Rapamycin treatment is associated with an increased apoptosis rate in experimental vein grafts

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Received 2 September 2004; received in revised form 10 November 2004; accepted 11 November 2004; Available online 15 December 2004

Abstract

Objective: Rapamycin is an immunosuppressive agent with marked antiproliferative properties and is effective in reducing in stent restenosis and vein graft neointimal hyperplasia. Apoptosis is one mechanism counterbalancing cellular proliferation. We therefore investigated the role of apoptosis in rapamycin treated vein grafts in a mouse model. Methods: C57BL6J 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 200 μg of rapamycin were applied locally in pluronic gel. The control group did not receive local treatment. Vein grafts were harvested at 4 weeks postoperatively and underwent morphometric analysis as well as immunohistochemical analysis for apoptosis (TUNEL). Results: In grafted veins without treatment (controls) neointimal thickness was 50 (12–58) μm at 4 weeks postoperatively. In 200 μg rapamycin treated grafts the neointimal thickness was 17 (5–55) μm. Rapamycin treated vein grafts showed a significantly increased rate of apoptosis in the adventitia as compared with controls (P = 0.032). In the neointima the apoptosis rate was lower in both groups with no significant difference between rapamycin treated grafts and controls.

Conclusion: We conclude that treatment of experimental vein grafts with rapamycin is associated with an increased apoptosis rate in the vascular wall and a trend towards reduction of neointimal hyperplasia. These results suggest that apoptosis may be a beneficial antiproliferative component for the treatment of vein graft disease.

Keywords: Neointimal hyperplasia; Vein graft; Apoptosis; Bypass

1. Introduction

Vein grafts still are important conduits in coronary artery bypass grafting (CABG). However, neointimal hyperplasia and consecutive graft atherosclerosis remain a significant limit for the longevity of venous bypass conduits [1]. At present platelet inhibition and statin treatment represent standard clinical care for vein graft disease, and numerous experimental studies on this topic have been performed. Nevertheless only a few new treatment strategies for neointimal hyperplasia, such as E2F decoy in the PREVENT study (saphenous vein grafts in vascular surgery) are currently tested in clinical studies [2,3].

One of the potential therapeutic agents is rapamycin (Sirolimus), an immunosuppressive agent with marked antiproliferative properties [4,5]. Rapamycin is used as a coating of coronary artery stents and peripheral vascular stents to prevent restenosis [6,7].

Apoptosis is a mechanism that counterbalances cell proliferation. Many human diseases involve exaggerated apoptosis (e.g. neurodegenerative diseases, viral hepatitis) or too little apoptosis (e.g. neoplasia, autoimmune diseases) [8,9].

Biochemically apoptotic cell death is characterized by caspase activation, and caspase-mediated protein cleavage, as well as DNA fragmentation. Morphologically apoptosis is characterized by shrinkage of the cell, fragmentation into membrane-bound apoptotic bodies, and phagocytosis by neighboring cells (macrophages and parenchymal cells). On the contrary accidental cell death is characterized by swelling of the cell, plasma membrane rupture, disruption of cellular organelles, and inflammation [8,10].

It has been demonstrated that rapamycin induces apoptosis in tumor cell lines (e.g. multiple myeloma, acute lymphoblastic leukemia, B-cell lymphoma) and in thymocytes, but little is known about the microtopography of rapamycin-induced changes in the vascular wall [11-14].
Therefore, we aimed to test the hypothesis that treatment of experimental vein grafts with rapamycin increases the rate of apoptotic cells in the vascular wall.

2. Material and methods

2.1. 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 section 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 described previously [5, 15, 16]. In brief the vena cava from a donor mouse was interposed between two carotid artery stumps (everted over a nylon cuff) of the recipient mouse [Fig. 1]. In the treatment group 200 μg of rapamycin (Wyeth, Collegeville, USA) were applied into the perivascular spaces of the grafted vein. As a carrier we used 0.1 ml of 20% Pluronic-F-127™ gel (BASF, Germany). The control group did not receive local treatment.

2.2. Tissue preparation

For histological analysis the animals underwent autopsy at 4 weeks postoperatively (untreated animals additionally at 1 and 2 weeks postoperatively). The grafts were perfusion fixed with 4% phosphate-buffered formaldehyde via puncture of the left ventricle as previously described [16]. The interposed vein segments were cut out at the cuff ends and fixed with 4% phosphate-buffered formaldehyde. Consecutively the veins were formalin fixed and embedded in paraffin.

2.3. Histology and lesion quantification

Sections of 4 μm thickness were stained by elastica van Gieson silver impregnation for measurement of the intimal thickness. The measurements were done using OPTIMAS 5.0 image analysis software. For achieving reproducible results, the cross sections of the veins were divided into 4 quadrants.

In each quadrant 3 measurements were performed. The median value of all measurements was regarded as representative for the intimal thickness.

2.4. Immunohistochemistry (TUNEL)

Immunohistochemistry was carried out on paraffin embedded sections of animals 4 weeks postoperatively (and on untreated vein grafts additionally at 1 and 2 weeks postoperatively).

For staining of apoptotic cells a TUNEL (terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling) assay was used with diaminobenzidine as chromogen (Boehringer Mannheim Inc., Germany). This method identifies apoptotically degraded DNA. Slides were subsequently washed, and counterstained with methyl green. Only cells with distinct nuclear staining were considered.

The results were quantified by a pathologist (AT), who was blinded to the study, by counting the number of positively staining cells in medium power fields (200× magnification; 0.747 mm²/field). The number of counted cells was extrapolated to 0.8 mm². Staining intensity was not quantified, but only intensities at least two times stronger than background were taken into consideration.

2.5. Statistical analysis

The SPSS™ for Windows statistical software package (SPSS 10.0) 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 staining cell nuclei in the TUNEL assay were made by Mann-Whitney U test. Results were considered statistically significant at p values of less than 0.05.

3. Results

In grafted veins without treatment (controls, n=6) neointimal thickness was 50 (12-58) μm at 4 weeks
postoperatively. In 200 μg rapamycin treated grafts (n=7) the neointimal thickness was 17 (5–55) μm (P=0.15).

The time course of apoptosis in (untreated) vein grafts is shown in Fig. 2.

At 4 weeks postoperatively the TUNEL staining showed the highest apoptosis rate in the adventitia. In the rapamycin treated group the mean number of apoptotic cells was 95 (cells/0.8 mm²) whereas in controls it was 0.3 (cells/0.8 mm²). (Fig. 3) This difference was statistically significant (P=0.032, Table 1).

Apoptosis was also present in the media. In the rapamycin treated group the mean number of apoptotic cells was 20 (cells/0.8 mm²) whereas in controls it was 0.3 (cells/0.8 mm²). This difference, however, did not reach statistical significance (P=0.11).

In the neointima the apoptosis rate was lower in both groups with no significant difference between rapamycin treated grafts (0.7 cells/0.8 mm²) and controls (0.8 cells/0.8 mm²).

4. Discussion

Venous CABG conduits undergo a hypoxic phase after harvesting and are exposed to systemic blood pressure after grafting. Matsushita and coworkers recently demonstrated that hypoxia induces endothelial apoptosis [17]. Mechanical stretch can induce vascular smooth muscle cell apoptosis, and apoptosis of smooth muscle cells occurs in experimental saphenous vein grafts [18,19]. This is in accordance with our study where we also found presence of apoptosis after venous bypass grafting in the mouse. Nevertheless the apoptosis rate that we observed in our model was too low to provide a complete safeguard against the deregulated cellular proliferation in neointimal hyperplasia. In rapamycin treated veins we found a 10 fold higher rate of apoptotic cells compared with controls. This is in accordance with Roque et al. who found a 7 fold higher apoptosis rate in rapamycin treated porcine coronary arteries 4 weeks after balloon injury compared with controls [20].

In our study the time course of apoptosis in untreated vein grafts showed a maximum at 1 week postoperatively. This is in accordance with Malik and coworkers who demonstrated a peak of apoptotic cell accumulation early after balloon injury of pig coronary arteries and a rapid fall of apoptotic cell counts thereafter [21].

Interestingly we detected the highest apoptosis rate in the adventitia of the vein grafts, whereas the apoptotic cell count in the neointima was low. By comparison Roque et al., after a peak at 1 week, noticed a decrease of apoptosis in the neointima 4 weeks after arterial balloon injury [20]. On the other hand Durand et al. describe the highest apoptosis rate in the intima and media of atherosclerotic rabbit femoral arteries after balloon injury [22]. One reason for these different patterns may be the wall structure of arteries which consists of a much thicker medial layer than veins. Another reason is the type of injury to the vessel. In balloon injury models the inner layer of the artery is primarily damaged, whereas in venous bypass grafts the complete wall is continuously exposed to mechanical stress. In addition we applied the antiproliferative agent on the adventitial layer of the vessel and penetration potentially did not reach the intima. Nevertheless the adventitia is a very active site of remodeling (including proliferation of fibroblasts, and their transformation into myo-fibroblasts with consecutive deposition of extracellular collagen) after porcine coronary angioplasty. Additionally there is evidence that myo-fibroblasts migrate from the adventitia into the media and neointima. Thus adventitial cells are involved in the pathogenesis of neointimal hyperplasia [23].

As demonstrated by Suzuki and coworkers in early porcine experiments of drug eluting stent treatment the antiproliferative agent rapamycin exhibits its action at the primary site of application. A 50% reduction of neointimal area was noted in this study, however, there is no clear information available whether apoptosis is involved in this reduction [24].

Fig. 3. TUNEL staining at 4 weeks postoperatively for controls (A) and rapamycin treated (B) experimental vein grafts. Note the increased rate of positively staining cell nuclei (brown colour) in the rapamycin treated veins.

<table>
<thead>
<tr>
<th>Layer</th>
<th>Controls (n=4)</th>
<th>Rapamycin (n=6)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neointima</td>
<td>0.8 (0-2)</td>
<td>0.7 (0-3)</td>
<td>0.72</td>
</tr>
<tr>
<td>Media</td>
<td>0.3 (0-1)</td>
<td>20 (0-40)</td>
<td>0.11</td>
</tr>
<tr>
<td>Adventitia</td>
<td>5.3 (3-10)</td>
<td>95 (5-190)</td>
<td>0.032</td>
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In our current investigation the increased apoptosis rate in rapamycin treated vein grafts was associated with a trend towards reduced neointimal thickness. Results from Mayr et al. support our findings: They found an increased neointimal hyperplasia in vein grafts of p53 knockout mice. This increase in wall thickness was associated with a reduced apoptosis rate [25].

One limitation of the study is the relatively low number of experiments. Another limitation is the use of TUNEL stain alone as a diagnostic tool for apoptosis. Complementary detection methods, such as in situ ligation assays using Taq polymerase, or assays demonstrating caspase activation are beneficial to confirm the complex process of apoptosis.

5. Conclusion

We conclude that treatment of experimental vein grafts with rapamycin is associated with an increased apoptosis rate and a trend towards reduction of neointimal hyperplasia. These results suggest that apoptosis may be a beneficial component for the treatment of vein graft neointimal hyperplasia.

References


Appendix A. Conference discussion

Dr A. Pavia (Paris, France): What is the future of your study? Do you want to go to clinical use?

Dr Schachner: There is one important step necessary, and this is a large animal model. Because one limitation is, of course, that it’s done in mouse; and the second one that the vein grafts are very thin, so one would need to test the effects of perivascularly applied drugs in a large animal model.

Dr H. Shenbin (Montreal, Canada): It’s a very difficult animal model, I understand, and my question to you has to do with the methodology. When you did the two groups, was it the same person doing both groups? And do you think that perhaps time also may have an impact, in other words, the timing of the operation, whether you’ve done the controls before the rapamycin, may have influenced the severity and the intensity of the changes that you have seen, or have you done one-to-one, one-by-one, one rapamycin and one control at the same time?

Dr Schachner: These experiments have been performed by the same surgeon, not one by one, so one control, one rapamycin, but consecutively

Dr Shenbin: And did you do the controls first or the rapamycin first?

Dr Schachner: We did the controls first.
**Dr Shennib:** Do you think that there is a learning curve that may have influenced some of the results?

**Dr Schachner:** Not in this case, because the surgeon was experienced and did many of these operations before.

**Dr G. Lutter (Kiel, Germany):** Could you imagine that a longer duration of waiting for the results could have increased the neointimal hyperplasia to get a stronger statistical significance? You showed us a trend towards reduction of neointimal hyperplasia.

**Dr Schachner:** That’s right, at the time point 4 weeks postoperatively, we found a trend towards reduction with the treatment of rapamycin, whereas at earlier time points the difference reached statistical significance. This may be due to the release time of the drug. It’s applied locally at the time of operation and the pharmacological effect decreases.

**Dr Lutter:** Did you look for the drug release in your mice? Did you look for the drug release in the serum or in the local area?

**Dr Schachner:** No, we didn’t.