Microvascular Endothelial Cell Sodding of 1-mm Expanded Polytetrafluoroethylene Vascular Grafts

Karl M. Ahlswede, Stuart K. Williams

Abstract  The formation of an endothelial cell lining on the inner surface of polymeric grafts may reduce the inherent thrombogenicity of synthetic implants. Endothelial cell transplantation onto the luminal surface of grafts has been suggested as one method of creating new endothelial cell linings on grafts. The purpose of this study was to morphologically evaluate the very early events of healing (between 4 and 14 days) of 1-mm-internal-diameter expanded polytetrafluoroethylene (ePTFE) grafts that were treated with autologous microvessel endothelial cells at the time of graft implantation. We evaluated the development of new intimal linings in microvascular endothelial cell-sodded 1-mm ePTFE vascular grafts and compared their healing characteristics with non-cell-treated grafts by using a rat aortic graft model. Endothelial cells were isolated from intraperitoneal fat pads of female rats and transplanted onto the grafts by using a pressure sodding method. One-centimeter-long grafts were immediately implanted as interpositional grafts in the aorta. Non-cell-treated grafts were also implanted. Grafts were explanted 4, 7, and 14 days after implantation and were evaluated by light and scanning electron microscopy. Morphometric analysis of the graft surfaces revealed the cellular coverage in sodded grafts to be 93.7±8.7% and in nonsodded grafts, 1.1±1.9%. Areas not covered by cells exhibited thrombus and bare graft. The luminal lining of cells exhibited morphological characteristics, indicating they were antithrombogenic, based on morphological criteria, and exhibited characteristics of endothelium. We conclude that microvascular endothelial cell sodding of microvascular ePTFE grafts results in a dramatic acceleration of the formation of a luminal cell lining that covers more than 90% of the luminal surface of grafts by 7 days. While control grafts exhibited a thrombogenic surface, sodded grafts exhibited an antithrombogenic cell lining, as indicated by the absence of adherent platelets, white cells, and fibrin. (Arterioscler Thromb. 1994;14:25-31.)

Key Words • endothelial cells • vascular grafts • cell transplantation • hyperplasia • bypass grafts • thrombogenicity

The limitations associated with autogenous vein grafts in microvascular surgery have prompted the search for clinically acceptable synthetic microvascular (<2 mm) grafts. Methods to improve the biocompatibility of synthetic grafts, including the accelerated formation of a complete endothelial cell lining on the luminal surface, have been the focus of numerous research programs. The susceptibility of microvascular grafts to thrombosis and hyperplastic narrowing is accentuated because of the small luminal diameters of these grafts. Attempts at improving the biocompatibility of synthetic grafts have included new polymer designs, including chemical modification of the synthetic surface, and methods to increase the biological nature of grafts. Changes in the structure of polymers (eg, porosity) as well as the incorporation of growth factors have been suggested as factors to help stimulate the spontaneous formation of biological linings on prosthetic implants. Tissue and cell transplantation methods have been reported as methods of accelerating both the formation of new cellular linings on the blood flow surface of synthetic implants and healing the entire implant. All of these studies recognize that the regenerative, antithrombogenic characteristics of a biological lining on synthetic grafts have significant functional advantages over the relatively inert characteristics of bare polymer.

Microvascular endothelial cell sodding of synthetic grafts represents one method proposed for the accelerated formation of an antithrombogenic endothelial cell lining on synthetic grafts. This method involves the isolation of microvascular endothelial cells from microvascularized tissue and the immediate deposition of these cells onto the luminal surface of vascular grafts. Although various sources of microvascularized tissue have been investigated, adipose tissue has been actively explored as a source of cells because of its abundance in the body. Previous investigations have shown that microvascular endothelial cell sodding results in large numbers of cells on the inner lining of grafts at the time of implant. The methods for microvascular endothelial cell sodding of synthetic grafts can be performed in the operating room and have been reported to result in endothelial cell linings on human vascular grafts.

We evaluated microvascular endothelial cell-sodding techniques with 1-mm microvascular expanded polytetrafluoroethylene (ePTFE) grafts in a rat aortic graft model. We observed that sodded 1-mm grafts exhibited an accelerated formation of a luminal cellular lining and that this lining was associated with reduced platelet and cellular adherence. The reduced thrombogenic characteristics of this new cell lining compared with untreated polymer were observed on more than 90% of the graft.
surface after 7 days. We concluded that methods for microvascular endothelial cell sodding of larger-diameter vascular grafts, previously reported using canine models, are amenable to microvascular grafts with luminal diameters of 1 mm.

Methods

Animal Selection and Welfare

All animal studies were performed after approval of protocols by our animal review committee. National Institutes of Health (NIH) guidelines for the care and use of laboratory animals (NIH publication 85-23, revised 1985) were observed. Animals were housed in American Association for the Accreditation of Laboratory Animal Care–approved facilities, and all surgeries were performed and animals cared for immediately before and after surgery in areas designated for animal surgery. Female Sprague-Dawley rats weighing 250 to 375 g were used. For each explant time reported, six animals with patent grafts were evaluated; three of these animals received control grafts, and three received cell-sodded grafts.

Fat Retrieval

General anesthesia was induced and maintained by intraperitoneal injections of pentobarbital. Sterility was maintained, and a warming pad was used throughout the procedure. The animal was shaved, and a midline abdominal incision was made. The distal two thirds portion of fat surrounding the uterine horns was removed after ligation with 4-0 silk. The fat was minced into pieces and placed in Dulbecco’s cation-free phosphate-buffered saline (DCF-PBS, pH 7.4) containing 0.1% bovine serum albumin (BSA; Sigma Chemical Co, St Louis, Mo). If the animal was to receive a nonsodded graft, the abdomen was left open and aortic dissection followed. If the animal was to receive a sodded graft, the fascia and skin were each closed with running 4-0 silk, and the animal was placed under warming lights.

Microvessel Endothelial Cell Isolation

The fat suspension was drained, and the minced fat was placed in an enzyme solution at a concentration of 1 g fat/mL. The enzyme solution was composed of crude clostridial collagenase 2 mg/mL (Boehringer Mannheim) and BSA 2 mg/mL in DCF-PBS and was sterilized by 0.2-μm filtration. The mixture, in an Erlenmeyer flask with a small magnetic stirring bar, was placed in a 37°C shaking water bath and was agitated vigorously for 30 minutes. The digested tissue was centrifuged at 100g for 7 minutes. The resulting cell pellet was washed with 10 mL DCF-PBS containing 0.1% BSA followed by 100g centrifugation for 3 minutes. After repeating the washing and centrifugation, aliquots for determination of cellular concentration were removed, and cell numbers were determined by electronic cell counting. The amount of cell suspension yielding the desired number of cells was determined, and the appropriate volume of cells was centrifuged for 3 minutes at 700g. The cell pellet was suspended in 1 mL medium 199 (M-199; GIBCO, Grand Island, NY) containing 0.1% BSA and was used for sodding the vascular graft.

Graft Sodding

Coincident with the first washing of the cell pellet, the graft was treated with buffer solution. A steam-sterilized, 1-mm internal-diameter ePTFE vascular graft with a 30-μm inter nodal distance (Impra Inc) was attached to the sodding device. A 20-gauge mouse-feeding needle with a 2-mm bulb secured the graft, and the open end was clamped closed with a vascular clamp. The feeding needle was attached to a stopcock. M-199 (1 mL) containing 0.1% BSA was flushed through the graft, which was then placed in a bath of the same solution until cell isolation was completed. The complete sodding apparatus is illustrated in Fig 1.

When cell isolation was completed, a 1-mL syringe containing the desired number of cells in suspension was attached to the sodding device, and the suspension was flushed through the graft, followed by 0.3 mL M-199 and 0.1% BSA. Grafts were sodded with cells at a concentration of 3×10⁶ cells/cm². The stopcock was used to close off the needle and maintain any pressure generated by the sodding. The intraluminal pressure was less than 5 psi (260 mm Hg) during both the introduction of cells and the pressure-induced flow of medium through the graft. The sodded graft and apparatus were set aside while the aorta was dissected. The entire procedure, from the first skin incision to retrieve fat to the completion of graft sodding, was completed in less than 90 minutes.

Aorta Dissection and Graft Placement

Under an operating microscope, the midline incision was reopened, and the intestines were wrapped and placed to the left of the incision. The operating field and exposed organs were kept moist with saline solution. The retroperitoneum was entered to the right of the midline medial to the right ureter. The aorta was dissected free of the inferior vena cava and surrounding tissues from the renal arteries to the iliac bifurcation. All lumbar branches were divided after bipolar coagulation. Intravenous heparin was given via a right lumbar vein, and bleeding was controlled with bipolar coagulation. After 4 minutes of heparin circulation, the aorta was clamped and resected below the renal arteries (approximately 6 mm) to accommodate the 1-cm graft. The aortic stumps were flushed with saline and gently diluted by using microvascular forces.

The sodded or nonsodded vascular graft was trimmed to 1 cm in length. Using 10-0 nylon (Ethicon Co), the graft was placed with interrupted sutures. After unclamping, light pressure was held over the graft until hemostasis was obtained. A single intravenous injection of saline via the right lumbar vein was given that was equal to the estimated blood loss. The retroperitoneum was approximated, the intestines were re replaced, and fascia was closed with running 4-0 silk. The skin was closed with staples. The animal recovered under warming lights and was returned to the animal facility until graft explantation.

Graft Explantation

After induction of general anesthesia, the abdomen was opened for graft isolation and evaluation of patency. If the aorta was patent graft distal to the graft, access to the left ventricle was gained, and perfusion with DCF-PBS was performed until the aorta was clear of blood. Perfusion fixation with paraformaldehyde 4% in sodium phosphate buffer was performed. The graft was removed and kept in paraformaldehyde for 1 hour. After this period the graft was cut longitudinally, and one half of the graft was placed in paraformaldehyde and the other half in 3% glutaraldehyde in piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES) buffer.
Light Microscopy

Samples for light microscopy were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4). Samples were dehydrated, paraffinized, sectioned, deparaffinized, and stained with both hematoxylin and eosin and trichrome stains. Photomicrographs were obtained by using a Nikon Optiphoto light microscope.

En Face Evaluation of Explanted Grafts With bis-Benzimide Nuclear Staining

Explanted grafts were fixed in 4% paraformaldehyde, solubilized with 1% Triton X-100, and stained with the nuclear-specific stain bis-benzimide (BBI; Hoechst dye 33258). Samples were immediately evaluated under UV epifluorescent excitation.

Scanning Electron Microscopy

Samples for scanning electron microscopy were fixed with 3% glutaraldehyde in PIPES buffer (pH 7.4). Samples were washed with PIPES buffer, dehydrated in a graded series of acetone, and critical-point dried. Dry samples were sputter coated by using a gold target. Samples were observed, and photomicrographs were obtained by using a JEOL 820 scanning electron microscope.

The percentage coverage by endothelium was quantified by first obtaining 10 randomly selected photomicrographs of the midgraft area with an instrument magnification of 100×. Photomicrographs were subsequently analyzed by using an image analysis system (Image 1, Ardmore, Pa). The total area of each print covered by endothelial cells was first determined. The total area of each print was subsequently quantified, and the percentage of surface area covered by endothelium was calculated as (area covered by endothelium/total graft area) × 100.

Immunocytochemistry of Fat

Samples of intraperitoneal fat were frozen, and cryostat sections were obtained. Sections were subsequently stained with primary antibodies to either von Willebrand factor (Dako Corp), α-smooth muscle cell actin (Sigma), or cytokeratin peptide 18 (Sigma). Fluorescein isothiocyanate-labeled secondary antibodies were used. All sections were also treated with BBI to label nuclei.

Immunocytochemistry of Explanted Grafts

Sections from paraffin-embedded grafts were deparaffinized and treated with pronase to expose additional antigen. Sections were treated with primary and secondary antibodies as described above.

Statistical Analysis

Morphological data were analyzed by using an unpaired Student's t-test.

Results

The use of autologous fat for cell isolation and transplantation has several attractive attributes, including the availability and ease of procurement of this tissue. One concern, however, is the type of cells that exist in the tissue used for cell isolation and the possibility of mixed populations of cells after tissue digestion. We quantified the type and relative number of cells in freshly excised rat intraperitoneal fat by using immunocytochemical identification of cell types (Table 1 and Fig 2). Fig 2a illustrates the staining pattern and distribution of von Willebrand factor-positive cells; the distribution of nuclei in the same section is shown in Fig 2b. Quantification of cell distribution in fat indicated that the most predominant cell type, even before tissue digestion and cell isolation, was endothelium. Mesothelial cells, characterized by cytokeratin peptide 18 staining, and smooth muscle cells, characterized by α-smooth muscle cell actin expression, collectively represented less than 5% of the total cell population that was obtained from rat intraperitoneal fat.

While developing the 1-mm sodded graft model, we evaluated methods for cell deposition, graft implantation, and the healing of grafts as determined at explant. The deposition of cells onto 1-mm grafts was accelerated by the use of a pressure sodding technique. As shown in Fig 1, cells were deposited on the graft by using pressure deposition created by forcing buffer through the graft interstices. The pressure necessary to permit the flow of medium through the grafts was less than 5 psi (260 mm Hg). The distribution of cells on the luminal surface immediately after pressure deposition was evaluated by scanning electron microscopy (Fig 3a) and by light microscopy (Fig 3b). Both single cells and multicellular fragments were observed by scanning microscopy. These fragments were of the size characteristic of the microvascular vessels present in fat.

Implantation of both sodded and control grafts involved the dissection of the aorta free of the vena cava (Fig 4a), resection of the aorta (Fig 4b), and anastomosis of the grafts with nine interrupted 10-0 nylon sutures (Fig 4c). During the preliminary stages of this study, we observed a significant difference in survival between sodded graft and control graft groups. Our first studies revealed an animal survival rate of 93% (14 of 15) for control grafts, whereas only 17% (1 of 6) of the animals that received sodded grafts survived. The level of anticoagulation in this early study was 200 units heparin/kg animal body weight IV at the time of graft implant. All deaths in this first series were the direct result of hemorrhage at the graft-aorta anastomosis. Rather than reversing the anticoagulant effects of the heparin (ie, protamine), we reduced the level of anticoagulant to 100 units heparin/kg body weight and observed a corresponding reduction in anastomotic bleeding in rats with sodded grafts.

Table 1. Immunocytochemistry of Cryostat-Sectioned Rat Intraperitoneal Fat

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Total Cells Expressing Antigen, %</th>
<th>(Antigen-Positive Cells/No. of Cells Counted)</th>
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<tbody>
<tr>
<td>von Willebrand factor</td>
<td>92.4 (933/1010)</td>
<td></td>
</tr>
<tr>
<td>α-Smooth muscle cell actin</td>
<td>3.2 (32/1008)</td>
<td></td>
</tr>
<tr>
<td>Cytokeratin peptide 18</td>
<td>1.7 (17/1014)</td>
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Rat intraperitoneal fat was frozen, and cryostat sections were obtained. Sections were reacted with primary and secondary antibodies and subsequently stained with the nuclear stain bis-benzimide (BBI). Microscopic fields were evaluated using a 40× water immersion lens. Total number of cells in each field was determined by counting nuclei stained with BBI. The number of cells expressing specific antigens was then counted in the identical field using fluorescein isothiocyanate epifluorescent illumination. A total of at least 1000 cells were counted for each primary antibody. von Willebrand factor (vWF) expression was evaluated by using polyclonal antibody to human vWF, which cross-reacts with rat vWF. α-Smooth muscle cell actin and cytokeratin peptide 18 expression were evaluated by using monoclonal antibodies.
### Explant Evaluation

The presence of a cellular lining on sodded grafts was identifiable by scanning electron microscopy at 4 days, the earliest time of explant (Fig 5a). Light microscopic evaluation of the sodded grafts at 4 days (Fig 5b) revealed the presence of a thin lining of antithrombogenic cells, as shown by the lack of platelet, leukocyte, or significant fibrin deposition. When 4-day sodded grafts were stained with the nuclear stain BBI, the presence of cells was also confirmed; Fig 5c shows that the luminal surface of these grafts contained cells. Note that the elongated nuclei are a shape that would be expected for flow-oriented endothelium. Morphological evaluation of these grafts 7 days after implantation revealed the persistence of a cellular lining, as visualized by scanning electron microscopy (Fig 6a), light microscopy (Fig 6b), and nuclear staining with BBI (Fig 6c). The cellular lining remained morphologically similar for at least 14 days, which was the longest implant time evaluated during this study (Fig 7a). In contrast, the luminal surface of control (non-cell-treated grafts) did not exhibit a cellular lining at any of the explant times (ie, 4, 7, and 14 days). Morphologically, the surface of the control grafts exhibited loosely adherent platelets, leukocytes, and fibrin, as shown by light microscopy (Fig 7b) and scanning electron microscopy (Fig 7c). We did observe the initiation of pannus ingrowth in control grafts, but this cellular migration was less than 1 mm in all grafts examined.

A morphometric analysis was performed by analyzing the luminal coverage of patent grafts implanted for 7 days (Table 2). Nonsodded grafts (n=3) were compared with grafts that were sodded at a density of $3 \times 10^6$ cells/cm$^2$ (n=2). As shown, the percent of cellular, thrombus, and bare coverage was significantly different between the sodded and control groups at 7 days.

To further characterize the cells on the surface of sodded grafts, explanted grafts were subjected to immunocytochemistry (Fig 8). Cells on the luminal surface of the grafts reacted specifically with antibodies to von Willebrand factor.

### Discussion

Microvascular endothelial cell sodding has been reported as a method of accelerating the formation of an antithrombogenic cell lining on the luminal surface of prosthetic vascular grafts. In the present study we have described methods that use microvessel endothelial cell sodding with microvascular ePTFE vascular grafts. The use of pressure sodding dramatically reduced the time necessary to deposit endothelial cells on the luminal surface of these grafts. Furthermore, after implantation of grafts as aortic replacements, we observed the formation of a new antithrombogenic luminal cell lining. This new lining exhibited all of the morphological characteristics of the native endothelium lining adjacent native vessels. All of these results were compared with control (non-cell-sodded) grafts, which exhibited a thrombogenic surface at all explant times. We therefore believe that microvascular endothelial cell sodding of prosthetic vascular implants, previously reported for larger-diameter grafts (ie, >3 mm), $^{4,12,13,15-18}$ is applicable to microvascular grafts with internal diameters of 1 mm. Vascular
Photographs showing surgical technique for the implantation of 1-mm expanded polytetrafluoroethylene (ePTFE) grafts in the aortas of rats. a, Dissection of the aorta free from surrounding tissue and blood vessels; b, resection of the aorta and dilation of the vessel stumps; and c, implantation of the 1-mm ePTFE graft with nine interrupted sutures.

Grafts in the submillimeter range could also conceivably be treated with endothelium.

The observation of significant areas of cellular monolayers exhibiting reduced thrombogenicity as early as 4 days after graft treatment indicates that the cellular elements present in the original inoculum exhibit the ability to differentiate rapidly and exhibit arterial endothelial cell phenotype. The non-cell-covered areas of sodded grafts exhibited the presence of both platelets and fibrin. Nonsodded grafts were essentially devoid of antithrombogenic cell coverage, exhibiting predominantly platelets and fibrin deposits. One significant difference in sodded versus control grafts, observed extensively at the earliest times of explantation, was the observed localization of predominantly white cells in areas of sodded grafts devoid of a cellular monolayer. Whereas control grafts exhibited occasional white cells, the density of white cells on bare areas of sodded graft surfaces was significantly different. Herring et al.219 and Emerick et al.20 indicate that significant activation and attraction of leukocytes occur after implantation of endothelialized prosthetic grafts. We therefore suggest that a major factor in the early healing of endothelial cell transplanted grafts may be the sequestration of leukocytes to areas of graft that have not been completely endothelialized. This attachment of cells is often localized in areas surrounded by cell monolayers that exhibit minimal leukocyte adherence.

While developing this small-animal model of vascular graft endothelial cell transplantation, we observed a significant difference in the susceptibility of endothelial cell-sodded grafts to anastomotic bleeding when heparin anticoagulation was used. The extent of bleeding was significantly different between sodded and control grafts. The control grafts tolerated a higher concentration of systemic anticoagulation. The use of heparin in these studies was based on the historical need to use...
anticoagulants when prosthetic grafts are implanted. However, the presence of endothelial cells on the luminal flow surface of grafts that exhibited antithrombogenic characteristics at the time of implant may provide an explanation for the unusual bleeding observed with sodded grafts. The distinction between endothelial cell-sodding methods (cells placed directly on the flow surface) and seeding methods (cells mixed with blood or similar coagulum and layered on the flow surface) is critical to note, since a seeded surface will still exhibit a thrombogenic luminal flow surface. Our methods involved the pressure-accelerated deposition of cells on the surface of grafts. In addition, cells were forced into the luminal surface of the graft to a depth of approximately 50 μm, while fluid penetrated the full interstices of the graft and was observed as fluid droplets on the abluminal surface. It is important to note that this procedure did not result in any observed bleeding or fluid leakage in the interstices of the graft.

All incidences of bleeding were limited to the anastomotic sites. We therefore suggest that the use of heparin or other anticoagulant therapies may not be necessary when prosthetic grafts are implanted containing a flow surface treated with endothelial cell-sodding methods.

We conclude that sodding techniques previously developed for larger-diameter vascular prosthetic devices are amenable to microvascular ePTFE grafts. The development of a cellular lining on the luminal surface of sodded grafts was quite rapid, with nearly complete cellular coverage by 7 days. This makes the present study unique in the field of endothelial cell transplantation, in which the earliest explant times reported are characteristically longer than 3 weeks. Although the exact origin of cells on the luminal lining of sodded grafts is not known, we suggest that these cells represent the same cells present in the original inoculum. Complete, spontaneous endothelialization of ePTFE grafts has not been reported to occur this early in rat graft...
endothelial cell sodding of ePTFE vascular grafts

TABLE 2. Luminal Coverage of Sodded and Nonsodded 1-mm ePTFE Vascular Grafts Implanted for 7 Days

<table>
<thead>
<tr>
<th></th>
<th>Cellular Coverage, %</th>
<th>Thrombus Coverage, %</th>
<th>Bare Graft, %</th>
</tr>
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<tbody>
<tr>
<td>Sodded, 3x10^4</td>
<td>93.7±8.7</td>
<td>6.3±8.7</td>
<td>0</td>
</tr>
<tr>
<td>cells/cm²</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonsodded</td>
<td>1.1±1.9</td>
<td>74.1±17.9</td>
<td>24.8±17.0</td>
</tr>
<tr>
<td>P</td>
<td>&lt;.01</td>
<td>&lt;.05</td>
<td>&lt;.05</td>
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ePTFE indicates expanded polytetrafluoroethylene. One-millimeter vascular grafts were implanted either as control (nonsodded) or sodded (sodding density, 1x10^4 cells/cm²) grafts. Grafts were explanted at 7 days and evaluated by scanning electron microscopy. Percent cell coverage was quantified by analyzing 10 micrographs (original magnification, x 100) from each explant with an image analysis system. Grafts from three animals were analyzed for each study group. Numbers represent the mean±SEM for 30 evaluations (10 micrographs for each of three explanted grafts).

models. Moreover, in control grafts, we did not observe the formation of any significant pannus of cells at the anastomosis for a distance in excess of 1 mm. Although the present study used grafts limited to 1 cm in length, the sodding technique is amenable to grafts of much longer lengths, which may permit microvascular reconstructions using ePTFE of lengths greater than 1 cm.

While the present study was not designed to evaluate the development of hyperplasia in vascular grafts, it is significant to note the relative lack of hyperplasia observed in sodded vascular grafts. The cellular lining that was observed on the sodded grafts was a maximum of two cell layers thick, with the luminal surface covered by cells that morphologically and immunocytochemically resembled endothelium. Immunocytochemical studies of intact fat indicate this tissue contains predominantly endothelium and adipocytes. Since this source of tissue contains very few mesothelial cells, smooth muscle cells, or pericytes, hyperplasia may be delayed or limited because of the lack of cells to initiate this process. Alternatively, transplanted microvascular endothelium may serve to reduce the thrombogenicity of polymeric grafts as well as to reduce the occurrence of graft-associated intimal hyperplasia.

Fig 8. Fluorescence photomicrograph of the luminal surface of a sodded graft. Paraffin-embedded sections were deparaffinized and reacted with antibodies toward von Willebrand factor. Only cells on the luminal surface exhibit fluorescence. Bar=5 μm.

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