In Vivo Cell Seeding With Anti-CD34 Antibodies Successfully Accelerates Endothelialization but Stimulates Intimal Hyperplasia in Porcine Arteriovenous Expanded Polytetrafluoroethylene Grafts

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Background—The patency of AV expanded polytetrafluoroethylene (ePTFE) grafts for hemodialysis is impaired by intimal hyperplasia (IH) at the venous outflow tract. The absence of a functional endothelial monolayer on the prosthetic grafts is an important stimulus for IH. In the present study, we evaluated the feasibility of capturing endothelial progenitor cells in vivo using anti-CD34 antibodies on ePTFE grafts to inhibit IH in porcine AV ePTFE grafts.

Methods and Results—In 11 pigs, anti-CD34–coated ePTFE grafts were implanted between the carotid artery and internal jugular vein. Bare ePTFE grafts were implanted at the contralateral side. After 3 (n=2) or 28 (n=9) days, the pigs were terminated, and the AV grafts were excised for histological analysis and SEM. At 3 and 28 days after implantation, 95% and 85% of the coated graft surface was covered by endothelial cells. In contrast, no cell coverage was observed in the bare graft at 3 days, whereas at 28 days, bare grafts were partly covered with endothelial cells (32%; P=0.04). Twenty-eight days after implantation, IH at the venous anastomosis was strongly increased in anti-CD34–coated grafts (5.96±1.9 mm²) compared with bare grafts (1.70±0.4 mm²; P=0.03). This increase in IH coincided with enhanced cellular proliferation at the venous anastomosis.

Conclusions—Autoseeding with anti-CD34 antibodies results in rapid endothelialization within 72 hours. Despite persistent endothelial graft coverage, IH at the outflow tract is increased profoundly at 4 weeks after implantation. Further modifications are required to stimulate the protective effects of trapped endothelial cells. (Circulation. 2005; 112:12-18.)

Key Words: arteriovenous anastomosis ■ endothelium ■ grafting ■ hyperplasia ■ renal dialysis

The patency of AV expanded polytetrafluoroethylene (ePTFE) grafts for hemodialysis is severely compromised by intimal hyperplasia (IH) at the venous outflow tract, ultimately leading to graft thrombosis. The clinical relevance of AV graft failure is illustrated by the limited 1- and 2-year primary patency rates of 50% and 25%, respectively.1,2 No effective intervention currently is available to improve graft patency. Successful development of new strategies requires closer insight into the pathogenesis of IH formation. IH formation is thought to reflect an accumulation of several separate entities, including inflammatory, coagulatory, and hemodynamic factors.3–5 A crucial factor in both activation of coagulation and inflammation is the lack of a functional endothelial monolayer on the prosthetic graft, because the endothelium constitutes the first-line homeostatic defense mechanism by exerting anticoagulatory and antiinflammatory effects.6–8 Consequently, endothelial cell (EC) seeding at the luminal surface of prosthetic vascular grafts is a valuable strategy to improve graft patency.9,10 Indeed, graft seeding with autologous ECs has been shown to increase patency rates of prosthetic bypass grafts in clinical trials. However, implementation of in vitro EC seeding is hampered by the laborious procedures for harvesting, expansion, and application of ECs obtained from autologous veins or adipose tissue.

Bone marrow–derived endothelial progenitor cells (EPCs) have emerged as a promising source of autologous ECs. EPCs are a subset of CD34(+) cells with the potential to proliferate and differentiate into mature ECs.11 Recent studies have emphasized that circulating EPCs have the capacity to home to sites of vascular injury, thus promoting the process of

Receive date September 2, 2004; revision received February 17, 2005; accepted March 3, 2005.

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Circulation is available at http://www.circulationaha.org DOI: 10.1161/CIRCULATIONAHA.104.504407
reendothelialization. Moreover, in vitro seeding of prosthetic vascular grafts with CD34(+) cells markedly increased graft reendothelialization in animal models.

In the present study, we evaluated the feasibility of capturing EPCs in vivo using immobilized anti-CD34 antibodies on ePTFE grafts. If proved valid, this novel technique circumvents the laborious in vitro procedures mandatory for handling ECs. We hypothesized that in vivo “autoseeding” with immobilized anti-CD34 antibodies establishes a confluent, mature EC monolayer that may attenuate IH formation in the outflow tract of porcine AV ePTFE grafts.

Methods

Study Design

Eleven female Landrace pigs weighing 50.8±0.7 kg were used. In each pig, an anti-CD34–coated ePTFE graft was implanted between the carotid artery and the internal jugular vein at a randomly determined site. The same commercially available bare ePTFE graft was implanted at the contralateral side and served as an internal control. To study graft coverage, 2 pigs were terminated 72 hours after surgery. The remaining 9 pigs were terminated at 28 days of follow-up to evaluate cell retention on the graft and IH in the venous outflow tract. The study protocol was approved by the Ethics Committee on Animal Experimentation of the University Medical Center Utrecht, and animal care conformed to established guidelines.

Graft Coating

Anti–human CD34 monoclonal antibodies (IgG2a, epitope class III) were immobilized to the ePTFE graft material (Orbus Medical Technologies) with a proprietary multistep process (Sens). The first step involves functionalization of the surface. To this functionalized surface, a biopolymer is covalently coupled in a reaction that leaves immobilized polymer without adding any new species to the graft or the polymer. Next, the anti–human CD34 monoclonal antibody is covalently coupled to the polymer-coated graft through stable peptide linkages. The anti-CD34–immobilized ePTFE grafts have been shown to exhibit cross-reactivity in porcine graft explants, which were observed to have a rich population of EC marker–positive cells after only 4 hours.

Anesthesia

Before operation and termination, the animals were fasted overnight and premedicated with intramuscular ketamine hydrochloride 10 mg/kg, midazolam 0.4 mg/kg, and atropine 0.5 mg and intravenous thiopental sodium 4 mg/kg. They were then intubated and ventilated with a mixture of O2 and air (1:2). An ear vein was used for continuous administration of midazolam 0.3 mg · kg⁻¹ · h⁻¹, sufentanil 2.5 μg · kg⁻¹ · h⁻¹, and pancuronium 50 μg · kg⁻¹ · h⁻¹.

AV Graft Implantation

Starting 6 days before the operation, the pigs received acetylsalicylic acid 80 mg/d. Clopidogrel 225 mg was given 1 day before operation and continued at a dose of 75 mg/d until termination. The ePTFE AV grafts were created bilaterally between the carotid artery and internal jugular vein as described previously. In short, heparin 5000 IU IV was administered before manipulation of the vessels. After dissection of the common carotid artery and the internal jugular vein, papaverin 5 mg/mL was applied locally to prevent vascular spasm. Next, the carotid artery was clamped, and a standardized 8-mm arteriotomy was performed. An end-to-side anastomosis was created at an angle of 45° by use of a continuous suture of 8-0 polypropylene (Ethicon). All ringed ePTFE grafts were 5 mm in diameter and 7 cm in length. The venous anastomosis was created in a similar fashion.

Tissue Preparation and Histological Analysis

After 72 hours (2 pigs) or 4 weeks (9 pigs) of follow-up, pigs were anesthetized as described previously. Heparin 10 000 IU was administered before manipulation of the vessels. Next, the carotid artery was cannulated, and the grafts and adjacent vessels were perfused with saline for 3 minutes. Subsequently, the pigs were euthanized, and the grafts and adjacent vessels were excised. The ePTFE grafts were then cut into 2 pieces: the first 2 cm from the arterial anastomosis was fixed in 2% glutaraldehyde for SEM, and the remainder of the ePTFE graft and the adjacent jugular vein was immersed in formalin for histological analysis. After 24 hours of formalin fixation, the graft and jugular vein were cut into 5-mm blocks and embedded in paraffin. Histological analysis was performed on 5-μm-thick sections obtained from 4 different locations to determine intimal and medial areas: at the center of the ePTFE graft with an in-between distance of 5 mm to determine graft coverage (2 sites), at the center of venous anastomosis, and 5 mm proximal (ie, caudal) to the venous anastomosis. For morphometric analysis, sections were stained with elastin van Gieson (EvG). With the highest magnification that allowed visualization of the entire vein section in 1 field, the intimal and medial areas were manually traced at the venous anastomosis and proximal to the anastomosis. The intima was defined as the tissue area encompassed by the internal elastic lamina. Thrombus formation was discriminated from IH through the use of hematoxylin–eosin– and EvG-stained sections. In the sections of the venous anastomosis, 2 parts of the intimal area were distinguished: the intima covering the graft (shoulder region) and the intima located at the venous part of the anastomosis (cushioning region). For immunohistochemical analysis, sections were incubated in 1.5% hydrogen peroxide in methanol to block endogenous peroxidases. Next, sections were incubated in boiling 10 mmol/L citrate acid for 15 minutes and subsequently preincubated with 10% horse serum (Vector Laboratories). Serial sections were stained with murine antibodies against α-smooth muscle actin (Sigma) for vascular smooth muscle cells (VSMCs) at 1:1500 dilution, Ki-67 (Immunotech) for cellular proliferation at 1:100 dilution, and MAC387 (Serotec) for macrophages at 1:10 dilution. Subsequently, sections were incubated with a biotinylated anti-mouse IgG antibody (Vector Laboratories) for 1 hour. To visualize ECs, sections were incubated with lectin from bandeiraea simplicifolia BS-1 at 1:100 dilution (Sigma) for 1 hour. Immunoreactive materials were visualized by use of streptavidin-labeled HRPO, diaminobenzidine in 0.05 mol/L Tris-Cl mixed with 0.01 mol/L imidazole, and 0.1% hydrogen peroxide. Sections were counterstained with hematoxylin. Ki-67–stained sections from each venous anastomosis were used to measure the amount of proliferating cells within the intima. Therefore, 100 cells were counted per high-power field (2 at the shoulder region, 2 at the cushioning region). A proliferation index was defined as the number of positive cells divided by the sum of Ki-67–negative and –positive cells and expressed as a percentage.

Scanning Electron Microscopy

The integrity of the cellular coverage of the ePTFE grafts was also visualized by SEM. For this purpose, the ePTFE grafts were fixed in 2% glutaraldehyde and then dehydrated through increasing concentrations of ethanol (80% to 100%). The samples were dried with 2% glutaraldehyde and then dehydrated through increasing concentrations of ethanol (80% to 100%). The samples were then critical-point dried with liquid CO2, coated with a thin layer of platinum/palladium, and analyzed with an SEM (Philips XL30).

Statistical Evaluation

Data are presented as mean±SEM. SPSS 11.0 was used for all statistical calculations. To ascertain the significance of differences, we performed the Wilcoxon test. A value of P<0.05 was considered significant.

Results

In total, 22 grafts were successfully implanted in 11 Landrace pigs. In the 2 pigs terminated after 3 days, all grafts (n=4)
were patent at time of harvest. In the 9 pigs terminated at 4 weeks, grafts were patent in 6 pigs, whereas bilateral occlusion was observed in 3 pigs. Histological analysis of the occluded grafts showed recent thrombotic occlusion on top of extensive IH in the venous outflow tract. These occluded grafts were excluded for further analysis because it is impossible to reliably perform morphometric analysis of IH in occluded grafts. The remaining patent grafts (n=16) were included for final analysis.

Scanning Electron Microscopy
Three days after graft implantation, the anti-CD34–coated grafts showed a confluent monolayer of cells. These attached cells had an inhomogeneous phenotype, ranging from round to spreading and flattened cells. In contrast, the bare grafts showed only little cellular adherence. Twenty-eight days after graft implantation, most of the attached cells on the coated graft were flattened. In some areas, cells with a plateletlike phenotype were observed on top of the cellular layer on the coated graft. At this time point, partial coverage of cells was observed in the bare grafts also (Figure 1).

Histological Analysis

Graft Coverage
Histological analysis of the grafts confirmed complete cellular coverage of the anti-CD34–coated grafts 3 days after graft implantation. The adhered cells were identified as ECs by lectin staining (Figure 2). EC coverage at 3 days after implantation was ∼95% in the coated graft and <5% in the bare grafts. Four weeks after implantation, 88±5% of the surface in the coated grafts was covered by lectin-positive cells, which was significantly higher than in the bare grafts (32±8%; P=0.04). (Figure 3A and 3B).

Intimal and Medial Areas
EvG-stained sections from the venous anastomosis revealed prominent intimal thickening at 4 weeks after graft implantation (Figure 3C and 3D). At this location, a strong increase...
in intimal area was observed in coated grafts compared with bare grafts (the Table). Overall, the amount of microvessels in the intima was relatively low in both groups. The vast majority of microvessels were located in the adventitia of the recipient vein. In this vessel layer, microvessel formation also was comparable between groups. Approximately 90% of the intimal cells expressed the VSMC marker $\alpha$-actin (Figure 3E), whereas at the shoulder region of the venous anastomosis, a small amount of macrophages was observed in both groups. Thus, except for the size of the intimal area, no differences in composition of the IH lesions at the venous anastomosis were observed between groups. At the proximal vein (5 mm proximal to the toe of the venous anastomosis), the intimal and medial areas did not differ significantly between the 2 groups (the Table).

**Cellular Proliferation**

Ki-67–stained sections obtained from the venous anastomosis showed prominent cellular proliferation in the intima (Figure 3F). At the venous anastomosis, the proliferation index was significantly higher in the coated grafts (20.9±2%) compared with the bare grafts (13.3±2%; $P=0.027$). The higher proliferation rate in the coated grafts was due mainly to increased proliferation at the shoulder region (Figure 4).

**Discussion**

In the present study, we show that anti-CD34 coating of AV ePTFE grafts results in a rapid and almost complete coverage of the graft with lectin-positive cells within 72 hours after implantation in pigs. This cellular coverage of coated grafts persists for at least 4 weeks. Despite cellular coverage of the
luminal surface, IH is augmented at the venous anastomosis of anti-CD34–coated grafts at 28 days of follow-up compared with uncoated grafts. The present findings show that anti-CD34 coating is successful in promoting cellular coverage of grafts with lectin-positive cells, whereas these trapped cells are unable to attenuate IH formation at the venous outflow tract.

Graft Coverage With Endothelial (Progenitor) Cells

The concept of EC seeding of grafts is based on the assumption that these cells will constitute a biologically active lining that will attenuate activation of blood passing through the graft. Unfortunately, the complicated procedures for harvesting, expansion, and seeding of ECs in vitro have prevented this method from finding broader application in humans. EPCs are bone marrow–derived CD34(+/−) cells with the potential to proliferate and differentiate into mature ECs. Circulating EPCs have recently been shown to actively provide new ECs from the circulating blood to sites of endothelial denudation and/or injury. Promoting adherence of these “endogenous” circulating endothelial progenitor cells could in theory eliminate the need for in vitro seeding procedures. In the present study, we observed rapid coverage with lectin(+) cells from 3 days onward. Four weeks after implantation, the cellular coverage of anti-CD34–coated grafts remained almost confluent, whereas the histological appearance of the cells gradually changed to flattened. The degree of cellular coverage compares favorably to previous studies on in vitro graft seeding techniques with CD34(+) cells in which graft coverage did not exceed 65%. The efficiency of in vitro seeding techniques using mature ECs has also been limited by low levels of EC retention. Combined, these data illustrate that antibody-assisted auto-seeding of CD34(+) cells is associated with more confluent coverage of grafts, whereas it simultaneously offers a safer and easier technique for clinical use.

Graft Coverage and IH

Increased graft coverage should result in less activation of circulating blood cells and potentially contribute to paracrine mediators conveying antiinflammatory and antiproliferative effects downstream at the venous outflow tract. This line of events assumes that the attached cells are able to exert effects similar to the physiological actions of a normal endothelial lining. In contrast, despite complete graft endothelialization in the anti-CD34–coated grafts, we observe a 3-fold increase in IH at the venous anastomosis.

Concomitantly, a strong increase in cellular proliferation was present, predominantly at the shoulder region of the venous outflow tract. These data imply a proliferative rather than antiproliferative effect of the cells covering the graft. Our results are consistent with those of Kang et al, who showed aggravated restenosis after intracoronary infusion of peripheral blood stem cells in patients who underwent coronary stenting. Several factors may have contributed to the adverse effects in the anti-CD34–coated grafts. First, it has previously been reported that CD34(+) cells are able to...
differentiate into various cell types, including ECs. Thus, VSMCs can originate from bone marrow–derived CD34(+) progenitor cells, implying that differentiation of CD34(+) captured cells into VSMCs can contribute to the increased proliferation index in CD34-coated grafts. Whereas differentiation of CD34(+) progenitor cells into macrophages has been reported, we observed no significant difference in macrophage numbers between coated and bare grafts. Second, EPCs have the capacity to release potent proangiogenic growth factors such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor. The proliferative and migratory effects of these cytokines are not restricted to ECs but also include VSMCs. Enhanced secretion of proliferation factors from cells adhered to anti-CD34–coated grafts may have contributed to increased IH at the venous anastomosis. Third, interaction of ECs with subendothelial matrix components has been shown to be of pivotal importance for the protective function of ECs. Therefore, the absence of a physiological microenvironment may also contribute to the apparent lack of protective effects. Fourth, laminar shear stress has been shown to upregulate EC-specific markers such as VE-cadherin in EPCs. Therefore, the turbulent flow pattern in AV grafts may have deteriorated differentiation into ECs, especially in the anastomotic region. Finally, adequate maturation of captured CD34(+) cells may have been hampered by the binding of the immobilized antibody to the CD34 epitope.

Study Limitations

The adhered cells were characterized as endotheliumlike cells by their morphological appearance on SEM and by immunohistochemistry using lectin from bandeiraea simplicifolia, a marker for porcine ECs. However, extensive characterization of the adhered cells was not feasible because of the unavailability of monoclonal antibodies for porcine endothelial (progenitor) cells markers such as CD133, VEGFR-2, CD31, and VE-cadherin. Consequently, we cannot exclude that the adhered cells contain a heterogeneous population, including CD34(−) cells. Still, the uniform lectin-positive staining of the graft-covering cells and their morphological appearance on SEM suggest efficient and specific binding of cells with an endothelial phenotype.

We used an AV graft model because of the poor patency rate of hemodialysis access grafts. The process of neointimal formation in AV grafts is characterized by specific pathophysiological stimuli such as high turbulent flow and graft-vein compliance mismatch, which coincide with the arterialization process of the vein. Therefore, extrapolation of these data to arterial bypass grafts may not be valid.

The uremic milieu has a clear impact on the progression of vascular diseases in end-stage renal disease patients. In this respect, the decreased number and impaired function of EPCs in patients with chronic renal failure have recently been suggested to contribute to the accelerated progression of cardiovascular diseases in these patients. Because this study was performed on nonuremic pigs, one should be cautious in extrapolating these results to the human situation.

Clinical Implications and Future Directions

The present findings indicate that autoseeding with EPCs offers a potential strategy to establish graft endothelialization in AV grafts. It remains to be established whether and to what extent these captured cells can be stimulated to regain their functional capabilities. In this respect, it is interesting to note recent developments demonstrating the regulatory role of vascular endothelial growth factor, erythropoietin, and angiopoietins on maturation of endothelial progenitor cells. In addition, capturing a mixture of both CD34(+) and CD14(+)CD34(−) cells also holds promise for optimal EC differentiation and functionality of EPCs. These options need to be addressed in future studies.

Acknowledgments

This study was supported in part by a grant from the Dutch Kidney Foundation (CSP 6001). Clopidogrel was supplied by Sanofi-Synthelabo (Maassluis, the Netherlands). We thank Orbis Medical Technologies (Fort Lauderdale, Fla) for generously supplying the ePTFE grafts and manufacturing the anti-CD34–coated grafts. We acknowledge Cees Weeterings for his excellent assistance with the SEM.

References

whereas the trapped cells aggravate neointimal formation. Several factors can contribute to these adverse effects of anti-CD34 antibodies on ePTFE grafts. Although anti-CD34 coating resulted in complete graft endothelialization within 72 hours after surgery, a profound increase in intimal hyperplasia was observed in the venous outflow tract of anti-CD34 coated grafts. The present findings show that anti-CD34 coating is successful in promoting autoseeding of ePTFE grafts, whereas the trapped cells aggravate neointimal formation. Several factors can contribute to these adverse effects of stem-cell seeding, including differentiation of CD34(+) into VSMCs and the release of potent mitogens for VSMCs by the trapped cells. Further modifications of the graft coating are required to stimulate the protective effects of trapped endothelial cells.

**CLINICAL PERSPECTIVE**

The patency of arteriovenous grafts for hemodialysis is severely compromised by intimal hyperplasia at the venous outflow tract. The concept of endothelial cell seeding of expanded polytetrafluoroethylene (ePTFE) grafts is based on the assumption that these cells will constitute a biologically active lining that will attenuate activation of blood passing through the graft, thereby reducing the release of mitogens for vascular smooth muscle cells (VSMCs). Endothelial progenitor cells are bone marrow–derived CD34(+) cells with the potential to proliferate and differentiate into mature endothelial cells. In the present study, we evaluated the feasibility of “autoseeding” with endothelial progenitor cells using immobilized anti-CD34 antibodies on ePTFE grafts. Although anti-CD34 coating resulted in complete graft endothelialization within 72 hours after surgery, a profound increase in intimal hyperplasia was observed in the venous outflow tract of anti-CD34 coated grafts. The present findings show that anti-CD34 coating is successful in promoting autoseeding of ePTFE grafts, whereas the trapped cells aggravate neointimal formation. Several factors can contribute to these adverse effects of stem-cell seeding, including differentiation of CD34(+) into VSMCs and the release of potent mitogens for VSMCs by the trapped cells. Further modifications of the graft coating are required to stimulate the protective effects of trapped endothelial cells.
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_Circulation_. 2005;112:12-18; originally published online June 27, 2005;
doi: 10.1161/CIRCULATIONAHA.104.504407
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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