Effect of Short Pulsed Nonablative Infrared Laser Irradiation on Vascular Cells In Vitro and Neointimal Hyperplasia in a Rabbit Balloon Injury Model

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Background—Neointimal hyperplasia after PTCA is an important component of restenosis.

Methods and Results— Cultures of rabbit endothelial cells and smooth muscle cells (SMCs) were irradiated with different doses of nonablative infrared (1064-nm) radiation. Normalized viability index detected with nondestructive Alamar Blue assay and direct cell count were studied. Our experiments demonstrated dose-dependent cytostatic or cytotoxic effects of laser irradiation. We also evaluated the long-term effect of endoluminal nonablative infrared laser irradiation on neointimal hyperplasia in a rabbit balloon injury model. PTCA of both iliac arteries of 23 New Zealand White rabbits was performed. One iliac artery was subjected to intra-arterial subablative infrared irradiation via a diffuse tip fiber. The contralateral vessel served as control. The diet was supplemented with 0.25% cholesterol and 2% peanut oil for 10 days before and 60 days after PTCA. Morphometry after 60 days showed that intimal areas were 0.76±0.18 and 1.85±0.30 mm² in the laser and control arteries, respectively (P=2.2×10⁻¹¹).

Conclusions—We conclude that nonablative infrared laser inhibited neointimal hyperplasia after PTCA in cholesterol-fed rabbits for up to 60 days. (Circulation. 2001;104:1850-1855.)

Key Words: lasers ■ angioplasty ■ restenosis ■ muscle, smooth ■ cells

One of the major problems in interventional cardiology is the prevention of restenosis after percutaneous coronary interventions.¹ ² Balloon dilatation and/or stent implantation initiates complex molecular reactions that result in intimal hyperplasia, which alone after stenting or along with arterial remodeling in the case of PTCA leads to recurrent narrowing of the lumen within months after the procedure.¹ ² ³

In the past few years, vascular brachytherapy has gained increased popularity in the treatment of in-stent restenosis. A large number of experimental and clinical studies have demonstrated that endoluminal ionizing radiation reduces cellular proliferation and restenosis.⁴ ⁵ ⁶ ⁷ Edge effect, late thrombosis, and increased risk of myocardial infarction, however, represent significant limitations of this therapeutic modality.⁸ ⁹ Trials with radioactive stents also demonstrated that they delay rather then prevent neointimal hyperplasia.¹⁰

Most recently, local antiproliferative strategies, including pharmacological stent coatings (paclitaxel, rapamycin, etc), have demonstrated inhibition of smooth muscle cell (SMC) proliferation in vitro, reduced neointimal thickening in animal models of restenosis, and produced promising results in a pilot human study.¹¹ ¹² High local toxicity, however, may alter the endothelialization process after stent implantation and cause late complications.

Laser angioplasty, which initiated great enthusiasm decades ago, has failed to demonstrate good immediate and long-term results. A high rate of thrombosis and restenosis was noted, most likely due to thermal damage of the vascular wall.¹³ ¹⁴ ¹⁵

We hypothesized that short pulsed nonablative infrared laser irradiation (NIL) may inhibit the proliferation of SMCs in the absence of thermal tissue debulking and thereby reduce neointimal response to the arterial injury. This approach can be practical because the equipment for transmitting laser irradiation (fiberoptic catheters) to the arterial segment is both readily available and cost-effective compared with vascular brachytherapy.

The objective of this study was to evaluate the effect of NIL on viability and proliferation of endothelial cells (ECs) and SMCs in vitro and on neointimal hyperplasia in the rabbit balloon injury model.

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Methods

EC Isolation and Culture
For EC isolation and culture, rabbit aortic ECs were used. Cells were isolated according to established procedures, with minor modifications. Rabbit aortic ECs were harvested from freshly obtained aortas with a 0.2% solution of collagenase type 1A (Sigma Chemical Co). After removal from the luminal surface and centrifugation at 800 rpm for 5 minutes, the cells were rinsed twice with Ca\(^{2+}\)- and Mg\(^{2+}\)-free PBS, pooled, and resuspended in DMEM (HyClone Laboratories) supplemented with heat-inactivated 7.5% FCS (Gibco Life Technologies Inc) and 7.5% horse serum (Gibco), 2 mmol/L L-glutamine (Fisher Scientific), and a combination of Fungizone (Gibco) and penicillin/streptomycin (Fisher Scientific). After digestion with collagenase type 1A and centrifugation, the cells were rinsed with PBS. Cells were resuspended in medium 199 (HyClone Laboratories) supplemented with heat-inactivated 7.5% FCS and 7.5% human serum, 30 μg/mL EC growth supplement (Sigma Chemical Co), 50 U/mL heparin (Lyphomed Inc), 2 mmol/L L-glutamine, and antibiotics.

SMC Isolation and Culture
Rabbit aortic SMCs were isolated from freshly removed aortas that were stripped of fat and connective tissue, after which the endothelial layer was removed by careful stripping. The medial tissue was cut into 4×4-mm explants and plated on 60-mm Petri culture dishes. The explants were incubated in a standard air/CO\(_2\) tissue incubator with DMEM supplemented with 10% FCS and antibiotics. The isolated cells were routinely tested with an immunostaining procedure and passaged after treatment for 5 minutes with trypsin/EDTA solution. Cells between passages 4 and 7 were used for these studies.

Cell Quantification
The number of cells plated in each experiment was determined directly with a standard hemocytometer. After attachment, quantification of viable cells attached to the substrates was performed by Alamar Blue (AB) assay. AB was added to cell cultures at 1:10 vol/vol ratio and incubated for 3 hours at 37°C in the standard tissue culture incubator. Samples of media were divided into aliquots, and their fluorescence yield was obtained by measurement in a spectrophotofluorometer (model 650-10S, Hitachi) set at excitation and emission wavelengths of 560 and 590 nm. The resulting values were converted to cell numbers by use of a standard calibration curve.

Cell Viability Assay
The immediate effect of laser irradiation on vascular cells on polystyrene tissue culture substrate was measured into 96-well Dynatech pigmented plates. Various doses of irradiation were applied to ≥6 parallel wells in serum-containing medium. At the end of irradiation at 37°C, the wells were gently washed twice with the same warm medium to remove any nonviable unattached cells. The viable attached cells were counted by AB assay. Individual experiments were repeated ≥3 times. Normalized viability index was calculated as percentage of viable cells in experiments compared with 100% viability of control nonirradiated cells.

Cell Growth Evaluation
ECs were seeded onto 96-well Dynatech pigmented plate at an initial density of 10,000 cells/cm\(^2\) for evaluation of growth rate. After 24 hours, the number of cells was determined by AB assay, and EC and SMC were irradiated with various doses of infrared laser light. Each test dose was performed in 6 parallel wells. Quantification by AB assay was performed for 9 days, and culture medium was refreshed after each assay.

Laser Irradiation System
Short-pulse infrared (1064-nm) laser (Alien Technologies) was used in this study. Laser irradiation was transmitted from the laser to the cell cultures with 400-μm optical fibers. The laser was powered at 260 mJ, with pulse duration of 160 ns in all experiments. The cell cultures were irradiated with 5, 10, 20, and 30 pulses. During laser irradiation, the cells were kept in a standard air/CO\(_2\) incubator that was filled with humidified air containing CO\(_2\) before an irradiation session. The temperature of the incubator was kept at 37°C. The inner walls and bottom of the incubator were black anodized, so light reflections were minimized. No scattering of light to the site
of the other nontreated wells could be detected in these experiments. Control cells were placed in the field of laser irradiation with opaque covers for the same length of time as the cells receiving laser treatment.

The temperature of the cells was measured with a cromel/constantan thermocouple probe (Omega Engineering) with an outer diameter of 25.4 μm embedded into the cell culture. The thermocouple was connected to a model 450/AET, hand-held digital thermocouple thermometer (Omega Engineering) with a temperature range of −137°C to 205°C and sensitivity of ±0.1°C over the entire range. The accuracy of the probe was confirmed against the standard mercury-in-glass thermometer in a 37°C water bath.

No detectable heating was produced during irradiation experiments with pulse duration of 160 ns. After irradiation, cultures were incubated as previously indicated for each experiment.

In vitro observations provide critical-dose justification for long-term experiments to examine the influence of subablative laser on restenosis.

**Rabbit Balloon Injury Model**

Twenty-three adult New Zealand White rabbits were maintained on a diet supplemented with 0.25% cholesterol and 2% peanut oil for 10 days before and 60 days after treatment. Rabbits were fasted overnight before all surgical procedures. The marginal ear vein was cannulated with a 22-gauge angiocatheter and flushed with heparin to ensure patency. Anesthesia was induced with a mixture of ketamine (10 mg/kg) and xylazine (1 mg/kg). It was administered as a bolus injection via the marginal ear vein and then maintained with pentobarbital (12 mg/kg IV) given as a bolus injection to achieve the necessary effect. The abdomen and groin were shaved, and the skin was cleaned for surgery with Betadine. The groin was opened and the femoral artery carefully exposed.

Animals used in this study received humane care in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy

### Morphometric Analysis of the Harvested Arteries at 60 Days

<table>
<thead>
<tr>
<th></th>
<th>Control Laser</th>
<th>P</th>
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<tbody>
<tr>
<td><strong>Lumen, mm²</strong></td>
<td>0.49±0.09</td>
<td>1.92±0.21</td>
</tr>
<tr>
<td><strong>Neointima, mm²</strong></td>
<td>1.85±0.30</td>
<td>0.76±0.18</td>
</tr>
<tr>
<td><strong>EEL/EEL&lt;sub&gt;ref&lt;/sub&gt;</strong></td>
<td>1.09±0.08</td>
<td>1.41±0.08</td>
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<tr>
<td><strong>Injury score</strong></td>
<td>2.38±0.24</td>
<td>2.41±0.29</td>
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Data are mean area±SD.

Figure 3. Effect of 4 different doses of NIL on (A) EC and (B) SMC growth at 3, 6, and 9 days in culture after treatment: dose 1: 5 pulses; dose 2: 10 pulses; dose 3: 20 pulses; dose 4: 30 pulses. Laser power was 260 mJ per pulse, with duration of 160 ns. *P<0.05; **P<0.01.

Figure 4. Influence of NIL (dose 2) on EC and SMC proliferation. A, Control EC culture after 6 days. B, Irradiated EC culture at 6 days after treatment shows normal proliferation. C, Control SMC growth at day 6 in culture. D, Decreased SMC proliferation of irradiated SMCs 6 days after intervention.
purely hypercellular in nature. A through C, Movat stain. Intimal hyperplasia in this laser-treated segment is minimal and core in control vessel. F, Iliac artery with similar initial damage. D, Local treatment with endoluminal laser results and massive complicated plaque containing lipid- and foam cell–rich core. D, Local treatment with endoluminal laser results in fibrocellular hyperplasia without focal macrophage/foam cell accumulation in core. E, Iliac artery 60 days after balloon injury and local application of laser. Cross section shows representative fibrocellular intimal hyperplasia with focal macrophage/foam cell accumulation in core. IEL is less disrupted, and original tunica media is thinned, indicating less but comparable initial balloon injury at this site. C, Control segment shows concentric lesion with fibrocellular intimal hyperplasia and massive complicated plaque containing lipid- and foam cell–rich core. D, Local treatment with endoluminal laser results in minimal fibrocellular intimal hyperplasia with little variation in thickness. E, Severe lesion showing breaking of IEL and media, with extensive intimal hyperplasia and formation of lipid-rich core in control vessel. F, Iliac artery with similar initial damage. Intimal hyperplasia in this laser-treated segment is minimal and purely hypercellular in nature. A through C, Movat stain.

Figure 5. A, Iliac artery 60 days after balloon injury. Cross section shows representative fibrocellular intimal hyperplasia with focal macrophage/foam cell accumulation in core. IEL is partially disrupted, and original tunica media is thinned. B, Iliac artery 60 days after balloon injury and local application of laser. Cross section shows representative fibrocellular hyperplasia without significant foam cell accumulation. IEL is less disrupted than one in A, but tunica media is also thinned, indicating less but comparable initial balloon injury at this site. C, Control segment shows concentric lesion with fibrocellular intimal hyperplasia and massive complicated plaque containing lipid- and foam cell–rich core. D, Local treatment with endoluminal laser results in minimal fibrocellular intimal hyperplasia with little variation in thickness. E, Severe lesion showing breaking of IEL and media, with extensive intimal hyperplasia and formation of lipid-rich core in control vessel. F, Iliac artery with similar initial damage. Intimal hyperplasia in this laser-treated segment is minimal and purely hypercellular in nature. A through C, Movat stain.

of Sciences and published by the National Institutes of Health (NIH publication 85-23, revised 1985).

Experimental Protocol
All catheters were advanced with a 4F or 5F introducer sheath through a small incision in the femoral artery. The introducer catheter was continuously flushed (1 to 2 mL/min) with lactated Ringer’s solution. Immediately before the catheter was introduced, heparin (1000 IU) was administered via the introducer catheter as a bolus injection. Balloon dilatation of both iliac arteries was performed with a 3.0-mm balloon (1.3 to 1.4:1 balloon/artery ratio). The inflation protocol consisted of 2 cycles of inflation to 8.0 atm for 30 seconds each, followed by 1 cycle of inflation to 8.0 atm for 90 seconds.

Endoluminal Irradiation
After PTCA of both iliac arteries, arteries were randomly selected for control ( sham) or laser treatment. Dose 2 (10 pulses of 260 mJ, 160 ns) was applied because this dose was found earlier in vitro to be optimal to arrest SMC proliferation without altering EC growth.

Laser irradiation was performed with a diffuse-tip laser fiber advanced via the balloon catheter to the target area. The balloon was inflated to 2 atm to stabilize the fiber. During sham treatment, the laser source was not activated. Angiography was performed via an introducer catheter in all animals immediately before and after treatment and was then repeated via the aorta before the arteries were harvested for tissue processing. After treatment, the catheter was removed and the site of catheter introduction repaired with 1 stitch with a Prolene suture. Both groin incisions were closed, and the animals were allowed to recover for 60 days. Antibiotics were given as necessary.

Tissue Processing
After 60 days, the animals were anesthetized and heparinized. After the abdominal aorta was exposed, the animals were euthanized to ensure that blood was not delivered to the abdominal aorta. The aorta was then cannulated distal to the renal arteries. The femoral arteries and all side branches of the abdominal aorta were ligated. The entire vessel was then flushed with 100 mL PBS to remove blood from the lumen of the vessel. This was followed with 100 mL of 10% buffered formalin fixation at hydrostatic pressure (80 mm Hg) for 90 minutes. After fixation, the iliac arteries were dissected to reveal the treatment zone.

Tissue Staining
Each vessel was serially sectioned in 2-mm increments to produce nine 4-μm cross sections. The sections were embedded in paraffin and dehydrated, then stained for light microscopic analysis with (1) Movat’s pentachrome and (2) hematoxylin and eosin.

Morphometry
Histological cross sections of vessel segments were observed under a 20× objective of a light microscope. Cross sections were examined with a Nikon photomicroscope equipped with a JVC video camera. The camera was interfaced with a JVC color monitor and an IBM PC equipped with a video image analysis board (video Van Gough, Tecmar). The image on the monitor was digitalized with a customized software program to provide morphometric data, including intimal and luminal areas. The borders of the external elastic lamina (EEL), internal elastic lamina (IEL), neointima, and vessel lumen were traced on a digitizing board by 2 blinded observers, and the respective areas were calculated. Vessel remodeling was assessed by measuring the ratio (EELinj/EELref) of the EEL area at the site of maximal stenosis (EELinj) to the EEL area in the noninjured proximal segment (EELref). Animals were excluded if an occlusive thrombus was detected. Interobserver variability was < 5%. Vessel segments with maximal stenosis were selected for final analysis. The histomorphometric parameters measured on 4 to 8 sections per vessel were averaged and expressed as mean±SD.

Statistical Evaluation
In culture experiments, cell numbers were averaged for experiments performed under similar conditions and expressed as mean±SD. Comparison of means was performed with 2-way ANOVA tests. Morphometric measurements from control and laser-treated arteries were compared by 2-sample Student’s t tests. Data were expressed as mean±SD, and a value of P<0.05 was considered statistically significant.

Results

Effect on Cell Viability
We carried out a series of experiments in cell cultures of rabbit aortic ECs and SMCs using the AB assay. Experiments in tissue culture demonstrated that very low levels of laser irradiation were nontoxic for ECs and SMCs (Figure 1). We observed, however, that ECs were more resistant to infrared irradiation (Figures 1 and 2). A nontoxic effect was noted with higher power, although a higher dose of NIL was cytotoxic for both ECs and SMCs.
Effect on Cell Growth
In the next series of experiments, we investigated the effect of NIL irradiation on EC and SMC growth. The results are shown in Figure 3. Nonablative doses of NIL decreased SMC growth. In contrast, the experiments on ECs revealed that nontoxic doses of NIL did not decrease growth rate, and there was no difference in comparison with control cultures (Figures 3 and 4). Higher cytotoxic doses of NIL irradiation decreased ECs and SMC growth.

Effect on Neointimal Hyperplasia
One animal died during the procedure because of aortic perforation with a guidewire. In the remaining 22 rabbits, there were 2 more deaths at 6 and 11 days after the procedure from sepsis secondary to infected femoral cutdown. All other animals survived to 60 days. We observed 4 occlusive thrombi (3 in control arteries and 1 in a laser-treated vessel). In another 4 arteries (2 treatment and 2 control), the injury score was <1. All of these arteries were excluded from morphometric analysis.

Morphometry
Histological evaluation demonstrated slight medial necrosis, with the degrees of injury similar in both treatment groups. Morphometry showed that the luminal area was significantly greater in the arteries subjected to laser treatment (1.92±0.21 mm² in the treated arteries and 0.49±0.09 mm² in the control animals, \( P=9.1\times10^{-14} \)) (Table). Similar significant differences were demonstrated in the neointimal areas (0.76±0.18 and 1.85±0.30 mm² in laser and control arteries, respectively, \( P=2.2\times10^{-11} \)). The ratio EEL_{nl}/EEL_{ref} was greater in laser than control vessels (1.41±0.08 versus 1.09±0.08, \( P=9.4\times10^{-9} \)).

Discussion
This study demonstrates for the first time that NIL is capable of photobiomodulation of proliferative processes of vascular ECs and SMCs. In our experiments, SMCs were more sensitive to laser irradiation than ECs. We developed a therapeutic nontoxic dose of laser irradiation that does not affect EC growth but reduces SMC proliferation in the absence of heat. The main difference in comparison with ionizing radiation is its greater specificity to the vascular SMCs.

Several studies using low-level continuous-wave visible and infrared laser irradiation previously demonstrated inhibition or stimulation of cell growth, differentiation, motility, migration, and phagocytosis in vitro.\(^{18–29}\) Despite these observations, there is no universally accepted theory as to the mechanisms of laser modulation of cellular processes.

Detailed review of light-cell interactions is beyond the scope of this article. Briefly, it has been speculated that application of laser irradiation at a very low dose promotes the acceleration of electron transfer within some sections of the respiratory chain.\(^{26,28,30}\) Conversely, higher doses result in free-radical formation, resulting in disruption of templates for production of cell division regulatory proteins.\(^{26,28,31,32}\) Some recent studies demonstrated that mitochondria, and more specifically enzymes (eg, NADH B [nicotinamide adenine dinucleotide reduced form] and cytochromes), absorb laser radiation, which then varies the mitochondrial energy metabolism.\(^{30}\)
The present study also shows that endoluminal NIL has a significant effect on restenosis (Figures 5 and 6; Table).

In vivo endoluminal delivery of this laser irradiation reduces neointimal hyperplasia and possibly as an indirect effect positively affects the vascular remodeling in the rabbit balloon-injury model. It should be noted that the dose of laser irradiation used was very low and we did not observe any evidence of thermal damage to the vascular wall (Figure 5). There was no edge effect at distal zones of the treatment segments.

It appears that with endoluminal nonablative laser irradiation, it is possible to retain vascular patency by affecting SMC cycle progression and neointimal hyperplasia. SMCs at the same time remain viable without totally disabling the necessary healing process.

Relatively simple laser dosimetry and favorable influence on both intimal hyperplasia and arterial remodeling may represent an advantage over more destructive methods such as radiation or cytotoxic agents (taxol, actinomycin-D, rapamycin, etc).

The major effect of this mode of laser therapy seems to be control of hyperplasia and thus may provide an attractive option for the prevention of restenosis after stent implantation. Patients with hemodialysis access-shunt reocclusion are another potential target group. Moreover, we think that because of the close proximity of the arteriovenous shunt to the skin, a transcutaneous mode of delivery might be used.

Conclusions
In in vitro studies, we demonstrated that short pulsed NIL inhibits SMC proliferation in a dose-dependent fashion. When delivered endoluminally, NIL reduces neointimal hyperplasia in the rabbit injury model. This study, however, should not be regarded as definitive preclinical data, and further experiments in the porcine coronary model are necessary to further establish the efficacy of this therapy. Ultimately, these data may lead to the development of new methods for the prevention of restenosis.

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