Low-Energy Laser Irradiation Reduces Formation of Scar Tissue After Myocardial Infarction in Rats and Dogs

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Background—Low-energy laser irradiation (LELI) has been found to attenuate various biological processes in tissue culture and experimental animal models. The aim of the present study was to investigate the effect of LELI on the formation of scar tissue in experimentally induced chronic infarct in rats and dogs.

Methods and Results—Myocardial infarction (MI) was induced in 50 dogs and 26 rats by ligation of the left anterior descending coronary artery. After induction of MI, the laser-irradiated (LI) group received laser irradiation (infrared laser, 803-nm wavelength) epicardially. Control MI-induced non–laser irradiated (NLI) dogs were sham-operated, and laser was not applied. All dogs were euthanized at 5 to 6 weeks after MI. Infarct size was determined by TTC staining and histology. The laser treatment ($P<0.05$) lowered mortality significantly, from 30% to 6.5%, after induction of MI. The infarct size in the LI dogs was reduced significantly ($P<0.0001$) (52%) compared with NLI dogs. Histological observation of the infarct revealed a typical scar tissue in NLI dogs and cellularity in most of the LI dogs. Only 14.6 ± 3% of the mitochondria in the cardiomyocytes in the ischemic zone (4 hours after MI) of LI MI-induced rats were severely damaged, compared with 36 ± 1% in NLI rats. Accordingly, ATP content in that zone was 7.6-fold (significantly) higher in LI than in NLI rats.

Conclusions—Our observations indicate that epicardial LELI of rat and dog hearts after chronic MI caused a marked reduction in infarct size, probably due to a cardioprotective effect of the LELI. (Circulation. 2001;103:296-301.)

Key Words: ischemia ■ myocardial infarction ■ antioxidants ■ lasers

The approach to acute myocardial infarction (MI) has moved in the past decade from simple monitoring of coronary events to aggressive interventions in the processes underlying coronary thrombosis. Cardiac repair after infarct is a complex process involving diverse inflammatory components, extracellular matrix remodeling, and responses of the cardiomyocytes to the ischemia. The sequential events that take place in the myocardium after occlusion of the left anterior descending coronary artery (LAD) in experimental animals (including dogs) have been well documented. After necrosis of the cardiomyocytes and a rather long inflammatory phase, the ischemic zone is subsequently replaced by fibrotic tissue.

Many studies have been directed toward the use of drugs, growth factors, and various interventional technologies in reducing myocardial infarct size and improvement of heart function after MI in experimental animals and humans. For example, recombinant adenovirus–mediated transfer of genes encoding antioxidants to the myocardium has been demonstrated to attenuate after ischemic dysfunction in neonatal mice. Novel approaches to enhancing angiogenesis in the ischemic myocardium by introducing growth factors (mainly of the vascular endothelial growth factor family) were adapted and found to have a beneficial effect on patients with severe angina.

Low-energy laser irradiation (LELI) has been found to modulate various biological processes. Laser irradiation, for example, could cause an increase in mitochondrial respiration and ATP synthesis, accelerate wound healing, and promote the process of skeletal muscle regeneration after injury. Inflammatory response after injury was markedly decreased by laser irradiation, and neoformation of blood vessels in the injured zone of skeletal muscles was elevated. We recently showed that LELI induces synthesis of cell-cycle regulatory proteins in tissue cultures of satellite cells from skeletal muscle because of activation of early cell-cycle-regulatory genes. The effects of LELI on cardiac cells have been studied to a limited extent. Zhu et al recently showed that argon dye laser (660-nm wavelength) improves the functional recovery of cold-stored, isolated rat cardiomyo-
cytes. In the present study, we investigated the possibility that LELI may also attenuate the process of scar tissue formation after chronic occlusion of the LAD in rats and dogs.

Methods

Experimental Procedure and Laser Irradiation

The study comprised 50 dogs [26 mongrels, 1 to 2 years old (17 to 26 kg), and 24 beagles, 1 to 1.4 years old (15 to 19 kg)] and 26 Sprague Dawley rats, 8 to 10 weeks old. The Animal Use and Care Committee of the Faculty of Medicine of the Technion University approved the experimental protocol. The dogs were randomly assigned to the non–laser irradiated (NLI) and laser-irradiated (LI) groups. There were no statistical differences in infarct size or blood tests between the NLI and LI mongrel and beagle dog groups. Furthermore, the effect of LELI on infarct size was similar in the mongrel and the beagle dog groups (54% and 49% reduction, respectively). Anesthesia was induced with ketamine (10 mg/kg IV) and diazepam (1 mg/kg IV), and after intubation, dogs were maintained with isoflurane 1% and fentanyl (0.25 mg · kg⁻¹ · min⁻¹ IV). In all dogs, MI was induced by left thoracotomy followed by ligation of the LAD distal to the first diagonal branch. Blood samples from all dogs were collected before MI and then daily for 8 days after MI for determination of troponin T and catalase in the serum. The laser used for the irradiation was a diode (Ga-As) laser with a wavelength of 803 nm and power output of 400 mW (Lasotronic Inc) equipped with an aluminum-backed glass fiber optic. An infrared viewer (Lasotronic Inc) was used to trace the infrared irradiation on the myocardium, and a NOVA power-energy laser monitor (Ophir Optronics Ltd) was applied to measure the precise power density of the laser on the myocardium. Power density at the level of the myocardium was kept at 6 mW/cm², and laser irradiation was applied for 3 minutes (1.08 J/cm²). The above parameters of the LELI were chosen after a pilot study that was performed in dogs. The laser irradiation was applied as above at 4 to 6 locations to cover the total area at risk in the left ventricle (the pericardium was cut and folded laterally) and its vicinity through the thoracotomy. The first LELI was performed ~15 minutes after MI, and the second was applied 3 days after MI after thoracotomy, as in the first irradiation. The control MI-induced NLI dogs were sham-operated as above, but the laser was not turned on. All dogs were euthanized 5 to 6 weeks after MI except for 2 beagle dogs that were used for electron microscopy and were euthanized 3 weeks after MI. Control or LI dogs were randomly selected.

MI in 26 rats (8 to 10 weeks old, 250 to 300 g) was induced by chronic ligation of the LAD ~3 mm distal to where it branches off the aorta after thoracotomy. Laser irradiation was performed 10 to 15 minutes after MI through the intercostal muscles at the same power density (at the level of the myocardium) as in the dogs. All rats were euthanized 4 hours after MI. Twelve rats (6 LI and 6 NLI) were used for electron microscopy (see below), and 14 rats (8 LI and 6 NLI) served for ATP determination. Samples were taken from the ischemic zone in the left ventricle, homogenized in Tris 0.01 mol/L (pH 7.4)/2 mmol/L CaCl₂, buffer, and used for ATP determination.

Histology and Infarct Size Determination

The dogs received diazepam before euthanasia with an intravenous overdose of KCl containing heparin (400 IU/kg). The hearts were exteriorized and perfused with 500 mL of 2% triphenyltetrazolium chloride (TTC) solution in saline at 37°C at a flow rate of 50 mL/min. They were then perfused with 4% phosphate-buffered formalin as above, rinsed in formalin for 48 hours, and then transversely cut into 6-mm slices. Infarct size was determined by scanning of the slices and use of computerized morphometry software (Sigma Scan Pro 4.0, Jandel Scientific). The total volume of the infarct in each section was calculated as the volume of a trapezoid with upper and lower bases of the infarct area in each slice, multiplied by its height. The infarct size in each dog was defined as the sum of the volumes of all infarcts in all slices and was expressed as a percentage of the total volume of the left ventricle.

Five to 8 tissue samples bearing the infarcted area (endocardium to epicardium) were taken from each heart, and sections were prepared and stained with Masson’s trichrome to delineate fibrous tissue and immunohistochemistry.

Immunohistochemistry and Electron Microscopy

For desmin immunostaining, the peroxidase staining kit (No. 9543, Zymed Inc) was applied on paraffin serial sections of formaldehyde-fixed dog heart tissue. Sections were deparaffinized and hydrated in alcohol and incubated with peroxide 3% followed by blocking solution for 15 minutes. The sections were washed with PBS–Tween 20 solution and then incubated with mouse antidesmin (Biomakor) diluted 1:100 for 30 minutes at room temperature. Biotin–goat anti-mouse IgG was then applied for 30 minutes at room temperature after a washing as above. Horseradish peroxidase–streptavidin conjugate was then applied for 30 minutes, followed by incubation with chromogen solution for 10 minutes. Sections were counterstained with hematoxylin. Quantitative analysis of the percentage of positive desmin expression structures in the infarcted area and in the interphase between the infarcted and the noninfarcted area was determined by computerized morphometry and Sigma Scan Pro software. Data were statistically analyzed by nested ANOVA as described previously.²

Twelve rats and 2 dogs (1 LI and 1 NLI) were euthanized 4 hours and 3 weeks after MI, respectively. Random samples from the ischemic area were fixed in 3.5% glutaraldehyde in 0.2 mol/L sodium cacodylate buffer, postfixed in OsO₄, and embedded in epoxy resin. Thin sections from 4 different regions (randomly chosen) of each of the above samples (in rats) were cut, and photographs were taken from 6 fixed points (intersections of the grid bars) on each grid. The quantitative morphometric measurements of these photographs were performed by video imaging and Sigma Scan Pro 4.0 Software. The percentage (of total numbers) of damaged mitochondria (>50% elevation of volume relative to normal cristae and membranes ruptured) was recorded in each micrograph.

Analytical Procedures and Statistics

Blood samples were collected from the leg veins of each dog daily after MI. The troponin-T level in blood serum at each time point after MI was determined with the human immunoprecipitation kit (Boehringer-Mannheim). Troponin-T accumulation in the blood after MI was calculated from the area under the curve (arbitrary units) of the troponin-T quantitative data versus time. Catalase in serum was determined by oxygen electrodes with proper standards. One unit of catalase was defined as the amount of enzyme that produces 1 µmol of oxygen per minute. ATP was determined with the luciferin-luciferase assay kit (Sigma).

The SPSS software was used for statistical analysis. Tests were performed first for normality distribution, followed by parametric (Student’s t test) or nonparametric (exact Wilcoxon) tests. Fisher’s exact test (for mortality analysis) was also used.

Results

The LELI caused a highly significant (P<0.0001) reduction (52%) in infarct size in the LI dogs compared with control NLI dogs (Figure 1). The mortality rate after LAD occlusion was 6.5% in the LI dogs, which was significantly (P<0.05) lower than the mortality rate (30%) in the NLI dogs. Furthermore, mortality in the LI dogs generally occurred at longer time intervals after MI than in NLI dogs. Release of troponin T to blood was measured for 8 days after LAD occlusion. A 22% reduction in release of troponin T (area under curve) to the blood during the 8-day period after MI was evident in the LI dogs compared with the NLI dogs. This reduction was mainly due to a significantly (P<0.05) lower level (7.5±1.6 U [47% reduction]) of troponin-T release from the myocardium into the blood up to 48 hours after LAD occlusion in the
LI dogs compared with the accumulation (12.0\pm 2.6 U) in the blood in NLI dogs (Figure 2). There was also a highly significant ($P<0.005$) correlation ($r=0.73$) between troponin-T release up to 48 hours after LAD occlusion and infarct size in the LI dogs, whereas in the NLI dogs, there was no correlation. The enzymatic activity of catalase in blood serum of the dogs was also determined before and 24 hours after LAD occlusion. Catalase activity before MI was 2.0\pm 0.5 U/mL and 1.9\pm 0.3 U/mL in the NLI and LI dogs, respectively. At 24 hours after MI, the NLI dogs showed a 1.65-fold (not significant) elevation in activity, whereas in the LI dogs, elevation reached 2.90-fold, significantly ($P<0.05$) higher than before MI (Figure 3). There was a significant ($P<0.03$; $r=-0.5$) negative correlation between catalase activity 24 hours after LAD occlusion and infarct size in all dogs (Figure 4).

The histological characteristics of 5 to 7 regions in the infarcted zone in each NLI or LI dog were investigated. The histological sections from NLI dogs showed typical histopathological features of an infarct of 5 weeks after MI with inactive scar tissue consisting mostly of dense collagen with sparse and mature fibrocytes (Figure 5a). Histological analysis of the LI dogs revealed infarcts that were distinct from those in the respective NLI dogs in their large content of viable cells and loose matrix containing sparse collagen in the form of thin, wavy filaments (Figure 5b).

Desmin immunostaining was performed on serial sections of the general histology sections. The relative area occupied by desmin-expressing structures in the interphase zone (between the infarcted and the noninfarcted tissue) was significantly ($P<0.01$) higher (7.3-fold) in the LI dogs than in the NLI dogs (Figure 5c through 5e). The percentage of these structures within the infarct composed $8\pm 3\%$ in the LI dogs, compared with $1\pm 0.7\%$ in the NLI dogs. Desmin staining was weak in normal mature myocardium and confined to Z lines and intercalated disks (Figure 5f). Cells that demonstrated a strong positive homogeneous immunostaining of desmin in their cytoplasm were found within the infarcted zone of the LI dogs (Figure 5g). In more developed cardiomyocytes, usually organized in bulk in the infarcted area, desmin staining was also confined to the Z line (Figure 5h). Electron microscopic examination revealed cells in the infarcted area containing clusters of ribosomes and unorganized myogenic filaments in their cytoplasm, indicating cells that synthesize myogenic proteins de novo (Figure 5i).

At 4 hours after LAD occlusion in rats, the infarcted areas of NLI and LI rats were analyzed by electron microscopy. Disorganization of contractile proteins, vacuolization, and mitochondria with various degrees of distended and ruptured cristae and clearance of electron-dense matrix characterized the cardiomyocytes in the infarcted zone in NLI rat hearts (Figure 6a). In the LI rat hearts, the cardiomyocytes manifested minor disorganization of the myofibrillar proteins, vacuolization in the cytoplasm, and a limited number of damaged mitochondria (Figure 6b). The quantitative morphometric results indicated that $36.0\pm 0.5\%$ (mean $\pm$ SD) of the mitochondria in the NLI cardiomyocytes were severely damaged, compared with only $14.9\pm 2.6\%$ ($P<0.001$) in the LI cardiomyocytes (Figure 6c). Accordingly, the content of ATP in the ischemic zone of the LI rats 4 hours after LAD occlusion was 7.6-fold higher ($P<0.05$) than that of the NLI rats (Figure 6d).

**Discussion**

The results of the present study indicate that LELI causes a marked reduction in formation of scar tissue after induction of MI in dogs. The possible beneficial and cardioprotective...
The effects of the LELI were evident in this study from the electron microscopic examination of the rat ischemic zone 4 hours after LAD occlusion. There was an \approx 60\% reduction in the number of damaged mitochondria in the LI rats compared with NLI rats. These results correlate with the much higher level of ATP that was found in the ischemic area in the myocardium of the LELI rats compared with the NLI rats at the same time interval after LAD occlusion. Thus, it may be postulated that the LELI given within a short time interval after occlusion of the coronary arteries may attenuate the irreversible adverse effects that take place in the cardiomyocyte mitochondria in the ischemic zone and inhibit the very rapid decrease in ATP. Indeed, LELI has been found to increase mitochondrial respiration and ATP synthesis.8 Thus, the injured laser-irradiated cells may have a much slower rate of degeneration because of an increase in ATP production in the LI rats. The decrease in the number of injured cardiomyocytes may also markedly reduce the inflammatory response after LAD occlusion in the myocardium, as we previously showed for toad skeletal muscles after injury.9 This phenomenon may, in turn, reduce the adverse effects that have been attributed to the leukocyte infiltration that is part of the complex sequential processes that occur after MI.13

The reduction in troponin-T release to blood, as found in the present study during 48 hours after LAD occlusion in the LI dogs compared with NLI dogs, lends further credence to the contribution of LELI to the reduction of cardiomyocyte destruction in the ischemic zone during the initial phase after MI. Myocardial ischemic injury is also mediated, at least in part, by the generation and accumulation of reactive oxygen species in the cells under ischemic conditions, which also take part in their degenerative process. The present study demonstrated that catalase, which is one of the major enzymes in the antioxidative machinery in the cells, was significantly elevated in the serum of the LI dogs 24 hours after LAD occlusion compared with NLI dogs. It can be assumed that this elevation reflects the increase of catalase in the myocar-
dium at a short time interval after MI by LELI, as was previously shown for isolated cardiomyocytes. This elevated antioxidant activity in the dog ischemic myocardium is probably one of the mechanisms inhibiting cardiomyocyte degeneration and asserting a cardioprotective effect on these cells in the ischemic zone. The inverse significant correlation between the level of catalase after MI and infarct size in the dogs further supports the assumption that the antioxidant level in the ischemic myocardium at the early phase after LAD occlusion plays a major role in the complex processes that eventually lead to scar formation. Indeed, Woo et al demonstrated that induction of antioxidant production via gene transfer to the heart of neonatal mice improved its functional performance. Catalase was also found to increase within the myocardium 24 hours after whole-body heat stress. Thus, the possible link between LELI and heat-shock proteins associated with cardioprotection and infarct development cannot be ruled out. It may be postulated that LELI stimulated overproduction of heat-shock proteins in cardiomyocytes and thus increased their survival in the ischemic zone after LAD occlusion. Furthermore, it was shown that cardediol, a potent antioxidant, produces a high degree of cardioprotection in ischemic cardiac injury.

The histological appearance of the infarcts of the LI dogs was more cellular and less collagenous than that of the NLI dogs. It may be postulated that possibly more presumptive fibroblasts in the infarcted area are transformed into myofibroblasts after LELI. Indeed, Pourreau-Schneider et al., using electron microscopic methods and immunohistochemistry, previously showed that a direct and massive transformation of cultured fibroblasts into myofibroblasts was observed in tissue cultures 24 hours after He-Ne laser irradiation, whereas control cultures included only resting and active fibroblasts. Furthermore, we also found, in the present study, that a significantly higher percentage of cells within the infarcted and peri-infarcted zone of the LI dogs react positively to desmin than in the NLI dogs. Desmin was found in the cytoplasm of developing myogenic cells from skeletal muscles and is considered to be a marker for myogenic cells. On the basis of the above findings and the electron microscopic observations, one can assume that this phenomenon indicates the ability of the cells within the infarcted zone of the LI dogs and partially injured cardiomyocytes in the peri-infarcted zone to synthesize myogenic contractile proteins de novo.

Because treatment of the chronic complications that arise from the complex processes of cardiac repair after infarct has not yet been resolved, the results of the present study may also have clinical relevance. On the basis of our previous results showing that direct LELI on myoblasts in culture does not affect their differentiation in vitro and that the use of LELI in humans has no known deleterious effects, it can be postulated that the use of LELI after MI is probably safe. Our observations indicate that delivery of laser energy to the heart may have an important beneficial effect on patients after acute MI or other ischemic heart conditions that are not accessible to current revascularization procedures. Because LELI by itself does not destroy any additional precious myocardial tissue, it will be suitable for catheter-based interventions alone or together with diagnostic intraventricular navigation procedures. It can be postulated that LELI can be delivered to the myocardium in humans via fiber optics in the catheter of the nonfluoroscopic in vivo navigation and mapping technology currently in use in experimental animals and in humans. The LELI energy can also be applied during or after the procedure of balloon angiography by use of a catheter with a central canal bearing a fiber optic, through which the laser energy can be delivered transversely (360°) to the infarcted area. Furthermore, as an adjunct procedure to other, increasingly popular approaches for repairing ischemic myocardium (angioplasty, stenting, grafting, transmyocardial revascularization, etc), the cardioprotective effect of LELI may likewise be beneficial at minimal or no additional risk.

In conclusion, to the best of our present knowledge, the results of this study demonstrate for the first time a marked reduction in infarct size by LELI after MI in dogs. Thus, LELI can significantly attenuate the processes that take place after MI in dog myocardium and lead to formation of scar tissue. Because the next frontier in the treatment of ischemic heart disease lies in the development of substances for specifically targeted molecular therapeutics for patients after MI, the phenomenon described in the present study may also shed light on the possible nature of such substances.

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References

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