cell shear stress resistance when cells were seeded upon fibrin glue and fibronectin in addition to gelatin [117,131,132]. Thus, shear stress clearly affects endothelial cell seeding efficiency.

To assess further the role of shear stress upon endothelial cell retention and graft function, Ott and Ballermann examined grafts that were seeded with endothelial cells and then either cultured in the presence or absence of chronic low-level shear stress before a trial in a high shear *in vitro* circuit [133]. Shear stress pre-conditioned grafts had significantly greater retention of endothelium after subsequent exposure to high shear stress and markedly resisted clotting when compared to their un-preconditioned counterparts [133]. When pre-seeded grafts were subjected to chronic shear stress of 1 dyne/cm² for 3 days and then 25 dyne/cm² for 3 days, the grafts were noted to have higher retention of endothelial cells at 24 h post-implantation into rat aorta [134]. High shear stress pre-conditioned grafts also developed less neointimal thickening when compared to low-shear and static pre-conditioned grafts ranging from 41 to 60 to 158 μ m intimal thickness, respectively [134]. These data may correlate with *in vitro* data which describe increased cell differentiation and altered expression of growth factors such as PDGF and vasoactive compounds such as endothelin by endothelial cells exposed to shear stress, suggesting that pre-conditioning seeded grafts in flowing sys-

tems may enhance clinical graft performance [135]. In sum, the current data suggest that delaying the initial exposure of endothelial cell seeded grafts to shear stress but using shear stress pre-conditioning may augment the cellular retention and clinical performance of the seeded graft.

4.3.2. Seeded graft histopathology and functional assessment

Since Herring et al. described a confluent endothelial cell lining throughout the explanted endothelial cell seeded graft [37], additional studies have attempted to characterize the neointima that develops upon the seeded graft. Other seeding methods have produced less than complete endothelialization. For example, Graham et al., described subconfluent, 60–70% and 80% endothelialization at 2 and 4 weeks, respectively in their canine thoracoabdominal bypass graft model, in contradistinction to the only 10% terminal endothelialization of unseeded grafts [85]. The high levels of endothelialization found in seeded grafts placed in animal models have been difficult to duplicate when such grafts have been placed in humans, however [90]. Thus, explanted endothelial cell seeded grafts all seem to demonstrate varying degrees of persistent endothelialization.

In addition to looking for a persistent endothelium upon explanted seeded grafts, pathologic assessment of the various phases of the vascular response to injury of endothelial cell seeded grafts has been carefully undertaken. In terms of thrombosis, most studies have shown that endothelial cell seeded graft surfaces decrease levels of platelet and fibrinogen deposition. In several animal models, Indium 111 labeled platelet and mural fibrinogen deposition were diminished substantially by endothelial cell seeding, independent of the use of adjunctive antiplatelet therapy with aspirin [136–145]. Thus, the endothelial cell seeding of vascular grafts seems to potently inhibit platelet-rich thrombus deposition.

The inflammatory response to synthetic vascular grafts has been well described and includes leukocyte adherence via complement dependent and independent mechanisms as mentioned earlier. Although it was hoped that endothelial cell seeding might inhibit inflammatory cell adhesion, studies designed to demonstrate this phenomenon have not shown any appreciable differences in leukocyte

adhesion to bare polymer or endothelial seeded surfaces [144,145]. Moreover, leukocytes may have a role in reducing endothelial cell viability after exposure of seeded grafts to blood flow. In fact, leukocyte depletion via cyclophosphamide improves endothelial cell retention in canine carotid endothelial cell seeded grafts, implicating leukocytes as a mediator for endothelial cell depletion from seeded grafts [26]. The precise role of the immune response upon endothelial cell seeding efficacy in humans remains to be elucidated.

In addition to the endothelial lining, some studies report the formation of a cellular neointima comprised of smooth muscle cells underlying the endothelial monolayer in seeded grafts [37,146]. However, the finding of a hyperplastic neointima has not been consistently reported in all experimental models [85]. Finally, there does not seem to be a significant role for remodeling in the ultimate pathology of seeded grafts. Thus, it appears that the primary pathologic evolution of endothelial cell seeded vascular grafts involves platelet deposition, followed by leukocyte recruitment, particularly where incomplete endothelialization exists, with variable cellular neointima formation thereafter.

Studies to assess the biochemical function of seeded grafts have examined the production of certain key endothelial compounds after grafts are implanted into physiologic circulation. Seeded grafts express prostacyclin, t-PA, and thromboxane at significant levels, albeit less than native arteries [136,141,147,148]. However, no studies report clear correlation between expression of such compounds and graft patency. Further evaluation of the release of vasoactive compounds by endothelial cells seeded upon vascular grafts remains to be pursued.

4.3.3. *Clinical experience*

Although many trials in animals have indicated higher patency rates for endothelial cell seeded grafts compared to unseeded grafts, early clinical trials in humans of such grafts were not uniformly successful. Direct comparison of these clinical trials is difficult, as graft position, diameter, and endothelial seeding technique vary. Nevertheless, some important lessons have emerged. In 1985, Herring et al., first reported the implantation of an endothelial cell seeded vascular graft in a human [149]. Thereafter in 1987, these

investigators reported a larger series of 17 patients undergoing femoropopliteal bypass with ePTFE grafts lined with enzymatically harvested autologous endothelial cells in which patients receiving seeded grafts exhibited 2.5-fold higher graft patency rates compared to those receiving unseeded control grafts at 1 year post-implantation. This study indicated the clinical feasibility of using seeded grafts in humans. However, at nearly the same time, Zilla et al., reported a series of 18 patients undergoing distal femoropopliteal bypass grafting with autologously seeded grafts. In these patients, objective serum markers and platelet survival studies demonstrated only an incomplete endothelialization of seeded grafts, leaving the debate open as to whether endothelial cell seeding would prove clinically useful [90].

Despite such mixed early data, further research showed decreased platelet adherence in endothelial cell seeded grafts 6–12 months after lower limb arterial reconstruction in 23 patients [150,151]. Moreover, another group reported a prospective trial examining the safety and efficacy of using autologously seeded crural reconstruction grafts in which seeded grafts enjoyed patency rates nearly twice as high versus unseeded grafts at 30 days post-implantation [105]. Moreover, patients receiving seeded grafts had amputation rates of nearly 50% of control at 18 months [105]. Taken together, these early results of decreased platelet adherence and clinical success of endothelial cell seeded grafts laid the groundwork for larger clinical trials.

Two long-term clinical trials of endothelial cell seeded vascular grafts have been well documented. Leseche et al., reported an observational study of the use of a two-stage seeding procedure of autologous venous endothelial cells in above-knee femoropopliteal bypass grafts in 23 patients. Primary patency of seeded grafts was 95% at 3 months, 89% at 10 months and 48 months, and 67% at 67 and 76 months, respectively, demonstrating for the first time long-term patency of endothelial cell seeded grafts in humans [103]. The next long-term trial, performed at the University of Vienna, was reported serially in 1994 [101], 1997 [152], and 1999 [153]. Over the entire follow-up period of the trial, autologous venous endothelial cell seeded femoropopliteal bypass grafts demonstrated higher patency rates com-

pared to unseeded grafts at all time points. For example, seeded grafts showed 84.7% patency at 3 years and 73.8% at 7 years post-implantation vs. 55.4% and 0%, respectively for unseeded grafts. Overall, femoropopliteal reconstructions with endothelial cell seeded grafts enjoyed patency rates approaching 70% at 7 years [152]. Further analysis of these same patients out to 9 years demonstrates a persistent benefit in those patients who received endothelial cell seeded grafts in both infra- and supra-popliteal vascular reconstructions [153]. Consequently, in follow-up as long as 9 years, it appears as though endothelial cell seeded grafts remain patent in an appropriate treatment population, and further research in endothelial cell seeding will likely remain a top priority in vascular medicine.

4.3.4. Genetically modified endothelial cells

The seeding of genetically modified endothelial cells onto vascular grafts has emerged as one of the most promising techniques for harnessing cells as a drug delivery platform within the cardiovascular system. In 1989, Zwiebel et al., reported high levels of recombinant gene expression in rabbit endothelial cells [154]. That same year, Wilson et al., implanted synthetic vascular grafts seeded with retrovirally transduced endothelial cells in a canine model [155]. After these two studies, more attention was paid to seeding vascular grafts with genetically modified endothelial cells [156] to mark the proliferation of seeded endothelial cells upon vascular grafts and to follow the ability of transduced endothelial cells to replicate and remain functional under in vivo flow conditions [157].

Further investigation then turned towards using genetically modified endothelial cells to locally deliver high doses of vasoactive compounds. The first such studies evaluated endothelial cells expressing recombinant t-PA. The t-PA transduced endothelial cells adhered to synthetic grafts under in vitro flow conditions and continued to secrete recombinant t-PA [158]. Similar t-PA or glycosylphosphatidylinositol-anchored urokinase-type plasminogen activator transduced baboon endothelial cells seeded onto collagen coated vascular grafts reduced platelet and fibrin deposition in baboon femoral arteriovenous shunts [159]. These early results of intact secretion of genetically overex-

pressed compounds by seeded endothelial cells were tempered by data in sheep which demonstrated that the proteolytic effects of the t-PA secreted by the transduced endothelial cells diminished the cells' ability to adhere to vascular graft material in vivo over time [160]. While other groups reported a less profound impact of recombinant t-PA expression on cellular adherence [161], these results underscored the need for investigators to pay close attention to the nature of the recombinant gene products being delivered by seeded endothelial cells so as to not hinder their ability to adhere to a substrate. Further issues have been illustrated by other studies in dogs utilizing endothelial cells retrovirally transduced with a reporter construct which demonstrated that long-term recombinant gene expression may be more difficult to achieve than initially believed [162]. Nevertheless, enthusiasm for using genetically modified endothelial cells to locally deliver vasoactive compounds remains high.

4.4. Totally tissue engineered vascular prostheses

While endothelial cell seeding of vascular grafts has broadened the scope of cellular delivery for cardiovascular therapy, tissue engineering may revolutionize conventional vascular grafting. By applying the basic tenets of engineering to biological science, scientists have worked to develop synthetic organ substitutes [163]. Many groups have sought to produce synthetic solid organs [164], such as the liver, others have sought to construct arteries from their constituent cell types, primarily endothelial cells, vSMC's and fibroblasts [8]. If the intact blood vessel has a trilaminar structure, neurohormonal regulation, and the ability to withstand high pressure, all of these qualities are expected from tissue engineered blood vessels. Moreover, it would be advantageous to have all vascular cell types and cell-secreted products present within the tissue engineered prostheses as well. Most attempts have focused on the mechanical, rather than the biochemical aspects of the blood vessel. In this section, we will review some of the key advances in the development of the tissue engineered blood vessel.

In 1986, Weinberg and Bell produced a completely biological tissue engineered blood vessel with a Dacron mesh serving as a scaffold for bovine aortic

endothelial cells, smooth muscle cells and fibroblasts [165]. The resulting blood vessel exhibited surface expression of von Willebrand Factor and prostacyclin, confirming endothelial cell biochemical activity. However, these vessels were not able to sustain physiologic pressures and were thus not viable for *in vivo* vascular grafting. Next, L'Heureux et al., wrapped smooth muscle cells in a collagen gel forming a tube around which an outer sheet of fibroblasts was applied from without and endothelial cells were seeded from within [166]. While this graft resembled an artery morphologically, it was unable to withstand high pressures. Further studies by Matsuda et al. [167–174] resulted in a hybrid vascular construct which was tested in a canine carotid artery model and had high levels of patency at 6 months after implantation. However, these vessels sustained arterial pressures only if supported by a Dacron outermesh [168].

In 1998, L'Heureux et al. finally overcame low vessel strength by culturing smooth muscle cells and fibroblasts in the presence of ascorbate, Vitamin C [175]. Such a strategy resulted in a vessel constructed out of a sheet of smooth muscle cells grown upon the extracellular matrix of fibroblasts and wrapped around an ePTFE mandrel which was incubated for a week in a bioreactor prior to the addition of a roll of adventitial fibroblasts around the outer diameter of the smooth muscle cell 'media'. After another 8 weeks of incubation, the ePTFE was removed from within the tube, endothelial cells were seeded upon the inner, luminal surface, and another week was permitted to elapse while the endothelial cells matured. These tissue engineered blood vessels were then subjected to burst testing which revealed burst strengths of nearly 2600 mmHg, higher than that of human saphenous vein grafts. When grafted into a canine femoral arterial interposition model, three of six grafts remained patent at 7 days despite being xenograft tissue.

On the heels of this breakthrough, Niklason et al. with Robert Langer published their development of tissue engineered arteries pre-conditioned by pulsatile flow [176]. They described seeding bovine aortic smooth muscle cells into tubular biodegradable polyglycolic acid scaffolds secured inside a bioreactor. After seeding the tubes with smooth muscle cells for 30 min, the bioreactors were filled with media and the constructs were cultured under pul-

satile radial stress while beating at 165 beats/min with 5% radial distention for 8 weeks. Thereafter, histologic examination revealed that smooth muscle cells had migrated through and had subsumed the polymer scaffold rendering an internal luminal surface that would be seeded with bovine aortic endothelial cells. Continuous perfusion was then performed for 3 days to allow the endothelial layer to mature. The media was supplemented with ascorbate, copper, and amino acids to support smooth muscle cell extracellular matrix elaboration. Following this protocol, functional arteries with burst strengths in excess of 2000 mmHg were generated. These vessels were responsive to vasoactive compounds such as endothelin and prostaglandin F₂ α , and the smooth muscle cells of the vessels demonstrated higher levels of myosin heavy chains, indicative of a significant degree of differentiation. Finally, these vessels were grafted into right saphenous artery of Yucatan miniswine and remained patent at 4 weeks post-implantation.

Recently, Campbell et al. reported yet another method for tissue engineering of vascular grafts [177]. These investigators inserted silastic tubing into the peritoneal cavity of rats and rabbits. The resulting inflammatory reaction to the silastic covered the tubes with layers of myofibroblasts, collagen matrix, and a monolayer of mesothelial cells. By withdrawing the tubing from the laminar, multicellular remains of the inflammatory response and then everting this biological tube, these investigators discovered synthetic arteries with architecture similar to that of native blood vessels. The mesothelial cells mimicked endothelial cells. The myofibroblasts served as smooth muscle cells lying in a collagen and elastin-rich bed. And, the entire structure was surrounded by a collagenous adventitia. When placed as rabbit carotid interposition or rat abdominal aortic interposition grafts in the same animals in which they were initially incubated, these tissue engineered blood vessels remained patent for at least 4 months. The vessels also developed structures resembling elastic lamellar and high volume myofilaments conferring contractile responsiveness to pharmacologic agonists. By using peritoneal incubation, Campbell et al. have introduced a potential method for limiting immune-mediated rejection of tissue engineered vascular prostheses.

The totally biological tissue engineered blood

vessel stands as a testament to the innovation within the field of vascular biology. These tissue engineered prostheses progressively evolved from early synthetic grafts covered with endothelial cells and grafts comprised of different cell types into multi-layered, metabolically reactive, high strength vascular structures. The clinical potential for such tissue engineered structures is profound, as presently, there are limited native arterial conduits that can be used in vascular bypass grafting [4]. The presumably higher patency rates and longer half-lives of tissue engineered vascular prostheses may well prove to be a significant advantage in the clinical arena in the future. While further improvements regarding optimal culture conditions, polymer scaffolding structure, and integration into the vascular neurohormonal milieu remain to be achieved, these early studies have paved the way for new discoveries.

5. Endothelial cell delivery after percutaneous interventions

5.1. Percutaneous interventions – introduction

Percutaneous vascular interventions such as balloon angioplasty and endovascular stent placement induce a great deal of mechanical injury within the vascular wall [6]. In addition, the compression of the endoluminal surface of the vessel typically results in high degrees of endothelial denudation [13,29]. As previously discussed, the loss of the endothelium in these types of procedures likely serves as a determinant of late restenosis. As a result, a great deal of effort has focused upon restoring the endothelium rapidly after percutaneous interventions by either delivering supplemental endothelial cells intravascularly, promoting endogenous endothelial recovery, and/or applying perivascular endothelial cells.

5.2. Endothelial cell seeding of balloon/stent injured native vessels

The rapid restoration of the endothelium after mechanical injury may diminish the sequelae of vascular injury. The delivery of endothelial cells to the luminal interface of the mechanically injured vessel was thought to be one method for achieving early re-endothelialization after percutaneous inter-

ventions. However, early enthusiasm for exogenous seeding of endothelial cells upon mechanically injured arterial segments has been dampened by difficulty in percutaneous cell delivery. While synthetic vascular bypass grafts offer ready access and time for adequate cellular seeding prior to insertion into the circulation, percutaneous cell delivery techniques depend upon short duration, high pressure instillation of cell rich suspensions prior to restoration of normal blood flow. Despite these limitations, Nabel et al. demonstrated that porcine endothelial cells expressing a lacZ reporter gene could be successfully introduced via a catheter into balloon denuded iliofemoral arteries of syngeneic animals with extended adherence to the vascular wall [178]. In fact, Conte et al. demonstrated that 7 days after endothelial denudation and seeding with genetically modified endothelial cells in a rabbit model, up to 90% of the denuded arterial segment was re-endothelialized by the modified cells [179]. Others have shown similar success in accelerating re-endothelialization of vascular segments by catheter-mediated delivery of endothelial cells [180]. These experiments together illustrate the technical feasibility of endoluminal endothelial cell delivery.

While balloon denuded segments seeded with endothelial cells in this manner may be repopulated rapidly, subsequent studies in different animal models with either balloon or stent induced injury have shown that such re-endothelialization did not reliably inhibit intimal hyperplasia [181]. However, further research has shown that genetically modified endothelial cells seeded upon the interstices of metal stents can successfully be implanted with high levels of cellular viability and adherence to the vascular wall even after the restoration of normal blood flow [182–187]. Moreover, novel percutaneous endothelial cell delivery techniques using endothelial cells with internalized paramagnetic beads and external magnetic fields have also been described [188]. These data offer hope that delivery of exogenous, genetically modified endothelial cells may prove useful in combating restenosis.

While exogenous endothelial supplementation has not yet been shown to inhibit intimal hyperplasia reliably, studies in our laboratory in a stent-induced rabbit iliac arterial injury model have demonstrated that remnant endothelium following stent placement enhances vascular repair and reduces ultimate intimal

hyperplasia [13]. These experiments indicate that attempts at enhancing repopulation of endogenous endothelial cells may decrease post-intervention restenosis. Therefore, the injured blood vessel may benefit from supplementation of endothelial growth factors such as vEGF [189,190], bFGF [191], and estrogen [192,193]. Systemic administration of such locally potent compounds has produced accelerated endogenous re-endothelialization in animal models. However, such systemic therapy may have undesired, and perhaps even hazardous side-effects, and further study is warranted into local therapeutic delivery of growth factors which could locally restore a confluent endothelium.

Investigators have examined gene therapy approaches to enhancing re-endothelialization of denuded vascular segments. While gene therapy for obstructive vascular disease has been extensively reviewed elsewhere [194–197], we shall only mention a few reports that have shown accelerated endogenous re-endothelialization following gene transfer. The adenoviral transfection of balloon injured rabbit iliac arteries with phVEGF accelerated ipsi- and contralateral re-endothelialization with concomitant inhibition of intimal hyperplasia [190]. When stents coated with naked plasmid DNA containing the phVEGF gene were implanted, these vessels also exhibited accelerated re-endothelialization [198]. Similarly, others have demonstrated that overexpression of prostacyclin synthase by lipofection of the balloon injured rat carotid artery resulted in accelerated re-endothelialization and marked inhibition of intimal hyperplasia [199]. These data emphasize the need for further studies to assess gene therapy as an adjunct to endothelial cell delivery for proliferative arteriopathies such as restenosis.

5.3. Endothelial cell implants for restenosis

The complex trilaminar structure of the blood vessel wall raises the natural question as to whether vascular homeostasis requires architectural integrity. Indeed most attempts at fabricating artificial vascular grafts focus on creating a luminal endothelial monolayer, a muscular media, and a loose adventitial support. We wondered whether endothelial cells need to reside at the luminal interface for the endothelium to impose its biochemical control on the artery. Only

the technology of tissue engineering enabled us to examine this issue.

Endothelial cells injected into the perivascular space are either destroyed or migrate away [200]. In contrast, when the same cells are grown on three dimensional polymeric scaffoldings, they can grow to great density and retain immunoidentity, biochemical function, and normal growth kinetics [44,200,201]. Bovine aortic endothelial cells grown within Gelfoam[®] polymeric matrices produced an identical amount of essential endothelial compounds such as prostacyclin and heparan sulfate proteoglycans as cells grown in conventional tissue culture [200]. These constructs inhibited restenosis by nearly 10-fold over control, unseeded implants, when placed in the perivascular space around injured rat carotid arteries as seen in Fig. 2 [200]. Moreover, the endothelial cell seeded implants inhibited intimal hyperplasia greater than 3-fold more than perivascular heparin release devices (Fig. 2 [200]). These initial experiments demonstrated tissue engineered endothelial cells could reassert control over the vascular response to injury and could outperform heparin in slowing smooth muscle cell proliferation without requiring the restoration of the normal vascular architecture in which the endothelium resides at the lumen.

Further studies in our laboratory showed that this technology remains effective when standard means of pharmacoregulation fail in a more complex animal model. Both bovine/xenograft and porcine/allograft aortic endothelial cell implants inhibited intimal hyperplasia in the porcine carotid artery overstretch injury model while the heparin release devices that had been effective in the rat did not [201]. The similar levels of inhibition of neointimal hyperplasia by xeno- and allograft cells occurred despite modest elevation of antibodies to the xenograft cells [201]. These data suggest that the infusion of isolated endothelial derived compounds such as heparin may be sufficient for the inhibition of intimal hyperplasia in lower complexity models, but may be necessary though insufficient for the treatment of more sophisticated disease models. Perhaps then, only the cell-based delivery of all bioactive and bio-enabling compounds in physiologic doses and subject to physiological control can regulate complex diseases like experimental neointimal hyperplasia and clinical restenosis.

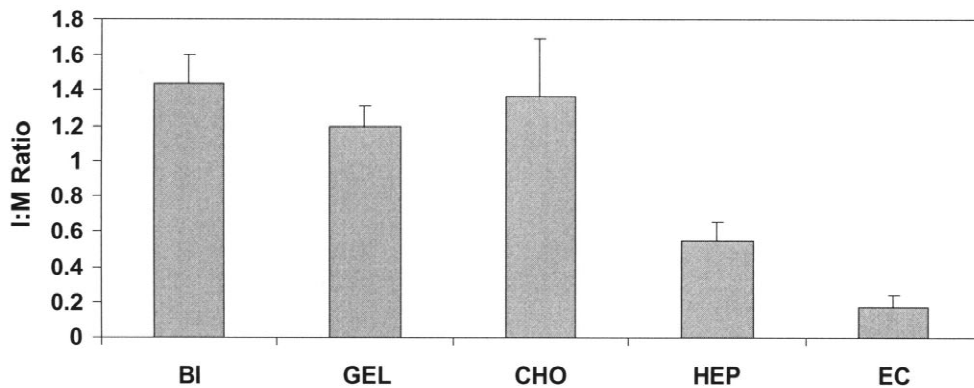


Fig. 2. Tissue engineered endothelial cells inhibit intimal hyperplasia in balloon injured rat carotid arteries. The ratio of the intima to media (I:M ratio) 14 days after balloon denudation injury (BI) of the rat common carotid artery was measured by digital planimetry as an index for restenosis. This value was unchanged when balloon-injured arteries were exposed to Gelfoam alone (GEL) or Gelfoam seeded with the heparan sulfate proteoglycan-deficient CHO-745 cells (CHO). Heparin administered perivascularly via sustained heparin release devices reduced intimal hyperplasia by 61.8%, and Gelfoam seeded with bovine aortic endothelial cells (EC) were 3.2-fold more effective than heparin, inhibiting intimal hyperplasia by 88.2%. (With permission of authors [200].)

6. Conclusions

Although obstructive atherosclerotic vascular disease stands as a major threat to public health worldwide, significant strides have been made towards improving the therapeutic modalities currently employed to treat atherosclerosis. However, as conventional mechanical revascularization therapies such as surgical bypass grafting or angioplasty exhibit their inability to adequately control the normal vascular response to injury, investigators have enlisted cell-based approaches to expand the clinical options for the treatment of vascular disease. A large body of data has been amassed establishing that the biochemical features of the blood vessel identify it as an active organ in which the secretory function of endothelial and other vascular cells are as important as their mechanical properties in their contribution to overall vascular homeostasis. In particular, the endothelial cell is uniquely poised to control multiple phases of the vascular response to injury because of its central role in vascular biology. This principle has been borne out by the ability of the endothelial cell to improve many current treatments for cardiovascular disease. For example, while conventional synthetic vascular grafts suffice for large vessel applications, small diameter grafts seeded with endothelial cells outperform bare synthetic grafts in humans. Moreover, the entire blood

vessel architecture can be recapitulated by the innovative techniques of tissue engineering endothelial cells and other vascular cells. Finally, native and recombinant endothelial cells have been shown to reassert physiologic control over post-angioplasty restenosis by resuming residence at their usual luminal location or even when tissue engineered to reside perivascularly. Such findings emphasize the intricate nature of the vascular system as far more than a bed of pipes and tubes. Rather, there exists an important interplay between vascular structure and function in which the endothelial cell plays a starring role. The innovative application of endothelial cells as a physiologically responsive drug delivery platform for cardiovascular therapy thus heralds an increasingly close tie between the fields of vascular biology, cardiovascular surgery, and clinical cardiology in the coming century.

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