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BASIC SCIENCE STUDIES

Chronic in vitro shear stress stimulates endothelial cell retention on prosthetic vascular grafts and reduces subsequent in vivo neointimal thickness

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Objective: **The absence of endothelial cells at the luminal surface of a prosthetic vascular graft potentiates thrombosis and neointimal hyperplasia, which are common causes of graft failure in humans. This study tested the hypothesis that pretreatment with chronic in vitro shear stress enhances subsequent endothelial cell retention on vascular grafts implanted in vivo.**
Methods: **Cultured endothelial cells derived from Fischer 344 rat aorta were seeded onto the luminal surface of 1.5-mm internal diameter polyurethane vascular grafts. The seeded grafts were treated for 3 days with 1 dyne/cm^2 shear stress and then for an additional 3 days with 1 or 25 dyne/cm^2 shear stress in vitro. The grafts then were implanted as aortic interposition grafts into syngeneic rats in vivo. Grafts that were similarly seeded with endothelial cells but not treated with shear stress and grafts that were not seeded with endothelial cells served as controls. The surgical hemostasis time was monitored. Endothelial cell identity, density, and graft patency rate were evaluated 24 hours after implantation. Endothelial cell identity in vivo was confirmed with cells transduced in vitro with β-galactosidase complementary DNA in a replication-deficient adenoviral vector. Histologic, scanning electron microscopic, and immunohistochemical analyses were performed 1 week and 3 months after implantation to establish cell identity and to measure neointimal thickness.**
Results: **The pretreatment with 25 dyne/cm^2—but not with 0 or 1 dyne/cm^2—shear stress resulted in the retention of fully confluent endothelial cell monolayers on the grafts 24 hours after implantation in vivo. Retention of seeded endothelial cells was confirmed by the observation that β-galactosidase transduced cells were retained as a monolayer 24 hours after implantation in vivo. In the grafts with adherent endothelial cells that were pretreated with shear stress, immediate graft thrombosis was inhibited and surgical hemostasis time was significantly prolonged. Confluent intimal endothelial cell monolayers also were present 1 week and 3 months after implantation. However, 1 week after implantation, macrophage infiltration was observed beneath the luminal cell monolayer. Three months after the implantation in vivo, subendothelial neointimal cells that contained α–smooth muscle actin were present. The thickness of this neointima averaged 41 ± 12 μm and 60 ± 23 μm in endothelial cell–seeded grafts that were pretreated with 25 dyne/cm^2 shear stress and 1 dyne/cm^2 shear stress, respectively, and 158 ± 46 μm in grafts that were not seeded with endothelial cells.**
Conclusion: **The effect of chronic shear stress on the enhancement of endothelial cell retention in vitro can be exploited to fully endothelialize synthetic vascular grafts, which reduces immediate in vivo graft thrombosis and subsequent neointimal thickness. (J Vasc Surg 1999;29:157-67.)**

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Endothelial cells serve anticoagulant and antiproliferative functions in blood vessels. Under physiologic conditions, endothelial cells adhere to the luminal vessel wall with sufficient strength to withstand the force of shear stress that is generated by flowing blood. The adhesive strength is generated by cell-matrix interactions, which are mediated, at least in part, by integrins that connect extracellular matrix–binding sites with intracellular focal adhesion plaques.[1] By contrast, endothelial cells that are seeded onto the luminal surface of prosthetic vascular grafts are not usually retained after implantation in vivo as a result of a lack of adhesive strength. Still, endothelialization of prosthetic vascular grafts is a goal for vascular reconstruction because seeded endothelial cells could potentially reduce graft failure caused by thrombosis and could inhibit cell proliferation associated with neointima-induced stenosis.[2,3] The molecular mechanisms that lead to vascular thrombosis and neointima formation are incompletely defined,[4,5] and neither local nor systemic pharmacologic treatments have improved clinical outcome sufficiently to overcome these problems.[6,7] Seeding of the luminal synthetic graft surface with endothelial cells[8] improves patency rates and reduces early thrombus formation in some animal models.[9-11] Nevertheless, there is a rapid loss of cells after exposure to shear stress in vitro[12] or implantation in vivo. For instance, Rosenman et al[13] reported that up to 40% of seeded cells are lost at 1 hour and that more than 80% are lost by 24 hours after implantation in vivo. Autologous human endothelial cells that are seeded onto vascular grafts fail to improve graft patency rates, presumably as a result of the loss of the seeded cells from the grafts.[9,14]

Prolonged exposure of endothelial cells to shear stress leads to cell flattening, which limits cell surface shear stress gradients,[15] reduces expression of the pro-proliferative mediators endothelin-1[16] and platelet derived growth factor–B,[17] and induces a nearly 40-fold higher density of Weibel-Palade bodies.[18] These findings are suggestive of endothelial cell differentiation. Chronic shear stress also promotes the adhesion of aortic endothelial cells to prosthetic vascular grafts in vitro.[19] Bovine aortic endothelial cells that were cultured for 6 days on the luminal surface of polyurethane grafts without shear stress were dislodged from the grafts by acute shear stress, whereas the cells that were similarly cultured on grafts but preconditioned with shear stress at 1 dyne/cm^2 for 3 days and then at 25 dyne/cm^2 for an additional 3 days were not dislodged. The rate of fibrin clot formation also was significantly less in the grafts that were pretreated with shear stress and that retained endothelium than in the grafts that were not pretreated from which the cells had been dislodged.[19]

The current study addressed the question of whether poor endothelial cell adhesion on prosthetic vascular grafts in vivo, which is currently a major obstacle to graft endothelialization, could be overcome by pretreating endothelial cells with shear stress for several days in vitro.

METHODS

Cell culture. Clonal rat aortic endothelial cell lines were derived from Fischer 344 rat aorta. A segment of the abdominal aorta was excised from a rat under anesthesia, inverted, and treated with 1 mg/mL collagenase (type IV; Sigma, St Louis, Mo) in RPMI 1640 medium at 37°C for 20 minutes. The dislodged cells were sedimented with centrifugation, washed three times with RPMI 1640, and then resuspended in endothelial cell basal medium (EBM; Clonetics, San Diego, Calif), supplemented with penicillin (5000 IU/mL), streptomycin (5000 μg/mL), heparin (1 μg/mL), fetal calf serum (15%), and bovine brain extract (3.1 mg/500 mL; Clonetics). The cells were plated on 3 to 5 gelatin-coated culture plates at a density of approximately 1000 cells per 70-cm^2 plate. On average, 5 to 10 endothelial cell colonies emerged on each plate. These were subcloned with cloning cylinders and expanded with supplemented EBM medium and gelatin-coated plates. All the endothelial cell lines were homogeneous clonal isolates that exhibited acetylated low density–lipoprotein uptake and contained von Willebrand's factor (vWF) with indirect immunofluorescence, as previously described.[20] Endothelial cells between passages 8 and 25 were used.

Shear stress. Shear stress was calculated from Poiseuille's equation: $\tau = 4\eta Q/\pi r^3$, where τ = shear stress, η = fluid viscosity, Q = medium fluid flow rate, and r = radius of the cylinder. The viscosity of the cell culture medium was measured with a Cannon-Manning tube viscometer (International Research Glassware, Kenilworth, NJ) and ranged from 0.0088 to 0.0092 poise.[19] The viscosity of whole blood is approximately 0.035 poise.[21]

Cell culture on vascular grafts with shear stress. Segments of untreated spun polyurethane grafts, (with a hardness of 50 on the Shore Hardness Scale D; Corvita Corporation, Miami, Fla), that had a 1.5-mm inner diameter and were 5 cm in length were coated with 4% bovine gelatin, air-dried, placed

into shear stress chambers,[18] and sterilized with ethylene oxide. The gelatin was cross-linked with 2.5% glutaraldehyde for 30 minutes at room temperature, followed by neutralization with 1% glycine. The grafts were washed three times with 10 mL of culture medium and then were seeded with 2 to 3 × 10^5 cells/cm^2. The cells were allowed to attach for 4 hours as previously described.[18] Shear stress then was initiated at 1 dyne/cm^2 for the first 3 days to allow cell attachment before exposure to higher levels of shear stress.[12] Shear stress then was increased to 25 dyne/cm^2 (arterial shear stress) or maintained at 1 dyne/cm^2 (venous shear stress) for an additional 3 days. In 10 grafts, the endothelial cells were seeded and allowed to attach but were implanted immediately without any in vitro culture or shear stress pretreatment because in vitro culture for 6 days without flowing medium leads to uniform death of rat aortic endothelial cells (data not shown). Control grafts that were not seeded with cells were processed identically and exposed to 25 dyne/cm^2 shear stress before implantation. Because such grafts uniformly underwent thrombosis during the first 24 hours after implantation, grafts that were neither coated with gelatin nor seeded with cells were used as additional controls for 1-week and 3-month time points.

Implantation of vascular grafts in vivo. Female Fischer 344 rats (Charles River Laboratories, Wilmington, Mass) that weighed from 150 to 175 gm underwent anesthesia with inhalation of methoxyflurane. After a lower midline incision was made, dissection was carried down to the aorta, a 1.5-cm section of the infrarenal aorta was clamped with an Acland clamp approximator (Microsurgery Instruments, Inc, Bellaire, Tex), and a piece of the aorta, approximately 0.7 cm in length, was excised. The ends of the aorta and the graft to be inserted were flushed with 0.5 mL of heparinized saline solution (25 U/100 mL). No systemic heparinization was performed. The grafts were divided into 1.0-cm sections for implantation in vivo, and a portion of each graft was taken for preimplantation analysis. All the grafts were kept in medium on ice and then washed in normal saline solution immediately before use. The grafts were sewn into the infrarenal aorta with interrupted 10-0 nylon sutures (MIC, Bellaire, Tex), with a standard microsurgical technique. The aortic cross-clamp time ranged from 24 to 57 minutes (35 ± 7 minutes, mean ± standard deviation) and did not differ between the groups. After the removal of the Acland clamp and the control of the suture line bleeding with light pressure, graft patency was confirmed with the presence of lateral pulsations. The surgical hemostasis time was defined as the time interval from the removal of the Acland clamp until the cessation of blood transudation through the graft interstices. Blood transudation was checked every 30 seconds. The abdomen was irrigated and closed with an absorbable suture, and 10 mL of sterile saline solution were injected intraperitoneally to circumvent volume depletion. The skin then was closed, and the animal was allowed to recover from the anesthesia. Food and water were made available immediately afterwards. All the animal care complied with the "Principles of Laboratory Animal Care" (formulated by the National Society for Medical Research) and the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 86-23, revised 1985) and received approval from the Johns Hopkins Animal and Care Use Committee, Animal Welfare Assurance No. A-3272-01. A single surgeon performed all the operations.

Graft removal and histology. The grafts were removed at 24 hours, 7 days, or 3 months after placement in vivo. The rats again underwent anesthesia, the abdomen was opened, and the grafts were inspected for patency. Patency was determined with the following methods: 1) a visual inspection for lateral pulsations; 2) the presence of visible intraluminal thrombus; 3) the presence of prograde flow in the distal vessel, also termed the strip test[22]; and 4) the presence of pulsatile blood flow after distal arteriotomy. After removal, the graft was flushed with 1 mL of saline solution, immersion fixed in 10% formalin, and paraffin embedded, and three to six 10-μm serial sections from the midpoint of each graft were stained with hematoxylin and eosin.[23] For those grafts that were examined by means of scanning electron microscopy, a 2-mm section of the mid-portion of the graft was placed into a formalin-paraformaldehyde buffer and processed as previously described.[18] For all the grafts that were kept in vivo for 24 hours, the number of cell nuclei per mm at the luminal surface was counted in three to six sections of the same graft. Intraexperimental variability for different sections of the same graft was routinely less than 10%. Cell retention then was defined as the percentage of cells/mm that remained at the luminal surface, with cells/mm on the same graft preimplantation serving as the reference. For the grafts that were left in place for 3 months, the thickness of the neointima was measured with an eyepiece micrometer, at eight equidistant points around the circumference of three to six serial cross-sections that were taken from the midpoint of each graft.

Immunocytochemistry. The sections also were processed for immunohistochemical examination, as previously described,[24] with purified rabbit polyclonal anti-vWF immunoglobulin G (IgG; 1:1000; Dako, Carpinteria, Calif), mouse anti-α–smooth muscle actin monoclonal IgG (1:400; Dako), and mouse monoclonal anti-rat macrophage IgG (Clone EDI, 1:300; Accurate, Westbury, NY). Endogenous peroxidase was quenched with 3% hydrogen peroxide for 20 minutes. The sections were treated with 0.4% pepsin (Sigma) in a 0.01-N HCL solution for 30 minutes at 37°C. For the detection of α–smooth muscle actin, the sections were microwave treated. Secondary antibodies were biotinylated goat anti–rabbit IgG (1:200; Vector Laboratories, Burlingame, Calif) for vWF staining and biotinylated goat anti–mouse IgG (1:300; Jackson ImmunoResearch, West Grove, Pa) for α-actin and macrophage staining. Detection was performed with 3,3´-diaminobenzidine with the ABC kit (Vector). Counterstaining was performed with Mayer's modified hematoxylin. Normal rat aorta served as positive control tissue for vWF and α–smooth muscle actin antibody, and normal rat spleen served as positive control for EDI antibody staining. As negative controls, the primary antibody was omitted or substituted with rabbit or mouse IgG (Sigma) from animals that had not undergone immunization. No staining occurred in the negative control sections. Indirect immunofluorescence was not performed as a result of the autofluorescence of polyurethane grafts (data not shown).

Cell transfection with β-galactosidase adenovirus. The *Escherichia coli* β-galactosidase (Lac-Z) gene was inserted into the transient expression vector pΔE1sp1B (Microbix Systems, Ontario, Canada) under the control of the cytomegalovirus promoter and upstream from a polyadenylation signal (pΔE1sp1BlacZ). The recombinant replication-deficient Lac-Z adenovirus (adeno–lac Z) was produced with in vitro homologous recombination in human embryonic kidney 293 (HEK 293) cells between pΔE1sp1BlacZ and plasmid pJM17. Individual plaques of adeno–lac Z were identified by X-gal staining in infected HEK 293 cells. These plaques were amplified and purified with ultracentrifugation through a CsCl gradient followed by dialysis against 10% glycerol in saline solution. The stock viral titer, determined in HEK 293 cells, was 2×10^{11} plaque-forming units/mL. After 5 days of shear stress treatment in vitro, the grafts were disconnected from the pump circuit and incubated with 2 mL adeno–lac Z virus, which was diluted to 5×10^{7} plaque-forming units/mL in EBM that contained 1.5% serum, for 2 hours at 37°C. The grafts then were washed with culture medium and reconnected to the pump circuit for the final 24 hours of shear stress treatment. The grafts then were implanted in vivo, as described above, and examined for X-gal staining at 24 hours and 7 days after implantation. A portion of the same graft was not implanted to evaluate β-galactosidase expression before implantation. After graft removal and flushing with saline solution, the graft was fixed with 0.5% gluteraldehyde and washed with saline solution. Staining was performed with X-gal for 18 hours at 37°C. The graft then was embedded in paraffin and serially sectioned; some sections were counterstained with eosin to stain cells that did not contain the β-galactosidase reaction product. False-positive staining was ruled out by performing β-galactosidase staining on grafts, which were seeded with cells that were not transduced with β-galactosidase complementary DNA, before and after implantation in vivo (data not shown).

RESULTS

In vivo retention of endothelial cells after shear stress treatment. Confluent monolayers of endothelial cells were observed lining the lumen of the grafts after pretreatment with 1 (n = 27) and 25 dyne/cm^2 (n = 60) shear stress before implantation in vivo (Fig 1*A*). Some cells also were present in the graft interstices before implantation (data not shown). Endothelial cells were similarly observed on the luminal surfaces of the grafts 24 hours after implantation in vivo (Fig 1*B,C*). These cells were immunoreactive with anti-vWF antibody (data not shown). Similarly, the cells that were observed in the interstices of the grafts 24 hours after implantation in vivo displayed vWF immunoreactivity (data not shown).

Quantitative analysis of luminal cell retention after 24 hours in vivo is shown in Fig 2*A*. Before implantation, the mean cell number per millimeter of luminal graft surface was 24.2 ± 7.9 in grafts that were pretreated with 25 dyne/cm^2 shear stress and remained 23.7 ± 8.5 after 24 hours in vivo (mean ± standard deviation; n = 7). The mean cell density at the luminal surface of the grafts that were pretreated with 1 dyne/cm^2 shear stress was 22.8 ± 4.9 cells/mm before implantation and 11.7 ± 2.9 cells/mm 24 hours after implantation in vivo (n = 6). On the grafts that were not exposed to shear stress, the cell density was 8.1 ± 2.4 cells/mm before implantation and 3.7 ± 2.4 cells/mm 24 hours after implantation in vivo (n = 4). Thus, endothelial cell

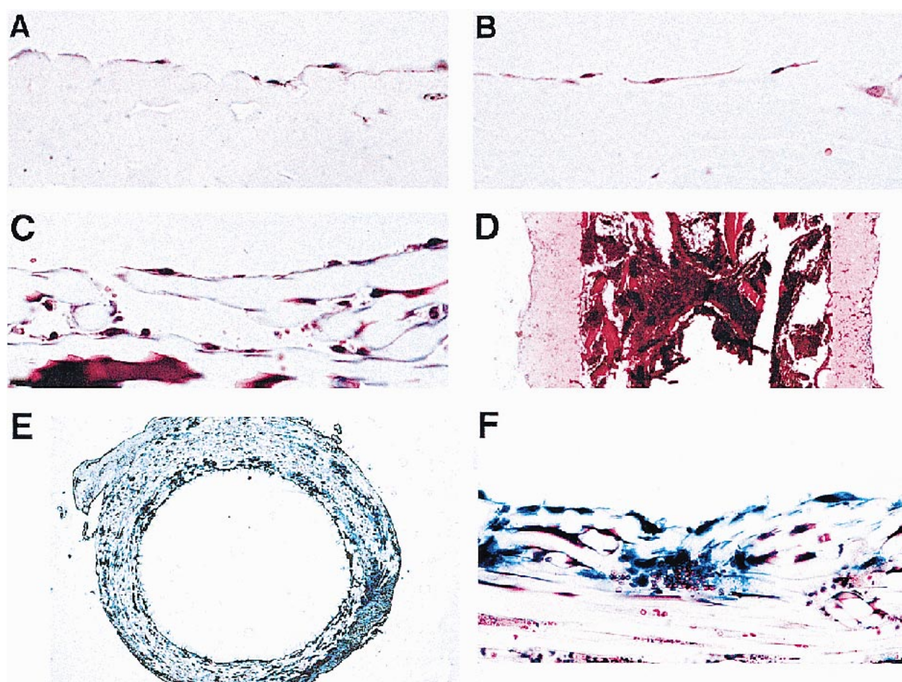

Fig 1. Light-microscopic appearance of endothelial cell–lined vascular grafts. Endothelial cell–seeded polyurethane graft treated with 25 dyne/cm^2 mean shear stress **(A)** before implantation and **(B)** 24 hours after implantation in vivo. **C,** Endothelial cell–seeded polyurethane graft treated with 1 dyne/cm^2 mean shear stress shown 24 hours after implantation in vivo. **D,** Gelatin-coated polyurethane graft without endothelial cells 24 hours after implantation in vivo. **E** and **F,** Polyurethane graft with shear stress (25 dyne/cm^2) treated endothelial cells that were also transduced with Lac-Z adenovirus, shown 24 hours after implantation in vivo. Magnification: **A** to **C** and **F,** 100×; **D,** 40×; and **E,** 20×.

retention 24 hours after implantation in vivo was much greater on the grafts that were pretreated with 25 dyne/cm^2 as compared with the grafts that were pretreated with 1 dyne/cm^2 shear stress ($P < .0001$, Student *t* test).

Effect of endothelial cells on thrombosis and hemostasis time. All the grafts that were pretreated with 25 dyne/cm^2 shear stress were patent 24 hours after implantation in vivo. Six of seven polyurethane grafts that were pretreated with 1 dyne/cm^2 shear stress and 4 of 5 grafts that contained endothelial cells but were not pretreated with shear stress remained patent 24 hours after implantation. Because all the endothelial cell–seeded grafts were precoated with gelatin, grafts that were similarly coated with gelatin but not seeded with endothelial cells were also implanted. These uniformly underwent thrombosis at 24 hours (n = 4; Fig 1*D*). Polyurethane grafts that were neither coated with gelatin nor seeded with endothelial cells remained patent (n = 7; data not shown).

The time to achieve surgical hemostasis was significantly longer for the endothelial cell–lined, shear stress–pretreated grafts as compared with the grafts without endothelial cells or the grafts that were seeded with cells but not exposed to shear stress (Fig 2*B; P* < .005, analysis of variance). The hemostasis times tended to be longer in the grafts that were pretreated with 25 dyne/cm^2, as compared with 1 dyne/cm^2 shear stress, although this difference did not achieve significance.

Evaluation of cell identity. To determine whether the cells that were observed at the luminal surface of the implanted grafts were those that were originally seeded onto the graft, the endothelial cells that were seeded onto the polyurethane grafts and pretreated with 25 dyne/cm^2 shear stress were infected with a replication-deficient adenovirus that carried the bacterial β-galactosidase gene. Before implantation, a confluent monolayer of endothelial cells was present on the luminal graft surface and cells were present within the interstices of the graft,

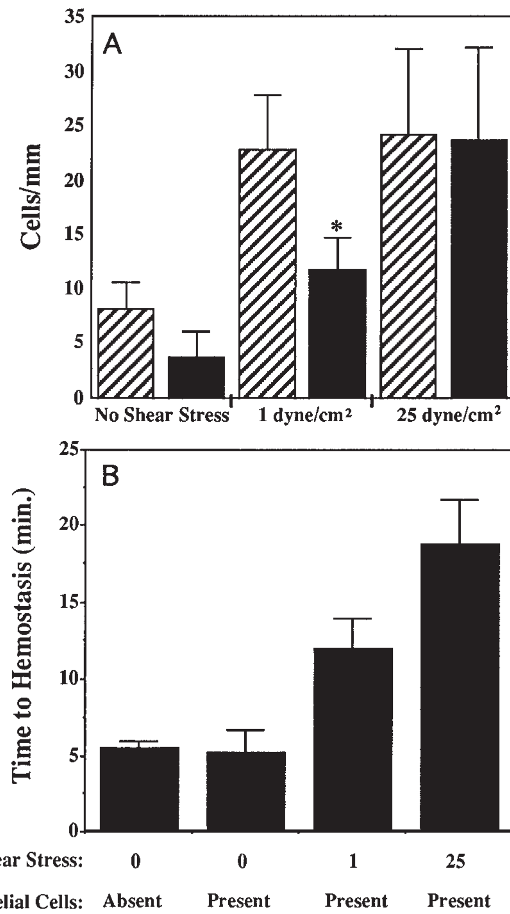

Fig 2. **A,** Endothelial cell density on polyurethane vascular grafts treated with 0, 1, or 25 dyne/cm^2 shear stress, before *(hatched bars)* and 24 hours after implantation in vivo (*solid bars*). **B,** Surgical hemostasis time after re-establishment of aortic circulation for unseeded grafts, seeded grafts not treated with shear stress, and seeded grafts treated with 1 or 25 dyne/cm^2 shear stress.
$*P < .0001$, Student *t* test.

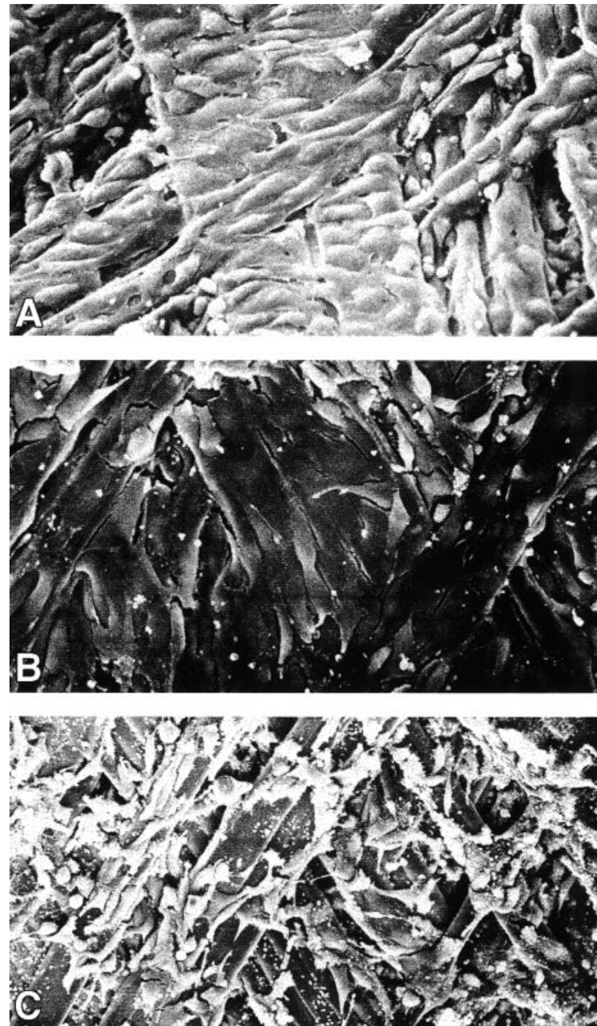

Fig 3. Scanning electron microscopy of endothelial cell–seeded polyurethane grafts. Endothelial cell–seeded graft treated with 25 dyne/cm^2 mean shear stress **(A)** before implantation and **(B)** 24 hours after implantation in vivo. **C,** Endothelial cell–seeded polyurethane graft treated with 1 dyne/cm^2 mean shear stress shown 24 hours after implantation in vivo. Magnification, 420×.

which was similar to the grafts that were not infected with adenovirus (n = 4; data not shown). All the observed cells exhibited β-galactosidase activity, and all the β-galactosidase staining was cell-specific. Twenty-four hours after implantation in vivo (Fig 1*E,F*), the mean luminal cell density was 104% of that before implantation on two separate grafts that were seeded with adeno LacZ–transduced endothelial cells—approximately 95% of the luminal cells observed on the grafts 24 hours after implantation stained blue (n = 2). At 1 week after implantation, the level of LacZ expression was significantly reduced as compared with the findings at 1 day after implantation (n = 2; data not shown), both because a lower percentage of cells in the graft expressed Lac-Z and because the level of expression in positive cells was reduced. Because the grafts contain many nonendothelial cells at 1 week, the fraction of Lac-Z expressing cells was not determined.

Scanning electron micrographs of grafts before implantation and 24 hours after implantation are shown in Fig 3. A confluent monolayer of endothelial cells was observed on all grafts that were pretreated with 25 dyne/cm^2 (n = 2; Fig 3*A*) or 1 dyne/cm^2 (n = 2; not shown) before implantation. The grafts that were pretreated with 25 dyne/cm^2 shear stress also contained confluent, undisrupted monolayers 24 hours after implantation (n = 2; Fig

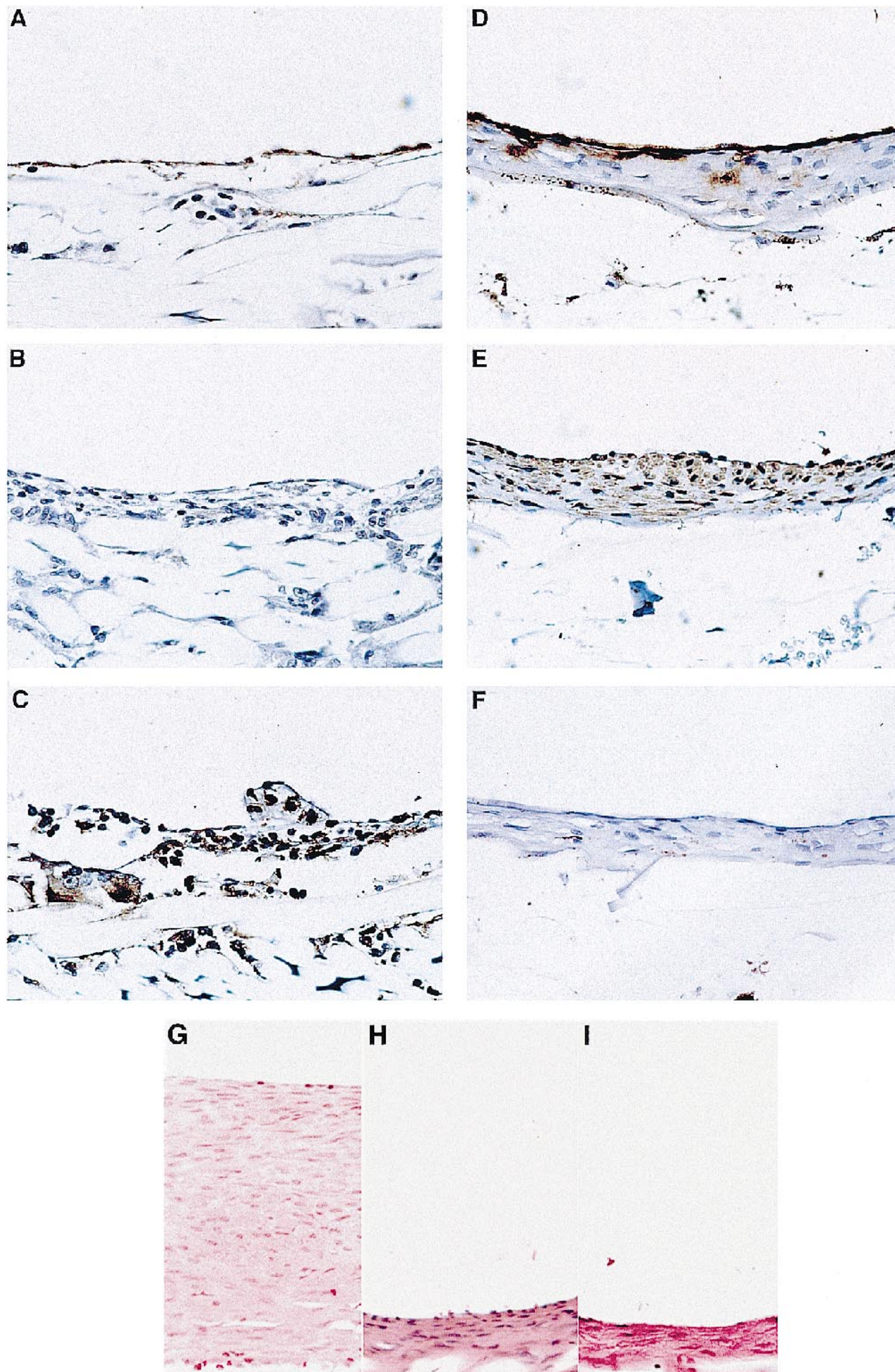

Fig 4. Nature of intimal cell layer on polyurethane grafts 1 week and 3 months after implantation in vivo. Grafts were harvested 1 week (**A** to **C**) or 3 months (**D** to **I**) after implantation in vivo. **A** and **D,** Anti–von Willebrand's factor antibody to identify endothelial cells. **B** and **E,** Anti-α smooth muscle actin antibody to identify neointimal myofibroblasts or smooth muscle cells. **C** and **F,** EDI antibody to identify macrophages. Magnification: **A** to **F,** 160×. **G** to **I,** Polyurethane grafts 3 months after implantation in vivo. **G,** polyurethane graft neither coated with gelatin or seeded with endothelial cells. **H,** Endothelial cell–seeded polyurethane graft pretreated with 1 dyne/cm^2 mean shear stress. **I,** Endothelial cell–seeded polyurethane graft pretreated with 25 dyne/ cm^2 mean shear stress. Magnification: **G** to **I,** 64×.

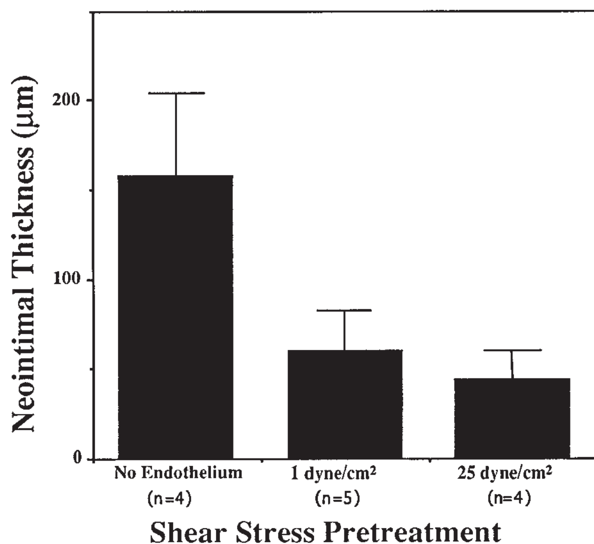

Fig 5. Quantification of neointimal thickness 3 months after graft implantation. Mean thickness of neointimal layer, measured at 8 equidistant points around circumference of 3 to 6 cross-sections taken from midpoint of graft was: **A,** 158 ± 46 μm (n = 4) for grafts without endothelial cells; **B,** 60 ± 23 μm (n = 5) for endothelial cell–seeded grafts pretreated with 1 dyne/cm^2; and **C,** 41 ± 12 μm (n = 4) for endothelial cell–seeded grafts pretreated with 25 dyne/cm^2 shear stress.

3*B*). The cells on these grafts appeared flatter than the cells on the same grafts before implantation. The endothelial cells on the grafts that were pretreated with 1 dyne/cm^2 were more rounded, and not all areas of the grafts were covered by cells (n = 2; Fig 3*C*). After implantation in vivo, platelet adhesion was observed on grafts that were pretreated with 1 dyne/cm^2 (Fig 3*C*) but not on grafts that were pretreated with 25 dyne/cm^2 (Fig 3*B*).

Characterization of the graft neointima 1 week and 3 months after implantation. One week after implantation in vivo, all the grafts that were seeded with endothelial cells and pretreated with shear stress contained a confluent monolayer of cells immunoreactive with anti-vWF antibody at their luminal surfaces (n = 2; Fig 4*A*). In grafts that were not seeded with endothelial cells, no cells were observed at the luminal surface 1 week after implantation (n = 2, data not shown). Also, at 1 week after implantation, cells were observed in most areas beneath the luminal monolayer. These were immunoreactive with antimacrophage (Fig 4*C*) but not with α–smooth muscle actin antibody (Fig 4*B*) and were observed in endothelial cell–seeded, shear stress pretreated grafts (n = 2). No macrophages were observed at 1 week after implantation in grafts that were not seeded with endothelial cells (n = 2; data not shown).

At 3 months after implantation, a neointimal layer was present in all the grafts (Fig 4), but this was much thinner in the grafts that were pretreated with 1 or 25 dyne/cm^2 and that contained adherent endothelial cells before implantation as compared with the grafts that had been implanted without endothelial cells (Fig 4*G*) or the grafts that had been seeded with endothelial cells and not treated with shear stress (data not shown). All the grafts that were previously seeded with endothelial cells contained a luminal cell monolayer that stained strongly with vWF antibody (n = 2; Fig 4*D*). The control grafts that were not seeded with endothelial cells also contained luminal endothelium 3 months after implantation (n = 4; data not shown). Subendothelial cell macrophages were not observed 3 months after graft implantation (Fig 4*F*). Instead, the subendothelial cell layer of all the grafts, whether seeded with endothelial cells or not, exhibited α–smooth muscle actin immunoreactivity (Fig 4*E*).

Quantification of neointimal thickness after 3 months in vivo (Fig 5) showed a neointimal layer 158 ± 46 μm in grafts without endothelial cells at the time of implantation (n = 4). By contrast, the neointima measured 60 ± 23 and 41 ± 12 μm in grafts that were seeded with endothelial cells and pretreated with 1 dyne/cm^2 (n = 5) or 25 dyne/cm^2 (n = 4) shear stress, respectively. The mean lumen diameter was reduced, on average, by 21% ± 6%, 8% ± 3%, and 5% ± 2% in unseeded, 1 dyne/cm^2 pretreated grafts, and 25 dyne/cm^2 pretreated grafts, respectively. Thus, the presence of shear stress pretreated endothelial cells at the time of graft implantation significantly reduced neointimal thickness and graft lumen narrowing 3 months later (P = .005, analysis of variance).

DISCUSSION

This study explored the effect of chronic in vitro shear stress pretreatment on endothelial cell retention in vivo. The implantation of shear stress pretreated endothelial cells on vascular grafts in vivo resulted in retention of a confluent endothelial cell monolayer, reduced immediate thrombogenicity, and reduced neointima thickness 3 months later.

The poor clinical performance of small diameter (<4 mm inside diameter) vascular grafts is caused, at least in part, by the absence of a functional endothelium.[25] Also, failure of larger prosthetic vascular grafts, particularly those used for vascular bypass grafting or

hemodialysis, is largely a result of neointimal hyperplasia at the anastomoses and elsewhere in the graft.[26] In humans, prosthetic vascular graft endothelialization is extremely slow and almost never complete.[27] It has been postulated that endothelialization of prosthetic graft material before implantation might reduce the incidence rate of graft thrombosis and the long-term rates of neointima formation.[28-30]

The extent of graft endothelialization is influenced by many factors, including graft material,[31] coating matrix,[32] and endothelial-cell growth factors.[33,34] Given that the adhesive strength of endothelial cells increased with chronic exposure to arterial levels of shear stress,[19] we postulated that shear stress pretreatment also enhances endothelial cell retention on vascular grafts implanted in vivo. To perform such in vivo studies, syngeneic rat endothelial cells were used.

Rat aortic endothelial cells that were seeded on the inner lumen of polyurethane grafts and exposed to shear stress for 6 days in vitro formed confluent endothelial monolayers (Fig 1 and 2). The cells in these monolayers appeared morphologically normal with scanning electron microscopy (Fig 3), even though the polyurethane grafts have no arterial media. The cells generally were oriented along the direction of flow, although in some areas the alignment was along the graft fiber (Fig 3A). The finding of nonuniform cell orientation on the fibers could reflect nonlaminar flow in areas where fibers overlap, focal cell migration into the graft interstices (Fig 1*C, E*), or plasticity of the cells after exposure to shear stress.[35] The lack of an underlying media also could alter cell alignment. In contrast to findings with shear stress pretreated grafts, the cell density on grafts that were seeded with endothelial cells and implanted immediately without shear stress pretreatment was much lower (Fig 2). Thus, full endothelialization of prosthetic vascular grafts was promoted when the cells were cultured in vitro for several days before implantation. Grafts that were seeded with endothelial cells and then implanted immediately have been used in patients, but low-density seeding did not alter long-term graft performance.[36]

Grafts that were exposed to shear stress at 25 dyne/cm^2 during the 3 days before implantation retained their luminal endothelial cells 24 hours and 1 week after implantation in vivo. This represents a much greater degree of graft endothelialization than previously reported.[13,37] The number of cells that remained on grafts, in vivo, was much less in those that were pretreated with 1 dyne/cm^2 shear stress, as compared with 25 dyne/cm^2 shear stress, during the 3 days before implantation. Thus, even though a similar degree of preimplantation endothelialization was achieved (Fig 2*A*), the level of in vitro shear stress pretreatment determines, at least in part, the degree of endothelial cell retention on polyurethane grafts. These results are consistent with previous findings that endothelial cells not exposed to shear stress were dislodged from polyurethane vascular grafts when exposed to acute shear stress.[19] That the cells retained at the luminal surface are those that were originally seeded can be concluded from the finding that grafts without cells do not spontaneously undergo endothelialization in 24 hours or 7 days and from the experiments (Fig 1*E,F*) that showed that endothelial cells transduced with adenovirus-LacZ before implantation are present 24 hours after implantation in vivo. Thus, in vivo endothelial cell retention on vascular grafts is enhanced significantly with in vitro pretreatment with arterial (25 dyne/cm^2) shear stress, which results in retention of a confluent intimal layer of endothelial cells 24 hours after implantation in vivo.

All the endothelial cell–seeded grafts were initially pretreated with gelatin because cells did not attach to uncoated grafts. Gelatin-coated grafts without endothelial cells uniformly underwent thrombosis within 24 hours (Fig 1*D*). Because gelatin activates platelets, the thrombosis of the grafts without endothelial cells was expected. The presence of the endothelial cells markedly inhibited the early thrombosis of gelatin-coated grafts, which is in keeping with the physiologic anticoagulant properties of endothelial cells. The time to achieve surgical hemostasis after the re-establishment of blood flow also was monitored. For the grafts that were not treated with shear stress, whether or not they were seeded with endothelial cells, hemostasis time was much shorter than that observed for endothelial cell–seeded grafts that were pretreated with shear stress (Fig 2*B*). Thus, fluid shear stress not only facilitates the lining of grafts with adherent endothelial cells, but shear stress–treated cells also exhibit the normal physiologic function of inhibiting local blood coagulation in vivo, as previously observed in vitro.[19]

Subendothelial macrophage accumulation in grafts at 1 week, but not at 3 months, after implantation (Fig 4*C* and 4*F*) suggests that the implanted endothelial cells support monocyte/macrophage chemotaxis. It is not yet clear whether the macrophages are recruited from the luminal or the abluminal compartment. Macrophage recruitment could be related to enhanced intercellular adhesion molecule–1 or to monocyte chemotactic protein-1 expression, which was previously shown to occur in response to acute shear stress.[38,39]

At 3 months after implantation in vivo, all the grafts, whether seeded with endothelial cells or not, had developed neointima, although the neointima was much thinner in the fully endothelialized grafts than in those that were implanted without endothelial cells (Fig 5). A previous study that showed that cell seeding does not reduce intimal thickness was performed in different animal species, with a different model of arterial injury at different time points of neointima formation, with venous endothelial cells, and with cells not conditioned by shear stress.[40] Our finding of a thinner neointima in endothelialized grafts supports the theory that endothelial cells not only serve anticoagulant functions in blood vessels but also inhibit myofibroblast proliferation or migration. Neointima formation that leads to stenosis and thrombosis is a significant cause of long-term graft failure,[2,3] including prosthetic vascular grafts used for dialysis.[41,42] This study suggests that graft neointimal hyperplasia can be minimized if a confluent, adherent monolayer of endothelial cells is established on the luminal surface of vascular grafts before implantation in vivo.

In summary, with the treatment of endothelial cells that were seeded on polyurethane vascular grafts with shear stress in vitro before implantation in vivo, full endothelialization of grafts was achieved and persisted after implantation in vivo. The presence of retained endothelial cell monolayers on grafts resulted in reduced immediate thrombogenicity and late neointimal thickness. Shear stress pretreatment, possibly in combination with other methods that improve endothelialization of prosthetic vascular grafts, could potentially be used in clinical medicine to reduce the incidence rate of graft thrombosis. In addition, it is tempting to speculate that pretreatment with shear stress might improve retention of endothelial cells that stably express genes of interest[43,44] for in vivo gene delivery.

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REFERENCES

1. Hanks SK, Polte TR. Signaling through focal adhesion kinase. Bioessays 1997;19:137-45.
2. LoGerfo FW, Quist WC, Nowak MD, Crawshaw HM, Haudenschild CC. Downstream anastomotic hyperplasia. A mechanism of failure in Dacron arterial grafts. Ann Surg 1983;197:479-83.
3. Imparato AM, Bracco A, Kim GE, Zeff R. Intimal and neointimal fibrous proliferation causing failure of arterial reconstructions. Surgery 1972;72:1007-17.
4. Wolf YG, Rasmussen LM, Ruoslahti E. Antibodies against transforming growth factor-beta 1 suppress intimal hyperplasia in a rat model. J Clin Invest 1994;93:1172-8.
5. Ferns GA, Raines EW, Sprugel KH, Motani AS, Reidy MA, Ross R. Inhibition of neointimal smooth muscle accumulation after angioplasty by an antibody to PDGF. Science 1991;253:1129-32.
6. Davies MG, Hagen PO. Pathobiology of intimal hyperplasia. Br J Surg 1994;81:1254-69.
7. Clowes AW, Reidy MA. Prevention of stenosis after vascular reconstruction: pharmacologic control of intimal hyperplasia—a review. J Vasc Surg 1991;13:885-91.
8. Schneider PA, Hanson SR, Price TM, Harker LA. Durability of confluent endothelial cell monolayers on small-caliber vascular prostheses in vitro. Surgery 1988;103:456-62.
9. Herring MB, Smith J, Dalsing M, Glover J, Compton R, Etchberger K, et al. Endothelial seeding of polytetrafluoroethylene femoral popliteal bypass: the failure of low-density seeding to improve patency. J Vasc Surg 1994;20:650-5.
10. Stanley JC, Burkel WE, Ford JW, et al. Enhanced patency of small-diameter, externally supported Dacron iliofemoral grafts seeded with endothelial cells. Surgery 1982;92:994-1005.
11. Schneider PA, Hanson SR, Price TM, Harker LA. Preformed confluent endothelial cell monolayers prevent early platelet deposition on vascular prostheses in baboons. J Vasc Surg 1988;8:229-35.
12. Miyata T, Conte MS, Trudell LA, Mason D, Whittemore AD, Birinyi LK. Delayed exposure to pulsatile shear stress improves retention of human saphenous vein endothelial cells on seeded ePTFE grafts. J Surg Res 1991;50:485-93.
13. Rosenman JE, Kempczinski RF, Pearce WH, Silberstein EB. Kinetics of endothelial cell seeding. J Vasc Surg 1985;2:778-84.
14. Fasol R, Zilla P, Deutsch M, Grimm M, Fischlein T, Laufer G. Human endothelial cell seeding: evaluation of its effectiveness by platelet parameters after one year. J Vasc Surg 1989;9:432-6.
15. Barbee KA, Davies PF, Lal R. Shear stress-induced reorganization of the surface topography of living endothelial cells imaged by atomic force microscopy. Circ Res 1994;74:163-71.
16. Malek A, Izumo S. Physiological fluid shear stress causes downregulation of endothelin-1 mRNA in bovine aortic endothelium. Am J Physiol 1992;263:C389-96.
17. Eng E, Ballermann BJ. Acute arterial shear stress downregulates PDGF-B message in glomerular capillary endothelial cells chronically conditioned to venous shear stress [abstract]. J Am Soc Nephrol 1996;7:1561.
18. Ott MJ, Olson JL, Ballermann BJ. Chronic in vitro flow promotes ultrastructural differentiation of endothelial cells. Endothelium 1995;3:21-30.
19. Ott MJ, Ballermann BJ. Shear stress-conditioned, endothelial cell-seeded vascular grafts: improved cell adherence in response to in vitro shear stress. Surgery 1995;117:334-9.
20. Ballermann BJ. Regulation of bovine glomerular endothelial cell growth in vitro. Am J Physiol 1989;256:C182-9.
21. Kraiss LW, Kirkman TR, Kohler TR, Zierler B, Clowes AW. Shear stress regulates smooth muscle proliferation and neointimal thickening in porous polytetrafluoroethylene grafts. Arterioscler Thromb 1991;11:1844-52.
22. Acland R. Signs of patency in small vessel anastomosis. Surgery 1972;72:744-8.
23. Dardik A, Henry EJ, Ballermann BJ. Gelatin increases adherence of polyurethane vascular grafts to glass slides. J Histotechnology. 1998;21:245-7.

24. Choi ME, Liu AL, Ballermann BJ. Differential expression of transforming growth factor-beta receptors in rat kidney development. Am J Physiol 1997;42:F386-95.
25. Williams SK. Endothelial cell transplantation. Cell Transplant 1995;4:401-10.
26. Kanterman RY, Vesely TM, Pilgram TK, Guy BW, Windus DW, Picus D. Dialysis access grafts: anatomic location of venous stenosis and results of angioplasty. Radiology 1995;195:135-9.
27. Rahlf G, Urban P, Bohle RM. Morphology of healing in vascular prostheses. Thorac Cardiovasc Surg 1986;34:43-8.
28. Herring M, Gardner A, Glover J. A single-staged technique for seeding vascular grafts with autogenous endothelium. Surgery 1978;84:498-504.
29. Swedenborg J, Bengtsson L, Clyne N, et al. In vitro endothelialisation of arteriovenous loop grafts for haemodialysis. Eur J Vasc Endovasc Surg 1997;13:272-7.
30. Pasic M, Muller-Glauser W, Odermatt B, Lachat M, Seifert B, Turina M. Seeding with omental cells prevents late neointimal hyperplasia in small-diameter Dacron grafts. Circulation 1995;92:2605-16.
31. Jensen N, Lindblad B, Bergqvist D. In vitro attachment of endothelial cells to different graft materials. Eur Surg Res 1996;28:49-54.
32. Miwa H, Matsuda T. An integrated approach to the design and engineering of hybrid arterial prostheses. J Vasc Surg 1994;19:658-67.
33. Weatherford DA, Sackman JE, Reddick TT, Freeman MB, Stevens SL, Goldman MH. Vascular endothelial growth factor and heparin in a biologic glue promotes human aortic endothelial cell proliferation with aortic smooth muscle cell inhibition. Surgery 1996;120:433-9.
34. Doi K, Satoh S, Oka T, Matsuda T. Impregnation of basic fibroblast growth factor on a microporous small caliber graft enhances vascularization. ASAIO J 1996;42:M394-8.
35. Sottiurai VS, Sue SL, Breaux JR, Smith LM. Adaptability of endothelial orientation to blood flow dynamics—a morphologic analysis. Eur J Vasc Surg 1989;3:145-51.
36. Herring MB. Endothelial cell seeding. J Vasc Surg 1991;13:731-2.
37. Walluscheck KP, Steinhoff G, Kelm S, Haverich A. Improved endothelial cell attachment on ePTFE vascular grafts pretreated with synthetic RGD-containing peptides. Eur J Vasc Endovasc Surg 1996;12:321-30.
38. Nagel T, Resnick N, Atkinson WJ, Dewey CF Jr, Gimbrone MA Jr. Shear stress selectively upregulates intercellular adhesion molecule-1 expression in cultured human vascular endothelial cells. J Clin Invest 1994;94:885-91.
39. Shyy JYJ, Li YS, Lin MC, et al. Multiple cis-elements mediate shear stress-induced gene expression. J Biomech 1995; 28:1451-7.
40. Conte MS, Choudhry RP, Shirakowa M, Fallon JT, Birinyi LK. Endothelial cell seeding fails to attenuate intimal thickening in balloon-injured rabbit arteries. J Vasc Surg 1995;21:413-21.
41. Taber TE, Maikranz PS, Haag BW, et al. Maintenance of adequate hemodialysis access. Prevention of neointimal hyperplasia. ASAIO J 1995;41:842-6.
42. Himmelfarb J, Saad T. Hemodialysis vascular access: emerging concepts. Curr Opin Nephrol Hypertens 1996;5:485-91.
43. Dichek DA, Anderson J, Kelly AB, Hanson SR, Harker LA. Enhanced in vivo antithrombotic effects of endothelial cells expressing recombinant plasminogen activators transduced with retroviral vectors. Circulation 1996;93:301-9.
44. Dunn PF, Newman KD, Jones M, et al. Seeding of vascular grafts with genetically modified endothelial cells. Secretion of recombinant TPA results in decreased seeded cell retention in vitro and in vivo. Circulation 1996;93:1439-46.

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