Tissue engineered perivascular endothelial cell implants regulate vascular injury

(atherosclerosis/polymer-based controlled delivery/restenosis/tissue engineering/vascular repair)

ARUNA NATHAN*, MATTHEW A. NUGENT†, AND ELAIZER EDELMAN*§

*Harvard–Massachusetts Institute of Technology, Division of Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge, MA 02139; †Department of Medicine, Cardiovascular Division, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115; and §Departments of Biochemistry and Ophthalmology, Boston University School of Medicine, Boston, MA 02118

Communicated by Robert W. Mann, Massachusetts Institute of Technology, Cambridge, MA, May 3, 1995

ABSTRACT Molecular biomaterial engineering permits in vivo transplantation of cells and tissues, offering the promise of restoration of physiologic control rather than pharmacologic dosing with isolated compounds. We engrafted endothelial cells on Gelfoam biopolymeric matrices with retention of viability, normal growth kinetics, immunoreactivity, and biochemical activity. The production of heparan sulfate proteoglycan and inhibition of basic fibroblast growth factor binding and activity by engrafted cells were indistinguishable from endothelial cells grown in culture. Perivascular implantation of Gelfoam-endothelial cell scaffolds around balloon-deneded rat carotid arteries reduced intimal hyperplasia 88.1%, far better than the isolated administration of heparin, the most effective endothelial mimic compound. In concert with a reduction in intimal area, cell proliferation was reduced by >90%. To our knowledge, there have been no previous reports of extracorporeal cell implants controlling neovascular proliferative disease. Tissue engineered cells offer the potential for potent methods of vascular growth regulation and insight into the complex autocrine-paracrine control mechanisms within the blood vessel wall.

Though the hallmark of the accelerated arteriopathies that follow angioplasty and vascular bypass grafting is the proliferation of smooth muscle cells and their accumulation within the tunica intima, it is the loss of normal endothelial function that heralds these events and may stimulate them to occur (1, 2). The arterial endothelium serves as a transport barrier, a biochemical filter, and a regulator of many vascular phenomena. The most potent vasodilators, thromboresistant compounds and inhibitors of smooth muscle cell proliferation, are endothelially derived. Vascular smooth muscle cell accumulation within the intima ceases with restoration of the endothelium (3, 4) and regression of intimal hyperplasia is maximized where endothelial restoration is maximized (5). Autologous endothelial cell transplantation (6–8) and implantation of endothelial cell-seeded interposition grafts (9, 10) or endovascular stents (11) have populated the surface of denuded arteries. These approaches may recreate the endothelial barrier. However, inhibition of intimal hyperplasia has yet to be demonstrated, perhaps because contact with circulating blood limits the efficiency and duration of cell seeding (6–12). Moreover, it is not clear that the endothelial control of vascular injury and smooth muscle cell proliferation requires that the endothelial cells reside at the luminal interface. The biochemical regulation imposed by the endothelium may be equipotent to or of far greater importance than its barrier function and may be active even if these cells are at a distance from the lumen.

To address this question we engrafted cells on three-dimensional biopolymeric scaffolds and placed them in the perivascular space of denuded blood vessels. Engrafted endothelial cells remained viable in large numbers within matrices of the porous collagen-like Gelfoam material, with full retention of their biologic activity and the biochemical markers of normal vascular cells. More importantly, though the perivascular space is significantly distant from the denuded intimal/ luminal interface, cells implanted in the perivascular area inhibited intimal hyperplasia to a greater extent than any other intervention and without the need for restoration of the endothelial luminal barrier. The desired density of cells can be established in vitro and implanted in vivo within the protected environment of the biopolymer scaffolding. Tissue engineered perivascular cell implantation may, therefore, allow us to examine fundamental issues in vascular biology, such as the regulatory role of the endothelium, and to develop alternative therapies for vascular injury.

MATERIALS AND METHODS

Cell Engraftment. Gelfoam has long been used as an implantable surgical sponge and more recently as a scaffolding for cell growth (13). This material, isolated from porcine dermal gelatin, was supplied in blocks (Upjohn) and cut into 2.5 × 1.0 × 0.3 cm pieces and hydrated by autoclaving for 10 min in Hanks' balanced salt solution (HBSS). Upon cooling, each block was placed in a 17 × 100 mm polypyrrole tube containing 2 ml of an endothelial cell suspension in Dulbecco's modified Eagle's medium (DMEM) or CHO-745 cell suspension in Ham's F-12 medium (0.6 × 10^5 cells per ml). Medium was supplemented with 1 g of glucose per liter and 10% calf serum. Studies were performed with bovine aortic endothelial cells as we have reproducibly harvested and cultured these cells with complete retention of their biochemical regulation of vascular smooth muscle cell proliferation (14). Mutant Chinese hamster ovary (CHO-745) cells served as control cells as they produce no detectable heparan sulfate (15) and, as a result, unlike endothelial cells, do not inhibit basic fibroblast growth factor (bFGF) binding to or mitogenesis of cultured vascular smooth muscle cells (14). The culture tubes containing the blocks were gently agitated to disperse the cells and then incubated at 37°C for up to 15 days at a 45° angle in humidified 5% CO_2/95% air. Growth medium was changed on days 3, 7, and 12. On days 0, 2, 4, 7, 10, and 15, the number of cells attached to the Gelfoam was determined after the blocks were washed four times with HBSS and digested with collagenase (1 mg/ml). Cell viability was checked at each of

Abbreviations: BrdUrd, 5-bromo-2'-deoxyuridine; CHO, Chinese hamster ovary; I:M, intima/media area ratio; bFGF, basic fibroblast growth factor.

†To whom reprint requests should be addressed: The Biomedical Engineering Center, 20A-127, Massachusetts Institute of Technology, 18 Vassar Street, Cambridge, MA 02139.

8130
these points in time by trypan blue exclusion. The preservation of the immune identity of cells within Gelfoam blocks recovered 14 days after placement in tissue culture or around rat carotid arteries was determined by immunostaining for the endothelial marker von Willebrand factor as described in detail (16).

**Proteoglycan Production.** The amount of heparan sulfate in conditioned medium produced by cells cultured on Gelfoam or tissue culture polystyrene was measured. Gelfoam films containing endothelial cells or CHO-745 cells were incubated in culture medium containing no antibiotics and no calf serum for 24 hr at 37°C. As a control, identical Gelfoam films without cells were incubated in the same media. The medium was collected, centrifuged (5000 × g), dialyzed extensively against water, and concentrated by lyophilization. Total sulfated glycosaminoglycan was determined using dimethylmethylene blue (17), and the amount of heparan sulfate was assessed after samples were treated with heparinase. The endothelial and CHO-745 cells grown on Gelfoam were also radiolabeled with $^{35}$SO$_4$ (100 μCi/ml; 24 hr; 1 Ci = 37 GBq) to visualize the metabolic synthesis of heparan sulfate by the cells. The medium was collected and centrifuged, and the $^{35}$SO$_4$-proteoglycan was separated from the free $^{35}$SO$_4$ by vacuum filtration through cationic nylon membranes (18). Filters containing $^{35}$SO$_4$-proteoglycan were incubated in nitrous acid (0.45 M sodium nitrite combined with 3.6 M acetic acid) and counted to determine the amount of radioactivity incorporated within heparan sulfate.

**Biologic Effect.** The biologic effects of cell engrafts were tested in culture and in vivo. The ability of conditioned medium from endothelial cell-CHO-745 cell-Gelfoam to block bFGF-induced smooth muscle cell mitogenesis and 121I-labeled bFGF (121I-bFGF) binding to heparan sulfate proteoglycan of confluent, quiescent vascular smooth muscle cell monolayers was determined (14). In vivo potency was assessed by evaluating the ability of Gelfoam (2.5 × 1.0 × 0.3 cm each) containing endothelial cells, CHO-745 cells, or no cells, to alter intimal hyperplasia when wrapped around endothelium-denuded rat common carotid arteries (16, 19, 20). The strip ends overlapped, ensuring complete encircling of the artery. Focalar planes were sutured closed to further immobilize the device. To compare the results of anticipated physiologic secretion of endothelial cell products to the pharmacologic dosing of a proven endothelial-cell analog, hydrogel films were formulated (21) to release heparin alone at a dose, 2.5 ± 0.1 μg/day, similar to the daily release of heparan sulfate proteoglycan from the engrafted cells (1.2 ± 0.1 μg/day). This dose and mode of delivery have previously been demonstrated to maximize heparin's inhibition of neointimal hyperplasia (20, 22, 23).

On the 14th postoperative day, animals were euthanized and perfused clear via the left ventricle with Ringer's lactate solution followed by immersion fixation with Carnoy's fixative (60% methanol/30% chloroform/10% glacial acetic acid). The location of the implanted films was marked with India ink, and the films were recovered with the entire length of the intact arteries. The carotid arteries were harvested and cut into five equal segments, three including the Gelfoam wrap and one segment each above and below the wraps. Segments were paraffin embedded and 6-μm sections were obtained along the length of each segment. After staining with hematoxylin/eosin or von Hefez's elastin stain, the intimal, medial, and adventitial areas, the intima/media area ratio (I:M) and the percent of luminal occlusion were calculated using computerized digital planimetry with a dedicated video microscope and customized software. Cell proliferation was assayed using immunocytochemical identification of the thymidine analog-5-bromo-2'-deoxyuridine (BrdUrd), which had been injected intraperitoneally, at 50 mg/kg, 3 and 7 days after surgery and 1 hr prior to sacrifice (16, 22-24).

Statistical comparisons were performed using analysis of variance (ANOVA) and subsequent differences among groups using Student's t test. Data were rejected as not significantly different if P values of >0.05 were observed. Data line fits were established using a linear regression and correlation model.

**RESULTS**

**Growth Kinetics, Immunologic Identification, and Biochemical Activity of Engrafted Cells.** Bovine aortic endothelial cells, and mutant CHO cells (CHO-745) were cultured in Gelfoam matrices. Engrafted cells lined the interstices of the three-dimensional collagen-like Gelfoam matrix (13) and followed a growth pattern similar to that observed for cells harvested from intact blood vessels and cultured on polystyrene (Fig. 1). Cell viability, as evaluated by trypan blue exclusion, remained at 90% ± 2.3% for the endothelial cells and 93.1% ± 1.7% for the CHO-745 cells over the 15-day culture course. The preservation of the immune identity of the implanted cells was documented when Gelfoam implants with endothelial cells were recovered 14 days after placement around the common carotid artery of laboratory animals. Immunostaining for the endothelial marker von Willebrand factor in the engrafted cells was unchanged from primary cultured cells (Fig. 1 b and c).

Engrafted cells remained viable with full retention of biochemical secretory ability and biologic potency as well as immune identity. Cells cultured on Gelfoam produced nearly identical amounts of total sulfated glycosaminoglycan and heparan sulfate as that produced when the same cells were grown on polystyrene dishes. In addition, there was no significant difference in the profile of the proteoglycan when resolved on 5% SDS/PAGE. For cells grown on Gelfoam and tissue culture polystyrene, the majority of the proteoglycan migrated as a band >600 kDa relative to protein standards. When compared to CHO-745 cells grown on Gelfoam, the engrafted endothelial cells produced an 11.1-fold greater amount of glycosaminoglycan (4.1 ± 0.3 μg per 10$^6$ cells per day for endothelial cells vs. 0.37 ± 0.02 μg per 10$^6$ cells per day for CHO-745 cells). While 29.1% of the glycosaminoglycan produced by the endothelial cells was heparan sulfate (1.2 ± 0.05 μg per 10$^6$ cells per day), CHO-745 cells produced no detectable heparan sulfate. Conditioned medium from Gelfoam-engrafted endothelial cells also inhibited binding of 121I-bFGF to heparan sulfate proteoglycan and the mitogenic effect of bFGF on vascular smooth muscle cells in a dose-dependent fashion (Fig. 2) (14). In contrast, conditioned medium from CHO-745 cells had no effect on binding and, similarly, no effect on growth factor-induced mitogenesis, as expected (Fig. 2).

**Engrafted Cell Implants Inhibit Intimal Hyperplasia and Cell Proliferation.** The in vivo potency of the endothelial cell engrafts was retained in addition to their in vitro effects. Balloon denudation of the carotid arterial endothelium led to an increase in the ratio of the area of the tunica intima to the area of the tunica media (I:M) to 1.44 ± 0.16 (Fig. 3). Arterial wall segments exposed to Gelfoam containing endothelial cells showed minimal hyperplastic disease. Intimal hyperplasia in arterial segments beneath endothelial cell-grafted Gelfoam was reduced to 0.17 ± 0.07. This modulating effect was specific to endothelial cells. Control CHO-745 cell engrafts had no statistically significant effect on intimal hyperplasia (I:M = 1.36 ± 0.32) compared to balloon injury alone or empty Gelfoam matrices (I:M = 1.20 ± 0.11, Fig. 3). Heparin has been identified as the gold standard inhibitor of smooth muscle cell proliferation and intimal hyperplasia, in major part because it resembles endothelial-cell-derived heparan sulfate proteoglycan (3, 14, 19, 25–29). Indeed, the perivascular release of heparin alone from hydrogel films (21) reduced
FIG. 1. (a) Bovine aortic endothelial cells and CHO-745 cells cultured on Gelfoam matrices followed the expected exponential growth curve. Each point represents the average cell number ± the SEM of duplicate determinations. Cell growth, expressed as cell number × 10^3, reached a plateau at about the 10th day, with average doubling times of 20.7 hr for CHO-745 cells (●) and 36.7 hr for endothelial cells (○). Cell viability was retained over the entire course of the experiment for both cell types. Photomicrographs of sections through the Gelfoam matrices containing endothelial cells (b) or CHO-745 cells (c) harvested 14 days after placement around the common carotid artery of laboratory animals and immunostained for von Willebrand factor illustrated the preservation of normal cell markers in engrafted endothelial cells and the retention of cells within the biopolymer matrix. The nonendothelial CHO-745 cells stained lightly with the counterstain but took up none of the antibody. (×320.) Seven animals were examined in each of the treatment groups.

proliferation to an IM of 0.55 ± 0.11. This result, however, was 3.2-fold less effective than the control exerted by engrafted endothelial cells despite hydrogel release of heparin at twice the rate of release of heparan sulfate proteoglycan from the endothelial implants.

Gelfoam-engrafted endothelial cells reduced cell proliferation as well as intimal hyperplasia. The numbers of proliferating cells relative to the total number of cells in the tunica media and intima were used as indices of proliferation. Gelfoam implants with engrafted endothelial cells caused a statistically significant decrease in cell proliferation in the tunica intima (29.4% reduction) and tunica media (26.3% reduction) relative to empty Gelfoam implants (Fig. 3e). Heparin infusion also decreased proliferation but the effect was not statistically significant at the number of animals and sections examined. Relative to balloon injury alone, heparin administration reduced BrdUrd immunostaining in the tunica intima by 11.0% and by 12.2% in the tunica media (Fig. 3e).

There was no evidence that the implants induced cellular or systemic rejection. The general health and appearance of the animals with the Gelfoam implants were unchanged from controls. Rats subjected to balloon injury alone gained 52.9 ± 3.6 g over the 14-day experimental period, and those animals that received a cell-free Gelfoam implant hydrated in either DMEM or Ham’s F-12 medium gained 48.0 ± 7.3 g. The animals implanted with endothelial-seeded implants gained 51.3 ± 2.8 g and the animals with CHO-745-laden Gelfoam gained 53.4 ± 6.5 g. Rats receiving perivascular heparin gained 56.7 ± 6.2 g.

DISCUSSION

Endothelial Regulation of Vascular Biology. Arterial endothelial cells form a continuous, selectively permeable, non-thrombogenic barrier between circulating blood and the arterial wall that controls many facets of vascular biology (1, 2). The anatomic continuity of the endothelial monolayer and the underlying vascular smooth muscle cells provides biochemical control of vascular physiology as well as structural integrity. Endothelial-derived products control blood flow and vessel tone (30, 31), thrombosis, platelet activation, adhesion and aggregation (32, 33), leukocyte adhesion (34–37), monocyte infiltration and smooth muscle cell migration (38, 39), and proliferation (3, 14, 19, 26, 29, 40–42). The homeostatic set point that determines the steady-state conditions for all of these parameters maintains the normal blood vessel in a quiescent state. Endothelial loss or dysfunction leads to an alteration in this balance and sets in place a sequence of events that culminates in the proliferation of normally quiescent smooth muscle cells. Restoration of the endothelium inhibits smooth muscle cell accumulation after denuding arterial injury (3, 4), and stimulation of endothelial restoration simultaneously maximizes regression of intimal hyperplasia (5). Yet, it is still not evident which of the many functions of the endothelium are required to reconstruct homeostasis. Nor do we understand why the infusion of analogs of endothelial products such as heparin (3, 14, 19, 25–29) and nitric oxide (40–42) successfully inhibits vascular cell proliferation in vitro.

FIG. 2. Endothelial cell-Gelfoam conditioned medium inhibited bFGF binding to (●) and mitogenesis for (□) vascular smooth muscle cells in a similar dose-dependent fashion. In contrast, conditioned medium from CHO-745 cells had no effect on binding (○) or proliferation (□).
with Harris' 3.2-fold were unchanged containing engrafted CHO-745 cells (b) or endothelial cells (c) (×290), immunostained for BrdUrd (dark brown-stained cells) and counterstained with Harris' hematoxylin (light blue), reveal the extent to which endothelial engrafts inhibited cell proliferation and intimal hyperplasia. The lumen of the artery is at the top of each panel and the dark arrows point to the internal elastic laminae that separate the neointima from the tunicae media. (d) The I:M after balloon denudation of the endothelium (BI) was used as a standard assessment of the extent of intimal hyperplasia. This value was unchanged when balloon-injured arteries were exposed to Gelfoam alone (GEL) or engrafts with the heparan sulfate proteoglycan-deficient CHO-745 cells (CHO). Heparin reduced intimal hyperplasia by 61.8% (I:M = 0.55 ± 0.11), and Gelfoam-engrafted endothelial cells (EC) were 3.2-fold more effective than heparin, inhibiting intimal hyperplasia by 88.2% (I:M = 0.17 ± 0.07). (e) Cell proliferation within the tunicae intima and media was reduced by Gelfoam-engrafted endothelial cells. By BrdUrd immunocytochemistry, 25.9% ± 2.2% of cells in the tunica intima and 9.5% ± 1.7% of cells in the tunica media were identified as proliferative. These numbers were not changed by the Gelfoam alone or Gelfoam-engrafted CHO-745 cells in a statistically significant fashion. Although heparin and the Gelfoam-engrafted endothelial cells reduced cell proliferation in both vascular wall compartments, at the numbers of animals and sections evaluated, only the latter reached statistical significance. Heparin reduced cell proliferation relative to balloon-injured arterial controls by 11% within the tunica intima (23.1% ± 3.0%) and the tunica media by 12.2% (8.4% ± 2.1%), and the engrafted endothelial cells were 2.2-fold more effective in the tunica intima (29.4% reduction compared to Gelfoam alone, 20.2% ± 2.4%) and 2.7-fold more effective in the tunica media (26.3% reduction, 6.3% ± 1.6%). Seven animals were examined in each treatment group and six animals received balloon injury alone. □, Intima; ■, media.

and proliferative vascular disease in animal models but has not proven effective in human disease to date. To determine whether the intact endothelial cell could exert increased control over vascular proliferation and to gain further insight into the means by which the endothelium exerts its control over vessel wall homeostasis, we investigated vascular repair following implantation of viable endothelial cells around injured blood vessels. Previous studies have proposed to restore growth control by recapitulating the endothelial lining of the artery (6–12, 43). These techniques have repopulated the surface of denuded arteries but inhibition of intimal hyperplasia has yet to be demonstrated, perhaps because the techniques of cell seeding are still plagued by a delay in complete restoration of the endothelial lining and the transient residence of cells at the vascular surface. Loss of seeded cells arises from exposure to physical and immune forces imposed by contact with circulating blood. It is not clear, however, that the restoration of vascular homeostasis requires endothelial cells at the luminal interface. The biochemical regulation imposed by the endothelium might be active even if these cells are not at the luminal border. We now demonstrate the ability to engraft endothelial cells on three-dimensional biopolymer scaffolds with preservation of their viability, normal growth characteristics, immunologic markers, biochemical activity, and physiologic effects.

**Specificity of Endothelial Cell Engrafts.** The biologic effects of engrafted endothelial cells were cell-specific and superior to the administration of a single presumptive pharmacologic analog of an endothelial product, heparin. Conditioned medium from Gelfoam-engrafted endothelial cells, but not heparan sulfate proteoglycan-deficient CHO-745 cells, inhibited bFGF binding to and mitogenesis for vascular...
smooth muscle cells in a dose-dependent fashion (Fig. 2). Similarly, only endothelial cell engrafted induced intimal hyperplasia (Fig. 3). Gelfoam alone or seeded with CHO-745 cells had no statistically significant effect on cell proliferation or intimal hyperplasia. Heparin is one of the most effective antiproliferative agents for vascular smooth muscle cells (3, 14, 19, 25-29). Yet, when this drug was released at twice the rate of heparan sulfate proteoglycan production by the endothelial cells, intimal hyperplasia (Fig. 3d) and smooth muscle cell proliferation (Fig. 3e) were also reduced but 3.2-fold less effectively than with the endothelial cell grafts.

The control exerted by the endothelial cell implants appears to result solely from the biochemical effects of the engrafted cells. Immunostaining with endothelial cell-specific markers detected no evidence for early recovery of endogenous endothelial cells or the migration of engrafted cells from their biopolymer scaffoldings to the arterial lining in any of the recovered arterial segments. The effects of the engrafted cells were localized in a similar manner to the focal effects observed with the perivascular release of other compounds (16, 20, 44). There was no evidence of a systemic or local immune response or graft rejection that might alternatively explain the results.

Thus, it appears that endothelial control of vascular proliferation stems in large part from biochemical regulation rather than mechanical barrier function and that the combined secretion of many compounds at physiologic doses by the intact cell is superior to pharmacologic administration of isolated endothelial cell-derived products. It is hoped that additional work with species-specific cells, novel polymer materials, and genetically modified cells with regulated secretory activity will enhance further the therapeutic and research potential of this approach.

We thank Dr. Jeffrey Esko for CHO-745 cells and Mr. Chris Colburn and Upjohn for Gelfoam samples. This work was supported in part by grants from the National Institutes of Health (GM/HL 49039 and AG00294, E.R.E.), the Burroughs-Wellcome Fund in Experimental Therapeutics (E.R.E.), the Whitaker Foundation for Biomedical Engineering (E.R.E.), and the American Heart Association (G130-522-923, M.A.N.).