Venous neointimal hyperplasia in polytetrafluoroethylene dialysis grafts

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Background. Vascular access dysfunction is the most important cause of morbidity and hospitalization in the hemodialysis population in the United States at a cost of $1 billion per annum. Venous neointimal hyperplasia (VNH) characterized by stenosis and subsequent thrombosis accounts for the overwhelming majority of pathology resulting in polytetrafluoroethylene (PTFE) dialysis graft failure. Despite the magnitude of the problem and the enormity of the cost ($1 billion), there are currently no effective therapies for the prevention or treatment of venous neointimal hyperplasia in PTFE dialysis grafts.

Methods. Tissue samples were collected from the graft-vein anastomosis of stenotic PTFE grafts during surgical revision. Specimens were graded using standard light microscopy and immunohistochemistry for the magnitude of neointimal hyperplasia and for the expression of specific cell types, cytokines, and matrix proteins.

Results. VNH was characterized by the (1) presence of smooth muscle cells/myofibroblasts, (2) accumulation of extracellular matrix components, (3) angiogenesis within the neointima and adventitia, and (4) presence of an active macrophage cell layer lining the PTFE graft material. Platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF) were expressed by smooth muscle cells/myofibroblasts within the venous neointima, by macrophages lining both sides of the PTFE graft, and by vessels within the neointima and adventitia.

Conclusions. Our results suggest that macrophages, specific cytokines (bFGF, PDGF, and VEGF), and angiogenesis within the neointima and adventitia are likely to contribute to the pathogenesis of VNH in PTFE dialysis grafts. Interventions aimed at these specific mediators and processes may be successful in reducing the very significant human and economic costs of vascular access dysfunction.

Key words: vascular access, hemodialysis access, stenosis, thrombosis, angiogenesis.

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Hemodialysis vascular access dysfunction is the single most important cause of morbidity in the hemodialysis population (currently 200,000 and growing at a rate of 7% per annum) [1]. It has been estimated that vascular access dysfunction is responsible for approximately 20% of all hospitalizations in the end-stage renal disease population [2] at a cost of $1 billion per annum. The most common form of vascular access procedure performed in chronic hemodialysis patients in the United States is the arteriovenous polytetrafluoroethylene (PTFE) graft, comprising as much as 83% of all hemodialysis accesses [3]. The current one-year primary patency rate for PTFE dialysis grafts is 40 to 50%, with a two-year patency rate of approximately 25% [4, 5]. Graft thrombosis is the cause of 80% of all vascular access dysfunction, and in over 90% of thrombosed grafts, the underlying pathology is a stenosis either at the venous anastomotic site or in the downstream (proximal) vein [6]. Despite the enormity of this problem, however, there are currently no effective interventions for either the prevention or treatment of venous stenosis in PTFE dialysis grafts [7, 8]. This is probably caused by a lack of knowledge about the pathophysiology underlying venous stenoses in PTFE grafts, especially at a cellular and molecular level [7].

Few studies have attempted to analyze the pathology of venous stenosis in PTFE dialysis grafts. Swedberg et al demonstrated that smooth muscle cells and extracellular matrix (ECM) proteins are important components of this lesion [9], while Rekhter et al have shown that there is a significant amount of angiogenesis within these specimens [10].

Recent studies in a variety of experimental arterial models of endothelial and smooth muscle injury have suggested that macrophages, endothelial cells, and smooth muscle cells/myofibroblasts are all involved in the response to injury that is responsible for the development of neointimal hyperplasia [11, 12]. Potential mediators thought to play a role in this process include basic fibroblast growth factor (bFGF) [13], platelet-derived growth factor
(PDGF) [14], vascular endothelial growth factor (VEGF) [15], and ECM proteins [16]. However, it is not clear that the mechanisms ascribed to arterial injury also apply to venous injury following PTFE graft anastomosis [17].

Therefore, we analyzed the distribution of specific cell types, cytokines, and matrix proteins from human vein samples in patients with PTFE graft thrombosis. Cytokines and matrix proteins were chosen based on their putative involvement in other vascular injury syndromes [11, 12]. Identification of the mediators present in these failed grafts may provide insight into potential therapeutic targets for venous stenosis in PTFE dialysis grafts.

**METHODS**

**Patients**

Clinical information was available on 7 of the 11 patients in our study. Within these constraints, the mean age of our patients was 62.6 years (range 41 to 85). Six were African American, and one was Caucasian. Six out of seven were male. Type II diabetes mellitus (four patients), hypertension (two patients), and nonrecovery from acute tubular necrosis (1 patient) were the cause of end-stage renal disease. All seven patients were hypertensive. The mean cholesterol level was 167 mg/dL (range 125 to 187), and the mean triglyceride level was 296 mg/dL (range 93 to 516). The graft in question was the first permanent access in all patients. One patient had a previous cuffed catheter. The average time between graft placement and the surgical revision (time of specimen acquisition) was 37 months (8 to 66 months). The average number of thrombotic episodes in this cohort was three (range 1 to 6).

**Collection of samples**

Discarded segments from the venous end of 11 PTFE grafts were collected at the time of graft revision surgery of thrombosed PTFE dialysis grafts. While we tried to obtain upstream graft (just before the graft-vein anastomosis) and downstream (proximal) vein (beyond the graft-vein anastomosis and towards the heart) in each of the samples, this was not always possible because of the surgical constraints associated with refashioning the thrombosed graft. Excised tissue was fixed in formalin and then embedded in paraffin using standard techniques. The number of tissue blocks from each sample varied from one to six. This was dependent on the amount of graft with attached vein that individual surgeons were able to obtain at the time of graft revision surgery. Each block was 3 to 4 mm in thickness. Normal vein of similar caliber to veins in the forearm was obtained at the time of multiorgan harvest.

**Molecules analyzed**

The expression of the following cell types, cytokines, and matrix proteins was analyzed in our samples. Table 1 shows details about the antibodies used.

**Cell types.** Specific antibodies recognizing smooth muscle cells and myofibroblasts [α-smooth muscle actin (α-SMA) and desmin], endothelial cells [von Willebrand factor (vWF)], proliferating cells (Ki-67), and a macrophage marker (PGM-1) were used in this study. We chose to examine expression of these cell types in view of the important role they played in other models of vascular injury [11]. Of note, SMA and desmin are differentiation markers for cells of a smooth muscle cell phenotype [18, 19].

**Cytokines.** The expression of PDGF-B, bFGF, and VEGF was analyzed based on their documented role in the pathogenesis of arterial neointimal hyperplasia [11, 20].

**Matrix/structural proteins.** Collagen IV, fibronectin, tenascin, and laminin are all ECM proteins. Except for tenascin, their counter-receptors belong to the integrin family of molecules. ECM proteins play a key role in modulating cellular proliferation through feedback mechanisms, and previous studies suggest a role for these molecules in experimental models of neointimal hyperplasia [16, 19, 21].

**Antibody specificities**

All of the antibodies used in this study are commercially available (Table 1). We have previously documented the specificity of the anticytokine antibodies with peptide inhibition studies for the anti-PDGF-B antibody. Western blots against cell extracts known to express bFGF, for the anti-bFGF antibody, and a comparison of the staining patterns with two other VEGF antibodies, for our anti-VEGF antibody [22].

**Immunohistochemistry**

A standard automated streptavidin biotin technique was used (Ventana 320ES automated immunostainer, Tucson, AZ, USA). Briefly, following deparaffinization and hydration, slides were washed and underwent protease digestion (if required for a particular primary antibody). Slides were then incubated with the primary antibody for 32 minutes with the biotinylated secondary antibody blend (anti-rabbit Ig, anti-mouse IgG, and anti-mouse IgM) for eight minutes and with the streptavidin/horseradish peroxidase for eight minutes (Table 1). All incubations were performed at 37°C with constant movement and with appropriate washes between each step. The slides were then developed with a diaminobenzidine/hydrogen peroxide mixture for four minutes, counterstained with hematoxylin, dehydrated with graduated alcohol and xylene, and mounted using a xylene-based
Figure 2. Within individual specimens, separate scores were given for those samples that contained PTFE graft (upstream graft) and those that did not (downstream vein). Thus, in the Results section, when reference is made to findings at the site of upstream PTFE graft, it indicates the pattern of staining either at the graft-vein anastomosis or just before the anastomosis (Fig. 1, areas B and C; PTFE graft would always be present in such a sample), while findings that refer to downstream (proximal) vein indicate the pattern of staining in the venous segment beyond the graft and towards the heart (Fig. 1, area A; no PTFE graft would be present in these samples). Each parameter analyzed (whether cell type, cytokine, or matrix protein) was assessed at a number of different sites within the specimen. For example, VEGF expression was scored within (1) the adventitial vessels overlying upstream graft and downstream vein, (2) the macrophage/giant cell layer lining both sides of the graft, (3) infiltrating macrophages within the graft, and (4) stromal cells (smooth muscle cells/myofibroblasts) and microvessels within the neointima of both upstream graft and downstream vein.

**Statistical analyses**

Although this study was designed essentially as a descriptive analysis, a statistical analysis comparing the expression of specific cell types, cytokines, and matrix proteins in the upstream graft (Fig. 1, areas B and C and Table 2) was performed in comparison with the downstream vein (Fig. 1, area A and Table 2). This comparison was only made between the same parameter at different sites. This was done to ensure that the semiquantitative score at the two different sites indicated the same level of expression (for example, in the comparison of adventitial angiogenesis in upstream graft vs. downstream vein, a score of 2+ indicated the same number of microvessels at the two sites). The expression of the same cellular phenotype at different sites in the specimens was also compared, but only when the quantitation of the scoring system was identical (for example, adventitial angiogen-

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**Table 1. Antibody specificities**

<table>
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<th>Antibody against</th>
<th>Source</th>
<th>Clone</th>
<th>Dilution</th>
<th>Epitope recognized</th>
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<td>DAKO</td>
<td>1A4</td>
<td>1:200</td>
<td>Terminal decapeptide of α-smooth muscle actin</td>
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<td>Endothelial cells</td>
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<td>Polyclonal</td>
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<td>von Willebrand factor</td>
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<td>Proliferating cells</td>
<td>AMAC Inc.</td>
<td>MIB-1</td>
<td>1:50</td>
<td>Ki-67 nuclear antigen in G1, S, G2, M phases</td>
</tr>
<tr>
<td>Macrophages</td>
<td>DAKO</td>
<td>PG-M1</td>
<td>1:50</td>
<td>110 kD glycoprotein encoding the CD68 antigen</td>
</tr>
<tr>
<td>Smooth muscle (desmin)</td>
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<td>DE-R-11</td>
<td>1:50</td>
<td>18 kD rod piece of the 53 kD intermediate filament desmin</td>
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<td>basic FGF</td>
<td>Oncogene</td>
<td>Polyclonal</td>
<td>1:100</td>
<td>Rabbit polyclonal raised against aa 40-63 of human bFGF</td>
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<td>PDGF</td>
<td>Santa Cruz</td>
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<td>1:125</td>
<td>Raised in rabbits against aa 1-30 at the N terminus of human PDGF-B</td>
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<tr>
<td>VEGF</td>
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<td>Polyclonal</td>
<td>1:8</td>
<td>Raised in rabbits against aa 1-20 at the N terminus of human VEGF</td>
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<td>Collagen IV</td>
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<td>COL-94</td>
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<td>Recognizes an epitope on the α1 and α2 chains</td>
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<td>Sigma</td>
<td>LAM89</td>
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<td>Purified human laminin</td>
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<tr>
<td>Tenascin</td>
<td>DAKO</td>
<td>TN2</td>
<td>1:150</td>
<td>Purified human tenascin; Does not cross-react with fibrinogen</td>
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</tbody>
</table>

Abbreviations are defined in Appendix.
Fig. 2. Scoring scale for angiogenesis (microvessel formation). (a) (vWF ×300, neointima) A single microvessel (score = 1+). (b) (vWF ×300, neointima) A few scattered microvessels (score 2+). (c) (vWF ×300, adventitia) A large number of microvessels (score 3+). (d) (vWF ×300, adventitia) Density of microvessels needed to have a score of 4+.

Fig. 3. Normal vein. (a) Note the thin one-to two-layer intima (thin arrows) and three-to four-layer media (thick arrows) of normal vein (hematoxylin and eosin ×400). (b) Relative absence of endothelial cell staining in the adventitia and media of normal vein. Note the intense staining of the single layer endothelium (vWF ×1500). (c) Thickness of normal venous (between thin arrows, V) and arterial (double-headed arrow, A) intima media (SMA ×117). (d) Thickness of the venous neointima and media in a dialysis patient with venous stenosis (SMA ×117). At an identical magnification to (c), the venous neointima (d; double-headed arrow, N) is 20 times thicker than the intima media of normal vein (c; between thin arrows). Also, note that the venous media (d; M, length of bar) is as thick as the arterial media in (c), indicating arterialization of the vein.

RESULTS

Normal vein

Hematoxylin and eosin. Normal vein of a similar caliber to our stenotic samples is shown in Figure 3a. Note the one cell-layer-thick intima (thin arrows) and the three to four cell-layer-thick media (thick arrows; Table 2).

Cellular phenotypes. (1) SMA was present in the media of normal vein (between thin arrows, Fig. 3c). (2) vWF was expressed by the endothelial cell layer (Fig. 3b) and occasionally by vessels within the adventitia. (3) There were no Ki-67–positive proliferating cells in normal vein. (4) Macrophages were rarely present within the adventitia of normal vein. (5) Desmin was present within the venous media only. Also note the pronounced difference in size between normal vein and the samples from dialysis patients with venous stenosis (Fig. 3c compared with d).

Cytokines. (1) bFGF was present within the venous media and intima. (2) PDGF was present within the venous media. (3) VEGF was present within the venous
media and intima on smooth muscle cells and endothelial cells.

Matrix proteins. Tenascin, collagen IV, and fibronectin were present within adventitial vessels in normal vein. Fibronectin was also present within the intima of normal vein.

Venous stenosis specimens (Table 2)

*Upstream PTFE graft (H and E).* A number of distinct layers were visible on these sections (Fig. 4a). These included: (1) a false adventitia on the outside of the graft that often had very prominent angiogenesis; (2) a macrophage and foreign body giant cell layer on both the adventitial and luminal sides of the PTFE graft; (3) PTFE graft material infiltrated by occasional mononuclear cells; (4) a prominent region of venous neointimal hyperplasia (extent of arrow in Fig. 4a; mean score ± SEM = 1.89 ± 0.35) comprising smooth muscle cells and myofibroblasts, ECM components, and a large number of microvessels; and finally (5) in many cases, an endothelial layer that lined the neointima.

**Table 2.** Different expression of cell types, cytokines and matrix proteins in the upstream graft and downstream vein

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Stenosed graft</th>
<th>Stenosed vein</th>
<th>Normal vein</th>
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<td>Cell types</td>
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<td>H/E neointima</td>
<td>1.89 ± 0.35</td>
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<td>SMA neointima</td>
<td>2.00 ± 0.31</td>
<td>4.00 ± 0.37</td>
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<tr>
<td>Desmin neointima</td>
<td>0.33 ± 0.17</td>
<td>1.11 ± 0.42</td>
<td>0</td>
</tr>
<tr>
<td>Mo adventitia</td>
<td>2.00 ± 0.62</td>
<td>1.33 ± 0.42</td>
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<tr>
<td>Mo neointima</td>
<td>2.50 ± 0.50</td>
<td>2.00 ± 0.45</td>
<td>0</td>
</tr>
<tr>
<td>vWF neointima</td>
<td>1.29 ± 0.42</td>
<td>1.80 ± 0.58</td>
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</tr>
<tr>
<td>vWF adventitia</td>
<td>2.86 ± 0.34</td>
<td>2.60 ± 0.25</td>
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<tr>
<td>Ki-67 adventitia</td>
<td>2.13 ± 0.48</td>
<td>0.83 ± 0.31</td>
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<tr>
<td>Ki-67 neointima</td>
<td>2.00 ± 0.38</td>
<td>1.17 ± 0.31</td>
<td>0</td>
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<tr>
<td>Cytokines</td>
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<tr>
<td>bFGF adventitia (ves.)</td>
<td>2.25 ± 0.37</td>
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<td>bFGF neointima (stromal)</td>
<td>2.14 ± 0.40</td>
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<td>PGF adventitia (ves.)</td>
<td>1.88 ± 0.39</td>
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<td>PGF neointima (stromal)</td>
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<td>PDGF neointima (ves.)</td>
<td>1.50 ± 0.33</td>
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<tr>
<td>VEGF adventitia (ves.)</td>
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<td>Matrix proteins</td>
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<td>Tenascin adventitia (ves.)</td>
<td>2.00 ± 0.84</td>
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<td>Tenascin neointima</td>
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<td>FN adventitia (ves.)</td>
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<td>FN neointima (stromal)</td>
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<td>FN neointima (ves.)</td>
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<td>Laminin adventitia (ves.)</td>
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<td>Laminin adventitia (ves.)</td>
<td>3.67 ± 0.21</td>
<td>3.67 ± 0.33</td>
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Abbreviations are defined in Appendix.

a Statistically significant differences
b >0.5 separation in score indicating a possible trend

**Downstream (proximal vein (H and E).** As for the sections that contained PTFE graft, a number of distinct layers were present in the downstream vein (Fig. 4b), with the obvious exception of PTFE graft material and the associated macrophage/giant cell layer. These included (1) a true adventitia with significant vascularity; (2) a thickened venous media; and (3) significant venous neointimal hyperplasia (mean expression score 3.80 ± 0.37), which was made up of smooth muscle cells/myofibroblasts, ECM components, and microvessels.

**Cellular phenotypes (Table 2)**

*α-Smooth muscle actin.* This was present mainly in the region of VNH, both in the downstream vein (4.00 ± 0.41; Fig. 3d) and in the setting of upstream PTFE graft (2.00 ± 0.81). Surprisingly, double-labeling studies for SMA and Ki-67 indicated that, at least at the time of sampling, the vast majority of SMA-positive cells in some samples were not actively proliferating (Fig. 4f). The media of downstream vein were strongly positive for SMA (Fig. 3d).

**Desmin.** There was strong expression of desmin within the venous media (5.00 ± 0.00) and occasionally on the abluminal side of the neointima in both downstream vein (1.11 ± 0.42) and upstream graft (0.33 ± 0.17).

**Macrophages.** Maximal macrophage (Mø) infiltration was in the area of the macrophage/giant cell reaction, layering both sides of the graft (Fig. 4e, g). In addition, Mø were also present in smaller numbers within the actual graft material and within the adventitia (upstream graft = 2.00 ± 0.62, downstream vein = 1.33 ± 0.42) and neointima (upstream graft = 2.5 ± 0.50, downstream vein = 2.0 ± 0.45).

**von Willebrand factor (endothelial cells).** This endothelial cell marker identified microvessels, both within the adventitia (upstream graft = 2.86 ± 0.34, downstream vein = 2.60 ± 0.25) and neointima (upstream graft = 1.29 ± 0.42, downstream vein = 1.80 ± 0.58; Fig. 4c, d).

**Ki-67 (proliferating cells).** The largest number of Ki-67-positive cells were in areas of microvessel formation (on endothelial and smooth muscle cells) within the adventitia (upstream graft = 2.13 ± 0.48, downstream vein = 2.60 ± 0.25). Ki-67-positive cells were also present within stromal-type cells in the neointima (upstream graft = 2.00 ± 0.38, downstream vein = 1.17 ± 0.31; Fig. 4d, f). Some microvessels within the neointima demonstrated active angiogenesis (identified by double-labeling studies for Ki-67 and vWF; Fig. 4d).

**Cytokines (Table 2)**

*Basic fibroblast growth factor.* bFGF was present on neointimal stromal (smooth muscle cells/myofibroblast)-type cells (upstream graft = 2.14 ± 0.40, downstream vein = 1.40 ± 0.40) and neointimal microvessels (up-
Fig. 4. Cell types and cytokines (adventitia to left and lumen to right). (a) PTFE graft (hematoxylin and eosin ×200). Note the significant venous neointimal hyperplasia (extent of arrow) between the graft (G) and the lumen (L). (b) Downstream vein (hematoxylin and eosin ×200). Note the presence of microvessels (thin arrows) within the adventitia (A). Also note the thickened (arterialized) media (M, double-headed arrow) and the significant amount of neointimal hyperplasia (N, bar). Note the prominent angiogenesis within the neointima (arrows), as assessed by this endothelial cell marker. (d) Downstream vein; neointima (vWF + Ki67 ×800). High-power view of a microvessel within the neointima of downstream vein. Note the distinct colocalization of blue (endothelial) and brown (proliferating) cells indicating active endothelial cell proliferation (angiogenesis). (e) Upstream graft; neointima (PG-M1 ×2000). High-power view of a macrophage giant cell adjacent to the neointimal surface of PTFE graft (G). Also note the large number of macrophages in this area (thin arrows). (f) Downstream vein; neointima (SMA + Ki-67 ×1000). High-power view of a portion of the neointima stained for smooth muscle cells (brown) and proliferating cells (blue). Note that almost all the active cellular proliferation in this specimen (arrows) is occurring within the neointimal microvessels (angiogenesis). At the time that this specimen was harvested, there was no ongoing smooth muscle cell proliferation (g) PTFE graft; adventitia (bFGF ×500). Note the strong expression of bFGF in adventitial vessels (thick arrow) and by the macrophage giant cell layer (thin arrow) lining the graft. (f) Downstream vein; media and neointima (PDGF ×400). There is strong expression of this cytokine in the venous media (M) and by smooth muscle cells/myofibroblasts within the neointima (N, bar).

Fig. 5. Matrix and structural proteins. (a) PTFE graft (tenascin ×200). There is strong expression of tenascin in the region of the macrophage giant cell layer (thin arrow) surrounding PTFE graft (G) and on the abluminal side of the neointima (thick arrow). (b) PTFE graft (collagen IV ×160). Collagen is present as expected within the walls of prominent adventitial blood vessels (thin arrows) and in this particular sample within the luminal portion of the neointima (thick arrow). (c) Downstream vein; neointima (fibronectin ×500). There is strong diffuse expression of fibronectin by ECM components. (d) Downstream vein; neointima (laminin ×500). Laminin is a prominent component of microvessels (arrows) within the neointima.
stream graft = 2.57 ± 0.48, downstream vein = 1.80 ± 0.4). bFGF was also present on the adventitial vessels overlying both upstream graft (2.25 ± 0.37; Fig. 4g) and downstream vein (2.40 ± 0.40). There was intense staining for bFGF by the macrophage giant cell layer lining both sides of the graft (3.13 ± 0.30; Fig. 4g) and by infiltrating mononuclear cells within the graft (3.17 ± 0.40). In some samples, the PTFE graft itself stained strongly for bFGF (Fig. 4g), suggesting that this cytokine might be diffusing through the interstices of the graft. 

**Platelet-derived growth factor.** PDGF was present in adventitial vessels in both upstream graft (1.88 ± 0.40) and downstream vein (2.17 ± 0.48). There was strong staining for PDGF by the macrophage giant cell layer on both sides of the graft (2.38 ± 0.42) and by macrophages that had infiltrated into the graft material (1.50 ± 0.54). Within the region of VNH, PDGF was present on stromal cells (upstream graft = 1.88 ± 0.52; downstream vein = 3.00 ± 0.71; Fig. 4h) and microvessels (upstream graft = 1.50 ± 0.33; downstream vein = 2.80 ± 1.48).

**Vascular endothelial growth factor.** VEGF was present in the adventitial vasculature overlying both upstream graft (3.00 ± 0.42) and downstream vein (4.00 ± 0.71). VEGF was also present in the macrophage/giant cell layer lining both sides of the graft (2.75 ± 0.56) and in macrophages that had infiltrated into the graft material. As for bFGF and PDGF, VEGF was present in stromal-type cells (upstream graft = 2.71 ± 0.42; downstream vein = 2.50 ± 0.30) and microvessels (upstream graft = 3.33 ± 0.42; downstream vein = 3.50 ± 0.87) within the venous neointima.

**Matrix and structural proteins (Table 2)**

- **Tenascin.** Tenascin was present within adventitial vessels overlying both upstream graft (2.00 ± 0.84) and downstream vein (2.25 ± 0.48; Fig. 5a). Tenascin appeared to be secreted by the macrophage/giant cell layer lining both sides of the graft. Within the neointima, tenascin was present within the ECM in upstream graft (3.20 ± 0.20) and downstream vein (2.75 ± 0.48). While the expression of tenascin appeared to be more on the abluminal surface in some samples (Fig. 5a), this was not consistent throughout the study. Tenascin was also expressed by microvessels within the neointima. The hypertrophied venous media had strong expression of tenascin (3.25 ± 0.63).

- **Collagen IV.** Collagen IV was expressed by adventitial vessels in both upstream graft (2.50 ± 0.87) and downstream vein (3.20 ± 0.20; Fig. 5b). Collagen IV was also present within the neointima of upstream graft (3.00 ± 0.45) and downstream vein (3.00 ± 0.00) and in microvessels within the neointima (upstream graft = 3.00 ± 0.58; downstream vein = 3.00 ± 0.58).

- **Fibronectin.** Fibronectin was expressed by adventitial vessels in both upstream graft (3.33 ± 0.49) and downstream vein (4.17 ± 0.40; Fig. 5c). Fibronectin was also present on stromal-type cells (upstream graft = 4.00 ± 0.22; downstream vein = 3.40 ± 0.87, Fig. 5c) and microvessels (upstream graft = 4.86 ± 0.14; downstream vein = 3.50 ± 1.19) within the neointima. Fibronectin was present on the macrophage and foreign body giant cell layer lining upstream graft (1.5 ± 0.96) and also appeared to be secreted into the actual matrix of the graft material.

**Laminin.** Laminin was expressed by adventitial vessels in both upstream graft (3.88 ± 0.13) and downstream vein (3.67 ± 0.21; Fig. 5d). Laminin was present within the neointima of upstream graft (2.43 ± 0.30) and downstream vein (2.67 ± 0.33), and in microvessels within the neointima (upstream graft = 3.67 ± 0.21, downstream vein = 3.67 ± 0.33). Laminin was also present in stromal elements of the neointima, especially at the site of what appeared to be needle track injuries.

**Statistical analyses**

Quantitative results are presented in Table 2. VNH was more pronounced in downstream vein as compared with upstream graft by hematoxylin and eosin analysis (P = 0.005). Within this downstream lesion, there was greater expression of the smooth muscle cell marker SMA actin as compared with upstream graft (P = 0.004). Finally, there was significantly more angiogenesis in the adventitia of upstream graft as compared with the neointima at the same site (P = 0.038; Table 2).

Because of the small number of samples available, we could have missed important differences as a result of inadequate power. Therefore, possible trends were identified (mean separation >0.5) toward differences between the upstream graft and downstream vein (Table 2). A brief summary of these trends between upstream graft and downstream vein is given below.

**Cellular phenotypes.** Desmin had stronger expression on the abluminal side of downstream vein as compared with downstream graft. Macrophages were generally more prominent within the adventitia of upstream graft. Ki-67 (proliferating cells) were present in the greatest numbers in the adventitia and neointima of the upstream graft as compared with the downstream vein. There were relatively more microvessels in the neointima of downstream vein as compared to upstream graft.

**Cytokines.** bFGF was increased within the neointima (stromal cells and microvessels) of upstream graft as compared with downstream graft. PDGF, in contrast, was increased within the neointima (stromal cells and microvessels) of downstream vein. VEGF was increased in the adventitial vessels of downstream vein as compared with those of upstream graft.
DISCUSSION

Despite the clinical importance of hemodialysis vascular access dysfunction, there has been little research on the pathogenesis of venous neointimal hyperplasia in the specific setting of dialysis access grafts. Therefore, the expression of specific cell types, cytokines, and matrix proteins in dialysis access specimens with venous stenosis were documented in an attempt to understand better the pathogenesis of this lesion. We believe that this is a first step toward the development of more effective measures for the prevention and treatment of this condition.

Initiating events

An important initiating event in the pathogenesis of VNH is thought to be hemodynamic stress (specifically a low shear stress [23, 24] caused by turbulence and compliance mismatch), which then activates (alters the phenotype of) smooth muscle cells and endothelial cells [25] at the graft-vein anastomosis. Our study suggests a second potential initiating factor, namely the macrophage giant cell layer that lines both sides of PTFE graft and has intense expression of all three cytokines discussed in this article (especially bFGF; Fig. 4g). We believe that PTFE graft material functions as a foreign body (perhaps made worse by the repeated punctures for dialysis), which attracts activated macrophages to the site of the lesion. These activated macrophages then produce cytokines such as bFGF, PDGF, and VEGF, which are known to cause smooth muscle cell, fibroblast, and endothelial cell differentiation, proliferation, and migration [11].

Smooth muscle cell proliferation

Table 2 demonstrates the presence of active cellular proliferation within stromal-type cells in the neointima. There were significantly more smooth muscle cells within the neointima of downstream vein as compared with the upstream graft, which suggests a preferential migration of smooth muscle cells from the venous media of the downstream vein into the neointima. Interestingly, in some specimens with a large number of smooth muscle cells within the venous neointima, double-staining experiments revealed active proliferation within endothelial cells lining microvessels (angiogenesis, discussed later in this article), but not in the smooth muscle cells (Fig. 4f), which formed the bulk of the neointimal lesion. This could be due to the fact that migration of smooth muscle cells from the media, rather than in situ intimal proliferation, is the key driving force for VNH. An alternative explanation could be that our samples can only provide a snapshot of a single time-point that is at the end of the natural history of the disease process. Thus, it is possible that active smooth muscle cell proliferation within the intima of our samples at an earlier time point has gone unrecognized by the present study. While approximately 50 to 75% of stromal-type cells within the neointima were positive for SMA, this was not the case for desmin, which was present on less than 10% of neointimal cells (mainly at the abluminal border). In their model of coronary venous grafting, Shi et al [18] and O’Brien et al [19] have suggested that SMA-positive, desmin-negative cells are likely to be adventitial fibroblasts that have transformed into smooth muscle-type cells and have migrated into the neointima. This is an important concept as it suggests that the changes in the adventitia could be influencing final luminal stenosis (discussed later in this article). We plan on closely examining this theory in a large animal model of VNH (abstract: Roy-Chaudhury et al, J Am Soc Nephrol 9:181, 1998).

Adventitial remodeling

This study clearly documents the presence of cellular proliferation, ECM protein accumulation, and cytokine expression within the adventitia of both upstream graft and downstream vein. In particular, there was increased microvessel formation in the adventitia surrounding upstream graft as compared with the neointima at the same site. These findings are in keeping with recent studies that indicate that activation and migration of adventitial cell types are an important determinant of vascular stenosis [18, 19]. Specifically, some studies suggest that adverse adventitial remodeling (constriction) could be as important as neointimal volumes in determining final luminal stenosis [26]. With regard to possible clinical application, therefore, our study suggests that therapeutic intervention in experimental models should be targeted at both the adventitia and the neointima.

Angiogenesis

A consistent finding in our study was the presence of microvessels, both within the neointima and adventitia of upstream graft and downstream vein (Fig. 4 c, d, f, g). In addition, many of our specimens also had evidence of active endothelial and pericyte proliferation (angiogenesis) within microvessels in the neointima (Fig. 4 d, f) and adventitia. These results are in keeping with those of Rekhter et al, who demonstrated that maximal cellular proliferation occurred in vascular regions of the neointima [10]. Of note, microvessels have also been found within the neointima and adventitia in coronary atherosclerosis [27] and in experimental models of coronary stenosis [28]. The importance of neointimal and adventitial angiogenesis is emphasized by recent seminal studies that have demonstrated that both neoplastic [29, 30] and non-neoplastic [31] tissue growth can be reduced by inhibiting angiogenesis. Our primarily descriptive study (which examines tissue at a single time point only) unfortunately does not allow a cause and effect relationship between
angiogenesis and neointimal hyperplasia to be identified. Further studies in an experimental animal model are needed to establish whether anti-angiogenic agents could be an effective therapeutic modality for VNH.

Cytokines

Platelet-derived growth factor and bFGF are important mediators of the smooth muscle cell proliferation that characterizes neointimal hyperplasia in balloon angioplasty models [13, 14]. bFGF and VEGF are also potent angiogenic growth factors [32]. All three cytokines were present on smooth muscle cells and on vessels, within the adventitia and neointima of upstream graft and downstream vein in our study. There was also intense expression of these cytokines by the macrophage giant cell layer lining PTFE graft (Fig. 4g). With regard to scoring cytokine expression on a semiquantitative scale, we accept that this may not always correlate with true cytokine activity, but it does allow us to have some idea of the cytokine program in place at a particular site. It is important to note that graft thrombosis had already occurred in our clinical specimens. While it is possible that the thrombus itself could have had an effect on cytokine expression, we would like to point out that there are strong similarities between our human specimens and our validated pig model in which the grafts are not thrombosed (abstract; ibid). Also, because of the fact that this was essentially an immunohistochemical analysis, we are unable to clarify whether the staining patterns for cytokine expression were due to increased production in situ or alternatively due to changes in the pattern of deposition and clearance.

We believe that these three cytokines could be primarily responsible for the smooth muscle cell and endothelial cell proliferation seen in our samples. bFGF and PDGF also play an important role in the accumulation of ECM proteins, as antibodies to these two molecules have been shown to decrease matrix protein volume in a model of neointimal hyperplasia [20]. Table 2 suggests a trend toward a greater expression of bFGF in upstream graft, while PDGF expression appears to be relatively more intense in the setting of downstream vein. While this suggests a specific role for particular cytokines at different sites of the lesion, a larger number of samples is needed to analyze these trends. In view of the possible key role for cytokines such as bFGF, PDGF, and VEGF in the cellular proliferation and matrix accumulation that characterizes VNH, we speculate that targeting these particular cytokines locally could result in future novel therapies for this lesion.

Although identification of a specific pathogenetic mechanism for VNH is beyond the scope of the present descriptive analysis, the differential expression of cellular phenotypes and cytokines does suggest a number of biological processes that could play an important role in the pathogenesis of VNH in dialysis access grafts. These include cellular proliferation, microvessel formation, and cytokine expression (perhaps the key factor) by smooth muscle cells, endothelial cells, and macrophages, resulting in the activation and proliferation of these cell types. Based on the results from this study, we are currently testing specific interventions in a pig model of VNH (abstract; ibid). In conclusion, we believe that venous stenosis in PTFE dialysis grafts could be the ideal clinical target for novel locally delivered therapeutic interventions against neointimal hyperplasia, in view of the accessibility of both the patient (on hemodialysis three times a week) and the lesion (superficially located).

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APPENDIX

Abbreviations used in this article are: bFGF, basic fibroblast growth factor; FN, fibronectin; H/E, hematoxylin and eosin; Mô, macrophages; PDGF, platelet-derived growth factor; PTFE, polytetrafluoroethylene; SMA, α-smooth muscle actin; VEGF, vascular endothelial growth factor; VNH, venous neointimal hyperplasia; vWF, von Willebrand factor.

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