Time Course of Smooth Muscle Cell Proliferation in the Intima and Media of Arteries Following Experimental Angioplasty

Hartmut Hanke, Thomas Strohschneider, Martin Oberhoff, Eberhard Betz, and Karl R. Karsch

Smooth muscle cell (SMC) proliferation is known to be an important factor for the development of restenosis after percutaneous transluminal coronary angioplasty. To determine the time course of intimal and medial SMC proliferation and morphological changes after experimental angioplasty, an intimal atheroma was produced with repeated weak electrical stimulations in the right carotid artery of 45 male New Zealand White rabbits. Angioplasty was subsequently performed in 35 rabbits, and the proliferative responses were analyzed with histomorphological and immunohistological criteria at 3, 7, 14, 21, 28, and 42 days after intervention. A hemodynamic relevant stenosis after angioplasty was found in eight (23%) of 35 dilated arteries. In five rabbits the stenosis was due to a mural thrombus, and in three animals restenosis was caused by intimal SMC proliferation. In all dilated arteries the intimal wall thickness increased from 13±5 intimal cell layers (after electrical stimulation) to 35±14 cell layers during 28 days after angioplasty (p<0.05). Later than 4 weeks after angioplasty, no additional increase of intimal thickening occurred. Application of bromodeoxyuridine 18 and 12 hours before excision of the vessels allowed determination of the percent of cells undergoing DNA synthesis in the intima and media using monoclonal antibody against bromodeoxyuridine. SMCs were identified by α-actin staining. Immunohistological quantification of intimal SMC proliferation showed a maximum of cells undergoing DNA synthesis within the first 7 days after angioplasty (p<0.01). In contrast, medial proliferation of SMCs was delayed and showed a small but significant increase 21 days after dilatation (p<0.05). Twenty-eight days after angioplasty, SMC proliferation in the media as well as in the intima was normalized and comparable to the control group without angioplasty. (Circulation Research 1990;67:651–659)

Percutaneous transluminal angioplasty, as described by Dotter and Judkins,1 has rapidly gained acceptance as a therapeutic option in the treatment of peripheral atherosclerotic lesions. Since Grünzig2 introduced percutaneous transluminal coronary angioplasty (PTCA) in 1978, this technique has also become a successful method in the treatment of patients with coronary artery disease.3 The clinical application of PTCA, however, is limited by the occurrence of restenosis in 30–40% of patients.4–6

Transluminal angioplasty, performed with an inflatable balloon, is associated with endothelial denudation and early accumulation of platelets and fibrin,7,8 splitting of the intima and media,9–11 stretching of the medial layer, and overdistention of the adventitia.12,13 Platelet adhesion and aggregation induced by endothelial injury after balloon angioplasty has been shown to result in the release of several mitogens,14 including epidermal growth factor and platelet-derived growth factor. These components, as well as expression of fibroblast growth factor and activation of macrophages, are thought to stimulate migration and proliferation of smooth muscle cells (SMCs) in the dilated artery.15–18 In addition, stimulated SMCs are also capable of producing intrinsic growth factors.19

SMC proliferation after PTCA was found to be important for the development of restenosis in several experimental and human postmortem studies.20–22 Another major cause of restenosis is the formation of mural thrombi and their fibrocellular organization at the site of vessel wall injury.13,23

The aim of this study was to determine the time course of intimal and medial SMC proliferation in an
in vivo model. Because fibromuscular plaques can be induced by local transmural electrical stimulations in rabbit carotid arteries, we used this model for plaque development. To study the dynamic process of SMC proliferation after angioplasty, bromodeoxyuridine (BrdU), a thymidine analogue that has been shown to enable determination and quantification of the extent of DNA synthesis of SMCs in vivo, was used.

**Materials and Methods**

**Induction of an Atheroma in Carotid Arteries of Rabbits**

A fibromuscular atheroma was produced by weak electrical stimulation of the carotid artery wall in 45 male New Zealand White rabbits (3.2–4.0 kg). Two graphite-coated gold electrodes were attached to the adventitia of the common carotid artery under general anesthesia (8 mg metomidate-HCl and 0.1 mg fentanyl-base/kg body wt). The electrodes were held in position by a Teflon cuff on each side of the artery. Thin, subcutaneously placed leads from the electrodes were connected to a small plastic socket attached to the skull. This socket was connected to an external stimulation unit with long thin leads so that the animals could move freely during the stimulations. To produce plaques of comparable sizes, the stimulation procedure of the arteries was standardized. Constant-current DC impulses (15 msec/impulse, 0.1 mA, 10 Hz) were applied twice daily for 30 and 15 minutes with a time interval of 8–10 hours between the stimulation cycles for a period of 28 days in each animal.

During the 28 days of electrical stimulation, all animals received a commercially available 0.5% cholesterol diet (Fa. Altromin, Lage, FRG). It has been shown that the combination of electrical stimulation and additional cholesterol-rich diet results in a typical fibromuscular atheroma.

**Study Protocol**

After 28 days of electrical stimulation, transluminal dilatation of the plaque was performed under general anesthesia. After preparation of the carotid artery, the exposed vessel was ligated at least 3 cm distal from the implanted electrodes. Angioplasty was performed with a 2.0-mm balloon catheter (Micro-Hartzler, ACS, Calif.), which was introduced by direct arteriotomy, then advanced into the region of the plaque under microscopic control. The angioplasty catheter was inflated twice with 5 atm for 30 seconds. Between both dilatations the balloon was deflated for 30 seconds.

After removal of the deflated catheter, the small incision of the arteriotomy was closed, and arterial blood flow was restored. To avoid bacterial infections, all animals were on antibiotic therapy during 3 days after the procedure. No animal received a therapy with heparin or drugs inhibiting platelet aggregation or after intervention. After the procedure, a commercially available standard diet without cholesterol (Fa. Altromin) was fed to the rabbits.

To study the time course of SMC proliferation and the chronological sequence of morphological changes, the animals were killed 3, 7, 14, 21, and 28 days and 6 weeks after transluminal angioplasty. A minimum of five animals was used in each group.

Ten control animals were separated into two groups of five rabbits each. One group of rabbits was electrically stimulated for 28 days and served as a control group without angioplasty. Five other rabbits were used as sham controls to exclude an additional effect on SMC proliferation during the procedure of angioplasty. The animals in the sham-operated group underwent a stimulation period of 28 days. Arteriotomy without inserting the catheter was performed. These animals were killed 7 days after intervention.

**Methods for Immunohistological Examination**

To determine the extent of SMCs undergoing DNA synthesis of mitosis, BrdU, a thymidine analogue, was given to each animal 18 and 12 hours before excision of the vessels. As described by Strohschneider et al.,

100 mg/kg body wt BrdU and 75 mg/kg body wt deoxycytidine (both from Sigma GmbH, Deisenhofen, FRG) were given as subcutaneous neck depot 18 hours before the animals were killed. In addition to this neck depot, 30 mg/kg body wt BrdU and 25 mg/kg body wt deoxycytidine were injected 18 and 12 hours before perfusion fixation. With this technique, serum concentration of BrdU has been shown to be constant, with a concentration of at least 8 mmol/ml.

Before excision the arteries were perfused in situ with 500 ml of 0.1 M cacodylate-buffered 2% paraformaldehyde solution at a pressure of 60–80 mm Hg via a catheter inserted into the left ventricle. The excised vessels were embedded in paraffin and prepared for histological and immunohistological examination. The embedded vessels were cut into cross sections beginning at the distal end of the dilated region until maximal plaque size in the dilated area was reached. In the area of balloon dilatation, semithin cross sections were assessed and used for histological and immunohistological analysis.

The incorporation of BrdU occurring during the 18 hours of the labeling period in the DNA of replicating cells allowed determination and quantification of SMC proliferation in the dilated arterial segment. A monoclonal antibody against BrdU (Bio Cell Consulting, Grellingen, Switzerland) was used to identify these cells. Immunohistological detection of BrdU-labeled cells was performed in semithin cross sections with the avidin-biotin method and combined staining with hemaluma.

To identify proliferated cells in this area as SMCs, we performed immunohistological staining (fluorescein isothiocyanate–labeled immunofluorescence [Sigma GmbH] and avidin-biotin method) by using a monoclonal antibody against α-actin (Renner,
Dannstadt, FRG). It has been shown that this antibody is α-actin smooth muscle specific.30 Arterial SMCs express predominantly the α-actin isoform of smooth muscle.31–33 Thus, detection of α-actin in the proliferated segment allows differentiation of SMCs from other cells.

To develop a valid method for quantification of SMC proliferation, all cells in the intima and media were counted separately in cross sections of 18 representative vessels; three sections with approximately the same plaque size (10, 20, 30, 40, and 50 cell layers) and normal vessels were included in each of these six groups.

A calibration curve was calculated (Figure 1) and used as a reference to determine the total number of intimal and medial smooth muscle cells in each cross section. For quantification of SMC proliferation after angioplasty, all BrdU-labeled cells were counted separately in the intima and media of four subsequent cross sections in the region of the maximal plaque size. The percent of DNA synthesis in SMCs in the media and intima was determined as the relation between BrdU-labeled cells and the total cell number.

Estimation of the small intestine mucosa–labeling index was used in each animal to control the incorporation of BrdU in replicating cells. In all animals DNA synthesis was found in approximately 30% of all cells in the small intestine mucosa.

In addition to the immunohistological staining of BrdU and α-actin, the embedded sections of the dilated region were stained with hemalaune and eosin. The extent of intimal cell layers was determined by counting the number of cell nuclei on the perpendicular line between endothelium and lamina elastica interna at the area of maximal plaque size.

**Statistical Evaluation**

Results are expressed as mean±SD. The statistical significance of differences between normal and dilated carotid arteries was determined with unpaired Student's t test. Analysis of variance was used to determine the significance of differences comparing dilated arteries. Differences were considered significant if p<0.05.34

**Results**

**Morphological Results**

After 28 days of electrical stimulation, microscopic examination has shown a plaque located beneath the anode of the electrode in control animals (Figure 2). The endothelial lining was intact, and quantification of intimal SMC layers in the region of maximal plaque size displayed a mean of 12.7±4.8 cell layers in the control vessels. Figure 3 shows a progressive and continuous increase of intimal thickening in all dilated arteries from 12.7±4.8 cell layers of the electrically stimulated control group to 32.6±13.8 cell layers at 4 weeks after angioplasty (p<0.05, t test). However, later than 4 weeks after angioplasty no additional increase of the number of intimal SMC layers occurred.

Histological examination revealed a stenosis of more than 50% of lumen diameter caused by an intimal hyperplasia in three of 35 animals (Table 1). In these cases an excessive proliferation of SMCs resulted in a neointima with a maximum of 50, 52, and 54 cell layers (Figure 4). Focal intimal splitting or medial disruption was not found in these segments. In five other animals, a complete thrombotic occlusion was found. The mural thrombi observed 7 days and later after angioplasty showed evidence of fibromuscular organization, and all of these thrombi were partially recanalized. Medial disruption was seen in two other animals, and both were associated with mural thrombi smaller than 50% of lumen diameter.

In four dilated arteries, a focal splitting of the intima with shearing off of the endothelial layer was observed.

**Quantification of Smooth Muscle Cell Proliferation After Labeling With Bromodeoxyuridine**

In control animals without angioplasty, quantification of intimal and medial SMC proliferation showed no statistical differences between the normal (intima, 0.9±0.5%; media, 0.4±0.3%) and the sham-operated group (intima, 0.7±0.6%; media, 0.3±0.2%). For
statistical evaluation, both groups were summarized to one group (intima, 0.8±0.6%; media, 0.3±0.3%).

A total of 30 dilated arteries was used for determination of SMC proliferation. Animals with evidence of mural thrombi were excluded from SMC analysis.

Quantification of intimal SMC proliferation revealed a significant increase in the number of cells undergoing DNA synthesis at 3 (9.6±4.6%) and 7 (8.4±4.4%) days after angioplasty (p<0.01) and a decrease of proliferation between 7 and 14 days after intervention (Figures 5 and 6). Analysis of variance revealed a significant decrease of DNA synthesis between 7 and 14 days after angioplasty (p<0.01).

In contrast to the intimal SMC proliferation, medial SMC proliferation was moderate and had a small but significant increase of cells undergoing DNA synthesis at 21 days (p<0.05) after angioplasty (Figure 5).

Twenty-eight days after dilatation the extent of SMCs undergoing DNA synthesis in the media and intima was normalized and reached the values of preintervention measurement. All data regarding the quantification of SMC proliferation and morphological changes are summarized in Table 1.

**Discussion**

The early and late morphological changes after transluminal angioplasty have been reported in several experimental and postmortem studies, but little is known about the dynamic process of cellular proliferation and its implication for the development of restenosis after PTCA.20–22,35,36

**Animal Model**

We produced an atheroma in the rabbit carotid artery before angioplasty by repeated local electrical stimulations.24 The electrostimulation method has the distinct advantage to induce atherosclerotic plaques by maintaining the integrity of the endothelial lining in the stimulated area.37 In addition, this method allows the development of local intimal fibromuscular atheromata under standardized conditions. The mechanism by which chronically applied electrical impulses induce migration and proliferation of SMCs is complex.38–40

The size of plaque has been shown to depend on the duration of electrical stimulation. During the first 2 weeks of electrostimulation, plaques grow considerably faster than in the next 2 weeks. After 28 days
of electrical stimulation, plaque size has reached its maximum.  

However, endothelial permeability to macromolecules is enhanced during the stimulation period and occurs predominantly beneath the anode of the electrode.  

Kling et al demonstrated leukocyte and monocyte migration through the endothelial lining into the subendothelial space during the early stage of plaque development with the identical method.  

Electrostimulation of carotid arteries probably initiates the atherosclerotic process by an increase of endothelial permeability and activation of leukocytes, macrophages, and SMCs. In rabbits fed a normal diet, electrostimulation results in an intimal fibromuscular plaque. In animals fed an additional cholesterol-enriched diet, the plaque becomes a typical fibromuscular atheroma during the 28 days of electrostimulation.  

By this method, however, it is impossible to induce a total or subtotal stenosis. Thus, in comparison to the situation in patients after angioplasty, balloon dilatation of the atheromata in our study might result in a lesser degree of arterial wall damage. This could possibly result in a reduction of SMC migration and proliferation in the postangioplasty phase.  

However, it is difficult to find an appropriate animal model for experimental angioplasty. Rabbits, pigs, and dogs have been used in several studies, but atherosclerosis does not naturally develop in these animals as it does in humans. A high-cholesterol diet (2%), important for the production of an atheroma after local endothelial denudation with a balloon, induces very high serum levels of cholesterol and is associated with a larger number of foam cells in the neointima.  

Endothelial denudation by balloon injury, however, leads to a damage of the natural endothelial layer, and it might be a problem for differentiation between additional effects of angioplasty from the primary injury.  

Quantification of Smooth Muscle Cell Proliferation by Bromodeoxyuridine Labeling  

The percentage of replicating SMCs after angioplasty was determined using BrdU to analyze the time course of the DNA synthesis in the dilated arterial segment. Both [3H]thymidine and BrdU are capable of producing toxic effects and can possibly influence cell proliferation in experimental studies. To minimize cytotoxic effects, BrdU labeling was limited in our study to a time interval of 18 hours before excision of the vessels. Quantification of SMC proliferation in the sham-operated and control animals without angioplasty showed no statistical difference, demonstrating that the increased DNA synthesis of SMCs after angioplasty was due to the effect of balloon treatment only. Furthermore, our results demonstrate that intimal SMC proliferation is a

![Figure 3](http://circres.ahajournals.org/)

**FIGURE 3.** Quantification of intimal cell layers (SMCs) after transluminal angioplasty in 30 dilated carotid arteries after immunohistological staining of α-actin. Values are expressed as mean±SD from five animals in each experimental group and 10 control animals.

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**Table 1. Morphological and Immunohistological Examination**

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Animals (No.)</th>
<th>Restenosis by SMC proliferation (No./total)</th>
<th>Mural thrombus (No./total)</th>
<th>Intimal SMC layers* (No.)</th>
<th>Intimal BrdU-labeled cells* (%)</th>
<th>Medial BrdU-labeled cells* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>28 ES (control)</td>
<td>10</td>
<td>0/10</td>
<td>0/10</td>
<td>13±5</td>
<td>0.8±0.6</td>
<td>0.3±0.3</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>0/5</td>
<td>0/5</td>
<td>16±9</td>
<td>9.6±4.6†</td>
<td>1.3±1.8</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td>0/6</td>
<td>1/6</td>
<td>19±9</td>
<td>8.4±4.4†</td>
<td>2.1±2.1</td>
</tr>
<tr>
<td>14</td>
<td>6</td>
<td>0/6</td>
<td>1/6</td>
<td>23±9</td>
<td>1.2±0.5</td>
<td>1.1±1.0</td>
</tr>
<tr>
<td>21</td>
<td>7</td>
<td>1/7</td>
<td>2/7</td>
<td>25±18</td>
<td>1.1±0.5</td>
<td>2.1±1.6‡</td>
</tr>
<tr>
<td>28</td>
<td>6</td>
<td>1/6</td>
<td>1/6</td>
<td>33±14‡</td>
<td>0.3±0.2</td>
<td>0.5±0.4</td>
</tr>
<tr>
<td>42</td>
<td>5</td>
<td>1/5</td>
<td>0/5</td>
<td>29±14‡</td>
<td>0.2±0.1</td>
<td>0.3±0.2</td>
</tr>
</tbody>
</table>

SMC, smooth muscle cell; BrdU, bromodeoxyuridine; ES, electrical stimulation.

*Mean±SD.

†Statistically significant to control group (p<0.01, t test).

‡Statistically significant to control group (p<0.05, t test).
dynamic process with a peak value of DNA synthesis during the first 7 days after balloon angioplasty.

The high number of proliferating cells early after dilatation results in a continuous increase of intimal cell layers. The number of SMCs undergoing DNA synthesis, however, already decreases between 7 and 14 days after angioplasty. The phenomenon of increasing cell layers during 4 weeks after angioplasty in spite of a decreasing DNA synthesis is explained by proliferation of a large number of cells in the early stage. The cells proliferate further at a lower level until cell division of SMCs approaches baseline level between 21 and 28 days after interventional treatment. In comparison to the intimal proliferation, medial SMCs present an increase of DNA synthesis up to 21 days after angioplasty. This prolonged increase of medial SMC proliferation can be explained in part by direct arterial injury of the medial layer induced by the intervention. Additionally, substitution of previously migrated SMCs into the intimal layer might lead to this increased medial SMC proliferation during a period of 21 days.

Determination of intimal and medial SMC proliferation with thymidine analogue substances after arterial balloon injury has been previously demonstrated in rats only.\textsuperscript{36,46} Clowes and Clowes\textsuperscript{46} found

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{Time course of intimal and medial smooth muscle cells undergoing DNA synthesis after angioplasty. Results of 30 dilated arteries after labeling with bromodeoxyuridine are shown. Values are expressed as mean±SD from five animals in each dilated group and 10 control animals.}
\end{figure}
maximal SMC thymidine indexes in the media at 4 days and in the intima at 7 days after balloon injury. The model of balloon injury by an inflated balloon catheter in normal arteries results in complete denudation of the endothelium. This might lead to activation of medial SMCs with migration and proliferation into the intimal layer with a prolonged increase of proliferation. By electrical stimulation, balloon angioplasty of a preexisting intimal plaque was performed in our experimental setting. Thus, intimal SMCs, which already proliferated during plaque development, start with a second proliferation as a response to the balloon dilatation before medial SMCs were activated. This time-dependent reaction of SMCs caused by the injury induced by balloon dilatation appears to be uniform and was observed in all dilated arteries.

Our findings are also in agreement with several autopsy reports, which have described an intimal hyperplasia in all patients whether or not restenosis occurred after PTCA. If increased proliferation of SMCs results in a moderate intimal hyperplasia only, or in excessive hyperplasia causing a hemodynamic significant restenosis may be due to activation of macrophages and platelets, expression of growth factors, and extent of the interventional injury. Additional unknown factors, for example, different subpopulations of SMCs existing in the arterial vessel wall, may be involved in these complex processes. In summary, our model of experimental angioplasty appears to be useful in determining the extent and time course of SMC proliferation and might allow further analysis of new interventional and pharmacological treatments to reduce the incidence of restenosis. Considering the high level of intimal SMCs undergoing DNA synthesis during the first 7 days after transluminal angioplasty and the prolonged increase of SMC proliferation in the media, our results could have important clinical implications in the prevention of restenosis after PTCA.

With regard to the development of a pharmacological treatment, it appears important and necessary to reduce migration and proliferation of SMCs with antiproliferative drugs in the early stage, after successful transluminal angioplasty.

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**KEY WORDS** • smooth muscle cell • bromodeoxyuridine • angioplasty • atherosclerosis • mitosis
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