

## **Bypass Graft Disease: Analysis of Proliferative Activity in Human Aorto-Coronary Bypass Grafts**

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### **ABSTRACT**

**Background:** Aortocoronary bypass graft disease with its increasing clinical signification represents an unsolved problem in cardiological and heart surgery practice. Late occlusion of autologous saphenous vein grafts occurs against a background of medial and neointimal thickening due to migration and proliferation of smooth muscle cells and the later appearance of atherosclerotic plaques. To clarify the role of cellular proliferation in humans we characterized the cellular composition and proliferative index in 30 stenotic saphenous vein grafts.

**Methods:** 30 stenotic vein grafts and 25 control veins were explanted during re-do heart surgery procedures. Time between initial surgical intervention and explantation was 3-168 month (mean 94,8 month). The total area and cell count of the neointima, media and adventitia was calculated computer assisted. Actively proliferating cells were identified using antibody to Ki-67 and by double-label immunocytochemistry with  $\alpha$  SMC actin, CD 31 (endothelial cells), CD 68 (makrophages) and CD 45 (T-lymphocytes).

**Results:** Active proliferation was detected in different cell types with a mean proliferation index of 0.15% ,0.18% and 0.086% for the neointima, media and adventita. Only 9% of the proliferating cells in the neointima were SMC (not identified cells 40%); corresponding 14% SMC (not identified cells 33%) were detected in the media. Endothelial cells were the predominante proliferating cell type in all sites of the vessel wall.

**Conclusion:** 1. Proliferation occurred at low level. While proliferation may play an important role in early lesions our data imply low proliferation activity in advanced graft lesions. Other mechanism like production and deposition of extracellular matrix (ECM) in the neointima are responsible for the lumen reduction of bypass grafts. 2. The high portion of unidentified cells may represent SMC or other cell types at different stages of differentiation; this requires further investigation. 3. The identification of

proliferating macrophages and T-lymphocytes implicate an inflammatory component in the development of human bypass graft lesions.

## INTRODUCTION

Aortocoronary bypass graft disease with its increasing clinical significance presents an unsolved problem in cardiological and cardiac surgery practice. Apart from arterial grafts autologous saphenous vein remains the conduit of choice especially in patients with multivessel disease [Cooper 1996]. The long-term patency of these grafts is limited because of the development of vein graft stenosis. By 10 years after surgery only 60% of vein grafts are patent and only 50% of patent vein grafts are free of significant stenosis. Approximately 20% of the patients require further revascularization 10 years after initial bypass surgery [Motwani 1998]. Late occlusion of autologous saphenous vein grafts occurs against a background of medial and neointimal thickening due to migration and proliferation of smooth muscle cells and the secondary appearance of atherosclerotic plaques. This vessel wall thickening may be regarded as an intrinsic adaptation of the vein to the increased wall tension, cyclic stretching, and high shear stress caused by the higher blood pressure and flow velocities in the arterial circulation [Bulkley 1977, Atkinson 1985, Brody 1989, Gibbons 1995]. Further predisposing risk factors for progression of saphenous vein graft disease are classic risk factors such as hyperlipidemia, insulin resistance and hypertension [Eritsland 1995].

Vein graft stenosis has been attributed to the process of myointimal thickening and smooth muscle cell proliferation seems to be the basic event in neointima formation [Newby 1996]. On the basis of contradictory results found in the cellular proliferation rate in different lesions in humans and animals there is an ongoing debate about the significance of proliferation in human vascular lesions [O'Brien 1993, Pickering 1993, Brandl 1997, Zou 1998]. Especially stenotic lesions in human bypass grafts used in coronary bypass surgery were not analyzed with regard to the proliferation activity. Furthermore a new classification of histological lesions types seen during bypass graft disease was developed.

In the present prospective study, the cell cycle-related antigen Ki-67 was analyzed for topographic determination of cell turnover in distinct regions of human stenotic aortocoronary bypass grafts specimens harvested during re-do surgery.

## METHODS

### *Tissue preparation*

Thirty saphenous vein grafts were explanted during re-do heart surgery procedures and 25 control veins were harvested from the same patients. The specimens were obtained from 27 patients (4 female, 23 male) and the time between initial surgical procedures and explantation ranged from 3-168 month (mean 94.8 month).

After explantation the specimens were immediately placed in 4% formalin. Following paraffin embedding the samples were serially cut into 3  $\mu$ m thick sections. The explanted bypass grafts were divided into proximal, medial and distal area and sections were histologically analysed. Sections with the highest degree of stenosis were used for this study.

### *Immunocytochemistry*

Single and double immunocytochemistry were performed on serial sections for identification of proliferating cells. The identity of Ki-67 positive cells was determined by double-label immunocytochemistry with antibodies to SMC  $\alpha$ -actin, CD 31 (endothelial cells), CD68 (macrophages) and CD 45 (T-Lymphocytes).

Proliferative activity was detected using the antibody MIB1 targeting the Ki-67 antigen (Dianova; Hamburg, Germany). After deparaffinization endogenous peroxidase

was blocked by 3% H<sub>2</sub>O<sub>2</sub> for 20 minutes. Antigen retrieval was performed in a microwave oven for 10 minutes using a citrate buffer followed by incubation in a moist chamber with the primary antibody (clone MIB1, 1:20, in 1% blocking reagent), at 4°C for 12 hours after a PBS rinse for 5 minutes. The secondary antibody was applied with the Super Sensitive Detection Kit (Biogenex, San Ramon, USA) following the manufacturer's instruction. The detection reaction was carried out with 0.1% 3,3 diaminobenzidine (DAB) (Roche; Mannheim, Germany) followed by a counterstaining with hematoxylin.

### ***Double-label immunocytochemistry***

For identification of proliferating cells, serial sections were double immunostained with MIB1 combined with cell specific antibodies. For characterization of smooth muscle cells (clone 1A4, 1:200 in 1% BSA, Sigma; St. Louis, USA), macrophages (clone PG-M1, 1:100 in 1% BSA, Dako; Hamburg, FRG), endothelial cells (clone JC/70A, ready to use, Dako) and T-cells (clone OPD4, 1:25 in 1% BSA, Dako) the primary antibodies were incubated for 1 h at room temperature in a moist chamber. After a PBS rinse for 10 minutes the LSAB system (LSAB-2-Kit, alkaline phosphatase, DAKO) was performed following the manufacturer's instruction and finally a fuchsin chromogen (New fuchsin red, DAKO) was applied for 20 minutes to yield a red reaction product at the site of the target antigen in the cytoplasm. A counterstaining with hematoxylin was used to visualize the nuclei in the tissue sections.

### ***Area measurement and cell counting***

The total number of Ki67 positive cells per slide was manually counted under light microscopy at 400X magnification separately for each layer with the observer blinded to the clinical details. A proliferation index was calculated by dividing the number of Ki67 positive cells by the total number of cells X100. For every section the total number of cell nuclei and area measurement was determined for intima, media and adventitia. Analysis of area measurements were performed using the Adobe Photoshop software (Version 5.0; Adobe Systems, San Jose, Calif., USA) and specifically designed plug-ins for determination of point size and count (The Image Processing Toolkit, Version 1.0.0, Reindeer Games, Charlotte, USA). For area measurement whole sections were scanned and transferred to a Macintosh computer (Macintosh 7600) equipped with a digital imaging board. Vessel lumen, intima, media and adventitia were sequentially marked using the *lasso* tool and the *edit/fill* mode. The number of pixels was assessed using the histogram tool and then converted to a square micrometer value by comparison to pixel values of defined areas on a micrometer-slide.

Using the *magic wand* tool and the *select similar* command of the Photoshop software all nuclei were selected in the vessel wall and counted as previously described in detail. The double-labeled slides were counted manually to determine the total number of Ki67 positive cells and for the cell specific markers. The ratio between the total number of specifically double-labeled cells to the total number of Ki67 positive nuclei was used to indicate the percentage of either endothelial cells, macrophages, smooth muscle cells or T-lymphocytes.

### ***Statistical analysis***

Data are expressed as mean $\pm$ SEM. Statistical significance was calculated by the nonparametric Mann-Whitney U test for independent groups using the Prism 3.0 software (GraphPad Software Inc., San Diego, USA).

## RESULTS

### *Lesion types*

Three major different histological characteristics could be distinguished and led to the following classification:

Type I. adaptive proliferative intimal thickening (n=9)

Type II. fibrous intimal thickening (n=11)

Type III. advanced atherosclerotic lesions (n=10)

The characteristics and the histological appearance are summarized in Figure 1.

### *Vessel layer measurement and cell count*

The data for area, cell number and cell density measurement of normal and stenotic veins are reported in Figure 2 A-C. As expected, the lumen reduction in stenotic veins was caused by extensive growth of the neointima. This is reflected in the intima/media ratio, which was significantly higher in stenotic bypass grafts (Figure 2 A,  $p < 0.05$ ). In contrast, a comparable, statistically significant increase was noted concerning cell density in neointimal and medial layers (Figure 2 C) when compared to native veins.

The adventitia was analysed in the same manner but due to a non-uniform procurement of vein grafts, the area and cell number measurements were of no physiologically relevant significance. However, there was a significant increase of cell density in stenotic bypass grafts compared to native veins.

### *Cell proliferation in native and stenotic bypass grafts*

Although the measurement of proliferation rates in our series of explanted venous bypass grafts yielded out low levels in the neointima ( $0.14 \pm 0.048$ ), media ( $0.18 \pm 0.096$ ) and adventitia ( $0.09 \pm 0.025$ ) there was a statistically significant difference when compared to native veins. The topographical correlation of proliferative activity is shown in Figure 3.

When the proliferative profile was plotted as a function of time since graft implantation no statistical correlation was found (data not shown). There was no statistical significance in proliferation activity between the different types of lesions.

### *Characterization of proliferating cells*

Despite the large predominance of  $\alpha$  actin positive SMC's in the neointima, SMC's contributed only 9% to the pool of proliferating cells (Table 1). This percentage was slightly larger in the media (14%, Table 1). A high percentage of proliferating cells were identified as endothelial cells in the neointima (42%), in the media (50%) and in the adventitia (83%). Histologically, proliferating endothelial cells were associated with newly formed microvessels (Figure 1 C) In the neointima a substantial number of proliferating cells were macrophages (9%), while this cell population was virtually non-existent among the proliferative cell pools of media and adventitia. In concordance with the literature quite a few proliferating cells were recognized by none of the cell-specific antibodies. This cell pool was 40% in the neointima, 33% in the media, and 5% in the adventitia (Table 1).

## DISCUSSION

The principal findings of this study are that proliferative activity in human stenotic vein graft specimens occurs at very low levels (Figure 3). Similarly low levels of cellular proliferative activity have been reported on human carotid and coronary atherosclerotic plaques [Brandl 1997]. Using antibodies against cell cycle related antigen Ki67 Brandl studied the cell proliferation in carotid endarterectomy specimens and

found an overall proliferation index of  $0.45 \pm 1.05$  morphologically localized to macrophage-rich areas of high cellular density. O'Brien performed PCNA immunocytochemistry on human primary and restenotic coronary atherectomy tissue and also found low replication rates [O'Brien 1993]. Westerband studied the cell replication (PCNA) in human infrainguinal vein graft stenosis and calculated a mean proliferation index of 1.34% [Westerband 1997].

The variation in proliferative activity between the different lesions may reflect the time course of lesion progression in peripheral vein grafts studied by Westerband and coworkers (proliferation index 1.345). They showed a faster atherosclerotic progression, as reflected by a much shorter interval between implantation and stenosis (3-8 month) than the bypass grafts studied in this study (mean interval 94 month). Furthermore, studies on experimental restenosis in animal models have demonstrated high proliferative activity in the initial period after grafting and a gradual decline in proliferative activity in later periods. In our study there was no difference in proliferation activity between the different morphological appearance of the bypass grafts. It is possible that there is a very early peak of proliferation activity that is not registered in this study. Therefore further detailed studies are needed to determine the degree of proliferation in human lesions during stages of development.

In our study the cell turnover was estimated by immunocytochemical detection of the nuclear antigen Ki-67 expressed in close relation to the cell cycle. The monoclonal antibody MIB1 against Ki67 is currently being used in routine surgical pathology as a reliable proliferation marker and prognostic indicator for several malignant tumors. Compared with PCNA which has been shown to give positive results in nonreduplicative DNA synthesis as well, there is a high specificity of Ki-67 expression for cycling cells as documented by autoradiographic  $^3\text{H}$ -thymidine incorporation. Another disadvantage using PCNA is that PCNA positivity over a wide range depends on the conditions of tissue fixation, a possible source of inconsistent and less distinct labeling results [Rowlands 1991; Scott 1991].

In addition to assessing the degree of cellular proliferation in human vein graft lesions, the secondary purpose of this study was to determine the identity of actively proliferating cells. By use of antibody double labeling the proliferating cells were identified not only as smooth muscle cells but also as endothelial cells, macrophages and T-lymphocytes. Only 9% of the proliferating cells in the neointima where SMC's and in the media 14% of replicating cells occurred as SMC's.

A high portion of actively proliferating cells in the vein grafts could not be identified by any of the cell markers used. These unidentified mesenchymal cells may represent SMC's or other cell types at various stages of differentiation.

The main portion of Ki-67 positive cells in the adventitia was ECs with 83%. The presence of EC proliferation has been previously reported in human primary and restenotic coronary lesions and in peripheral venous bypass grafts. The precise role of EC replication is unclear but it may be a necessary component of healing after injury due to vein graft harvesting as well as a response to arterialization.

Of particular interest was the observation that the majority of proliferating cells in all layers of the stenotic vein grafts were endothelial cells associated with microvessels. We presume that this reflects a specificity of bypass graft lesions, linked to the adaptation of the graft to the altered hemodynamic conditions of a high-pressure arterial circulation. If indeed neovascularization can be linked to bypass graft stenosis, than prophylactic interventions might be aimed at the interruption of angiogenesis. Such therapeutic tools are available and are currently being tested for the use as anti-neoplastic agents [Kuzuya 1995].

As preventive therapies to date have not been successful in limiting graft threatening intimal thickening in human vein grafts, a detailed understanding of the biology of smooth muscle growth, migration and the biosecretory products causing

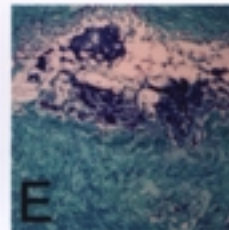
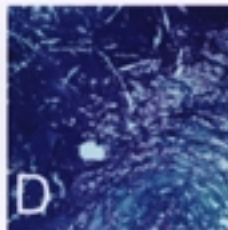
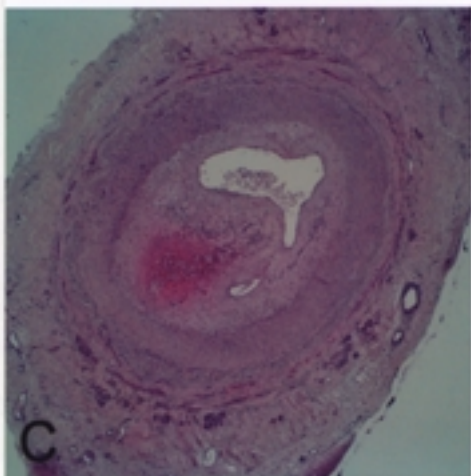
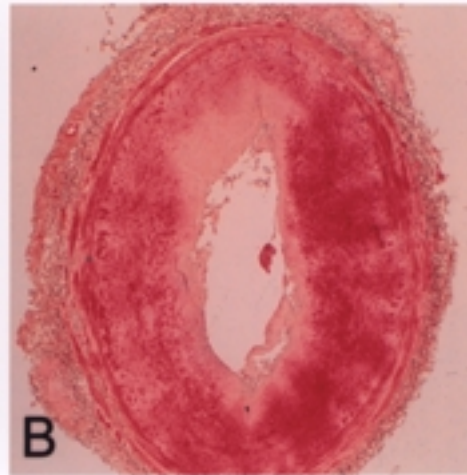
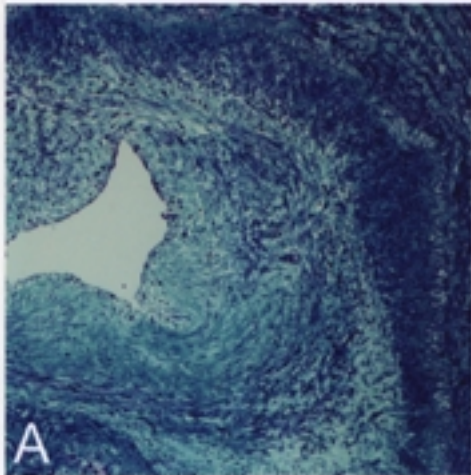
intimal thickening may require a redefinition of the basic process of adaptive remodeling of veins transplanted into the arterial circulation.

## REFERENCES

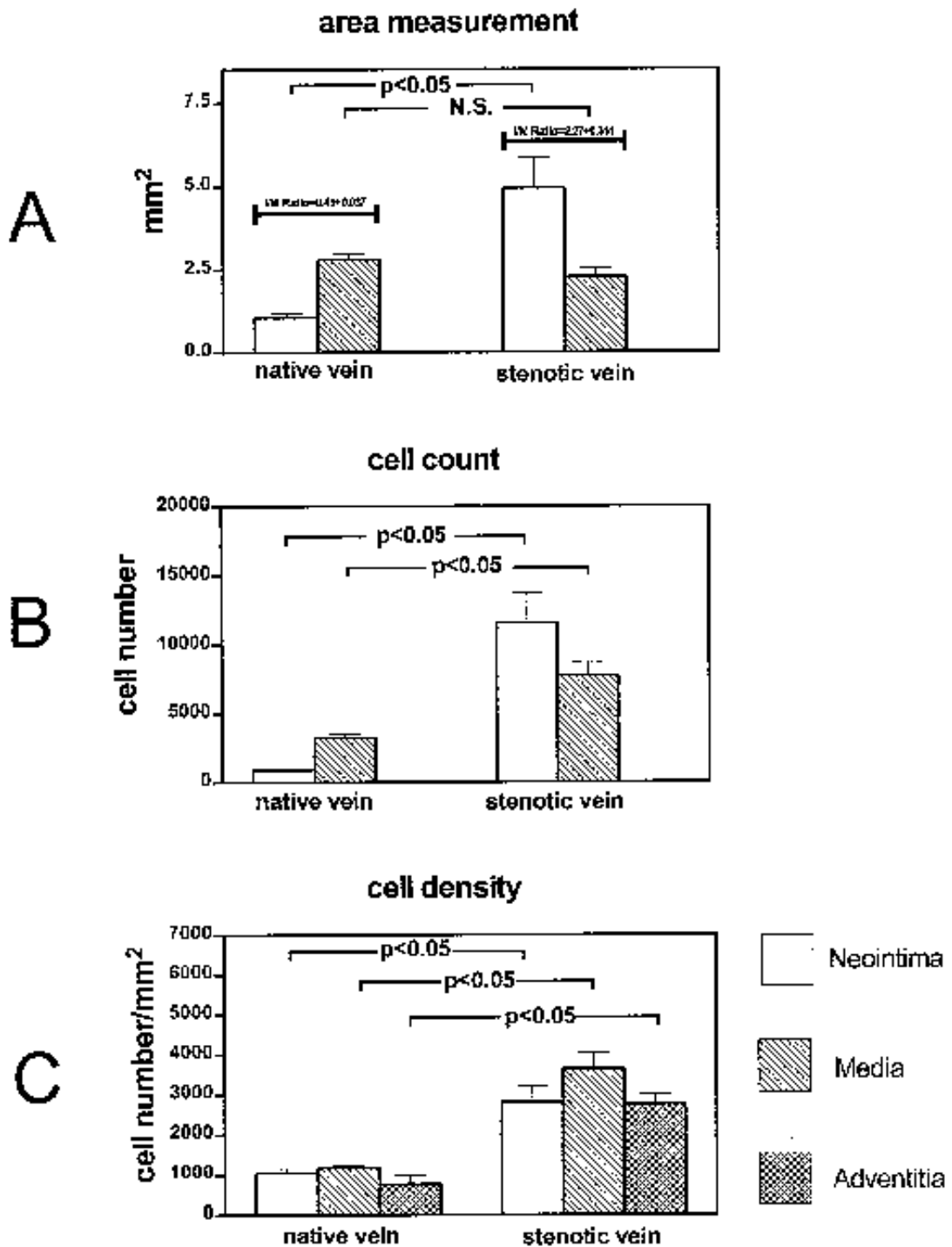
1. Atkinson, J.B., Forman, M.B., Vaughn, W.K., Robinowitz, M., McAllister, H.A. and Virmani, R., Morphologic changes in long-term saphenous vein bypass grafts. *Chest* 88: 341-8, 1985.
2. Brandl, R., Richter, T., Haug, K., Wilhelm, M.G., Maurer, P.C. and Nathrath, W., Topographic analysis of proliferative activity in carotid endarterectomy specimens by immunocytochemical detection of the cell cycle-related antigen Ki-67. *Circulation* 96: 3360-8, 1997.
3. Brody, J.I., Pickering, N.J. and Fink, G.B., Immunocytochemical features of obstructed saphenous vein coronary artery bypass grafts. *J Clin Pathol* 42: 477-82, 1989.
4. Bulkley, B.H. and Hutchins, G.M., Accelerated "atherosclerosis". A morphologic study of 97 saphenous vein coronary artery bypass grafts. *Circulation* 55: 163-9, 1977.
5. Cooper, G.J., Underwood, M.J. and Deverall, P.B., Arterial and venous conduits for coronary artery bypass. A current review. *Eur J Cardiothorac Surg* 10: 129-40, 1996.
7. Eritsland, J., Arnesen, H., Fjeld, N.B., Gronseth, K. and Abdelnoor, M., Risk factors for graft occlusion after coronary artery bypass grafting. *Scand J Thorac Cardiovasc Surg* 29: 63-9, 1995.
8. Gibbons, G.H., The pathogenesis of graft vascular disease: implications of vascular remodeling. *J Heart Lung Transplant* 14: 149-58, 1995.
9. Kuzuya, M., Satake, S., Esaki, T., Yamada, K., Hayashi, T., Naito, M., Asai, K. and Iguchi, A., Induction of angiogenesis by smooth muscle cell-derived factor: possible role in neovascularization in atherosclerotic plaque. *J Cell Physiol* 164: 658-67, 1995.
10. Motwani, J.G. and Topol, E.J., Aortocoronary saphenous vein graft disease: pathogenesis, predisposition, and prevention. *Circulation* 97: 916-31, 1998.
11. Newby, A.C. and George, S.J., Proliferation, migration, matrix turnover, and death of smooth muscle cells in native coronary and vein graft atherosclerosis. *Curr Opin Cardiol* 11: 574-82, 1996.
12. O'Brien, E.R., Alpers, C.E., Stewart, D.K., Ferguson, M., Tran, N., Gordon, D., Benditt, E.P., Hinohara, T., Simpson, J.B. and Schwartz, S.M., Proliferation in primary and restenotic coronary atherectomy tissue. Implications for antiproliferative therapy. *Circ Res* 73: 223-31, 1993.
13. Pickering, J.G., Weir, L., Jekanowski, J., Kearney, M.A. and Isner, J.M., Proliferative activity in peripheral and coronary atherosclerotic plaque among patients undergoing percutaneous revascularization. *J Clin Invest* 91: 1469-80, 1993.
14. Rowlands, D.C., Brown, H.E., Barber, P.C. and Jones, E.L., The effect of tissue fixation on immunostaining for proliferating cell nuclear antigen with the monoclonal antibody PC10. *J Pathol* 165: 356-7, 1991.
15. Scott, R.J., Hall, P.A., Haldane, J.S., van Noorden, S., Price, Y., Lane, D.P. and Wright, N.A., A comparison of immunohistochemical markers of cell proliferation with experimentally determined growth fraction. *J Pathol*: 165, 173-8, 1991.
16. Westerband, A., Mills, J.L., Marek, J.M., Heimark, R.L., Hunter, G.C. and Williams, S.K., Immunocytochemical determination of cell type and proliferation rate in human vein graft stenoses. *J Vasc Surg* 25: 64-73, 1997.
17. Zou, Y., Dietrich, H., Hu, Y., Metzler, B., Wick, G. and Xu, Q., Mouse model of venous bypass graft arteriosclerosis. *Am J Pathol* 153: 1301-10, 1998.

**Figure 1**

Light photomicrographs showing the morphology of human vein graft stenoses **A** Masson Goldner stain. Luminal narrowing caused by an adaptive proliferative intimal thickening (type 1). **B** Immunolabeling for  $\alpha$ -smooth muscle actin. Note the fibrous cap (type 2) **C** Advanced atherosclerotic lesions (type 3). **D** Core of extracellular lipid includes cholesterol crystals. **E** Macrophage foam cells

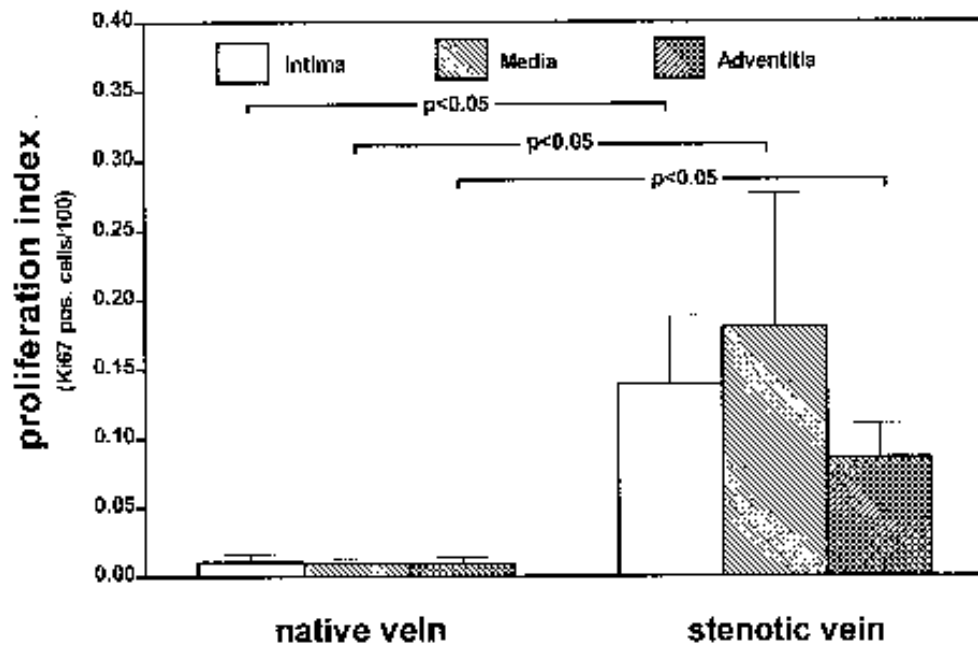


**Figure 2**  
 Bar graph showing the area (a), cell number (b) and cell density (c) in the different layers of the vessel wall for native and stenotic venous bypass grafts.



**Figure 3**

Bar graph showing the proliferation index in the intima, media and adventitia for native and stenotic bypass grafts.



**Table 1.** Proliferating cell types

	<u>EC</u>	<u>SMC</u>	<u>M</u>	<u>T</u>	<u>N</u>
Neointima	42%	9%	9%	0%	40%
Media	50%	14%	1	2%	33%
Adventitia	83%	6%	2%	4%	5%

EC = endothelial cells

SMC = smooth muscle cells

M = macrophages

T = T-lymphocytes

N = not identified proliferating cells