Endothelial Implants Inhibit Intimal Hyperplasia After Porcine Angioplasty

Helen M. Nugent, Campbell Rogers, Elazer R. Edelman

**Abstract**—The perivascular implantation of tissue-engineered endothelial cells around injured arteries offers an opportunity to study fundamental vascular physiology as well as restore and improve tissue function. Cell source is an important issue because the ability to implant either xenogeneic or allogeneic cells would greatly enhance the clinical applications of tissue-engineered grafts. We investigated the biological and immunological responses to endothelial cell xenografts and allografts in pigs 4 weeks after angioplasty of the carotid arteries. Porcine or bovine aortic endothelial cells were cultured within Gelfoam matrices and implanted in the perivascular space of 42 injured arteries. Both porcine and bovine endothelial cell grafts reduced the restenosis index compared with control by 54% and 46%, respectively. Perivascular heparin release devices, formulated to release heparin at twice the rate of release of heparan sulfate proteoglycan from endothelial cell implants, produced no significant reduction in the restenosis index. Endothelial cell implants also reduced occlusive thrombosis compared with control and heparin release devices. Host immune responses to endothelial implants were investigated by immunohistochemical examination of explanted devices and by immunocytochemistry of serum samples. The bovine cell grafts displayed infiltration of leukocytes, consisting primarily of lymphocytes, and caused an increase in antibodies detected in serum samples. Reduced cellular infiltration and no humoral response were detected in animals that received allografts. Despite the difference in immune response, the biological effects of xenografts or allografts did not differ significantly. (Circ Res. 1999;84:384-391.)

**Key Words:** tissue engineering ■ restenosis ■ perivascular ■ heparin ■ thrombosis

The blood vessel is a structure of extraordinary sophistication. The physical continuity of vascular cells and tissues that make up the blood vessel wall contributes to its structural integrity while also maintaining homeostasis through biochemical regulation. The endothelial monolayer that lines the normal blood vessel serves as a 2-fold bioregulator of vascular physiology. The endothelium provides structural integrity to the blood vessel by forming a continuous, selectively permeable, thromboresistant barrier between circulating blood and the arterial wall. At the same time, it also produces and supplies products that control blood flow, vessel tone, thrombosis, platelet activation, adhesion, and aggregation, leukocyte adhesion, monocyte infiltration, and smooth muscle cell migration and proliferation. Endothelial injury such as occurs after angioplasty not only removes the physical barrier but also interferes with the biochemical regulatory potential of the blood vessel and sets into motion a sequence of events that leads to the proliferation and migration of smooth muscle cells, resulting in obstructive arterial lesions. This process of restenosis leads to critical narrowings in 30% to 50% of patients after angioplasty, the failure of bypass grafts, and proliferative vascular disease in transplanted organs.

Tissue engineering enables the development of biological substitutes that restore, maintain, or improve tissue function while also providing substrates by which to examine structure-function relationships for specific tissues or organs. Cells may be implanted at sites distant or in different configurations from their original state, providing an opportunity to examine added benefits of cell secretory function to regulation of tissue biology above that imposed by preservation of tissue architecture. This may be especially important in vascular biology, in which the autocrine, paracrine, and endocrine function of the endothelium is rapidly emerging. Innovative studies have attempted to recreate the structure of the blood vessel by autologous endothelial cell transplantation, or endovascular stents. Yet, the question remains as to whether reestablishing biochemical control of vascular homeostasis also requires reestablishing the ordered architecture of the blood vessel. We have demonstrated through the use of tissue-engineered endothelial cells that the biological effect of these cells on blood vessel regulation is maintained even when they are implanted at a distance from the lumen. Engrafted endothelial cells on 3-dimensional polymer matrices, implanted in the perivascular space of injured rat carotid...
arteries, reduced intimal thickening by 88%. This therapy was 3-fold more effective than the isolated administration of heparin, an inhibitor of smooth muscle cell proliferation in vitro. These experiments supported the hypotheses that endothelial control over vascular repair is derived from the secretion of endothelial cell–based products and need not emanate from the luminal surface.

In the present study, we have addressed important questions relating to the biological effects of perivascular endothelial cell graft implantation. First, we examined whether allotransplantation of endothelial cell grafts was effective in controlling vascular repair in a porcine carotid artery model of vascular injury, thought to be less responsive to certain growth-regulatory agents than simpler models. Second, we explored whether xenotransplantation, a central issue in developing safe and practical clinical strategies, was more or less effective than allotransplantation. We now report that perivascular tissue-engineered endothelial cell implants exert profound control over intimal growth after arterial injury in pigs. Furthermore, despite an increased immune response to cross-species transplantation, beneficial control of vascular repair was maintained. These results provide insight into how the endothelium controls vascular homeostasis and are a further step toward the development of clinically viable strategies for modulating vascular repair after injury.

Materials and Methods

Cell Engraftment

Bovine aortic endothelial cells (BAEs) were isolated from freshly excised aortas of 3- to 4-week-old calves (Area and Sons, Hopkinton, Mass) as previously described. Porcine aortic endothelial cells (PAEs) were obtained from Cell Systems (Kirkland, Wash). Cells were maintained at 37°C in a humidified 5% CO2/95% air incubator and were seeded at a density of 3 × 10^4 cells/mL PAE cell suspension at passage 6 in DMEM supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), glutamine (2.0 mmol/L), and 10% calf serum (all from Life Technologies, Inc). Gelfoam was supplied sterile by Upjohn and cut into 3.0-cm blocks. The Gelfoam blocks were hydrated in PBS before implantation. The cells were placed at 37°C in a humidified 5% CO2/95% air incubator and allowed to adhere for 2 hours. The cells were placed at 37°C in a humidified 5% CO2/95% air incubator and allowed to adhere for 2 hours. The cell-loaded Gelfoam blocks were subsequently placed in 17 × 100-mm polypropylene tubes containing 2 mL of medium and incubated for up to 2 weeks at a 45° angle. Growth medium was changed every 72 hours. The number of cells adhered to the scaffolds was determined after the blocks were washed with HBSS (Life Technologies, Inc) and digested with collagenase (1 mg/mL, type I, Worthington Biochemical Corp). Cell viability was checked by trypan blue exclusion as well as a LIVE/DEAD viability/cytotoxicity kit supplied by Molecular Probes in which live and dead cells within the matrices were visualized by confocal laser-scanning microscopy (BioRad MRC 600). Endothelial cell-Gelfoam matrices were implanted postconfluent, because this is optimal for vascular smooth muscle cell growth inhibition.

Heparin Release Devices

Poly(dl-lactide-glycolide) with a copolymer ratio of 70/30 (lactide/glycolide) and molecular mass of 100 000 Da was obtained from Polysciences. Heparin (160 U/mg, from porcine intestinal origin) was obtained from Pharmacia & Upjohn. Heparin-loaded microspheres were prepared by a solvent extraction method carried out in a double-emulsion system. A microsphere-alginate suspension was poured into a Petri dish and freeze-dried. The film was then cross-linked by immersion in 3% CaCl2 for 30 minutes. Devices were formulated to release heparin at the highest possible dose before bleeding complications ensued. This dose (~3.0 μg/d) and mode of delivery have previously been demonstrated to maximize the inhibition by heparin of neointimal hyperplasia and was twice the rate of release of heparin sulfate proteoglycan (HSPG) from the endothelial implants. The heparin devices demonstrated a consistent release profile over a 25-day period after an initial burst in the first 10 to 24 hours. Therefore, devices were prereleased for ~16 hours in a saline solution containing 1.0 mmol/L CaCl2.

Animals

The ability of the endothelial cell Gelfoam grafts to reduce intimal hyperplasia and thrombosis when wrapped around balloon-injured porcine carotid arteries was assessed. The carotid artery was chosen for these experiments because it provided surgical access with minimal trauma in a well-described model. This study conformed to the guidelines specified in the National Institutes of Health Guide for Care and Use of Laboratory Animals and was approved by the Institutional Animal Care and Use Committee of the Veterans Association Medical Center (West Roxbury, Mass). Twenty-seven male domestic pigs (40.1 ± 3.4 kg) were obtained from Animal Biotech, Inc (Danboro, Pa). Anesthesia was induced with intramuscular ketamine (1000 mg), xylazine (150 mg), and atropine (0.6 mg) and maintained with inhaled isoflurane (0.5% to 1.5%) via an endotracheal tube. All animals also received intravenous ceftazolin (300 mg, before and after surgery) to prevent infection. The intra-arterial pressure and ECG were continuously monitored throughout the procedure.

Surgical Procedure

Right femoral arterial access with an 8F sheath was obtained via cutdown, and an 8.0-mm-diameter angioplasty balloon (Cordis) was advanced to the common carotid artery under fluoroscopic guidance. Angiography was performed and recorded by cineradiography. The right and left carotid arteries were injured by 30-second balloon inflations between 8 to 10 atm (5 inflations per side, in overlapping segments). The balloon to artery ratio (1.26 ± 0.03) did not vary significantly between treatment groups. After final angiography to assess vessel patency, a midline neck incision was made, and both left and right common carotid arteries were isolated and gently wrapped with Gelfoam containing BAEs (n = 8 arteries, 2 arteries per animal), PAEs (n = 10 arteries, 2 arteries per animal), or no cells (n = 10 arteries, 2 arteries per animal); heparin release devices (n = 6 arteries, 2 arteries per animal); or nothing (n = 8 arteries, 2 arteries per animal). The carotid sheath was closed to immobilize the device and sutured to facilitate the location of implants at sacrifice. In another group of animals, to determine if the placement of an implant next to an artery induced injury, a 4.0-mm-diameter angioplasty balloon (Cordis) was expanded in the left and right carotid arteries so as not to fracture the internal elastic lamina. The balloon to artery ratio (0.95 ± 0.05) was expanded in the left and right carotid arteries and treated with Gelfoam containing BAEs (n = 4 arteries, 2 arteries per animal), no cells (n = 4 arteries, 2 arteries per animal), or left unwrapped (n = 4 arteries, 2 arteries per animal).

Tissue Processing

On the 28th postoperative day, animals were euthanized with intravenous potassium chloride (40 mEq), and the carotid arteries were perfused at 100 mm Hg for 10 minutes with 4% paraformaldehyde in 0.1 mol/L PBS (pH 7.4) to fix the arteries in situ. The arteries with attached implants were then isolated, and the vessel was divided into 3 10-mm-long segments: proximal to the wrap, at the wrap (middle), and distal to the wrap. The segments were paraffin-embedded. Five-micrometer sections were obtained from the proximal, middle, and distal segments and stained with Verhoeff’s elastin stain. Morphometric analysis was performed on all segments. The intimal (I), medial (M), and lumen (L) areas as well as the internal elastic lamina (IEL)
circumference and IEL fracture length (F) were measured using computerized digital planimetry with a video microscope and customized software (Figure 1). Vessel size was assessed by measuring the area circumscribed by the outer border of the external elastic lamina (EEL area). Morphometric measurements were made by an observer who was blinded to the treatment groups. The extent of injury was represented by the fracture length of the IEL, normalized for the size of the artery by the circumference of the IEL: injury index = F/IEL. Intimal hyperplasia was also normalized by the total artery wall area: I/(I+M). A restenosis index was then calculated taking into account the degree of injury: restenosis index = [I/(I+M)]/F/IEL. The residual lumen was also measured, which reflected the change in vessel geometry after injury and repair. In a normal artery, the ratio approximates 1; as the extent of intimal proliferation increases, the vessel lumen is altered, and the ratio decreases. The residual lumen was defined as L/(L+I). Dissected arteries, as determined by frank rupture of the EEL and complete thrombosis, were excluded from all analyses (n=5 arteries).

Immunohistochemistry
To examine the cellular response to device implantation, the explanted devices were subjected to immunohistochemical analysis. Five-micrometer paraffin sections were cut and antigen retrieval performed by microwave heating for 10 minutes in a 0.01 mol/L citrate buffer, pH 6.0. Leukocytes, T and B lymphocytes, and monocytes/macrophages were identified by an avidin-biotin peroxidase complex method. The primary antibodies were mouse anti-porcine CD45, to identify leukocytes (Serotec, Raleigh, NC; 1:10 dilution); rabbit anti-human CD3, to identify T cells (Dako Corp, Carpinteria, Calif; 1:50 dilution); mouse anti-human CD79a, to identify B cells (Dako Corp; 1:25 dilution); and mouse anti-human MAC387, to identify monocytes/macrophages (Serotec; 1:50 dilution). Purine spleen was used as a positive control, and rabbit mouse IgG was used as a negative control. Primary antibodies were applied for 1 hour at room temperature, and all sections were counterstained with Mayer’s hematoxylin solution (Sigma Chemical Co). Neutrophils were identified by their multilobed nuclei. For every specimen, 4 nonoverlapping fields (∗600) were examined per arterial cross section. Each specimen was graded, on the basis of the number of positively stained cells per field, as negative, weakly positive, moderately positive, or strongly positive. The results for each treatment group were averaged. Artery and Gelfoam sections were also stained with rabbit anti-human von Willebrand factor (vWF, Dako Corp; 1:1000 dilution) to identify endothelial cells.

Fluorescence Immunocytochemistry
Sera were collected from pigs on the 28th postoperative day and tested for a humoral response to the material or the transplanted endothelial cells. Sera were collected from pigs that received Gelfoam endothelial cell implants, Gelfoam implants without cells, or no implants and tested for antibody production against either the same lot of endothelial cells as those used for implantation or Gelfoam containing no cells. Cells were grown to confluence on glass coverslips and fixed in 3% paraformaldehyde. Because the nuclear stain, propidium iodide, binds to both RNA and DNA, samples containing cells were incubated with 100 μg/mL RNase (Worthington Biochem Corp) at 37°C for 30 minutes before staining. All samples were then blocked with rabbit serum (Life Technologies, Inc) for 30 minutes at room temperature, followed by nonfat dry milk (5.0% in PBS, blotting grade, BioRad Laboratories) for 30 minutes at room temperature. After washing with PBS, samples were incubated with undiluted sera from animals that received implants or sera from animals that received no implants. Control porcine sera were also obtained from Life Technologies. After 2 hours at 4°C, the samples were incubated for 1 hour with FITC-conjugated rabbit anti-pig IgG (diluted 1:20, Sigma Chemical Co) and propidium iodide (10 mg/mL, 1:100 dilution, Calbiochem). Controls for nonspecific staining were stained only with the secondary antibody. The samples were examined with a confocal laser-scanning microscope. Six fields were selected for each sample containing cells by locating confluent areas of cells with propidium iodide nuclear staining. The intensity of immunofluorescence was measured using customized software.

Statistical Analysis
All data are presented as mean±SE. Statistical analysis comparing treatment groups used a nonpaired t test. Values of P<0.05 (2-tailed analysis) were considered significant.

Results

Growth Kinetics and Viability
BAEs and PAEs cultured within Gelfoam matrices lined the interstices of the 3-dimensional matrix and followed a growth pattern similar to that observed for cells cultured on tissue culture dishes (Figure 2). Cell doubling was observed approximately every 36 hours. Cell viability, as evaluated by trypan blue exclusion and a LIVE/DEAD cytotoxicity kit, remained at 95% over the 2-week culture course.

Endothelial Cell Implant Inhibition of Intimal Thickening and Thrombosis
The pigs used in the present study were randomly selected to receive one of the following treatments after balloon injury:
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Of the 27 pigs used, one that belonged to the sham group died without cells, heparin release devices, or no implant (sham). Gelfoam-BAE or Gelfoam-PAE implants, Gelfoam implants and medial disruption.

Figure 3. Photomicrographs of cross sections of arteries show the effects of perivascular endothelial cell grafts on neointimal formation 28 days after balloon injury of porcine carotid arteries (Verhoeff's elastin stain, magnification ×20 for panels A, C, and E; magnification ×40 for panels B, D, and F). Porcine (A and B) and bovine (C and D) endothelial cell grafts reduced intimal thickening when compared with control arteries (E and F). Black arrows point to the neointimal lesions; white arrows point to IEL and medial disruption.

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Gelfoam-BAE or Gelfoam-PAE implants, Gelfoam implants without cells, heparin release devices, or no implant (sham). Of the 27 pigs used, one that belonged to the sham group died 24 hours after surgery (96% survival rate) and was excluded from analysis. All neck incisions of the remaining pigs healed well and all animals gained weight throughout the 28-day postoperative period. Morphometric analysis of the proximal, middle, and distal segments of each artery revealed no significant differences in the injury response between the 3 segments for any of the treatment groups. Therefore, measurements made from 3 sites on each vessel were averaged so that each vessel resulted in 1 data point. Arteries with an intact IEL were deemed uninjured and were excluded from analysis (n = 2 arteries).

Four weeks after injury to the carotid arteries, extensive neointimal proliferation or occlusive organized thrombus was observed at the site of vessel injury in control animals. The restenosis index of control animals receiving sham carotid exposure (1.30 ± 0.20) or Gelfoam (1.41 ± 0.30) did not differ significantly. Arteries wrapped with Gelfoam containing either BAEs or PAEs showed a significant decrease in both the restenosis index and thrombosis (Figure 3, Table 1). The restenosis index in arterial segments treated with BAEs or PAEs implants was reduced by 46% to 0.70 ± 0.08 (P < 0.05) or by 54% to 0.60 ± 0.06 (P < 0.05), respectively (Figure 4), compared with sham animals. There was no significant difference between the restenosis indices of the animals that received PAE or BAE implants. The perivascular release of heparin from hydrogel films at twice the rate of release of HSPG from endothelial cell implants did not significantly reduce the restenosis index compared with control arteries (1.12 ± 0.23). Gelfoam matrices containing endothelial cells also significantly reduced thrombosis (Figure 4). Extensive occlusive organized thrombosis was observed in 4 arteries (40%) of the control Gelfoam group, 2 arteries (33%) of the sham group, 1 artery (25%) of the heparin group, and none of the arteries of either the Gelfoam-PAE group (0%, P < 0.05 compared with control arteries) or the Gelfoam-BAE group (0%, P < 0.05 compared with control arteries). There were no significant differences in EEL area between treatment groups (Table 1), making an effect of Gelfoam with or without cells on vessel remodeling unlikely. vWF staining of sectioned arteries from each of the treatment groups revealed complete reendothelialization in only 2 arteries, 1 from the heparin group

![Image of photomicrographs](http://circres.ahajournals.org/)

**Figure 3.** Photomicrographs of cross sections of arteries show the effects of perivascular endothelial cell grafts on neointimal formation 28 days after balloon injury of porcine carotid arteries (Verhoeff’s elastin stain, magnification ×20 for panels A, C, and E; magnification ×40 for panels B, D, and F). Porcine (A and B) and bovine (C and D) endothelial cell grafts reduced intimal thickening when compared with control arteries (E and F). Black arrows point to the neointimal lesions; white arrows point to IEL and medial disruption.

### TABLE 1. Histopathological Characteristics of Porcine Carotid Arteries After Balloon Injury

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Empty Gelfoam Matrices</th>
<th>Sham-Operated Animals</th>
<th>Heparin Release Devices</th>
<th>PAE Implants</th>
<th>BAE Implants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of vessels, n</td>
<td>10</td>
<td>6</td>
<td>4</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Occluded vessels, %</td>
<td>40</td>
<td>33</td>
<td>25</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EEL area, mm²</td>
<td>10.94 ± 0.41</td>
<td>10.92 ± 0.82</td>
<td>9.13 ± 0.98</td>
<td>10.74 ± 2.05</td>
<td>9.92 ± 1.04</td>
</tr>
<tr>
<td>Media area, mm²</td>
<td>4.61 ± 0.22</td>
<td>3.51 ± 0.11</td>
<td>4.75 ± 0.49</td>
<td>4.0 ± 0.36</td>
<td>4.95 ± 0.23</td>
</tr>
<tr>
<td>Intima area, mm²</td>
<td>2.09 ± 0.93</td>
<td>1.7 ± 0.8</td>
<td>1.07 ± 0.29</td>
<td>0.85 ± 0.18</td>
<td>0.55 ± 0.05</td>
</tr>
<tr>
<td>Lumen area, mm²</td>
<td>3.55 ± 0.29</td>
<td>3.60 ± 1.0</td>
<td>2.99 ± 0.59</td>
<td>3.91 ± 1.16</td>
<td>3.10 ± 0.77</td>
</tr>
<tr>
<td>IEL length, mm</td>
<td>7.85 ± 0.45</td>
<td>8.42 ± 0.55</td>
<td>8.40 ± 0.55</td>
<td>7.60 ± 1.10</td>
<td>7.26 ± 0.81</td>
</tr>
<tr>
<td>Fracture length, mm</td>
<td>1.73 ± 0.30</td>
<td>2.02 ± 0.30</td>
<td>1.34 ± 0.05</td>
<td>1.90 ± 0.22</td>
<td>1.23 ± 0.15</td>
</tr>
<tr>
<td>Residual lumen (ratio)</td>
<td>0.63 ± 0.07</td>
<td>0.68 ± 0.13</td>
<td>0.74 ± 0.07</td>
<td>0.82 ± 0.02</td>
<td>0.85 ± 0.04</td>
</tr>
<tr>
<td>Injury index*</td>
<td>0.22 ± 0.04</td>
<td>0.24 ± 0.04</td>
<td>0.16 ± 0.02</td>
<td>0.25 ± 0.02</td>
<td>0.17 ± 0.01</td>
</tr>
<tr>
<td>Restenosis index †</td>
<td>1.41 ± 0.30</td>
<td>1.3 ± 0.20</td>
<td>1.12 ± 0.23</td>
<td>0.70 ± 0.08‡</td>
<td>0.60 ± 0.06†</td>
</tr>
</tbody>
</table>

*Injury index = IEL fracture length/IEL circumference.
†Restenosis index = (I/I + M)/I/IEL.
‡P < 0.05 compared with control arteries.
and 1 from the control Gelfoam group. Therefore, it is also unlikely that rates of reendothelialization explain the differences observed in intimal thickening between treatment groups.

Non–Balloon-Injured Arteries
Six additional pigs were randomly selected to receive a surgical procedure in which a 4.0-mm balloon was advanced and inflated in the left and right carotid arteries. These pigs served as a control to determine if placement of the perivascular implants induced injury or vascular inflammation to the arteries independent of balloon-induced injury. Because this balloon did not touch the arterial wall, no fracture of the IEL was anticipated. Morphometric analysis confirmed the absence of injury to any of the arteries (data not shown), and there was no apparent difference in tissue integrity or vascular inflammation between the noninjured arteries that received implants and the arteries that did not.

Cell-Mediated Immune Response and Device Fate
Immunological studies revealed evidence of cellular infiltration into and around the devices at 28 days. Table 2 summarizes the expression of CD45 (leukocyte marker), CD3 (T-cell marker), and CD79a (B-cell marker) positive cells as well as the presence of monocytes and macrophages within or surrounding the explanted grafts. In the Gelfoam-BAE group, T cells were abundant within the graft, whereas B cells were found at the periphery of the graft (Figure 5). Macrophages and a few neutrophils had also infiltrated into the BAE grafts. T cells and B cells were found in or around both the Gelfoam-PAE and control Gelfoam explants, although there were markedly fewer positively stained cells compared with the xenografts (Figure 5). Monocytes and macrophages were

![Figure 4. Effects of endothelial cell implants on the restenosis index and thrombosis of balloon-injured porcine carotid arteries.](image)

**Figure 4.** Effects of endothelial cell implants on the restenosis index and thrombosis of balloon-injured porcine carotid arteries. Restenosis index = [(I/I[1])/(F/IEL)]. A, Bar graph shows that endothelial cell implants decreased the restenosis index by 54% and 46% for porcine and bovine cells, respectively. *P<0.05 vs control arteries. B, Bar graph shows a decrease in occlusive thrombosis for arteries wrapped with endothelial cell grafts compared with control and heparin-treated arteries. *P<0.05 vs control arteries.

![Figure 5. A, CD3 (T-cell) staining of Gelfoam-BAE explant.](image)

**Figure 5.** A, CD3 (T-cell) staining of Gelfoam-BAE explant. On the 28th postoperative day, marked cellular infiltration is found in the graft. B, CD3 staining of Gelfoam-PAE explant. On the 28th postoperative day, fewer CD3-positive cells are found within the matrix, magnification ×100. C, Photomicrograph of a section through a Gelfoam matrix containing endothelial cells harvested 28 days after implantation and stained for vWF. The figure illustrates few remaining endothelial cells within the polymer matrix after the 28-day period. Brown cells are vWF-positive cells, magnification ×600.

### Table 2. Expression of CD45-Positive Cells, CD3-Positive Cells, CD79a-Positive Cells, and Monocytes/Macrophages

<table>
<thead>
<tr>
<th>Explants</th>
<th>Gelfoam</th>
<th>PAE</th>
<th>BAE</th>
<th>Alginate</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45 (leukocyte)</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++*</td>
</tr>
<tr>
<td>CD3 (T cell)</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++*</td>
</tr>
<tr>
<td>CD79a (B cell)</td>
<td>±</td>
<td>±*</td>
<td>+*</td>
<td>+*</td>
</tr>
<tr>
<td>Monocytes/macrophages</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+*</td>
</tr>
</tbody>
</table>

* + + indicates strongly positive; +, moderately positive; ±, weakly positive; and –, negative.
* Positive cells surround graft and were not found within the matrix.
also much less abundant in the PAE group, and none were found in the control Gelfoam group. T cells, B cells, and macrophages were found surrounding the heparin release device; however, no positively stained cells were found within the device. Few neutrophils were found in the Gelfoam, Gelfoam-PAE, and alginate groups. Sections through explanted Gelfoam-containing PAEs or BAEs, stained with vWF, revealed only sparse endothelial cells remaining within the matrices (Figure 5). Although endothelial cells were detected within the explants, cell loss had occurred during the 28-day period. Migration of the implanted cells to the arterial lining was not detected in any of the arterial segments.

**Xenogeneic and Allogeneic Humoral Immune Response**

The results of sera immunofluorescence after xenograft and allograft implantation are shown in Figure 6. In both sets of experiments, control sera were from sham animals or from porcine sera obtained from Life Technologies. There was no difference in fluorescent intensity between these 2 controls. The same lot of BAEs that was used for implantation was also cultured as a monolayer on coverslips. Sera from animals that received BAE implants tested against BAEs showed a significant increase in fluorescent intensity (89.6 ± 1.7) compared with sera from animals that did not receive an implant (38.2 ± 12.4; *P* < 0.05). The increase in fluorescent intensity was not detectable in sera dilutions above 1:50. Sera from animals that received Gelfoam or Gelfoam-BAE implants tested against Gelfoam showed no greater immunofluorescence (8.0 ± 5.3) compared with sera from animals that did not receive an implant (7.2 ± 4.2). This reaction indicates an increased amount of antibodies against cell surface antigens located on the implanted bovine endothelial cells in pigs 28 days after BAE graft implantation. Sera from pigs that received PAE implants were tested by an identical set of experiments (Figure 6). Sera from animals that received PAE implants and tested against PAEs (56.4 ± 4.2) or Gelfoam (3.5 ± 2.0) showed no significant increase in fluorescent intensity compared with control (63.6 ± 4.4 and 10.4 ± 6.2, respectively). These results show that after 28 days, an increase in antibodies to either PAEs or Gelfoam was not detected in sera from pigs that received PAE implants.

**Discussion**

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. Restenosis remains the major limitation of all interventional procedures. Cell culture and small animal data support the role of endothelial-based products in regulating repair after injury. Unfortunately, there is a lack of correlation between small animal models and human vascular diseases. For example, despite the data supporting the regulatory role of heparin-like compounds, exogenous heparin preparations have yet to show any clinical benefit in vascular proliferative diseases. These observations may stem from the increased complexity of the lesions with increased injury or higher species. More complex lesions may not be controlled by the isolated administration of a single product.

The swine as an experimental model of vascular injury offered advantages in the present study on restenosis. The carotid arteries were easily accessible to surgical manipulation and standard catheterization techniques. In addition, compared with smaller animal models, the swine model has more in common with humans with respect to platelet coagulation and histological characteristics. Tissue-engineered endothelial cells have been proposed as a strategy to restore, maintain, and improve blood vessel function. The isolation and subsequent transplantation of endothelial cells onto polymeric surfaces were first reported by Herring et al as a means to improve the long-term patency of small-diameter bypass grafts. Since this study, there have been numerous reports of endothelial cell growth on polymer surfaces. Seeding endothelial cells at the luminal interface is difficult to achieve in practice, applies a limited number of endothelial cells during periods of intense injury and response, and may not be necessary.
for the secretion of biochemical vasoregulatory compounds. Engraftment of PAEs or BAEs within the matrices of a 3-dimensional polymer scaffold allowed us to implant a high density of endothelial cells in the perivascular space of balloon-injured porcine carotid arteries. The hypothesis of the present study was that, in this position, the cells could maintain biochemical control without the need for restoration of the barrier function and in a manner superior to the perivascular administration of a single isolated endothelial product.

Both BAEs and PAEs were grown in Gelfoam matrices with preservation of their viability and normal growth characteristics. Endothelial cells grown in Gelfoam also retain their normal biochemical activity. The amount of glycosaminoglycans and heparan sulfate produced by cells grown on Gelfoam was statistically similar to the amount produced by cells grown on tissue culture dishes. When implanted around injured arteries, porcine and bovine endothelial cell grafts reduced the restenosis index by 54% and 46%, respectively. Neither Gelfoam alone nor heparin released from perivascular devices had an effect on the restenosis index. Heparin is an inhibitor of cultured smooth muscle cell growth, and natural heparin-like compounds may also be central to the in vivo reparative process to limit accelerated arteriosclerosis. However, the inhibitory effects of heparin on smooth muscle cell proliferation in pig models have been inconsistent. It appears from the data obtained in the present study that endothelial control over vascular repair in a porcine model resulted from the secretion of all the cell-based products, not solely from a single agent. In a normal blood vessel, the endothelium is able to maintain a delicate balance between growth promotion and inhibition, vasoconstriction and vasodilation, by synthesis and release of a number of factors. These factors include prostanooids, nitric oxide, endothelins, angiotensins, eicosanoids, glycosaminoglycans, and a myriad of growth factors. Our data reflect the cooperative action of these factors, released to injured arteries from perivascular endothelial cell grafts, at inhibiting intimal thickening and thrombosis in a porcine model.

The increasing appreciation for the potential of tissue-engineered implants requires that we investigate host responses to both xenografts and allografts. The ability to implant either xenogeneic or allogeneic cells would greatly enhance the clinical applications of tissue-engineered grafts. The data in the present study suggest that the implantation of cross-species endothelial cell grafts elicited both a cell-mediated and humoral immune response in experimental animals. T and B lymphocytes, macrophages, and neutrophils were found within or surrounding the explanted xenografts after 28 days. The majority of infiltrating cells were T cells, as indicated by anti-CD3 staining. The rest of the infiltrating cells were macrophages. Sera obtained at 28 days from pigs that received BAE implants displayed increased cell surface staining to cultured BAEs at low titers. The antigenic specificity of this humoral response remains as yet unclear. Cellular infiltrates were not as abundant in same-species endothelial cell grafts, with fewer T and B lymphocytes, macrophages, and neutrophils found in or around the explanted allografts. Moreover, a humoral immune response to allogeneic cells was not detected.

The transplantation of cells across immunological barriers has previously been reported. For example, allogeneic chondrocytes embedded within a collagen matrix were implanted in rabbits without eliciting a significant immune reaction. The successful transplantation of the cells was attributed to protection from antibodies and infiltrate by the nonvascularized matrix. However, a recent study investigated the immune response to xenograft cartilage transplants, previously thought to be immunologically protected. Although the grafts did not result in hyperacute rejection, they did undergo chronic rejection after several weeks, characterized by infiltrating T lymphocytes, macrophages, and a humoral response. The endothelial xenografts in the present study also resulted in a chronic rejection mechanism demonstrated by leukocyte infiltration and a humoral response, evident at 1 month. Although there was also evidence of a chronic immune response to the allografts, the lesser amount of cellular infiltrate and lack of a humoral response at 28 days may indicate prolonged graft survival compared with the xenografts. The insignificant difference in biological effects between PAE and BAE grafts suggests that the grafts are able to affect thrombus formation, cellular recruitment, and subsequent smooth muscle cell proliferation before undergoing rejection or cell loss from other causes. Further exploration of how tissue-engineered endothelial cell grafts control vascular repair, particularly in settings of more chronic vascular injury, will afford insight into the structure and function of the blood vessel wall and into how experimentally effective techniques may be brought to clinical fruition.

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