

Perivascular Endothelial Implants Inhibit Intimal Hyperplasia in a Model of Arteriovenous Fistulae: A Safety and Efficacy Study in the Pig

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Key Words

Anastomosis · Perivascular endothelial implants · Endothelium · Intimal hyperplasia · Arteriovenous fistula

Abstract

Vascular access complications are a major problem in hemodialysis patients. Native arteriovenous fistulae, historically the preferred mode of access, have a patency rate of only 60% at 1 year. The most common mode of failure is due to progressive stenosis at the anastomotic site. We have previously demonstrated that perivascular endothelial cell implants inhibit intimal thickening following acute balloon injury in pigs and now seek to determine if these implants provide a similar benefit in the chronic and more complex injury model of arteriovenous anastomoses. Side-to-side femoral artery-femoral vein anastomoses were created in 24 domestic swine and the toxicological, biological and immunological responses to allogeneic endothelial cell implants were investigated 3 days and 1 and 2 months postoperatively. The anastomoses were wrapped with polymer matrices containing confluent porcine aortic endothelial cells (PAE; n = 14) or control matrices without cells (n = 10). PAE implants significantly reduced intimal hyperplasia at the anastomotic sites compared to controls by 68%

(p < 0.05) at 2 months. The beneficial effects of the PAE implants were not due to differences in the rates of reendothelialization between the groups. No significant immunological response to the allogeneic endothelial cells that impacted on efficacy was detected in any of the pigs. No apparent toxicity was observed in any of the animals treated with endothelial implants. These data suggest that perivascular endothelial cell implants are safe and reduce early intimal hyperplasia in a porcine model of arteriovenous anastomoses.

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Introduction

Vascular access failure is the major complication in providing care to patients on hemodialysis therapy [1]. Arteriovenous dialysis access fistulae frequently develop stenoses and occlusions at the anastomosis, commonly on the venous anastomotic side [2, 3]. Histologic examination of segments removed from patients with anastomotic stenosis revealed extensive intimal hyperplasia consisting of smooth muscle cells and extracellular matrix [2]. In a recent clinical study of radiocephalic arteriovenous fistulae, stenoses were found in all 25 of the fistulae studied at 3 months [4]. The current therapy for arteriovenous fistu-

la stenosis is either surgical revision or angioplasty with or without stenting. Surgical treatment may be risky in these typically multimorbid patients [5], and the long-term results of angioplasty and stenting are generally disappointing due to their own failure rates [6–8]. The goal of vascular access for hemodialysis, therefore, is to maintain the original fistula site with little or no intimal hyperplasia and thus allow for flow rates to support the dialysis treatment.

The exact causes of intimal hyperplasia at the anastomotic site of an arteriovenous fistula are not completely understood. Isolation of veins and arteries followed by exposure of the vein segment to arterial blood flow and pressure can cause unavoidable ischemia and reperfusion injury [3, 9]. Surgical manipulation such as suturing can also result in direct trauma to the endothelium and smooth muscle cells of the media in both veins and arteries [3, 9]. These phenomena are different from the response of vascular tissue to the dramatic, acute injury that occurs after angioplasty. For example, while endothelial damage most likely plays a role in both types of vascular injury, anastomotic intimal hyperplasia may also represent an intrinsic adaptive response of the medial smooth muscle cells themselves to the increase in wall stress [3, 10, 11]. Previous studies in other labs have suggested that the degree and type of vascular damage may be very important in the stimulation of intimal proliferation [12]. It therefore remains unclear whether the complexity of these lesions makes them resistant to those therapies found to be effective in simpler models of vascular injury.

Perivascular endothelial implants have proven effective at diminishing intimal thickening 3 months after angioplasty of porcine carotid arteries [13–15]. In the present study, we investigated the vascular response to injury in arteriovenous anastomoses wrapped with allogeneic endothelial implants. We hypothesized that by delivering physiologic levels of normal endothelial growth-regulatory compounds to the adventitia and media of anastomotic sites, perivascular endothelial cell implants could control vascular repair in a chronic injury model. Additionally, through the use of standard hematology, clinical chemistry and coagulation parameters as well as microscopic and macroscopic pathology, we evaluated the safety of the perivascular implants. We now report that the application of endothelial implants to the adventitia of arteriovenous anastomoses resulted in no apparent toxicity and significantly reduced the intimal hyperplasia index at the anastomotic sites 2 months after surgery.

Materials and Methods

Formulation and Testing of Endothelial Implants

Porcine aortic endothelial cells (PAE; Cell Applications, San Diego, Calif., USA) were cultured in Gelfoam as previously described [13–15]. $4.0 \times 1.0 \times 0.3 \text{ cm}^3$ blocks of sterile Gelfoam (Pharmacia & Upjohn, Kalamazoo, Mich., USA) were seeded with 1.5×10^5 cells per sponge. The number of cells attached to the Gelfoam was determined after digestion with collagenase (Worthington Biochemical Corp., Freehold, N.J., USA), and cell viability was assessed by trypan blue exclusion. Endothelial cells were grown to confluence before implantation. Microbiological and functional testing was performed on representative samples before implantation. Functional testing was also performed on in vitro cohorts up to 2 weeks after implantation. The production of heparan sulfate by endothelial cells cultured in Gelfoam sponges was used as a marker of cell function. Conditioned medium was collected from postconfluent endothelial cells cultured in Gelfoam. Total sulfated glycosaminoglycan levels in conditioned media was determined using dimethylmethylene blue, and the amount of heparan sulfate was assessed after enzymatic digestion [16]. Sterility and endotoxin testing was performed to demonstrate that the implants were free from microbial contamination.

In vivo Safety and Efficacy of Endothelial Cells Cultured on Gelfoam

The safety of the endothelial implants and their ability to reduce intimal hyperplasia when wrapped around arteriovenous anastomoses were assessed. This study conformed to the United States Department of Agriculture regulations and National Research Council guidelines and to the guidelines specified in the National Institutes of Health 'Guide for Care and Use of Laboratory Animals'. The Institutional Animal Care and Use Committees of Harvard Medical School (Boston, Mass., USA) and Charles River Laboratories (Worcester, Mass., USA) approved the study. Twenty-four male and female domestic pigs, $27.5 \pm 1.3 \text{ kg}$, were obtained from Animal Biotech Inc. (Doylestown, Pa., USA) and from Charles River Laboratories (Pittsfield, N.H., USA). Animals were treated daily with aspirin (5–10 mg/kg) beginning 3 days prior to the day of surgery. All pigs received intramuscular buprenorphine (0.03 mg/kg) on the day of surgery. Anesthesia was induced with intramuscular xylazine (2 mg/kg), atropine (0.04 mg/kg), butorphanol (0.55 mg/kg) and ketamine HCl (25 mg/kg). Intravenous heparin was administered prior to surgery as a 100 U/kg bolus and maintained until the end of surgery by a 35 U/kg/h continuous infusion. The pigs were intubated and anesthesia was maintained with isoflurane inhalant (0.5–1.5%) via an endotracheal tube. Additional bolus doses of heparin were administered as necessary to maintain an activated coagulation time of $\geq 200 \text{ s}$.

Surgical Procedure

Right femoral arterial access was obtained via cut down. The femoral artery and vein were both mobilized at a distance of approximately 15 cm from their passage through the femoral ring distally. Adventitia from both vessels was removed at the site of their transection. The vessels were connected to each other in a continuous fashion to complete a side-to-side anastomosis (fig. 1). After completion of the anastomosis, blood flow through the fistula was confirmed. The anastomosis was then gently wrapped with two Gelfoam sponges containing PAE ($n = 14$ anastomoses, 1 per animal) or no cells ($n = 10$ anastomoses, 1 per animal). The implants were placed only at the anastomosis and did not extend beyond the suture site. After treat-

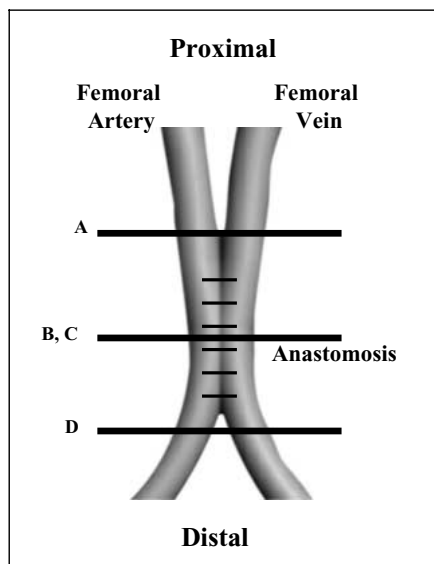


Fig. 1. Diagram of an arteriovenous fistula between the right femoral artery and the femoral vein. Histological sections were obtained at the anastomotic site (B, C) and 5 mm distal (D) and 5 mm proximal (A) to the anastomosis. Sections B and C were used to calculate the intimal hyperplasia index, as shown in table 1.

ment with Gelfoam was complete, the wounds were closed in layers to eliminate dead space.

Tissue Processing

On the 3rd, 29th or 57th postoperative day, animals were terminally anesthetized with sodium pentobarbital (65 mg/kg) and then perfused with PBS followed by formalin to fix the tissue in situ. All animals received heparin as a bolus injection (100 U/kg) prior to perfusion. The anastomotic sites, surrounding tissues, contralateral normal veins and arteries and draining lymph nodes were isolated from all pigs, trimmed, embedded in paraffin, sectioned and stained with hematoxylin and eosin. The anastomotic sites were sectioned as shown in figure 1. Sections were obtained through the Gelfoam/anastomotic sites and approximately 5 mm proximal and 5 mm distal to the Gelfoam/anastomotic sites from all animals. The slides were examined and scored by two board-certified veterinary pathologists. Sections from the anastomotic sites were also stained with mouse anti-porcine CD31 (PECAM-1; 1/100 dilution; Serotec Inc., Raleigh, N.C., USA) as previously described to assess reendothelialization [15]. Contralateral normal femoral vein and arteries were used as positive controls and mouse IgG was used as a negative control. Additional sections were obtained from animals euthanized at 1 and 2 months and stained with Verhoeff's elastin stain to assess the degree of intimal hyperplasia. A comprehensive necropsy, defined as the macroscopic examination of the external surface of the body, all orifices and the cranial, thoracic and abdominal cavities and their contents, was performed on animals euthanized after 57 days.

Morphometric Analysis

Histomorphometric analysis was performed on sections stained with Verhoeff's elastin stain from the anastomotic sites of animals

euthanized at 1 and 2 months. The intimal, medial and lumen areas were measured using computerized digital planimetry with a video microscope and customized software. The extent of intimal hyperplasia was determined by normalizing the intimal area by the total vessel wall area, as follows: $[I/(I + M)]$, where I represents the intimal area and M represents the medial area. The residual lumen was also measured and defined as follows: $L/(L + I)$, where I represents the intimal area and L represents the lumen area. Vessel size at the anastomotic site was assessed by measuring the area circumscribed by the outer border of the external elastic lamina (EEL) of both the vein and artery.

Serum Antibody Analysis

Sera were collected from pigs prior to surgery and at 3 days, 1 week, 3 weeks, 1 month, 6 weeks and 2 months. Total antibody binding to the same strain of endothelial cells as those used for implantation and to porcine lymphocytes (porcine T cells; PTC) pooled from domestic pigs was measured by flow cytometry following incubation with porcine serum and fluorescein isothiocyanate (FITC)-labeled goat anti-swine immunoglobulin (IgG). PAE or PTC (Ficolled blood passed through a Nylon wool column and pooled from 11 domestic pigs) were seeded at 2×10^5 and 4×10^5 cells/well, respectively. The cells plus appropriate serum (10 μ l serum per 1×10^5 cells) were incubated at 4°C with rocking for 30 min, and unbound antibodies were removed by washing three times with 200 μ l of FACS buffer. The cells were then incubated for 30 min at 4°C with a 1/50 dilution of FITC-conjugated goat anti-swine IgG (50 μ l/well). The cells were washed with FACS buffer and transferred to tubes for FACS analysis.

Hematology, Clinical Chemistry and Coagulation

Blood was also collected at the following time points for standard hematology, clinical chemistry and coagulation analysis: day 1 (prior to surgery) and 3 days, 1 month and 2 months after surgery. Potassium EDTA-anticoagulated blood samples were processed and analyzed for hematology parameters, serum samples were analyzed for clinical chemistry and citrated plasma samples were analyzed for coagulation parameters.

Statistical Analysis

All data are presented as mean \pm SE. Statistical analysis comparing treatment groups was performed using a nonpaired Student's t test. Values of $p < 0.05$ were considered significant.

Results

Microbial and Activity Testing of Gelfoam Implants

PAE-Gelfoam sponges were assayed for cell number, viability and heparan sulfate production. The number of PAE that could be recovered from Gelfoam sponges increased exponentially over time from $1.25 \times 10^5/\text{cm}^3$ on day 0 to $10\text{--}12 \times 10^5/\text{cm}^3$ when they reached confluence between days 10 and 11. Thereafter, the cell number was fairly constant between days 13 and 28. The viability of the cells cultured in Gelfoam was approximately 95%. Conditioned medium was prepared from in vitro cohorts of the implanted PAE-Gelfoam sponges at three

different time points after the PAE achieved confluence, i.e. days 13, 21 and 28. Significant levels of heparan sulfate were detected at each time point and the levels remained consistent during the postconfluence interval, i.e. 1.5 ± 0.1 , 1.4 ± 0.2 and 1.2 ± 0.1 μg of heparan sulfate/ 10^6 PAE on days 13, 21 and 28, respectively. Sterility and endotoxin tests were performed on both control and cell-containing sponges. There was no evidence of elevated endotoxin levels or microbial growth after 14 days of incubation.

Safety of Perivascular Endothelial Cell Implants

The pigs used in this study were randomly selected to receive one of the following treatments after the creation of side-to-side anastomoses: PAE-Gelfoam or Gelfoam without cells. Animals were euthanized at 3 days ($n = 2$ Gelfoam control, $n = 4$ PAE-Gelfoam), 1 month ($n = 4$ Gelfoam control, $n = 4$ PAE-Gelfoam) or 2 months ($n = 4$ Gelfoam control, $n = 6$ PAE-Gelfoam). All incisions healed well and all animals gained weight throughout the respective postoperative period. There were no unscheduled deaths during the time course of the study and all fistulae remained patent until the time of sacrifice. There were no notable differences in macroscopic changes detected in PAE-Gelfoam-treated animals compared to control animals at any of the three time points that could be attributed to the cell-containing implant. Microscopic changes were observed in animals from both groups and were considered common postsurgical sequelae unrelated to the actions of the implants. A full panel of hematological, serum chemistry and coagulation parameters was evaluated in this study. All mean hematologic and clinical chemistry values from both groups were within expected ranges. In addition, there were no significant differences in the values from animals treated with PAE-Gelfoam when compared to controls. All test values from both groups relating to coagulation analysis were within the expected ranges. There were no significant differences in these values from animals treated with PAE-Gelfoam when compared to control animals. Normal contralateral femoral arteries and veins were compared to anastomosed femoral arteries and veins in the same animal to rule out pathological findings due to vascular abnormalities specific to a particular animal. No abnormalities were found by histology in any of the contralateral arteries and veins isolated from animals sacrificed after 3 days or 1 or 2 months.

Efficacy of Perivascular Endothelial Cell Implants

Morphometric analysis was performed on pigs euthanized at 1 and 2 months (table 1). Four weeks after

Table 1. Histopathological characteristics of porcine arteriovenous anastomotic sites

Characteristics	Control Gelfoam	PAE-Gelfoam
<i>One month</i>		
Number of anastomoses	4	4
Intimal area, mm^2	2.35 ± 0.89	1.54 ± 0.69
Medial area, mm^2	8.02 ± 1.02	7.98 ± 1.4
Lumen area, mm^2	11.31 ± 5.01	10.74 ± 2.31
EEL area, mm^2	21.32 ± 6.68	20.26 ± 4.23
Residual lumen	0.84 ± 0.02	0.90 ± 0.04
Intimal hyperplasia index	0.20 ± 0.06	0.13 ± 0.04
<i>Two months</i>		
Number of anastomoses	4	6
Intimal area, mm^2	6.22 ± 2.67	1.34 ± 0.25
Medial area, mm^2	9.95 ± 2.08	10.01 ± 2.80
Lumen area, mm^2	18.59 ± 5.33	18.93 ± 7.32
EEL area, mm^2	34.76 ± 8.85	30.28 ± 10.13
Residual lumen	0.69 ± 0.10	0.89 ± 0.04
Intimal hyperplasia index	0.38 ± 0.04	$0.12 \pm 0.02^*$

Residual lumen and the intimal hyperplasia index were calculated by the formulas detailed in Materials and Methods. * $p < 0.05$ compared to control arteries.

implantation, cell-containing implants had reduced the intimal hyperplasia index at the femoral anastomotic site by 35% ($p =$ not significant). The extent of intimal hyperplasia, as measured by the intimal hyperplasia index, for control and treated anastomotic sites was 0.20 ± 0.06 and 0.13 ± 0.04 , respectively. Eight weeks after implantation, the extent of intimal hyperplasia remained essentially unchanged in treated anastomoses. However, more extensive neointimal hyperplasia was observed at the anastomotic sites in control animals. The intimal hyperplasia index in the anastomotic segments treated with PAE implants was significantly reduced by 68% to 0.12 ± 0.02 ($p < 0.05$), in contrast to that in control animals (0.38 ± 0.04) (fig. 2). Although the residual lumen was greater for PAE-treated compared to control anastomotic sites at both 1 and 2 months, the difference did not reach significance (table 2).

We did not observe significant intimal formation in the proximal vein or artery or in the distal arterial sections in any of the animals (control or treated) at either 1 or 2 months in this study. We did observe intimal hyperplasia at the distal vein site in animals at both 1 and 2 months. More extensive intimal hyperplasia was noted in control veins compared to treated veins at both 1 and 2 months (0.45 ± 0.5 vs. 0.09 ± 0.07 , $p < 0.05$, and 0.45 ± 0.12 vs.

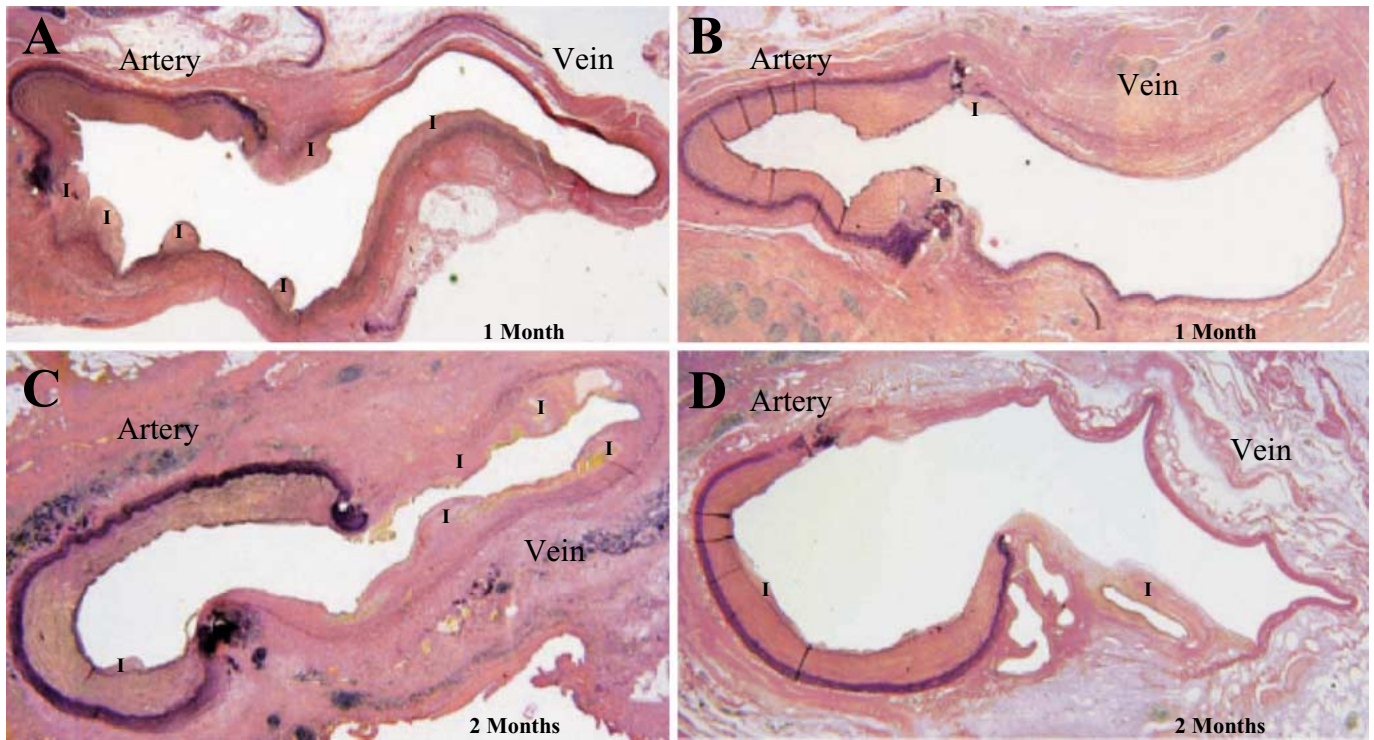


Fig. 2. Representative photomicrographs of cross-sections of the anastomotic sites show the effects of perivascular endothelial cell implants on neointimal formation 1 and 2 months after surgery. Intimal hyperplasia generally occurred more frequently on the venous side of the anastomosis. When compared to control anastomoses (A, C), perivascular endothelial cell implants (B, D) reduced intimal hyperplasia at 1 and 2 months, respectively. I = Intimal thickening. Verhoeff's elastin stain. $\times 20$.

Table 2. Average severity of acute and chronic inflammation in femoral arteriovenous fistulae

Time, days	Perivascular inflammation		Luminal inflammation			
	Gelfoam	PAE-Gelfoam	artery		vein	
			Gelfoam	PAE-Gelfoam	Gelfoam	PAE-Gelfoam
3 (acute)	1.8 \pm 0.8	1.7 \pm 0.2	2.0 \pm 0.3	1.0 \pm 0.2	2.0 \pm 0.3	0
29 (chronic)	1.6 \pm 0.4	1.9 \pm 0.1	1.0 \pm 0.1	1.3 \pm 0.2	1.0 \pm 0.1	1.2 \pm 0.2
57 (chronic)	2.1 \pm 0.8	1.8 \pm 0.1	1.4 \pm 0.4	1.3 \pm 0.1	1.2 \pm 0.2	1.2 \pm 0.1

Acute inflammation was marked by granulocytes, primarily neutrophils, while chronic inflammation was marked by macrophages and lymphocytes. Average severity = severity of group/incidence. 0 = None; 1 = minimal; 2 = moderate; 3 = marked; 4 = severe.

0.19 \pm 0.04, $p = 0.06$, respectively). There was no significant difference in EEL area between treatment groups at either of the time points evaluated (table 1), making an effect of the cellular implants on vessel remodeling unlikely. There was an increase in EEL area, suggesting positive remodeling, in the animals of both groups at 2 months

when compared to their respective group at 1 month. PECAM-1 staining of anastomotic sections from each treatment group revealed complete denudation of the arterial and venous endothelium 3 days postoperatively (fig. 3). Sections through Gelfoam containing PAE and explanted after 3 days stained positive for PECAM-1

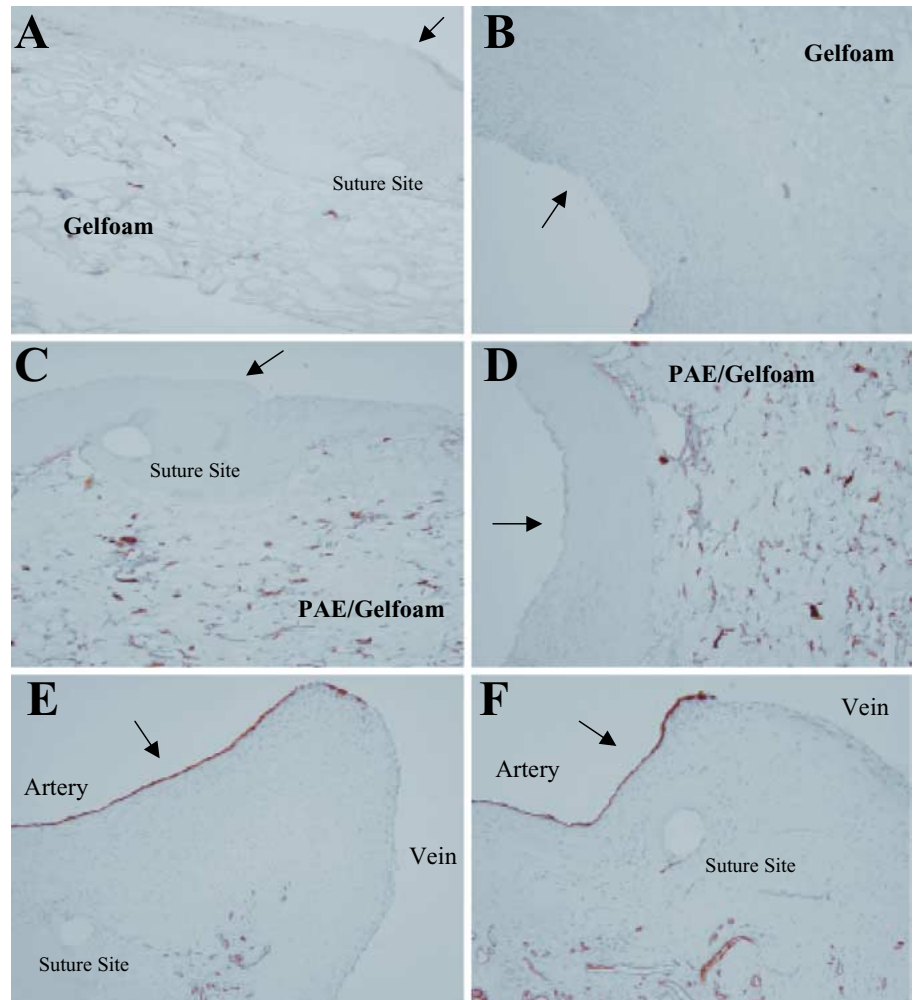


Fig. 3. Photomicrographs of PECAM-1 staining of porcine femoral anastomotic sections 3 days (A–D) and 2 months (E, F) after injury. Brown cells are PECAM-1-positive cells. After 3 days, the arterial and venous segments were completely denuded in both control (A, B) and PAE-treated (C, D) groups. Arrows point to denuded endothelium. Gelfoam containing PAE and explanted after 3 days stained positive for PECAM-1 (C, D). Control Gelfoam showed no positive staining (A, B). After 2 months, the arterial endothelium had completely regenerated in both PAE-treated (E) and control (F) groups. Arrows point to regenerated arterial endothelium. The venous endothelium still remained partially denuded at 2 months. Note the highly vascularized anastomotic sites in both groups at 2 months. Gelfoam, with or without cells, was variably present in animals from both groups at 1 month and was not located in any animals after 2 months. $\times 100$.

(fig. 3). Empty control Gelfoam showed no positive staining. After 1 month, the endothelium had begun to regenerate, primarily in the arterial segment of the anastomotic sections from both treatment groups. After 2 months, the endothelium had completely regenerated in the arterial segments from both treatment groups (fig. 3). Reendothelialization of the venous segments, however, was not complete in any animals from either treatment group after 2 months. PECAM-1 staining also revealed that at 2 months, the anastomotic sites from both groups had become highly vascularized when compared to anastomoses at 3 days (fig. 3). Contralateral femoral veins and arteries, used as positive controls, showed a complete endothelium by PECAM-1 staining at all time points. These results suggest that it is unlikely that the beneficial effects observed in PAE-treated anastomoses were due to early recovery of luminal endothelium in the treated animals.

Cellular Immune Response

Mild inflammation was present in the adventitia of the implant sites in animals from both groups at all time points. Acute inflammation, which consisted mainly of granulocytes (primarily neutrophils), was present only in animals euthanized after 3 days (table 2). Chronic inflammation, which consisted of macrophages and lymphocytes, was observed only in animals euthanized after 1 and 2 months (table 2). There were no differences in acute or chronic perivascular inflammation between control Gelfoam- or PAE-Gelfoam-treated anastomoses in this study. These results are similar to those previously reported for perivascular allogeneic endothelial cell implants in a porcine model of balloon angioplasty [13]. Mild inflammation was also present in the lumen of the implant sites in animals from both groups at all time points except for the venous segment of PAE-treated ani-

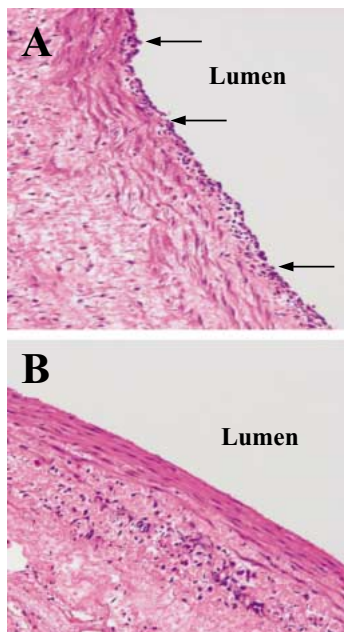


Fig. 4. Photomicrographs of sections of porcine femoral veins 3 days after the creation of arteriovenous fistulae stained with hematoxylin and eosin. A A control vein with moderate neutrophil infiltration in the lumen. Neutrophils were identified by their trilobed, segmented nucleus and numerous cytoplasmic granules. B A vein that was wrapped with PAE-Gelfoam. Note the absence of inflammatory cells in the lumen. $\times 100$.

mals at 3 days (table 2). Less inflammation was noted in the femoral artery of PAE-treated animals compared to femoral arteries in control animals (an average difference of 1 severity point). Evidence of acute inflammation was not detected in any of the venous sections of PAE-treated animals (fig. 4). In contrast, moderate inflammation was present in the veins of control animals (average severity grade 2.0) (fig. 4).

Serum Antibodies That Bind to Porcine Endothelial and T Cells

The average relative concentrations of antibodies binding to donor PAE or to PTC pooled from domestic pigs before and after implantation of PAE-Gelfoam are shown in figure 5. The fluorescence measurement is expressed as the mean channel shift. The mean channel shift for a given day is equal to the mean channel for that day minus the mean channel for day 1 (prior to surgery). Two separate FACS analyses were performed on each sample and the mean channel shifts of the two separate experiments were averaged. The mean channel shift of the post-surgical sera was then compared to the mean channel shift

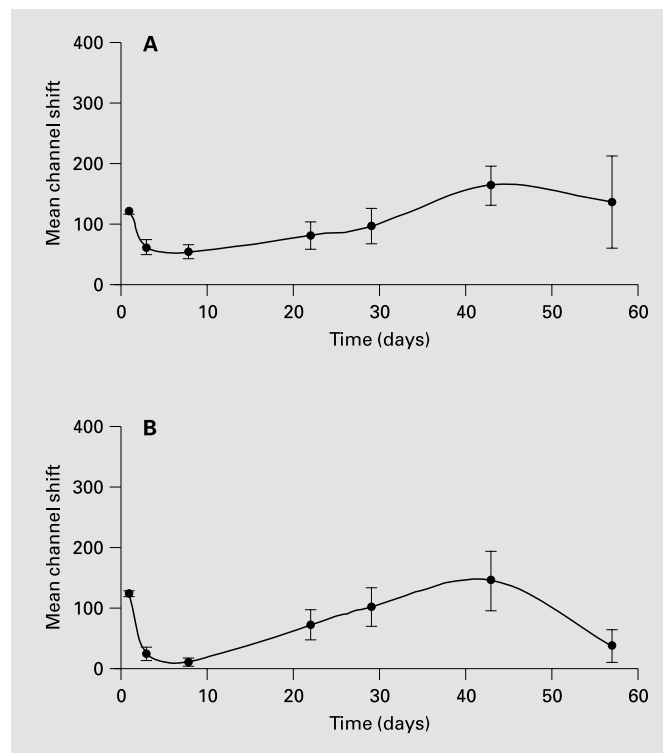


Fig. 5. Graph of average allogeneic humoral responses. Sera were obtained from animals prior to surgery (day 1) and 3 days, 1 week, 3 weeks, 1 month, 6 weeks and 2 months after implantation and tested against the same endothelial cell strain used for implantation (PAE) and against PTC pooled from domestic pigs. The mean channel shift for postsurgical sera was compared to the average standard deviation of two experiments for day 1 sera. A Reactivity to donor PAE of sera from animals treated with PAE implants and euthanized at 1 or 2 months. On average, an insignificant increase in antibodies binding to PAE was detected 6 weeks and 2 months after implantation, when compared to presurgery levels. B Reactivity to PTC of sera from animals treated with PAE implants and euthanized at 1 or 2 months. On average, a similar insignificant increase in antibodies binding to PTC was detected at 6 weeks but not at 2 months. No significant increase in antibody levels was detected in sera obtained from control animals at any of the time points tested or in any of the treated animals euthanized at 3 days or 1 month.

for day 1 (which is zero) plus the average standard deviation of day 1 for the two experiments. Any serum with a mean channel shift above the average standard deviation for the day 1 sera is considered to be positive for antibodies. The day 1 value in figure 5 is therefore equal to the day 1 average standard deviation for all animals. All other time points are equal to the average mean channel shift for all animals at that time point.

The mean channel shift for postimplant sera from animals treated with Gelfoam only did not exceed the aver-

age deviation for the day 1 sera at any of the time points tested, and therefore they were considered negative for anti-PAE and anti-PTC antibodies (data not shown). All of the pigs treated with PAE-Gelfoam that were euthanized on day 3 or 29, as well as 1 animal euthanized on day 57, were negative for anti-PAE and anti-PTC antibodies at all of the time points tested. One animal treated with PAE-Gelfoam and euthanized on day 57 was positive for antibodies to PAE and PTC on days 29 and 43. Levels of both anti-PAE and anti-PTC antibodies in day 57 serum had decreased to below the standard deviation for day 1 sera. Sera from 2 animals treated with PAE-Gelfoam and euthanized on day 57 were positive for antibodies to PAE on days 22, 29, 43 and 57 and for antibodies to PTC on days 22, 29 and 43. The levels of anti-PTC antibodies had decreased by day 57 such that 1 pig was negative for PTC antibodies and 1 pig's levels had decreased by 90%.

These analyses indicate that sera from 3 pigs implanted with PAE had increased levels of both anti-PAE and anti-PTC antibodies compared to baseline. On average, the increased levels of antibodies were not statistically significant. Because the sera from these pigs were positive for both PAE and PTC antibodies, it is likely that the antibodies are binding to common cell surface antigens such as swine leukocyte antigen molecules expressed on the cell surface of the donor endothelial cells.

Discussion

Vascular access complications are the greatest cause of morbidity in hemodialysis patients in the United States, and a satisfactory long-term pharmacologic means of preventing stenosis due to intimal hyperplasia has yet to be found [2, 17]. Injury to artery and vein endothelium during the creation of arteriovenous fistulae influences patency and occlusion rates [3]. In addition to the physical trauma associated with cutting and suturing veins and arteries, increased wall stress and shear force may also cause physical and/or biochemical injury to the endothelium [9–11, 18]. It has been suggested that arterial pressure may alter the normal production of endothelial growth-regulatory compounds as well as produce morphological and biochemical changes in the media of the vein [19].

The endothelial cell implants described here represent a novel approach to addressing this problem. In this study, we examined the role of endothelial cells in the vascular response to injury after creating arteriovenous anastomoses through the use of perivascular endothelial implants. When implanted around surgically created arterio-

venous anastomoses, porcine endothelial cells significantly reduced the intimal hyperplasia index compared to controls 2 months after surgery. The extent of intimal hyperplasia in treated anastomoses remained essentially unchanged between 4 and 8 weeks (intimal hyperplasia index: 0.13 ± 0.04 and 0.12 ± 0.02 , respectively), while there was an almost 2-fold increase in control animals during this same time period (0.20 ± 0.06 and 0.38 ± 0.04 , respectively). Moreover, we demonstrated that the inhibition of intimal hyperplasia observed in treated anastomoses was not due to an early recovery of the luminal endothelial cells in the treated animals. The completely denuded endothelium regenerated at similar rates in both groups.

It is known that confluent endothelial cells release a variety of biological agents that in combination may inhibit smooth muscle cell proliferation [20]. Seeding the cells and allowing them to proliferate within a polymer support matrix (Gelfoam) allowed for the implantation of postconfluent endothelial cells. In fact, the endothelial cells cultured in Gelfoam were evaluated *in vitro* prior to implantation for their ability to produce significant levels of heparan sulfate. The controlled adventitial delivery of endothelial-derived compounds such as heparan sulfate and fibroblast growth factor-2 has been shown to have effects on smooth muscle cell proliferation following vascular injury in animal models [21, 22]. Deposition of compounds delivered in this manner within the blood vessel wall was rapidly distributed circumferentially and was substantially greater than that observed following intravenous injection of the same compound [21]. In the present study, it is possible that antiproliferative compounds released from the transplanted endothelial cells are able to move throughout the blood vessel wall in a similar manner and have effects at a distance from their site of release.

Analysis of tissue sections from acute PAE-treated animals revealed less inflammation in the lumen of the arterial segments compared to control arterial segments and no inflammatory cells in the lumen of the PAE-treated venous segments. The importance of immune mediation in the vascular response to injury is becoming increasingly well appreciated. Several animal studies have shown infiltration of neutrophils at the blood vessel wall within hours of vascular injury [23, 24]. A study of balloon-injured rabbit iliac arteries demonstrated that neutrophil, and not macrophage, infiltration preceded neointimal thickening in injured arteries [25]. The study also demonstrated a correlation between inhibition of neutrophil infiltration and inhibition of medial smooth muscle

proliferation. While the mechanisms by which neutrophils affect vascular repair are not completely understood, it is known that they contribute to tissue injury through the release of reactive oxygen species and proteases [26]. It has also been shown that cultured vascular smooth muscle cells are stimulated to proliferate when cocultured with neutrophils or neutrophil-conditioned media [27]. The data in the present study suggest that perivascular endothelial cells, or factors released by the cells, may provide control over intimal formation by influencing early events such as neutrophil infiltration at the lumen.

The source of cells for use in cellular implants depends upon many factors, most importantly the desired function of the implant and technical feasibility. The ability to isolate, culture, characterize and manipulate the endothelial cells were decisive factors in developing the allogeneic implants described in the present study. Moreover, if applied in a clinical setting, time constraints and the possibility of dysfunctional endothelial cells in a person with vascular disease would preclude the use of autologous tissue to formulate the endothelial implants. The increasing appreciation of the potential use of allogeneic tissue-engineered implants requires an investigation of host immune responses. The data in the present study suggest that the implantation of Gelfoam containing allogeneic endothelial cells around arteriovenous anastomoses elicited only a mild cell-mediated immune response. This response was similar to that seen with control Gelfoam and mirrors that which was previously shown for perivascular allogeneic endothelial cells placed around balloon-injured porcine carotid arteries [13]. Gelfoam is a marketed hemostatic sponge used to control bleeding in certain types of surgical

procedures, such as the creation of arteriovenous fistulae for hemodialysis access.

A humoral immune response to the implanted endothelial cells was detected in 3 of 14 treated animals. A significant increase in the titer of circulating antibodies to the donor porcine endothelial cells that had not returned to presurgery levels after 2 months was observed in only 1 of the treated pigs. Because the serum from these animals was positive for both PAE and PTC antibodies, the activity is attributed to common cell surface antigens, presumably swine leukocyte antigen, located on both cell types. There was no correlation between the humoral immune response and the effects on intimal hyperplasia in the animals with increased levels of circulating antibodies, suggesting that the implants were able to affect the response to injury before eliciting effective immune responses. Further exploration of the control mechanisms of endothelial implants and further characterization of the immune response in settings of chronic treatment in sensitized animals would provide additional insight into the use of endothelial implants in clinical applications with arteriovenous fistulae.

Acknowledgments

We are grateful to Desmond White from Curis Inc. for his expert technical assistance. This research was supported by a National Institute of Standards and Technology Advanced Technology Program Award (70NANB9H3003) to Curis Inc., Cambridge, Mass., USA, and by the National Institutes of Health (GM/HL 49039, HL 60407) and an Established Investigator Award from the American Heart Association to E.R.E.

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