Evidence of Vascular Growth Associated With Laser Treatment of Normal Canine Myocardium

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Background. Transmyocardial laser revascularization is a new therapy for patients with refractory angina. Although clinical studies suggest that transmyocardial laser revascularization decreases angina and may improve regional blood flow, the underlying mechanisms are not elucidated. We hypothesized that one mechanism may relate to stimulation of vascular growth in laser-treated regions.

Methods. Transmyocardial laser revascularization channels were made with holmium:yttrium-aluminum garnet or carbon dioxide lasers in eight normal canine hearts; animals were sacrificed 2 to 3 weeks later and examined for vascular density and for evidence of smooth muscle proliferation.

Results. The original channels were infiltrated by gran-

There is a growing appreciation for the fact that there are many patients with coronary artery disease in whom symptoms of angina cannot be controlled by pharmacologic means and whose disease is not amenable to traditional invasive therapies (eg, bypass grafting or angioplasty). Accordingly, many new modes of therapy are currently under investigation. Among these investigational therapies is a surgical technique called transmyocardial laser revascularization (TMLR) [1]. With this technique, a laser is used to create channels through the myocardial wall so that, in principle, oxygenated blood from the ventricular chamber could flow directly to perfuse the myocardium. This type of physiology, which would obviate the need for coronary arteries, was intended to mimic the situation in reptilian hearts, which have extensive, naturally occurring endocardial channels that provide significant myocardial perfusion [2, 3].

Preliminary clinical studies of TMLR have revealed two interesting findings. First, subjective symptoms of angina are decreased shortly after operation and this effect does not diminish over time as would be expected for a placebo or thoracotomy effect [4]. Second, although clinical studies suggest that there may be an improvement in myocardial perfusion in treated regions of the heart by 3 months, which improves further by 6 months ulation tissue with associated vascularity. Vascular growth was stimulated immediately surrounding the channel remnant as evidenced by an increase in the number of vessels (approximately twice that of the control region) and an increase in the number of vascular cells staining positive for markers of cellular proliferation.

Conclusions. Transmyocardial laser revascularization leads to local vascular growth as early as 2 weeks after treatment. It remains to be determined whether this mechanism contributes to increased regional blood flow or to clinical benefits associated with this novel form of therapy.

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after therapy [5, 6], conflicting results of basic studies have raised questions as to whether myocardial perfusion can be improved in the acute and subacute settings [3, 7–9]. In view of the apparent time-dependent improvement in myocardial perfusion after laser therapy, it has been speculated that there is some effect that takes time to mature.

We hypothesize that one such effect might relate to the growth of new blood vessels, which potentially could be required for blood flow capacity to increase in the vicinity of laser treatment. To test this hypothesis, we looked for evidence of vascular growth in regions of laser-treated myocardium. Specifically, we determined whether there was an increase in the number of blood vessels and whether there was evidence of smooth muscle proliferation in laser-treated myocardium; both of these findings would signify active vascular growth [10]. Tissue samples obtained from canine myocardium 2 to 3 weeks after creating channels with two different clinically used lasers (a carbon dioxide [CO₂] laser and a holmium: yttriumaluminum garnet [YAG] laser) were examined. Previously, we have shown that the general features and appearance of acute and chronic (up to 6 weeks) laser channels made with these two lasers are similar, apparently differing only in that there is a greater amount of acute thermal injury surrounding holmium:YAG channels [11]. The results of the present study provide evidence of vascular growth within 2 to 3 weeks in the vicinity of laser-treated myocardium with both lasers.

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Material and Methods

All animals were cared for in accordance 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 of Sciences and published by the National Institutes of Health (NIH publication 85-23, revised 1985).

Transmyocardial laser channels were created in the hearts of 8 mongrel dogs (24 to 26 kg body weight) with either a holmium: YAG laser (n = 4) or a CO_2 laser (n = 4) according to the following procedures. Dogs were sedated with midazolam (0.1 mg/kg) and anesthesia was induced with an intravenous bolus injection of thiamylal (7 to 10 mg/kg). Anesthesia was maintained with 1.0% to 2.0% inhaled isoflurane. A left thoracotomy was performed and the pericardium was opened. An average of 12 channels (approximately 1 channel/cm²) were made in each heart in the region supplied by the left anterior descending coronary artery distal to the first diagonal branch. Transmyocardial penetration was confirmed by pulsatile bleeding during systole from the channel that was controlled either by manual compression or an epicardial U stitch (4-0 polypropylene suture). After the laser protocol was completed, the chest was closed in layers and the animals were allowed to recover from anesthesia. All animals were euthanized (pentobarbital, 100 mg/kg) between 2 and 3 weeks after the TMLR procedure. The locations of previous laser treatment were easily identified by the appearance of fibrous plaque on the epicardial surface of the heart. Transmural cubes of myocardium (containing either one or two channels) were excised, which were subsequently cut parallel to the epicardial surface (ie, perpendicular to the axis of the channels) at about 2-mm intervals. These sections were placed in labeled cassettes and processed as detailed further below.

Laser Parameters

The holmium:YAG laser (The CardioGenesis ITMR System; CardioGenesis Corp, Sunnyvale, CA) was set to deliver 2 J/pulse in bursts of three rapid succession pulses triggered to the R wave of the electrocardiogram. The laser energy was delivered to the myocardium through a fiberoptic cable (CardioGenesis Corp) with a 1.75-mm focusing lens at its tip, which is placed against the epicardial surface of the heart and advanced through the myocardium with each burst until penetrating into the ventricular chamber. The blunt surface of the probe prevents it from advancing through the myocardium on its own, thus ensuring that the channel is created by the laser energy and not due to mechanical forces exerted on the probe. Each channel required a total of three to five bursts for a total energy of 18 to 30 J/channel. The CO₂ laser (800 W, The Heart Laser; PLC Inc, Milford, MA) was used in the other hearts. The pulse duration of this continuous wave laser was set at 50 ms so that the laser delivered a 40-J pulse with each firing (the average energy used in the ongoing clinical trials), which was also timed to the R wave of the electrocardiogram. The CO_2 laser energy is delivered to the myocardium from the laser head through a wave guide with a hand piece on its end that is placed on the epicardial surface; each channel requires only one laser pulse to create the transmyocardial channel. In a previous study using the same laser parameter settings we have shown that in the acute setting the internal channel diameter is approximately 800 to 1,000 μ m with both laser systems and that the channels are surrounded by a rim of thermoacoustic injury, which is greater for the holmium:YAG laser [11]. We have also shown that the general histologic appearance of the channels after 2 to 3 weeks is essentially indistinguishable for these two lasers [11].

Fixation and Processing of Specimens for Histologic Evaluation

Samples were fixed in 10% neutral buffered formalin overnight and routinely dehydrated and embedded in paraffin. Serial sections 4 to 5 μ m thick were cut and stained with hematoxylin and eosin to evaluate the general morphology of lased and nonlased myocardium. Sister sections were stained using the Gomori trichrome technique with aniline blue counterstain. Standard immunohistochemical techniques were used to stain the tissue for vascular endothelium (to help identify vascular structures) and for markers of cellular proliferation (proliferating cell nuclear antigen, PCNA) to vessels that were undergoing active vascular growth.

Assessment of Histology Samples

To quantify the degree of vascularity and presence of proliferating cells, slides from 24 holmium:YAG laser channels and 26 CO₂ laser channels were submitted for quantitative analysis. As shown in Figure 1, two concentric ovals were constructed around the center of each laser channel remnant to delineate regions for quantitative analysis; the axes of the smaller of these ovals measured 1×0.6 cm (~0.5 cm²) and the axes of the larger oval measured 1.4×1.0 cm (~1 cm²). This oval shape was chosen to match to the generally elliptical shape of the channel remnants. The area inside the smaller oval excluding the channel remnant was defined as the area immediately surrounding the laser channel. The area between the two ovals was defined as the area neighboring the laser channel. The size of each channel remnant proper was calculated and this was excluded from the calculations described below because the purpose of the analysis was to look for evidence of increased vascularity and vascular growth in normal myocardium surrounding laser channels; as will be shown later that the channel remnants themselves uniformly contained a very high density of vascularity. Analysis was performed with the observer blinded to whether the sample came from a heart treated with the holmium:YAG laser or with the CO₂ laser. Samples from the left circumflex territory (which were remote from the region of laser treatment) were also obtained and examined from each animal and these served as control regions.

The number of arterial structures cut in cross-section

within the area immediately surrounding the channel remnant and in the neighboring area were counted on the factor VIII-stained sections. A vascular structure was counted as an "arterial structure" if it stained positive for factor VIII and was surrounded by at least one layer of smooth muscle cells. As indicated above, vessels within the core of the channel remnant or within fibrous tissue related to the channel remnant were not counted. Vessels on the boundary of the channel remnant (ie, arteries flanked on one side by granulation tissue and by normal myocardium on the other side) were included. Next, the number of smooth muscle or endothelial cells staining positive for PCNA were counted in these areas on sister sections stained with PCNA antibodies. Finally, the density of arterioles (arterioles/cm² myocardium) and the density of PCNA-positive cells were then calculated. All analyses were done under the microscope from the original slides.

Results

The general appearance of myocardium 2 weeks after laser treatment is shown in the typical examples of Figure 2 (holmium:YAG laser) and Figure 3 (CO₂ laser). For comparison, a typical sample of normal myocardium (obtained from the left circumflex, nonlased region) is shown in Figure 4; note that a sample containing a small artery was specifically identified for this sample. As we have observed previously, the trichrome-stained sections (Figs 2A, 3A) reveal that the original channel region has been invaded by granulation tissue that includes fibrosis and a large amount of vascularity. Because we have never identified any widely patent chronic channels with internal diameters similar to that of the original channel (800 to 1,000 μ m), we have referred to these regions where channels were made as "channel remnants." In the case of both examples, the vessels within the granulation tissue are of varying sizes and include capillaries, small arterioles, and frequently larger arteries with several layers of smooth muscle. There are vessels cut in crosssection (indicating that they are traveling in the epicar-



Fig 1. Low-power view of a typical laser channel remnant showing concentric ovals used to define the area immediately surrounding the channel remnant (0.6×1 cm, inner oval) and the neighboring region (1.0×1.4 cm, outer oval). Ovals and channel remnant are drawn with same scale to their relative sizes.



Fig 2. Trichrome stained (A) and factor VIII immunostained (B) histologic sections about 2.5 weeks after holmium:YAG laser treatment. The original channel region is invaded by granulation tissue that includes significant amounts of vascularity both within and surrounding the channel remnant. Arrows point to examples of factor VIII-positive staining arterial structures that are surrounded by at least one layer of smooth muscle. (×25 before 54% reduction.)

dial-to-endocardial direction) and also vessels cut longitudinally (indicating that they are traveling radially out from the channel). Also seen in Figure 2 are crosssections of larger muscular arteries within the core of the channel remnant. As seen in the accompanying sections stained for factor VIII (Figs 2B and 3B), the arteries were endothelial lined. The general appearance of channel remnants made with both lasers exhibited a rather wide range of characteristics. However, it was not possible to distinguish between channels made with the two lasers.

The factor VIII-stained tissue shown at low power magnification in Figures 2B and 3B also reveal vascularity on the border of the region of granulation tissue and in the area immediately surrounding the channel remnant, which again included vascular structures of various sizes. These vessels appeared to be originating from within the channel remnant and extend out into the surrounding normal myocardium. Arrows in these figures point to some of the smooth muscle-lined vessels found on the edge of the channel remnant and in the surrounding normal myocardium. Note that identification of a muscular layer in the smaller of these arteries, not always



Fig 3. Trichrome stained (A) and factor VIII immunostained (B) histologic sections about 2.5 weeks after CO_2 laser treatment. The original channel region is invaded by granulation tissue that includes significant vascularity both within and surrounding the channel remnant. Arrows point to examples of factor VIII-positive staining arterial structures that are surrounded by at least one layer of smooth muscle. (×25 before 54% reduction.)

apparent at low power, was performed at a magnification of $400\times$. Another example is shown in Figure 5A. This figure shows vessels extending beyond the channel remnant (which is off to the left of the photographic field) with multismooth muscle-layered vessels (near bottom) and a capillary (in upper portion of figure); note the normal myocardium surrounding the vessels in both of these regions. In comparison, vessels of these intermediate sizes (ie, small caliber vessels bigger than capillaries surrounded by layers of smooth muscle) occur relatively infrequently in normal myocardium (Fig 4B; also see quantitative analysis below).

Evidence that these vessels are new and actively growing is provided by the cell proliferation assay, as shown in Figure 5B. The arrows in the figure point to regions where numerous dark-stained PCNA-positive nuclei are seen. Note that positive PCNA staining is limited to the vessels in the area and is not seen at all in myocytes. This finding indicates that the cells in the wall of these vessels are being stimulated to proliferate; in comparison, positive PCNA staining in vessels (or myocardium) of normal myocardium is a very rare event (see Fig 6A and quantitative analysis below).

Within the core of channel remnants, positive PCNA staining was observed in a variety of cell types such as fibroblasts, inflammatory cells, and cells within the wall of forming vessels. The density of PCNA staining was always significantly higher within the channel remnant than in the surrounding tissue; this was observed for channel remnants associated with both types of lasers.

The appearance of PCNA-positive nuclei is more clearly shown on the higher power examples of smooth muscle cells within the walls of variously sized arteries in the area immediately surrounding the channel remnants shown in Figure 6; these examples are taken from different hearts treated either with the holmium:YAG laser (Figs 6B, 6C) and from different hearts treated with the CO₂ laser (Figs 6D, 6E). Note that in all of these cases normal myocardium is present next to the artery indicating that the vascular growth is not limited to the region of the channel remnant itself, but extends into the surrounding normal myocardium. For comparison, a similarly stained artery taken from the distant circumflex region, shown in Figure 6A, reveals no PCNA staining, which is the usual situation for arteries of normal adult myocardium; it is a very rare event to find a PCNApositive staining cell in normal myocardium or arteries of any size.

The extent of vascularity and frequency of PCNApositive staining nuclei within the vasculature were



Fig 4. Trichrome stained (A) and factor VIII immunostained (B) histologic sections of a region of normal myocardium showing that it is rare to find more than one multismooth muscle layered arterial structure in a given microscopic field. (\times 25 before 33% reduction.)



Fig 5. Factor VIII (A) and proliferating cell nuclear antigen (B) immunostained sections on the edge of a channel remnant showing multiple smooth muscle layered vessels near bottom and a smaller capillary structure toward the top. The arrows in B point to regions where there are several positive proliferating cell nuclear antigen staining nuclei. (\times 100 before 31% reduction.)

quantified as indicated in the Material and Methods section. The results of this analysis are summarized in Figure 7 with both vascular density (Fig 7A) and numbers of proliferating cells (Fig 7B) expressed as a number per square centimeter. Consistent with observations summarized, the vascularity was increased within the 0.6 \times

Fig 6. (A) Artery within normal myocardium revealing the usual finding that there are no proliferating cell nuclear antigen-positive stained nuclei. (B and C) Examples of proliferating cell nuclear antigenpositive arteries near holmium:YAG channel remnants. (D and E) Examples of proliferating cell nuclear antigen-positive arteries near CO_2 channel remnants. (×250 before 5% reduction.)



1.0-cm oval areas immediately surrounding the channel remnants compared with the control areas; furthermore, a similar degree of increased vascularity was seen with both lasers. However, this increase in vascularity did not extend to the neighboring areas (ie, the area between the smaller and larger ovals) around the channel remnants with either laser; vascular density exhibited in this region was similar to that of the remote circumflex territory.

Similarly, the number of PCNA-positive vascular cells in the area immediately surrounding the laser channels were statistically significantly greater than that of the control area (see Fig 7B). However, this density diminished to that of the control region beyond the inner oval surrounding the channel remnant. There was no difference in PCNA-positive vascular cell density between the two lasers.

Comment

Results of several clinical trials of TMLR suggest that this procedure provides significant symptomatic relief to patients suffering from otherwise untreatable angina [5, 6, 12]. There is also evidence that over time, myocardial perfusion is improved in regions treated with TMLR [5, 6, 13]. Larger randomized trials (TMLR versus continued medical management) are currently underway with different types of lasers whose goals are to prove objectively the clinical effectiveness and safety of TMLR. In contrast to the generally concordant reports of clinical studies, significant controversy has emerged in the scientific arena regarding the mechanism of the potential clinical benefits. Two issues at the center of these controversies include (1) whether the channels remain "patent" and (2) elucidation of the mechanisms of improved blood flow in the chronic setting.

With regard to channel patency, it is first important to note that there has been no reported observation of a chronic channel whose internal diameter is comparable with that made in the acute setting (ie, approaching 1 mm in diameter). In the present study we show, as in previous studies [8, 9, 14], that channels made in myocardium undergo a significant morphologic change within 2 to 3 weeks. The original channels (which are 800 to 1,000 μ m in diameter with both laser systems [11]) are infiltrated by granulation tissue with associated vascularity and lacunar spaces, creating what we have referred to as a channel remnant.

With regard to blood flow, results of most studies are consistent in showing very little or no blood flow potential in the acute setting [3, 7–9]. Thus, one of the initial hypotheses, that transmyocardial channels connect with preexistent myocardial sinusoids, appears not to be supported by available data. Furthermore, critical evaluation of available data suggest that myocardial sinusoids are neither prevalent anatomically nor physiologically significant structures in mammalian myocardium.

The results of the present study show that vascular growth is stimulated within and immediately surrounding the channel remnant detected as early as 2 weeks after creating the laser channels. The evidence supporting this observation was in two forms. First, there was an



Fig 7. Density of arterial structures (A) and proliferating cell nuclear antigen (PCNA) positive vascular cells (B) in regions immediately surrounding the channel remnants (solid bars), in the neighboring region (hatched bars), and in the remote (normal) myocardial regions (open bars). There is increased vascular density and PCNA positivity immediately surrounding the channel remnant that was similar for holmium:YAG and CO₂ laser treatment (*p < 0.001 compared with remote region by unpaired t test with Bonferroni correction for multiple comparisons.)

increase in the number of vessels with one or more layers of surrounding smooth muscle cells. These vessels were observed not only inside the channel remnant but also in normal myocardium up to 3 mm from the channel remnant center. Second, smooth muscle cells of these vessels stained positive for molecular markers of cellular proliferation (PCNA) indicating that these cells are being stimulated to enter the cell cycle, thus signifying active vascular growth. The PCNA-positive smooth muscle cells were also identified in regions of the arteries located both within and outside of the channel remnant.

There are several possible mechanisms that could lead to vascular growth as observed in the present study. Both holmium:YAG and CO_2 lasers create a rim of thermoacoustic injury around the channel lumen in the acute setting that incites an inflammatory response. Current evidence suggests that cells involved in the inflammatory response liberate cytokines, growth factors, and upregulate growth factor receptors, all of which are involved with vascular growth [15]. Further insights into the mechanisms will improve as understanding of the fundamental factors that regulate vascular growth improve.

The terms angiogenesis and vasculogenesis refer to different types of vascular growth, which may be observed in tissue [15, 16]. True angiogenesis is the process whereby new capillaries sprout from preexisting capillaries with subsequent migration and division of smooth muscle cells. Vasculogenesis (the development of new vessels in situ) consists of recruitment of circulating angioblasts and hematopoietic stem cells from the blood with the subsequent differentiation and proliferation of endothelial cells and smooth muscle cells (with the latter possibly derived from in situ fibromyoblasts) [17]. Finally, vascular remodeling is the phenomenon whereby vascular diameter can increase by as much as 20-fold by way of a complex sequence of events that involve intimal hyperplasia and changes in the surrounding myocardium to accommodate a larger vessel [15]. It is also noted that these processes may be occurring at the same time. Accordingly, when observing a specific vessel in the process of growing, particularly as in the present study in the setting of significant inflammation after injury, it may be difficult to classify unambiguously which of these two processes is occurring.

Increased myocardial vascularity after TMR has been commented on in several previous studies [2, 9, 14, 18-21]. It has also been recognized that the observed tissue responses, including the increased vascularity, may simply reflect the typical tissue response to inflammation caused by laser or even other types of injury. Yet, the analysis performed in the present study reveals potentially important insights into this process. First, previous studies have not distinguished between increased vascularity within the granulation tissue of the channel remnant itself and in the surrounding normal myocardium; the present study, which excluded an analysis of vascularity deep within the channel remnant, makes this important distinction. Second, our observation of growth of muscle-lined vascular structures in the surrounding normal myocardium is significant in that it indicates that the stimuli for vascular growth reach sites beyond the boundaries of the injury region proper; we were able to identify such growth up to 3 mm from the center of the channel remnant.

Although the present findings reveal vascular growth after laser treatment in normal myocardium, there are many fundamental questions that must be addressed to prove that this mechanism contributes to clinical benefits observed after TMLR. First, it can be questioned whether results obtained in normal myocardium can provide useful information regarding TMLR, when the technique is used clinically in the setting of chronically ischemic myocardium; there are several observations that suggest that they can provide useful information. There is mounting evidence that the histologic appearance of TMLR channels we and others [14] have observed in normal animal myocardium are similar to those seen in autopsy specimens [21, 22]. Further work is clearly needed to determine whether the present observations pertain to the chronic ischemic human myocardium to which TMLR is applied in the clinical setting. Second, it will need to be demonstrated that these vessels provide nutritive myocardial blood flow. Finally, it will be important to determine the anatomic connections of these vessels and their capacity to carry nutritive blood flow.

It is also noteworthy that Whittaker and colleagues [18] studied myocardial channels made with lasers and needles in normal rat myocardium after allowing the animals to survive for several months. After that period, acute ischemia was induced and the physiologic significance of the effect of the treatments was assessed. They showed that needle channels, but not laser channels, conveyed some protection to the myocardium. Needle channels tended to retain patent vascular communications with the left ventricular chamber, whereas laser channels did not. In either case, their histologic analysis failed to reveal any increase in capillary density in the surrounding myocardium. This observation led them to conclude, in contrast to the present study, that angiogenesis was not induced by these procedures. Myocardial protection attributable to needle channels in the absence of an angiogenic response lead the investigators to hypothesize that the protection could be related to blood flow from the left ventricular chamber. There are many differences between our study and that of Whittaker and colleagues that may contribute to the different conclusions. First, our histologic analysis did not examine capillary density, but rather focused on larger vessel growth in the area surrounding the channel remnants. Second, rat hearts were studied in the previous study, whereas in the present study we examined canine hearts, vet the diameters of the channels where comparable in both studies. It needs to be determined whether an approximately 1-mm diameter channel made in a heart with wall thickness of approximately 2 mm (rat heart) induces similar responses and behaves physiologically similarly to similarly sized channels made in a myocardial wall typically more than 1.2 cm (canine hearts).

In summary, the results of this histologic study provide evidence of active vascular growth in the vicinity of laser channels 2 to 3 weeks after their creation with a high frequency of proliferating smooth muscle cells. Many fundamental questions remain regarding whether diseased human myocardium responds similarly and how these new vessels may contribute to clinical benefits. Other controversial aspects relating to TMLR, such as whether blood flows from the left ventricular chamber to perfuse the myocardium directly, which were not addressed in the present study, will also require additional study to provide a comprehensive hypothesis of the sequence of events leading to the clinical benefits after laser treatment. In the meantime, encouraging preliminary results from several ongoing clinical trials continue to fuel a great deal of interest in the investigation of this novel form of therapy.

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