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Perivascular Treatment with Azathioprine Reduces Neointimal Hyperplasia in Experimental Vein Grafts

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ABSTRACT

Background. Azathioprine is an immunosuppressive and anti-inflammatory drug, and it has been shown to induce apoptosis in human T-lymphocytes. We investigated whether local treatment with azathioprine can inhibit neointimal hyperplasia in experimental vein grafts.

Methods. C57BL/6J mice underwent interposition of the inferior vena cava from isogenic donor mice into the common carotid artery using a cuff technique. In the treatment group azathioprine was perivascularly applied. The control group did not receive local treatment. Vein grafts were harvested at 1 and 2 weeks postoperatively and underwent morphometric analysis as well as immunohistochemical analysis for apoptosis (TUNEL).

Results. In grafted veins without treatment (controls), neointimal thickness was 10 μm (range, 6-29 μm), and 12 μm (range, 8-40 μm) at 1 and 2 weeks postoperatively, respectively. In azathioprine-treated grafts, the neointimal thickness was 2 μm (range, 1-5 μm) and 4 μm (range, 3-11 μm) at 1 and 2 weeks postoperatively, respectively. This reduction of neointimal thickness was significant at 1 week ($P = .001$) and 2 weeks ($P = .016$) postoperatively.

Azathioprine-treated vein grafts showed an increased rate of apoptosis in the vascular wall as compared with controls (593 [range, 26-783] versus 45 [range, 0-106] apoptotic cells/mm^2 at 1 week, $P = .063$; and 656 [range, 327-1270] versus 19 [range, 0-79] apoptotic cells/mm^2 at 2 weeks, $P = .016$).

Conclusion. We conclude that treatment of experimental vein grafts with azathioprine is associated with a reduction of neointimal hyperplasia and an increased apoptosis rate in the vascular wall. These results suggest that azathioprine may be useful for the prevention of vein graft disease after coronary artery bypass grafting.

INTRODUCTION

Vein grafts are still important and commonly used conduits in coronary artery bypass grafting (CABG). In some aspects they are excellent material; for example, veins are surgically easier to handle than arteries. In most patients, saphenous veins are available and easy to harvest. Vein grafts are not prone to vasospasm as arteries are, especially if vasopressors are used in the intensive care unit. Saphenous vein grafts provide the highest blood flow to the heart after CABG. Veins can be used to bypass coronary arteries with stenoses between 50% and 70%, but arterial grafts have decreased patency rates.

However, the major drawback of saphenous vein grafts in CABG is their decreased long-term patency rate as compared to that of arterial grafts. The so-called vein graft disease is caused by an atherosclerotic process that originates from neointimal hyperplasia. The formation of the neointima starts immediately after arterialization of the vein graft and continues for some weeks postoperatively. Several experimental models exist that develop these characteristic pathological features, one of which is the mouse model we used in this study.

Inflammation plays a key role in immediate damage of the grafted vein. The reason for this is an accumulation of blood cells (e.g., macrophages, platelets, lymphocytes) in the "wounded" vascular wall that release various proinflammatory substances and growth factors. Azathioprine has been clinically used as an anti-inflammatory drug (e.g., in chronic inflammatory bowel disease, pemphigus) for decades. In addition, azathioprine exerts immunosuppressive properties which are widely used after heart transplantation [Belgi 2002; Maltzman 2003; Schwab 2003; Schachner 2004a].

Our aim was to investigate the effects of perivascularly applied azathioprine on the development of neointimal hyperplasia and the amount and distribution of apoptotic cells in the wall of azathioprine-treated experimental vein grafts.

MATERIALS AND METHODS

Mice and Vein Grafting

C57BL/6J mice were purchased from Harlan-Winkelmann (Borchen, Germany). They were maintained at 24°C and received food and water ad libitum. All procedures were performed according to protocols approved by the Austrian Ministry of Science according to §8 of the law on animal experiments and all animals were treated according to the *Guide for the Use and Care of Laboratory Animals*, published by the National Institutes of Health (NIH publication no. 85-23, revised 1985).

The operation was performed as previously described [Zou 1998; Schachner 2004b]. In brief, the vena cava from a

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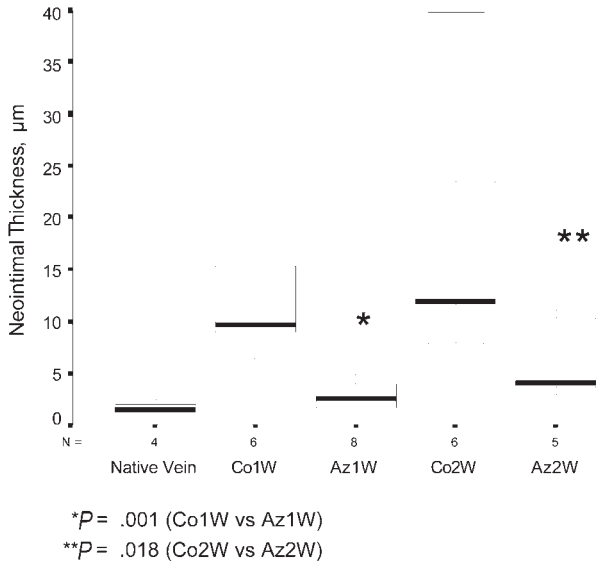

Figure 1. Development of neointimal thickness in control vein grafts (Co) and azathioprine-treated grafts (Az) at 1 (1W) and 2 (2W) weeks postoperatively compared with ungrafted native veins. Note the decreased neointimal hyperplasia in azathioprine-treated vein grafts.

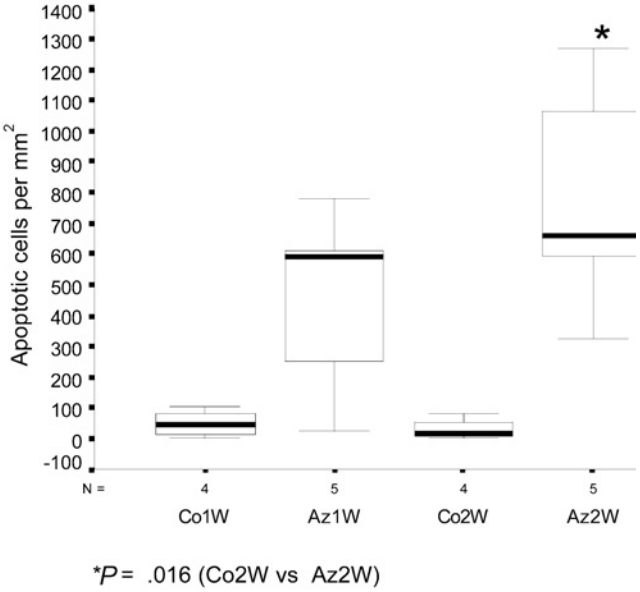

Figure 2. Apoptosis rate in the vascular wall in control vein grafts (Co1W, Co2W) and azathioprine-treated grafts (Az1W, Az2W) at 1 and 2 weeks postoperatively. Note the increased apoptosis rate in azathioprine-treated vein grafts.

donor mouse was interposed into the carotid artery (cuff technique) of the recipient mouse. In the treatment group, azathioprine lyophilisate (Imurek; GlaxoSmithKline, Brentford, England) was directly dissolved on the grafted vein to form a thin layer around the vessel. Depending on the size of the vein graft, approximately 1 mm^3 of lyophilisate was used. The control group did not receive local treatment.

Tissue Preparation

For histological analysis the animals underwent autopsy at 1 and 2 weeks postoperatively. The interposed vein segments were cut out at the cuff ends and fixed with 4% phosphate-buffered formaldehyde and embedded in paraffin.

Histology and Lesion Quantification

Sections of 3 µm thickness were routinely stained by hematoxylin, eosin, and elastica van Gieson. For assessment of the neointimal thickness, pictures were taken under microscope at a magnification of 1:400, and the measurements were done using Optimas 5.0 image analysis software (Media Cybernetics, Silver Spring, MD, USA). For achieving reproducible results, the cross sections of the veins were divided into 4 quadrants and in each quadrant 4 measurements were performed. The median value of all measurements was regarded as representative for the neointimal thickness.

Immunohistochemistry (TUNEL)

The paraffin embedded sections were immunohistochemically stained with the In Situ Cell Death Detection Kit (Roche, Indianapolis, IN, USA) to determine apoptotic cells in treated and untreated veins labelling DNA strand breaks (TUNEL assay; terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling). For antigen retrieval the slides were pretreated in citrate buffer (ph 6) in the microwave at 750 watts for 15 minutes and endogenous peroxidase was blocked by incubation in 5% H2O2 in methanol for 15 minutes. Afterward the TUNEL reaction mixture was freshly prepared as described in the instruction leaflet and the slides were incubated with it for 45 minutes in a humidified chamber. The following signal conversion was done by incubation with second antibody conjugated with horseradish peroxidase and by using diaminobenzidine as chromogene. Additionally, the slides were counterstained with hemalum and subsequently analyzed under a light microscope. Only cells with distinct nuclear staining were considered.

The results were quantified by 2 pathologists, who were blinded to the study, by counting the number of positively stained cells per high power field at 400× magnification. The number of counted cells was extrapolated to 1 mm^2. Staining intensity was not quantified, but only intensities at least 2 times stronger than the background were taken into consideration.

Statistical Analysis

The SPSS for Windows statistical software package (SPSS 10.0; Microsoft, Redmond, WA, USA) was used for analysis. Neointimal thickness is given as median and range. Comparisons of histological measurements of the intimal thickness and the amount of positively stained cell nuclei in the TUNEL assay were made by a Mann-Whitney U test. Results were considered statistically significant at P values <.05.

RESULTS

In all vein grafts (n = 5-8 in each group) neointimal hyperplasia developed at 1 and 2 weeks postoperatively. In grafted veins without treatment (controls) neointimal thickness was 10 µm (range, 6-29 µm) at 1 week postoperatively, whereas in azathioprine-

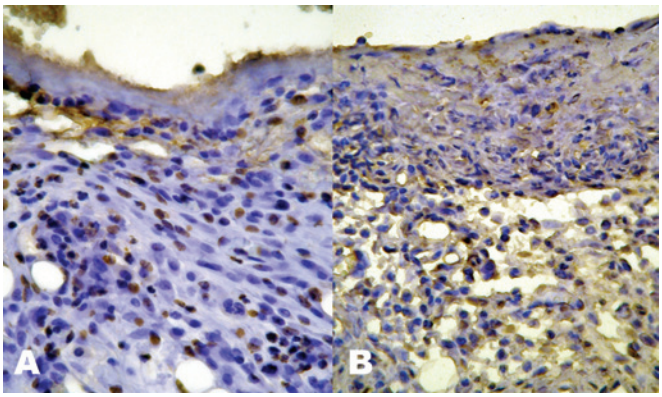

Figure 3. Apoptosis rate in (A) azathioprine-treated vein grafts and (B) mouse control vein grafts. Note the increased number of apoptotic cells in azathioprine-treated veins. (TUNEL stain, original magnification ×400.)

treated grafts it was 2 μm (range, 1-5 μm; $P = .001$). At 2 weeks postoperatively in controls neointimal thickness was 12 μm (range, 8-40 μm), whereas in azathioprine-treated grafts it was 4 μm (range, 3-11 μm; $P = .016$; Figure 1).

Azathioprine-treated vein grafts showed an increased apoptosis rate in the vascular wall as compared with controls (593 [range, 26-783] versus 45 [range, 0-106] apoptotic cells/mm^2 at 1 week, $P = .063$; and 656 [range, 327-1270] versus 19 [0-79] apoptotic cells/mm^2 at 2 weeks, $P = .016$; Figures 2 and 3).

The apoptosis rate was distributed in the different vein graft layers in the following pattern: adventitia > media > neointima.

DISCUSSION

Neointimal hyperplasia is among the early changes of an arterialized vein graft, and it is thought to be the pathological feature that eases vein graft atherosclerosis. Hence early inhibition of neointimal hyperplasia might reduce late vein graft disease.

Immunosuppressants have potentially beneficial effects with regard to prevention of neointimal hyperplasia, which is due to their antiproliferative properties. Mingoli et al [1996] saw a reduced dilatation, medial thickening, and lymphocyte infiltration in vein grafts of cyclosporine-treated dogs. We have previously reported that rapamycin treatment reduces neointimal thickness in experimental vein grafts in the mouse [Schachner 2004b]. In this study we saw that local treatment with azathioprine also inhibits neointimal hyperplasia. Arruda et al [2003] found a low amount of in-stent (bare metal stent) neointimal hyperplasia in renal transplant recipients under immunosuppressive treatment (mainly cyclosporine and azathioprine). One commonly accepted therapeutic mechanism of azathioprine is the inhibition of purine nucleotide biosynthesis. However, other mechanisms of action remain to be elucidated. For example, it has been demonstrated that azathioprine induces apoptosis in human T-lymphocytes [Maltzman 2003; Tiede 2003].

In an earlier study, we described the association between reduction of neointimal hyperplasia and an increased apoptosis rate in vein grafts [Schachner 2004a]. Now we report again the association of an increased apoptosis rate in the vascular wall with a reduced neointimal hyperplasia in azathioprine-treated vein grafts. This underlines the potentially beneficial role of apoptosis for inhibiting the early phase of vein graft disease. These results suggest that azathioprine may have a therapeutic potential for treatment of vein graft disease.

There are limitations for this study. The local application of azathioprine lyophilisate is followed by a time-limited effect of this drug. Carriers providing a longer release time of azathioprine would be desirable. There are no long-term experiments with azathioprine.

Nevertheless, in this animal model neointimal hyperplasia markedly develops during the first weeks after vein graft arterialization, and this part of vein graft disease was the specific focus of our study. The study is conducted in thin mouse vein grafts. Large animal studies would be necessary to prove the effects of azathioprine in bigger vein grafts.

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