Morphological Changes and Smooth Muscle Cell Proliferation After Experimental Excimer Laser Treatment

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**Background.** Little is known about the mechanism(s) in the development of restenosis after excimer laser angioplasty. Thus, the rationale of this study was to determine the time course of intimal and medial smooth muscle cell (SMC) proliferation and histomorphological changes after experimental excimer laser treatment.

**Methods and Results.** Laser ablation was performed in the right carotid artery of 34 New Zealand White rabbits after development of a fibromuscular plaque by repeated weak electrical stimulations. The vessels were excised 3, 7, 14, 21, 28, and 42 days after excimer laser treatment. Staining of α-actin was used to identify SMCs. In five rabbits (15%), a stenosis of more than 50% of luminal area was due to intimal proliferation of SMCs, and in four other rabbits, a total occlusion was due to organized thrombi. After the initial ablation of the preformed plaque (13±6 intimal SMC layers) a continuous increase of intimal wall thickness was found from 7±6 SMC layers at 7 days to 28±5 intimal SMC layers at 28 days after excimer laser ablation (p<0.01). After 42 days, no additional increase of intimal thickening occurred. After bromodeoxyuridine labeling, the extent of cell proliferation (percent of cells undergoing DNA synthesis) in the intima and media was determined using a monoclonal antibody against bromodeoxyuridine. Immunohistological quantification of SMC proliferation in the intima revealed a significant increase of cells undergoing DNA synthesis at 3 (p<0.05) and 14 (p<0.01) days after laser treatment. Medial proliferation of SMCs was delayed and had a significant increase 7 days (p<0.05) after intervention. Twenty-one days after laser treatment, SMC proliferation in the intima as well as in the media was normalized.

**Conclusions.** The proliferative response of SMCs after experimental excimer laser treatment will occur as a dynamic process with a maximum of SMCs undergoing DNA synthesis during 14 days after laser ablation, resulting in an increase of intimal thickening within 4 weeks after laser treatment. The extent of intimal hyperplasia due to SMC proliferation after excimer laser treatment is comparable with the effect of transluminal balloon angioplasty in this experimental model. *(Circulation 1991;83:1380–1389)*

A major limiting factor of balloon angioplasty is the high incidence of restenosis, which occurs in approximately 30% of cases.1–3

To improve the long-term efficacy for treatment of coronary artery disease, lasers for removal of atherosclerotic artery obstructions have been suggested as an alternative method.4–6

However, application of thermal lasers (i.e., argon, carbon dioxide, or neodymium:yttrium aluminum garnet [YAG]) is limited because of coagulation, necrosis of vascular tissue, perforation, and thrombosis.7–9 In contrast, experimental in vitro and in vivo studies10,11 have demonstrated that pulsed 308-nm XeCl excimer lasers can perform efficient tissue microablation with only minimal thermal injury of adjacent tissue. The ablative mechanism of excimer lasers is supposed to be based on “ablative photodecomposition” of tissue.12

Initial clinical results13–16 suggest that excimer laser atherectomy may become an alternative treatment for patients with coronary artery disease. However, little is known about the occurrence and inci-
ence of restenosis after excimer laser ablation. Thus, the rationale of this experimental study was to determine the temporal sequence of morphological changes after excimer laser ablation in atherosclerotic rabbit carotid arteries. In addition, the extent of vascular smooth muscle cell (SMC) proliferation was determined at different time intervals after laser treatment by bromodeoxyuridine labeling.

**Methods**

**Animal Model**

Fibromuscular plaques were produced before laser ablation by using the electrostimulation method as described by Betz and Schlote. For implantation of two graphite-coated gold electrodes at the carotid artery, 44 male New Zealand White rabbits were anesthetized with intramuscular injections of 8 mg metomidate HCl and 0.1 mg fentanyl base/kg body wt. After preparation of the right carotid artery, the electrodes were diametrically attached to the adventitia and held in position by an 8-mm-long Teflon cuff. Thin, subcutaneously placed leads were connected to a small plastic socket fixed at the skull. Connecting an external stimulation unit to the socket, constant direct-current impulses (15 msec/impulse, 0.1 mA, 10 Hz) were transmurally applied twice daily in the carotid artery of each rabbit for 30 minutes in the morning and for 15 minutes in the afternoon, with a time interval of 8–10 hours between the stimulation cycles. This protocol was carried out for 28 days. To produce atheromatous plaques by electrical stimulation, the rabbits were additionally fed with a commercially available 0.5% cholesterol diet (Altromin, Lage, FRG) during the stimulation period.

**Laser and Catheter**

A commercial XeCl excimer laser (MAX-10, Technolas Inc., Munich) emitting light at a wavelength of 308 nm with a pulse duration of 60 nsec was used. The laser was operated at 20 Hz. The laser beam was focused into a catheter device consisting of 20 quartz fibers of 100-μm core diameter each. The transmitted energy densities were measured before and after treatment with a conventional power meter and were considered to be above the ablation threshold at 40 mJ/mm².

The fiberoptic quartz fibers were arranged concentrically around a central lumen suitable for a 0.014-in. guide wire and fixed at the proximal and distal end to ensure a maximal catheter shaft flexibility. The cross-sectional diameter of the catheter device was 1.3 mm.

**Study Protocol**

After 28 days of electrical stimulation, 39 rabbits were anesthetized, the right carotid artery was prepared, and the exposed vessel was ligated by two clamps. Transversal arteriotomy was subsequently performed between both clamps. The carotid artery and the exposed lumen surface were kept moist with 0.9% NaCl solution during the whole procedure.

The laser catheter was inserted in the lumen of the artery under microscopic control. After removal of the cranial clamp, the catheter was manually advanced to the region of plaque. Excimer laser energy was applied while the catheter tip was slowly (4 mm in 15 seconds) advanced into the region of plaque, beginning 4 mm from the caudal end and terminating 4 mm from the cranial end of the implanted electrodes (Figure 1). Additional ablation was performed during withdrawal (16 mm in 20 seconds) of the catheter. After a second advance and withdrawal (each performed with a speed of 16 mm in 20 seconds) by continuous energy delivery, the laser catheter was removed. The cranial artery clamp was subsequently replaced. To reestablish arterial blood flow, the arteriotomy was closed with a 7-0 polypropylene suture. To avoid bacterial infections, all rabbits were on antibiotic therapy during the following 3 days. To reduce acute mural thrombus formation, a bolus of 700 IU/kg body wt standard heparin was given subcutaneously at 2 hours before laser treatment. In addition, all rabbits received 900 IU/kg body wt s.c. heparin twice daily for 72 hours after laser ablation. Cholesterol-containing diet was not prolonged, and all rabbits received a standard diet (Altromin).
To study the temporal sequence of morphological changes, the rabbits were killed 3, 7, 14, 21, 28, and 42 days after excimer laser treatment.

Ten control rabbits were separated into two groups of five rabbits each. Five rabbits that were electrically stimulated for 28 days without application of laser energy served as the control group. Five other rabbits were used as sham-operated controls to assess a possible mechanical injury derived from the catheter during the procedure of laser treatment. The rabbits in the sham-operated group, which had also received an electrostimulation period of 28 days, underwent arteriotomy and insertion of the catheter without application of laser energy. All of these rabbits were killed 7 days after the procedure.

**Tissue Analysis**

*Application of bromodeoxyuridine.* Bromodeoxyuridine (BrdU) labeling was performed in all rabbits to determine the extent of SMCs undergoing DNA synthesis at different time intervals after laser treatment. As described previously, 100 mg BrdU/kg body wt and 75 mg deoxycytidine/kg body wt were given as a subcutaneous neck depot 18 hours before the rabbits were killed. In addition to this neck depot, intramuscular injections (30 mg BrdU/kg body wt and 25 mg deoxycytidine/kg body wt) were given 18 and 12 hours before perfusion fixation.

*Perfusion fixation.* After application of an overdose of metomidate HCl and fentanyl base, a thoracotomy was performed. The carotid arteries were fixed in situ with perfusion of 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 immersion-fixed in 2% cacodylate-buffered paraformaldehyde for at least 6 hours and not fixed in distension.

*Histological examination.* The arterial segments below the Teflon cuff were used for histological analysis. The excised samples were embedded in paraffin and prepared for histological and immunohistological examination.

The embedded arterial segments were cut into series beginning at the caudal end of the laser-treated region to the maximal extent of plaque. The 4-μm-thick cross sections were then used for histological and immunohistological analysis.

Standard hemalune, hematoxylin, and eosin stains were prepared; stained sections were evaluated with respect to laser-specific alterations like vacuolization, carbonization, and intimal fissuring. Local reduction of SMC nuclei in the media was qualitatively determined. The extent of cell reduction was graded in three categories: minimal, less than 50% of medial cross-sectional area, and more than 50% of medial cross-sectional area. The extent of intimal cell layers was determined by counting the number of cell nuclei on the perpendicular line between endothelium and internal elastic membrane at the area of maximal plaque size.

Rabbits with evidence of thrombus formation were excluded from this analysis. The technique of immunohistological examination was previously described and is based on staining (avidin–biotin method) with a monoclonal antibody against BrdU (Bio Cell Consulting, Grellingen, Switzerland). This technique allows identification of all proliferated cells. In addition, immunohistochemical detection (fluorescein isothiocyanate–labeled immunofluorescence [Sigma GmbH, Deisenhofen, FRG] and avidin-biotin method) of the α-actin isoform of smooth muscle (monoclonal antibody from Renner, Darmstadt, FRG) was performed to identify the neointimal proliferated cells as SMCs. All BrdU-stained samples were morphometrically analyzed by the following method: The histological sections were projected onto a digital image analyzer (Olympus, Hamburg, FRG; software from Bilaney Consulting, Düsseldorf, FRG); the area of intima, media, and residual lumen was calculated (demarcated by the elastic internal and external lamina), and the extent of stenosis was determined:

\[
\text{% stenosis} = \frac{\text{intimal area} \times 100}{\text{intimal area} + \text{lumen}} \%
\]

All BrdU-positive cells and the total number of cells were separately counted in the intimal and medial cross-sectional area in two histological cross sections of each rabbit. The proliferative response of SMCs after laser treatment was calculated as the relation of BrdU-positive cells to the total cell number expressed in percent for both layers separately.

**Statistical Evaluation**

All values are expressed as mean±SD. The statistical significance of differences between control and laser-treated arteries was determined using two-tailed Student's *t* test. Differences were considered significant at *p*<0.05.

**Results**

**Histology**

In the control rabbits, histological examination after 4 weeks of electrical stimulation evidenced a fibromuscular intimal plaque due to SMC proliferation. Extent of the intima was 13±6 cell layers (Figure 2). In the sham-operated control group, intimal proliferation of SMCs resulted in a fibromuscular plaque consisting of 17±7 cell layers. The difference between both groups was not statistically significant.

Determination of the maximum number of intimal SMC layers showed an intimal wall thickness of 11±7 cell layers at 3 days and 7±6 cell layers at 7 days after laser treatment. After 7 days, a continuous increase of intimal SMC layers was found during the following 3 weeks. After 28 days, mean intimal wall thickness was 28±5 cell layers and significantly different from the control group without laser ablation (*p*<0.01). Forty-two days after treatment, however, no addi-
Histological cross section of a rabbit carotid artery after 28 days of electrical stimulation. Intimal proliferation of smooth muscle cells resulted in a fibromuscular plaque. Magnification, ×70; hemalaune combined with immunohistological staining of α-actin (avidin–biotin).

Morphometric analysis of all cross sections confirmed the histomorphological results. The mean percentage of stenosis decreased from 30±9% in the control group to 19±9% at 7 days after excimer laser atherectomy. Corresponding to the continuous increase of intimal SMC layers, the percentage of stenosis at 28 days after laser intervention was 45±18% (Table 1). However, these differences were not statistically significant.

A stenosis of more than 50% luminal reduction was found in nine of 34 laser-treated arteries (26%). In four rabbits, a total occlusion was found, which was due to mural thrombus formation. In four of the nine rabbits, the lesion was due to intimal proliferation of SMCs (Figure 4), and in one artery, a partial fibromuscular organized thrombus and additional considerable intimal SMC proliferation were found. In addition, mural thrombus formation not resulting in a stenosis greater than 50% was found in two arteries.

Evidence of vacuoles in the intimal layer was observed in 10 vessels. No vessel perforation occurred, but tissue ablation into the medial layer was found in 12 arteries. In 10 of these arteries, ablation was associated with a local reduction of SMCs in the media (Figure 5). In the remaining two rabbits with ablation of the medial tissue, this phenomenon was not observed (Table 1). However, evidence of carbonization and intimal fissuring was not found in any artery.

Quantification of Cell Proliferation After Excimer Laser Treatment

In control rabbits, determination of cells undergoing DNA synthesis by BrdU labeling displayed an extent of 0.9±0.5% in the intima and of 0.4±0.3% in the media
TABLE 1. Histomorphological and Immunohistological Findings After Excimer Laser Treatment in Rabbits

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<th>Stenosis (Intimal (%))</th>
<th>Cells undergoing DNA synthesis</th>
<th>Ablation into the media</th>
<th>Medial SMC nuclei reduction</th>
<th>Vacuoles</th>
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Calculation of cells undergoing DNA synthesis in the sham-operated rabbits showed no statistically significant differences in SMC proliferation in the intimal and medial layer.

As shown in Figure 6, quantification of intimal SMC proliferation revealed a significant increase during the first 14 days after excimer laser ablation (3 days: 2.9±1.7%, p<0.05; 7 days: 5.9±6.1%, p<0.25 [not significant]; 14 days: 5.6±2.3%, p<0.01). Twenty-one days after laser treatment, the extent of cells undergoing DNA synthesis was decreased and comparable with the control and sham-operated group (Figure 7). In contrast to intimal proliferation, medial proliferation only moderately increased and had a small but significant increase at 7 days (p<0.05) after intervention. Twenty-eight days after laser treatment, the extent of cells that have synthesized DNA in both layers was normalized.

**Discussion**

**Laser-Specific Effects and Vessel Wall Injury**

Several in vitro studies have demonstrated that with the energy emitted from a pulsed 308-nm excimer laser, precise and efficient ablation of atherosclerotic plaque with only minimal thermal injury of the adjacent tissue can be performed.10,12,28

In contrast to the acute mechanisms, only limited information is available about the chronic effects after excimer laser ablation in vivo.14

In a previous study, Prevosti et al29 reported fibrointimal ingrowth of craters induced by excimer laser ablation during 42 days in normal arterial vessels in dogs. The ablation of tissue in our study resulted in an initial decrease of intimal cell layers in the acute setting within the first 7 days after intervention, which, however, was not statistically significant for the total group. Interestingly, complete ablation of the intima and additional ablation and scarring of the medial layer, which occurred in 12 vessels, was associated with local reduction of cell nuclei in the adjacent tissue in the majority (10 of 12) of these arteries.

This phenomenon may be explained by local tissue necrosis, probably induced by either thermal injury or photoacoustic effects of excimer laser ablation.30 Similar effects, described as abnormal cell ablation, after mechanical injury, or enhanced uptake of stains in histological sections, were also observed after thermal laser angioplasty.29,31

**Time Course of SMC Proliferation and Incidence of Restenosis After Excimer Laser Ablation**

Our data demonstrate that proliferation of SMCs leads to a continuous increase of intimal wall thickness after excimer laser ablation. The increased activity of DNA synthesis of SMCs occurs as early as 3 days after intervention. As already shown for the induction of SMC proliferation after balloon angioplasty, expression of different growth factors including chemotactants,32–35 as well as activation of macrophages, leucocytes, and SMCs, may be involved in this process.36–38

Histomorphological determination of intimal plaque size in sham-operated and control rabbits did not show statistically significant differences, suggesting that the observed effects are indeed the result of application of excimer laser energy rather than pure mechanical vessel wall irritation. This is even more important, since we used the identical catheter device that was used in clinical trials in patients with coronary artery disease.13,14 Quantification of cells undergoing DNA synthesis revealed no significant difference between sham-operated and control rabbits, demonstrating that the increased SMC proliferation after excimer laser angioplasty was due to the effect of laser ablation only.

Experimental studies10,39 have documented that the uptake of thymidine analogue substances like [3H]thyminid or BrdU in SMCs proliferating after mechanical injury (balloon dilatation, endothelial denudation) displayed a maximum of DNA synthesis within 7 days after the initial injury. Thus, to exclude a possible

<table>
<thead>
<tr>
<th>Study group</th>
<th>Rabbit number</th>
<th>SMC layers</th>
<th>Stenosis (%)</th>
<th>Cells undergoing DNA synthesis</th>
<th>Ablation into the media</th>
<th>Medial SMC nuclei reduction</th>
<th>Vacuoles</th>
</tr>
</thead>
<tbody>
<tr>
<td>42 days after laser (n=5)</td>
<td>29</td>
<td>20</td>
<td>47.5</td>
<td>Intimal (%) 1.0</td>
<td>Medial (%) 0.8</td>
<td>(2/5)</td>
<td>(2/5)</td>
</tr>
<tr>
<td></td>
<td>34</td>
<td>25</td>
<td>66.0</td>
<td>0.2</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>29</td>
<td>29.7</td>
<td>0.2</td>
<td>0.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>18</td>
<td>17.0</td>
<td>0.7</td>
<td>0.5</td>
<td>+</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>+</td>
<td>II</td>
</tr>
<tr>
<td>Mean±SD</td>
<td>23±5§</td>
<td>41±21</td>
<td>0.5±0.4</td>
<td>0.5±0.2</td>
<td>(2/5)</td>
<td>(2/5)</td>
<td>(1/5)</td>
</tr>
</tbody>
</table>

SMC, smooth muscle cell; - , absence; +, presence; I, minimal; II, <50% of medial cross-sectional area; III, >50% of medial cross-sectional area.

*No bromodeoxyuridine labeling.
†Local mural thrombus formation (<50%).
‡Vessel occlusion by thrombus formation.
§p<0.05 compared with control group by t test.
¶p<0.01 compared with control group by t test.

...
Figure 4. Cross section of a laser-treated artery 28 days after excimer laser ablation. Upper panel: Intimal thickening with significant reduction of the cross-sectional lumen area (magnification, ×10; hematoxylin and eosin stain). Lower panel: Histological section of the same vessel with a higher magnification (×100) demonstrating that the stenosis is predominately due to intimal proliferation of smooth muscle cells (fluorescein isothiocyanate–labeled immunofluorescence of α-actin).
mechanical side effect on the proliferative response of SMCs, the number of cells undergoing DNA synthesis in the sham-operated group was determined at 7 days after sham intervention.

Furthermore, our results demonstrate that intimal and medial SMC proliferation is a dynamic process with a maximum of DNA synthesis during the first 14 days after excimer laser treatment. However, the variability in vessel wall reaction 7 days after laser treatment was considerable, resulting in a large standard deviation in this group.

In a recent study, determination of intimal SMC proliferation using BrdU labeling after conventional balloon angioplasty in the same rabbit model demonstrated a maximal proliferation of intimal SMCs at 7 days after dilatation. In comparison with balloon angioplasty, the proliferative response of intimal SMCs after excimer laser ablation is delayed. The maximal rate of SMC proliferation after excimer laser ablation appears to be reduced to approximately 50%, as observed after balloon angioplasty. However, SMC proliferation after excimer laser ablation was prolonged up to 14 days after intervention. This resulted in a continuous increase of intimal thickening during 28 days. Thus, the absolute extent of intimal cell layers at 28 days after excimer laser treatment is comparable with the increase of intimal cell layers after balloon angioplasty within the same period. Incidence and morphology of stenosis (>50%) due to intimal SMC proliferation is comparable in both studies.

These results strongly support the concept of intimal hyperplasia due to proliferation of SMCs of the medial and intimal layer as a uniform process after vessel wall injury.

**Limitations of the Method**

It is extremely difficult to find an appropriate animal model of advanced atherosclerosis; therefore,
all experimental studies are limited and cannot be transferred to the situation in humans. The advantage of the model of electrical stimulation in producing atherosclerotic lesions is the induction of fibromuscular plaques of comparable size under standardized conditions and by maintenance of the integrity of the endothelial layer. This fibromuscular plaque might be comparable with the early stage of atherosclerotic lesions in humans.

This model, however, is limited since it is impossible to induce a high degree of stenosis or even subtotal lesion in rabbit carotid arteries.

Study Implications

In the present in vivo study, excimer laser ablation of fibromuscular plaques resulted in only moderate laser-induced thermal vascular injury. Excimer laser ablation into the intimal and medial layer, however, resulted in proliferation of vascular SMCs. In addition, platelet deposition with mural thrombus formation in areas of laser treatment was observed.

The extent of intimal hyperplasia due to SMC proliferation after excimer laser treatment is comparable with the effect of transluminal balloon angioplasty in this experimental model.

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Key Words • excimer laser angioplasty • bromodeoxyuridine labeling • smooth muscle cells
Morphological changes and smooth muscle cell proliferation after experimental excimer laser treatment.

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