Arterialization of Human Vein Grafts Is Associated With Tenascin-C Expression

Kurt Wallner, MD,* Chen Li, MD, PhD,* Michael C. Fishbein, MD,† Prediman K. Shah, MD, FACC,* Behrooz G. Sharifi, PhD*
Los Angeles, California

**OBJECTIVES** This study was performed to test the hypothesis that tenascin-C (TN-C), an extracellular matrix (ECM) protein with counteradhesive chemotactic and vascular growth-promoting effects, is expressed in "arterialized" human saphenous vein grafts (SVGs).

**BACKGROUND** Tenascin-C is expressed in the vessel wall after vascular injury in the experimental model, where it has been implicated in the formation of neointima. Overexpression of TN-C has also been implicated in the development and progression of pulmonary hypertension. Saphenous vein grafts are exposed to hemodynamic stress when interposed in the arterial circulation and mechanical stress upregulates expression of TN-C, whereas stress-relaxation suppresses its synthesis. We hypothesized that the hemodynamic stress of increased arterial pressure could also induce TN-C expression in SVG.

**METHODS** We examined the expression of TN-C protein and mRNA in normal vein and "arterialized" human SVG using immunohistochemistry and in situ hybridization, respectively.

**RESULTS** TN-C protein was not detected in control human saphenous veins; however, it was uniformly and strongly expressed in the adventitia and media of patent human vein grafts, with minimal or no expression in the neointima (n = 27, 100%). In situ hybridization showed that TN-C mRNA was not detected in the neointima, but was strongly upregulated in the adventitia and media, corroborating immunostaining data (n = 10, 100%). Unlike patent SVG, TN-C was not expressed in the adventitia of occluded grafts, except for a low level of expression around the newly formed vessels in neointima (n = 5, 100%). Smooth muscle cell-specific staining demonstrated that the lack of expression of TN-C in occluded vein grafts is not due to the lack of presence of smooth muscle cells in the graft.

**CONCLUSIONS** These findings suggest that placement of a venous graft in the arterial system leads to expression of TN-C, which may in turn facilitate graft remodeling. Conversely, loss of flow and intravascular pressure, associated with vein graft occlusion, is accompanied by disappearance of TN-C expression. (J Am Coll Cardiol 1999;34:871–5) © 1999 by the American College of Cardiology

Although coronary artery bypass grafting (CABG) is an effective therapy for patients with coronary artery disease, about 50% of grafts close within 10 years (1,2). After the first year, most graft closures are due to intimal hyperplasia with or without subsequent atherosclerosis (3,4). Graft failure is the leading cause of recurrent ischemia and need for subsequent revascularization procedures in patients after CABG.

Extracellular matrix (ECM) proteins play a crucial role in the remodeling of blood vessels by affecting cell adhesion, growth, migration, apoptosis and morphology (5). One matrix protein that has been implicated in tissue remodeling is tenascin–C (TN–C). It is not generally found in normal adult tissue but is strongly expressed in a site-restricted fashion during both embryogenesis and wound healing. It is believed that TN–C is involved in cell growth, migration, differentiation and apoptosis, processes that are important in wound healing (6). Tenascin–C is not found in normal arteries, but it is highly expressed in balloon-injured arteries (7). We have demonstrated that the expression of TN–C in aortic smooth muscle cells (SMCs) is regulated by chemotactic factors (8). Furthermore, TN–C blocks adhesion of human and rat SMCs to fibronectin, and mediates migration of SMCs (9,10). There is no information about the expression and role of TN–C in human vein grafts. Consequently, the goal of this study was to establish the...
spatial distribution of TN-C in a series of excised patent and occluded human vein grafts, as a first step in assessing the potential role of TN-C in saphenous vein graft (SVG) remodeling and closure.

The normal saphenous vein consists of three layers (4). The normal intima is thin, overlying an often incomplete internal elastic lamina. The media is more heterogeneous than that of arteries of similar size, consisting of a mixture of mostly circularly arranged SMCs, ECM proteins and some elastic fibers with no distinct external elastic lamina. The adventitia is composed of abundant amounts of collagen with a lesser amount of elastic fibers. The presence of bundles of adventitial SMCs, not present in arteries of similar size, is conspicuous in the veins.

With aging, presumably related to hydrostatic pressure, saphenous veins in the lower extremities may develop intimal hyperplasia composed of primarily SMCs and collagen, medial hypertrophy and in some cases dilatation (varicose veins).

When implanted into the arterial circulation, the intimal proliferation and thickening is accelerated, a more distinct internal elastic lamina becomes apparent, with medial fibrosis, hypertrophy and adventitial fibrosis. Lipid deposition, inflammatory cell infiltration and calcification of the intima are often present as well. The remodeling and atheroma formation are responsible for the veins assuming characteristics of arteries, hence the term “arterialization.”

**MATERIALS AND METHODS**

**SVGs.** Thirty-two human vein grafts were obtained from 12 patients who underwent heart transplantation. As a control for vein tissue, we used eight sections from eight human saphenous veins obtained during coronary bypass surgery that were not used for coronary bypass. These specimens were fixed in 10% buffered formalin for 4 h and embedded in paraffin. The HXB 1005 polyclonal antibodies to purified human TN-C were a gift from Dr. H. P. Erickson (Duke University). Dr. Erickson has established the specificity of these antibodies. We further confirmed the specificity of the antibodies by Western blot using conditioned media derived from cultured SMCs. These antibodies are highly specific and do not cross-react with other matrix proteins found in the aorta, including fibronectin, vitronectin, collagen and laminin (9). Cell proliferation was evaluated in eight cases by immunohistochemistry using anti-PCNA antibodies (Dako Corporation, Carpinteria, California).

**Identity of the cells.** Antibodies used included SM-1 directed to smooth muscle α-actin, smooth muscle (SM) myosin heavy chain, h-caldesmon, desmin and vimentin (all from Sigma Chemical Co., St. Louis, Missouri). These antibodies were used at 1:50 dilution as recommended by the manufacturer. The anti-caldesmon antibodies have been shown to be specific for 120- to 150-kDa h-caldesmon and do not cross-react with skeletal or cardiac muscle or with 70-kDa nonmuscle caldesmon. The anti-desmin antibodies cross-react with skeletal, cardiac, visceral and SMCs.

**Immunohistochemistry.** Staining was performed by the avidin–biotin complex immunoperoxidase procedure. Briefly, slides were deparaffinized and treated with H2O2 to eliminate endogenous peroxidase activity. The sections were incubated for 2 h with primary rabbit anti-TN-C antibody (1:500 dilution) followed by repeated wash and 30-min incubation with biotin-conjugated goat anti-rabbit IgG (Dako) secondary antibodies. After washing, slides were incubated with 2.5 mg/ml streptavidin–peroxidase (Dako) for 30 min, developed with substrate, 3% amino-4-ethylcarbazolein N,N dimethylformamide (AEC) (Dako), counterstained with hematoxylin for 30 s and coverslipped. The preimmune serum was used as negative control.

**Riboprobe preparation and in situ hybridization.** In situ hybridization was performed essentially as described (11) with some modification. We generated human TN-C cDNA corresponding to the fibrinogen-like domain by subcloning it into pBluescript. We also generated a digoxigenin riboprobe, instead of radioactive probe, using DIG RNA Labeling Kit (Boehringer Mannheim, Mannheim, Germany) as recommended by the manufacturer. Tissue sections were deparaffinized, washed, prehybridized, hybridized and digested with RNase, and alkaline phosphatase-conjugated anti-digoxigenin antibody was used to detect the probe. Once the color intensity was attained, the reaction was stopped, and specimens were counterstained with Fast Green (Sigma).

**Statistical analysis.** The statistical analysis of the sections was performed using chi-square test. A p value < 0.05 was considered statistically significant.

**RESULTS**

In total, 60 different segments from 32 different human vein grafts were studied. Compared with control veins, all vein grafts showed more intimal hyperplasia. The magnitude of intimal hyperplasia varied greatly among the vein grafts. Overall, 14 vessel segments were classified as mildly diseased. All of these segments showed mild intimal proliferation and contained no atheroma. A total of 38 vessels were
moderately diseased and eight contained atheroma. Eight vessel segments were occluded.

Tenascin-C was not detected in control human veins (Fig. 1C). It was strongly expressed in patent vein grafts (Fig. 1A). All 50 (100%) patent vein graft segments expressed TN-C. Spatially, TN-C was concentrated in the adventitial and medial ECM, but the neointima did not stain. There was inconsistent staining of the endothelial cells. In contrast to patent grafts, TN-C either was not detected or was weakly expressed in occluded graft segments. (C) Lack of detectable expression of TN-C in control veins. Magnifications: A and B, 10×; C, 30×, reduced by 65%.

Table 1. The Expression of Tenascin-C in the Patent and Occluded Human Vein Grafts

<table>
<thead>
<tr>
<th></th>
<th>Control Vein</th>
<th>Patent Vein Graft</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive stain</td>
<td>n = 0</td>
<td>n = 52</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>Negative stain/</td>
<td>n = 8</td>
<td>n = 0</td>
<td></td>
</tr>
<tr>
<td>weak positive</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Occluded Vein Graft</th>
<th>Patent Vein Graft</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive stain</td>
<td>n = 0</td>
<td>n = 52</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>Negative stain/</td>
<td>n = 8</td>
<td>n = 0</td>
<td></td>
</tr>
<tr>
<td>weak positive</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n is the number of segments stained for tenascin-C. The statistical analysis of the sections was performed using chi-square test. A p value < 0.05 was considered statistically significant.

protein and mRNA, suggesting that TN-C remains close to the source of synthesis and does not diffuse away.

Because TN-C has been implicated as a comitogen for SMCs (12), we compared its expression pattern with PCNA-positive cells. PCNA positivity was found throughout the patent vein graft in all sections, concentrated primarily in the adventitia and neointima (Fig. 3A). PCNA positivity was found infrequently in the medial layer of the grafted segment. This suggests a high level of proliferation of adventitial and neointimal cells. We did not find a correlation between TN-C expression and PCNA positivity.

In contrast to patent veins, occluded veins stained weakly with anti-TN-C antibodies (Fig. 1B). This relative lack of expression cannot be explained by acellularity of the occluded veins, as cell-specific staining of serial sections showed that the occluded veins were highly cellular. Smooth muscle-specific myosin heavy chain antibodies strongly stained neointima and adventitia, whereas media stained weakly (Fig. 4A). The h-caldesmon antibodies consistently stained SMC bundles found in the adventitia and neointimal cells, but stained medial cells weakly (Fig. 4B). Staining
pattern with anti-desmin antibodies was similar to h-caldesmon (Fig. 4D). Anti-SM-α-actin (Fig. 4C) strongly stained SM bundles found in the adventitia and neointimal cells.

A similar staining pattern was found in the patent vein grafts. Anti-myosin (Fig. 5A), anti-caldesmon (Fig. 5B), anti-desmin (Fig. 5C) and anti-SM-α-actin (Fig. 5D) strongly stained SM bundles found in the adventitia and neointimal cells. We consistently observed that neointima and adventitial cells, particularly SM bundles, stained with the SM-α-actin, desmin and caldesmon antibodies, whereas medial cells were either weakly stained or were negative. Based on this staining pattern, we concluded that TN-C is not uniformly expressed by SMCs of the patent vein graft and that its expression by adventitial and medial cells, either SMCs or myofibroblasts or both, is subject to a different regulatory control than neointimal cells.

DISCUSSION

This report provides the first description of TN-C expression in human vein grafts. We found two novel features of TN-C expression. First, although TN-C was not expressed in control human veins, it was highly expressed in “arterialized” veins in a distinct spatial pattern. Tenascin-C expression was highly concentrated in the adventitia and media, but was absent from the neointima of the vein graft. We have previously demonstrated that TN-C blocks adhesion of aortic SMCs to fibronectin (9). Recently, we have demonstrated that TN-C facilitates migration of aortic SMCs (10). Consequently, we propose that the expression of TN-C in the SVGB adventitia and media most likely reduces interaction of cells with their surrounding matrix, promoting their migration.

In addition to its effect on SMC migration, TN-C may affect expression of genes that are important in vascular remodeling. For example, fibroblasts plated on a mixture of fibronectin and TN-C, but not fibronectin alone, showed an increase in the synthesis of four gene products: collagenase, stromelysin, a 92-kDa gelatinase and c-fos (13). The effect on metalloproteinase expression is reversed in the presence of a monoclonal antibody that reacts with the seventh and eighth fibronectin type III repeats, suggesting that these changes in gene expression may be related to the counter-adhesive activity of TN-C. Thus, we also hypothesize that the expression of TN-C serves to create a microenvironment that promotes “arterialization” and remodeling of vein grafts by promoting expression of metalloproteinases.
The second novel finding of our study is the correlation between TN-C expression and patency of the vein graft. This suggests that TN-C expression may be in part regulated by mechanical strain. There is a substantial body of data to support this hypothesis. Cells bind to ECM via specific cell surface receptors that activate signal transduction pathways within the cells and may act as mechanoochemical transducers (14). For example, bone cells remodel their matrix and reorient bone trabeculae in response to mechanical strain (15). Fibroblasts attached to a collagen matrix under mechanical strain produce more of the ECM protein TN-C and collagen XII than cells in a relaxed matrix (16). In vivo, both TN-C and collagen are specifically expressed in locations where mechanical strain is high. In addition, the chick TN-C gene promoter contains a novel cis-acting, strain-responsive element that causes enhanced transcription in cells attached to a strained collagen matrix. It is possible, therefore, that connective tissue cells, either SMCs or myofibroblasts, or both, sense force vectors in their ECM environment and react to altered mechanical needs by regulating the transcription of specific ECM genes. In blood vessels, chronic arterial hypertension leads to proliferation and hypertrophy of SMCs in blood vessel walls (17), possibly due to the synthesis of PDGF-BB after activation of a cis-acting element found in its promoter (18). We hypothesize that strain-induced and growth factor-induced remodeling of vein grafts is initiated, in part, by the induction of TN-C, which in turn, promotes SMC migration (10) and growth (12), leading to the formation of neointima and “arterialization” of the vein graft.

REFERENCES


