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EXPERIMENTAL STUDIES

Angiogenesis Is Enhanced in Ischemic Canine Myocardium by Transmyocardial Laser Revascularization

NORIYOSHI YAMAMOTO, MD, TAKUSHI KOHMOTO, MD, ANGUO GU, MD, CAROLYN DEROSA, BS, CRAIG R. SMITH, MD, DANIEL BURKHOFF, MD, PHD

New York, New York

Objectives. **This study sought to test whether transmyocardial laser revascularization (TMLR) stimulates angiogenesis in an animal model of chronic ischemia.**

Background. **TMLR relieves angina and may also improve blood flow in patients who are not candidates for traditional therapies. The mechanisms of these benefits are not fully defined.**

Methods. **Ischemia was created in 14 dogs by proximal left anterior descending coronary ameroid constrictors. TMLR was performed in the anterior wall (~1 channel/cm^2) of seven dogs; the remaining dogs served as the ischemic control group. Myocardial blood flow was measured (colored microspheres) at rest and during chemical stress (adenosine) in the acute setting and after 2 months.**

Results. **TMLR did not influence blood flow in the acute setting. After 2 months, resting blood flow increased comparably in the anterior wall in both groups to ~80% of normal. However, the TMLR-treated dogs demonstrated an ~40% increase in blood flow capacity during stress in the ischemic territory compared with untreated dogs (left anterior descending coronary artery/left circumflex coronary artery flow 0.53 ± 0.16 in the control group vs. 0.73 ± 0.08 in TMLR animals, $p < 0.05$). Vascular proliferation, assessed by bromodeoxyuridine incorporation and proliferating cell nuclear antigen positivity in endothelial and smooth muscle cells was about four times greater in the TMLR group than in the control group ($p < 0.001$). The density of vessels with at least one smooth muscle cell layer was ~1.4 times greater in the myocardium surrounding the TMLR channel remnants than in control ischemic tissue ($p < 0.001$).**

Conclusions. **In this canine model of chronic ischemia, TMLR significantly enhances angiogenesis as evidenced by the increased number of vessels lined with smooth muscle cells, markedly increased vascular proliferation and increased blood flow capacity during stress.**

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Significant advances have been made in the development of new surgical and percutaneous techniques to restore blood flow through diseased coronary arteries. However, most of these advanced therapies are primarily designed to treat relatively discrete vascular stenoses. It has become increasingly evident that there are a growing number of patients who suffer from debilitating angina who cannot be treated by these therapies, typically because the extent of the vascular disease is too diffuse. Accordingly, there is interest in developing alternative forms of therapy to relieve angina and improve blood flow to ischemic myocardium.

Transmyocardial laser revascularization (TMLR), one such therapy currently under active clinical investigation, has received a great deal of attention because results of several studies indicate that it provides significant relief of angina and may improve myocardial perfusion in otherwise untreatable patients (1–4).

During TMLR surgery, a laser is used to create channels through the myocardial wall that penetrate into the ventricular chamber. The original idea behind TMLR was to mimic the naturally occurring channels of reptile hearts, which permit a substantial amount of myocardial perfusion directly from the ventricular chamber (1,5), thus diminishing the reliance of myocardial perfusion on the epicardial arteries. However, results of recent experimental studies have indicated that laser channels made in mammalian hearts do not conduct blood (6,7). Furthermore, the channels do not remain patent, but are rapidly occluded by thrombus and infiltrated by granulation tissue within 3 weeks (6–11). (We use the term *patent chronic channel* to specifically denote a chronic channel whose inner dimensions approximate those of the channels at the time when they are created.) Accordingly, investigators have begun to consider other possible mechanisms by which clinical benefits could be achieved, with significant interest focused on the possibility that the TMLR stimulates angiogenesis. A recent study from our laboratory documented a doubling of arteriolar density in the normal myocardium immediately surrounding the TMLR channel remnants, with evidence of active vascular growth 3 weeks after the procedure (12). However, the degree

From the Departments of Surgery and Medicine, Columbia University, New York, New York. This study was supported in part by a research grant from CardioGenesis Corporation, Sunnyvale, California.

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Address for correspondence: Dr. Daniel Burkhoff, Department of Medicine, Division of Circulatory Physiology, Columbia University, 630 West 168th Street, New York, New York 10032. E-mail: db59@columbia.edu.

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Abbreviations and Acronyms

BrdU = bromodeoxyuridine
LAD = left anterior descending coronary artery
LCx = left circumflex coronary artery
MAP = mean arterial pressure
PCNA = proliferating cell nuclear antigen
SMA = smooth muscle actin
TMLR = transmyocardial laser revascularization

to which TMLR stimulates this angiogenic process under clinically relevant ischemic conditions, whether the new vessels persist for longer periods and whether the new vessels are capable of mediating an increase in local blood flow are all unexplored, fundamental questions.

To gain further insight into the potential mechanisms underlying the clinical benefits of TMLR, these questions were investigated in a chronic canine model of ischemia. An ameroid constrictor was placed on the proximal left anterior descending coronary artery (LAD) and TMLR channels were created in the anterior wall; a separate group of control dogs underwent ameroid placement without the TMLR procedure. Blood flow was measured with microspheres at rest and during adenosine-induced stress at the initial operation and 2 months later. Evidence of vascular growth was obtained through vessel counting and by detecting proliferation of vascular endothelial and smooth muscle cells in the tissue retrieved at the 2-month evaluation. Although there was no acute effect of TMLR on blood flow, this procedure significantly stimulated vascular growth in the chronically ischemic myocardium; this was associated with increased blood flow capacity during stress, a finding that mimics those obtained in the clinical setting (2,3). These studies provides evidence that angiogenesis may be a mechanism by which blood flow is improved by TMLR, which is emerging as a therapy for patients with severe coronary artery disease.

Methods

All dogs were cared for by a veterinarian 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 (National Institutes of Health publication 85-23, revised 1985) and the "Position of the American Heart Association on Research Animal Use" (November 11, 1984).

Surgical procedures. Fourteen adult mongrel dogs of either gender, weighing 21 to 26 kg, were anesthetized with an intravenous injection of thiopental sodium (15 mg/kg body weight). The anesthesia was maintained with 0.5 to 2.0% inhaled isoflurane. The femoral artery was cannulated to monitor arterial blood pressure and to take samples of arterial blood. A left thoracotomy was performed through the fifth intercostal space using a sterile technique. Two catheters were introduced into the left atrial appendage through a pursestring suture (4–0 polypropylene); one catheter was for injection of microspheres and the other for the infusion of adenosine. In the TMLR group (n = 7) transmural laser channels were created from the epicardial surface into the ventricular chamber over the LAD territory (anterior and anteroapical region of the heart). A fiberoptic cable with a 1.75-mm focusing lens (CardioGenesis Co.) connected to a holmium:yttrium-aluminum-garnet laser (CardioGenesis Corp.) was used to create the channels. The firing of the laser was synchronized with the R wave of the electrocardiogram to deliver a burst of three pulses (2 J/pulse) in rapid succession; three to five bursts (between 18 and 30 J total energy) were required to create each transmural channel. Channels were made with a density of ~1 channel/cm^2, and an average of 14 channels (range 11 to 19) were made in each heart.

All visible epicardial collaterals connecting LAD diagonals to the left circumflex coronary artery (LCx) or right coronary artery were ligated with a 4–0 stitch to minimize collateral flow to the LAD territory; an average of four such collateral vessels were identified in each heart. In order to assess the blood flow capacity of the remaining collateral circulation, 1 ml of mixed colored microsphere solution (15 μm diameter, $3 \cdot 10^6$ microspheres/ml in a saline suspension with 0.01% Tween 80 and thimerosal, Dye-Trak, Triton Technology, Inc.) was injected into the left atrium through the previously inserted catheter while temporarily clamping the LAD proximal to the first diagonal branch. To assess collateral flow during stress, microspheres of a different color were injected after left atrial adenosine infusion. The rate of infusion was titrated as in previous studies (13) to create an ~20% fall in mean arterial pressure (MAP); as with the baseline measurement, this infusion was performed while temporarily clamping the LAD. Blood samples were obtained during microsphere injections by a constant rate of withdrawal (7 ml/min) from the femoral artery using a syringe pump. After microsphere injections under both baseline and stress conditions, an ameroid constrictor was placed on the LAD at the same location (i.e., proximal to the first diagonal branch). The control group (n = 7) underwent the same procedures described earlier, except that no TMLR channels were made. After completion of the procedure, the chest was closed in layers and the dog was allowed to recover from anesthesia.

In order to provide an index of cellular proliferation at multiple time points after the initial surgery, bromodeoxyuridine (BrdU, 25 mg/kg, Sigma) was administered subcutaneously in four TMLR and four control animals on postoperative days 7, 14 and 28.

The final procedure was performed 8 to 9 weeks after the initial operation. The dogs were anesthetized and mechanically ventilated and a median sternotomy was performed. The same protocol of microsphere infusions under rest and adenosine stress conditions described earlier was performed using microspheres of two additional colors; the only difference was that the LAD was not clamped because, as expected from previous reports and as shown by direct evaluation after euthanasia,

every ameroid constrictor was completely occluded. After euthanasia (pentobarbital 100 mg/kg), the heart was removed and cut into small (~1 g) transmural samples from the LAD-laser channel region (one channel per sample) and from the LCx region. Three LAD and two LCx myocardial samples were submitted for immunohistochemical analysis; all other samples were submitted for microsphere analysis.

Microsphere analysis. Retrieval and quantitative analysis of the microspheres were performed exactly as described previously (6,14). In brief, tissue samples were digested and the microspheres were retrieved by filtration of the digestate. The dye on the microspheres was then itself digested into solution using dimethylformamide and the photometric absorption of the resulting sample was measured by a diode array spectrophotometer (model 8452A, Hewlett-Packard Co.). The composite spectrum was then resolved at the peak frequencies into the contributions from the individual colored microspheres using a matrix inversion technique (6,14). The number of microspheres in each sample was calculated according to the optical density at the wavelength corresponding to each dye color using standardization curves generated from known quantities of microspheres from the same batch of spheres. Regional myocardial blood flow (RMBF, ml/min per g) was calculated using the following equation:

$$\mathrm{RMBF} = \frac{F_{ref}}{M_{sample}} \times \frac{OD_{sample}}{OD_{ref}}, \qquad [1]$$

where F_{ref} represents the rate at which arterial blood is withdrawn from the femoral artery (i.e., of the reference sample, which was always 7 ml/min in our study); OD_{ref} represents the optical density of the dye solution obtained from this reference sample; M_{sample} represents the mass of the respective myocardial sample; and OD_{sample} represents the optical density at the corresponding wavelength of the dye solution obtained from the myocardial sample.

Tissue fixation and preparation. Myocardial samples destined for histologic evaluation were fixed in 10% neutral buffered formalin, left overnight and routinely dehydrated and embedded in paraffin. Serial sections, 4 to 5 μm thick, were cut and stained with Masson's trichrome stain to evaluate the general morphology of the lased and nonlased myocardium. Sister sections were stained using standard immunohistochemical techniques with antibodies against BrdU (15), PC10 proliferating cell nuclear antigen (PCNA) (16), and alpha smooth muscle actin (SMA) (17), using standard techniques.

Statistical analysis. All data are presented as mean value ± SD. The statistical significance of differences between groups was determined by the Student *t* test or analysis of variance with a Tukey post hoc test in cases when multiple groups were being compared. A p value < 0.05 was considered significant.

Results

Coronary blood flow in acute and chronic settings. Coronary blood flow to the LCx territory averaged 0.9 ± 0.24 ml/min per g in the control group and 1.30 ± 0.63 ml/min per g in the TMLR group (p = NS). With all visible epicardial collateral vessels between the LCx and LAD territory permanently ligated, and with the LAD itself temporarily clamped during the measurements, residual collateral flow into the LAD territory averaged ~25% of the flow in the LCx territory in both groups (Fig. 1). Adenosine infusion decreased MAP from 81 ± 11 to 69 ± 11 mm Hg (21% decrease) in the control group and from 85 ± 13 to 65 ± 11 mm Hg (24% decrease) in the TMLR group (p = NS). Despite the drop in MAP, coronary flow into the LCx territory increased to 1.89 ± 1.51 ml/min per g in the control group and to 2.02 ± 1.20 ml/min per g in the TMLR group (p = NS), indicating significant coronary vasodilation. However, because of the relatively limited reserve of the preexisting collateral vessels, flow into the LAD territory was only ~12% that of the LCx territory (Fig. 1); there was no difference in this response between the two groups. Thus, TMLR did not affect regional myocardial blood flow in the acute setting, neither at baseline conditions nor during the stress of a pharmacologically induced vasodilator challenge.

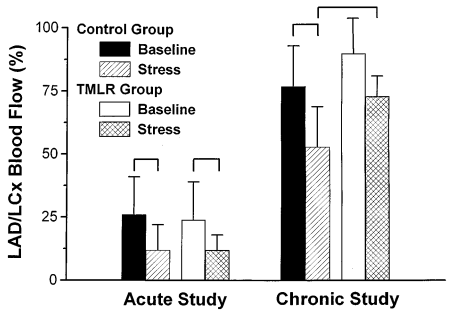

Figure 1. Color microsphere measurement of blood flow into ischemic anterior wall (LAD distribution) showed that flow was normal compared with flow in the normally perfused circumflex territory in control (n = 7) and TMLR-treated (n = 7) hearts. In the acute setting, TMLR had no influence on flow in the ischemic territory. During adenosine-induced vasodilatory stress, relative flow decreased further in the anterior wall, but again TMLR did not influence flow. In the chronic (2-month) setting, blood flow at rest increased comparably in both control and TMLR-treated groups, reflecting the normal process of angiogenesis that occurs in response to ischemia. However, during vasodilatory stress, blood flow was better maintained in the TMLR group, suggesting that angiogenesis was enhanced by TMLR. Normalized flow was significantly less during adenosine-induced stress than under corresponding flow at rest in all cases, except in the TMLR group in the chronic study where these two values did not differ. Also, analysis showed that in the chronic study during adenosine stress, relative flow was greater in the TMLR group than in the control group. **Bars** indicate pairs that were statistically different from each other based upon analysis of variance with the Student-Newmann-Keuls post-hoc test; $p < 0.05$ considered significant. See text for details.

After 2 months, the dogs were again anesthetized and the chest was opened and coronary blood flow measured at baseline and during the same pharmacologically induced stress using microspheres. Blood flow to the LCx territory at rest was unchanged from the acute setting, averaging 0.98 ± 0.36 ml/min per g in the control group and 1.14 ± 0.49 ml/min

per g in the TMLR group (p = NS). Flow to the LAD territory at baseline increased comparably in both groups: to 77 ± 16% of LCx flow in the control hearts and to 90 ± 15% in the TMLR hearts (p = NS, Fig. 1). Thus, although not returning to normal, there was a marked increase in collateral flow into the chronically ischemic bed; under these conditions there was no apparent effect of TMLR, although there was a trend for flow to be increased.

However, during adenosine-induced stress there was a statistically and physiologically significant difference in blood flow response in the LAD territory (Fig. 1). Adenosine infusion decreased MAP from 95 ± 12 to 76 ± 12 mm Hg (20% decrease) in the control group and from 102 ± 20 to 77 ± 19 mm Hg (25% decrease) in the TMLR group. Collateral flow into the LAD territory was only ~53% that of the LCx territory; this value was ~73% in the TMLR-treated dogs ($p < 0.05$). Statistical analysis showed that relative LAD flow was decreased during adenosine infusion in the control group, but there was no statistically significant difference between baseline and stress in the TMLR group. Importantly, the relative blood flow during adenosine-induced stress was significantly increased in the TMLR group compared with the control group. Thus, TMLR was associated with an almost 40% increase in blood flow reserve in the chronically ischemic myocardium (i.e., a 20-percentage point increase over the control value of 53%).

Histologic and immunohistochemical findings. The histologic appearance of myocardium 2 months after being treated with TMLR (Fig. 2) was similar to what has been reported at 2 weeks (6,7,18), except that there was no significant active inflammation. The channels, which were approximately 1 mm in diameter in the acute setting, were infiltrated with granulation tissue that included a significant amount of vascularity. Because the channels are not "patent" in the sense of retaining an internal bore with diameter comparable to that at the time of its creation, we have called these regions *channel remnants*. The regions of granulation were generally longer in the fiber direction, averaging 3.3 ± 0.7 mm in length and 1.3 ± 0.3 mm in width. The typical appearance of channel remnants is shown in the transverse and longitudinal sections of Figure 2A and 2D, respectively. Relatively large, mature arterial vessels frequently existed within the core of these channel remnants. SMA immunostaining of these tissue clearly revealed the vessels within the remnants (Fig. 2B and 2E). Simply on the basis of their high density within the channel remnant, it is evident that these are new vessels and thus represent the result of a significant angiogenic process. The typical examples shown in Figure 2C and 2F reveal that many smooth muscle and endothelial cells of these vessels incorporated BrdU. These findings confirm that these vessels had been actively growing during the times of BrdU administration (and also served as a positive control for the assay). For comparison, examples obtained from the remote, nonischemic LCx territory of these hearts show very little BrdU incorporation and, when present, was observed mostly in small vessels and capillaries (Fig. 3B). PCNA positive nuclear staining was rare in this and in all other

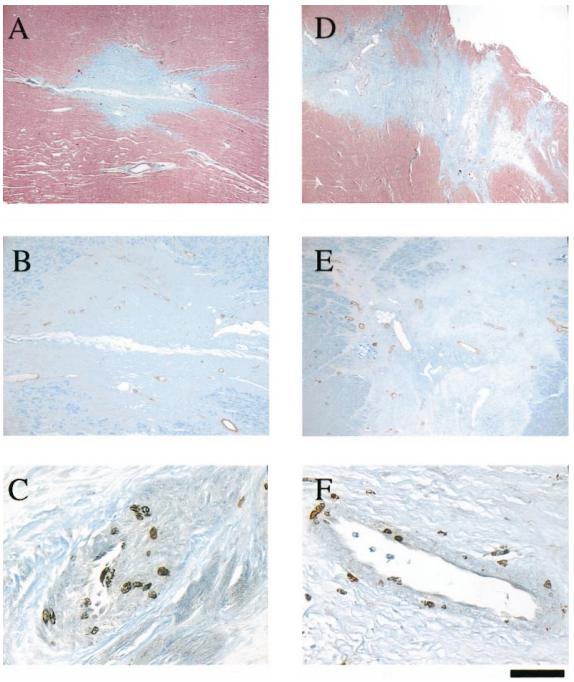

Figure 2. Histologic appearance of channel remnants 2 months after creation. **A,** to **C,** Sections of a transverse cut through a channel remnant. **D** to **F,** Sections of the endocardial side of a longitudinally cut sample. Trichrome stains (**A** and **D,** original magnification ×20) show the region to be infiltrated with granulation tissue without active inflammation at this time. Smooth muscle actin immunostaining (**B** and **E,** original magnification ×40) highlights vessels within the granulation tissue. Examples of BrdU incorporation (dark staining nuclei into smooth muscle and endothelial cells of vessels within channel remnants (**C** and **F,** original magnification ×400) indicate that these vessels had undergone significant growth after the TMLR procedure. **Calibration bar** shows 1 mm for panels **A** and **D;** 0.5 mm for **B** and **E;** 0.05 mm for **C** and **F**.

tissue examined (assay validated using canine intestinal mucosa as a positive control); this indicates that the phase of active vascular growth revealed by the BrdU staining was completed by 2 months after the TMLR procedure.

Vascular density appeared increased in the normal myocardium surrounding the channel remnants compared with the vascular density in the nonischemic LCx territory (Fig. 4, edge of channel remnant in the upper left corner of panel A). Significant amounts of nuclear BrdU incorporation, and therefore antecedent vascular growth, were noted in small (Fig. 4B and 4C), intermediate (Fig. 4D and 4E) and large (Fig. 4F) vessels outside of the channel remnants. As for the other regions examined, PCNA staining was rarely observed in these vessels, indicating low levels of active proliferation.

BrdU incorporation was also detected in the ischemic tissue of control hearts. An example of a new collateral vessel that formed a bridge between two preexistent arteries is shown in the low magnification, trichrome-stained tissue of Figure 5A. Immunostaining (Fig. 5B and 5C) revealed that many of the endothelial cells along the entire length of this vessel stained positive for BrdU incorporation, a finding that confirms that

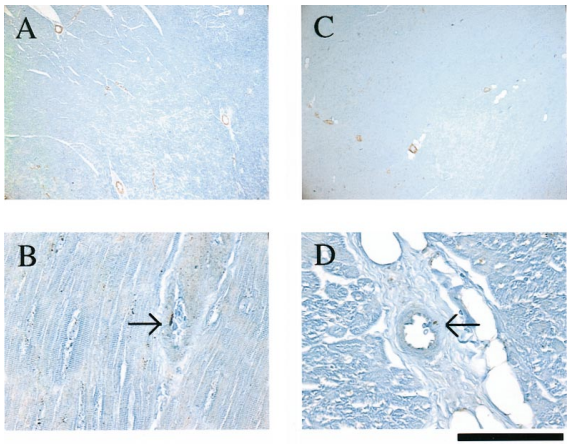

Figure 3. Samples from the nonischemic LCx territory obtained from TMLR-treated (**A** and **B**) and control hearts (**C** and **D**). SMA-stained samples (**A** and **C**) show the normal density of vessels in both groups of hearts (original magnification ×40). Sparse BrdU incorporation (**arrows** in panels **B** and **D**) indicates that, as expected in normally perfused myocardium, there was very little vascular proliferation in these areas. **Calibration bar** shows 1 mm for panels **A** and **C;** 0.1 mm for panels **B** and **D**.

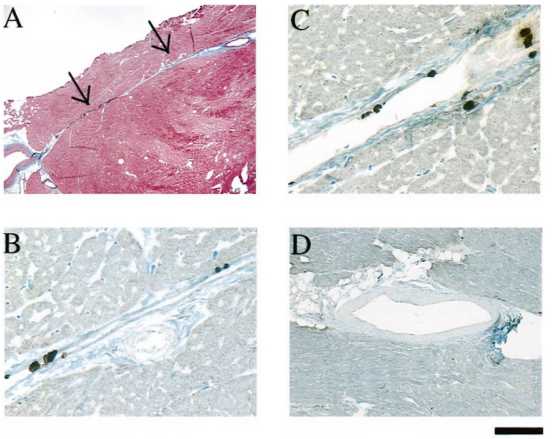

Figure 5. Trichrome-stained tissue in ischemic territory of a control (non-TMLR) heart shows a small, thin vessel **(arrows)** bridging two larger vessels. Examples of nuclear BrdU incorporation (**B** and **C,** original magnification ×400) into endothelial cells along the entire course of this vessel confirmed that this was a new, *bridging* collateral. By contrast, BrdU incorporation into larger arteries of these hearts was rarely observed, even in the ischemic territory (**D,** original magnification ×100). **Calibration bar** shows 1 mm for panel **A;** 0.05 mm for **B** and **C;** 0.20 mm for **D**.

this is a new vessel. As with the other examples, there was no PCNA staining observed in the cells of this vessel. Also as in this typical example, BrdU incorporation was rarely seen in the smooth muscle or endothelial cells of large arteries (Fig. 5D).

Tissue samples obtained from the remote, nonischemic tissue of the LCx territory of each heart were also examined. As shown in Figure 3, examination of low magnification views of SMA-stained samples revealed a relatively low density of arterial structures with one or more layers of smooth muscle in both control (Fig. 3C) and TMLR-treated (panel A) hearts; note that in creating these photographs, regions were specifically chosen so as to provide the maximal number of vessels in the field as possible. Furthermore, as reviewed earlier, BrdU positive staining was very rare in smooth muscle or endothelial cells of the vessels in the normal region; as shown in Figure 3B and 3D, when found it was typically present in only a single cell per vessel cross-section.

In order to provide a quantitative assessment of the various observations, we determined vascular density (number of vessels with at least one layer of smooth muscle cell/cm^2) and proliferating cell density (positive BrdU and positive PCNA vascular smooth muscle or endothelial cells/cm^2) in several areas of each heart. The results of this analysis are summarized in Figure 6. In control hearts, vascular density (Fig. 6C) was comparable in the LCx and anterior regions; this was also similar to the vascular density in the LCx region of the TMLR hearts. The vascular density in the myocardium immediately surrounding the channel remnants (contained between the edge of the channel remnant and an ellipse with a minor axis of 6 mm and a major axis of 10 mm) was approximately twice that of the LCx territory. Furthermore, in the region neighboring the channel remnant (with boundaries defined by the first ellipse and a second, concentric ellipse with a minor axis of 10 mm and a major axis of 14 mm), vascular density was still significantly increased compared with the LCx territory. The

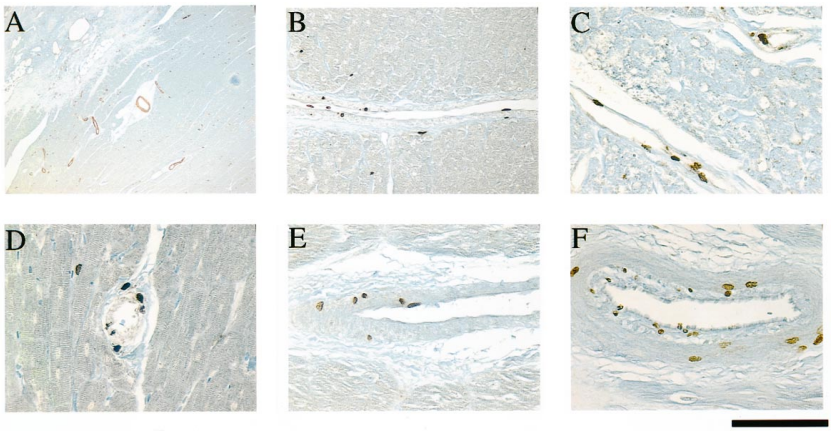

Figure 4. SMA stain (**A;** original magnification ×40) shows generally increased vascular density compared with normal and ischemic myocardium in the region surrounding the TMLR channel remnant (edge of channel remnant toward upper left corner of panel). Examples of BrdU incorporation into vessels in normal myocardium near the remnants in small (**B,** magnification ×100 and **C,** ×400) intermediate (**D** and **E,** ×400) and large (**F,** ×400) vessels indicate previous growth of these vessels and provide evidence for an angiogenic process. **Calibration bar** shows 1 mm in panel **A;** 0.25 mm in **B;** 0.1 mm in **C** through **F**.

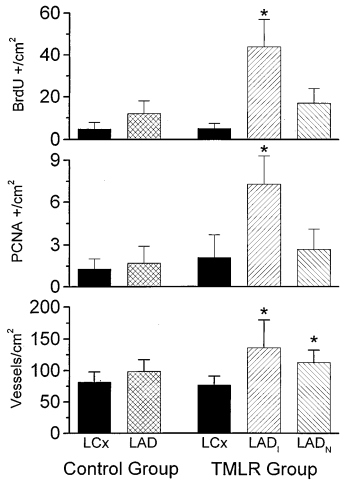

Figure 6. Quantitative analysis of BrdU, PCNA and vessel density in control and TMLR-treated hearts. LCx, data from nonischemic, normally perfused left circumflex territory; LAD, data from ischemic anterior wall of control hearts; LAD_I, data from anterior wall myocardium immediately surrounding channel remnants (i.e., confined between edge of remnant and an ellipse with minor and major axes of 0.6 and 1.0 cm, respectively); LAD_N, data from anterior wall myocardium neighboring the channel remnants (i.e., confined between the first ellipse and a second concentric ellipse with axes of 1.0 and 1.4 cm). Each **bar graph** represents mean value ± SD of observations made from 21 observations (3 from each heart studied). *$p < 0.05$ by analysis of variance with the Student-Newmann-Keuls post hoc test.

number of PCNA positive staining nuclei (Fig. 6B) was very low in all regions, although it was increased in the area immediately surrounding the channel remnant. Similarly, compared with all other regions in both groups of hearts, BrdU incorporation (Fig. 6A) was increased in the region immediately around the channel.

Discussion

Evidence has been accumulating that angiogenesis may be an important factor contributing to the clinical benefits observed after TMLR. Previously, this evidence has been derived from observations made in experimental tissue and in autopsy specimens that many, apparently new, blood vessels appear within the granulation tissue that invades the original channels (i.e., neovascularization within the channel remnants) (6–10,18–20). Such observations, however, provide limited support for the angiogenesis hypothesis because blood vessels within scar tissue by themselves may not contribute meaningfully to myocardial perfusion. More recently, we showed that by 2 weeks after creating transmyocardial laser channels in normal canine hearts active vascular growth in the normal myocardium surrounding TMLR channel remnants occurs (12), with vascular density increasing to approximately twice that of normal. Although these findings indicated that vessels are growing in the myocardium surrounding the treated region, questions remained as to whether this increased vascularity persists over longer periods, whether and to what degree such vessels could mediate an increase in blood flow and how myocardial ischemia might impact on this process.

Canine model of chronic ischemia. The present study was performed in a canine model of chronic ischemia created with the use of an ameroid constrictor (which gradually closes within 2 to 3 weeks) in combination with surgical ligation of all visible epicardial collateral vessels that connect to the LAD circulation. Microsphere assessment of regional blood flow in the control group confirmed that we were successful in creating significant anterior wall ischemia. As has been observed previously, collateral blood flow in the acute setting was ~25% of normal under conditions at rest, but only ~12% of normal during adenosine-induced vasodilation. In the chronic setting, however, blood flow at rest increased to ~80% of normal, demonstrating the natural increase in collateral flow over time due to the angiogenesis that was stimulated by chronic ischemic conditions (21,22); this same degree of recovery of blood flow at rest has been observed by other investigators in both porcine and canine models of chronic ischemia. Concordant with this increase in blood flow at rest, evidence of endothelial and, to a lesser extent, smooth muscle proliferation (BrdU incorporation) confirmed vascular growth, including growth in capillaries and bridging collateral vessels. Nevertheless, limitations of the blood flow capacity of this collateral network were exposed when the vasculature was challenged by a vasodilator, at which point blood flow into the collateral-dependent bed decreased to ~50% that of the control region.

Effects of TMLR. TMLR significantly influenced vascular growth patterns and collateral blood flow potential in the chronic setting. Compared with the ischemic region of the control hearts, there was a significant increase in the number of vessels in the region ~3 mm beyond the edge of the TMLR channel remnants. Although we observed the growth of capillaries and new small arteries similar to those observed in the ischemic areas of the control hearts, significant growth was also observed in larger arteries. The finding that many of the smooth muscle and endothelial cells of these vessels exhibited BrdU incorporation provide corroborating evidence that these vessels had been stimulated to grow after the TMLR procedure. Evidence of the functionality of these vessels was obtained from the microsphere blood flow analysis, which revealed an almost 40% increased blood flow capacity during vasodilatory stress. These findings suggest that TMLR significantly enhances the normal compensatory development of collateral vessel development. Histologic findings corroborated that TMLR enhances the normal angiogenic process, because significant growth of large vessels in the myocardium surrounding the channel remnants was routinely observed following TMLR and rarely observed in the ischemic myocardium of the control hearts.

Our previous studies have shown that blood does not flow through TMLR channels, neither in the acute setting nor 2 weeks after the procedure (6,7). Consistent with our previous studies, the current results obtained in the acute setting showed that the presence of TMLR channels did not affect blood flow. Thus, improved blood flow was not immediate, but

rather was gradual. Although not specifically studied in the chronic setting, the relatively rare observation of vessels extending from the channel remnant to the endocardial surface renders it unlikely that the source of the increased blood flow is derived from the ventricular chamber. Rather, increases in blood flow of the magnitude observed in the present study would more likely be mediated by collateral vessels communicating with the surrounding normal myocardium. Nevertheless, direct blood flow from the chamber has not been totally excluded and may be a contributing factor.

Underlying mechanisms. The factors responsible for stimulating angiogenesis after TMLR have not been elucidated. Myocardial injury created by the laser application results in an inflammatory response that is evident within days of the procedure and persists for about 4 weeks (9,10,18). Recent studies have revealed that several growth factors and cytokines commonly liberated by inflammatory cells promote angiogenesis (e.g., vascular endothelial growth factor [VEGF] and basic fibroblast growth factor [bFGF]) and up-regulates receptors for these factors. The general features of such a response are likely to not be unique to laser injury but would be expected with any type of injury affecting a similar amount of myocardium. These features stimulate budding and growth of small vessels from preexistent blood vessels ("true angiogenesis" [21]) and remodeling of preexistent vessels by endothelial and smooth muscle cell proliferation, leading to an increased luminal diameter. Another mechanism that may be involved is vasculogenesis, the formation of new blood vessels, which, until recently, was believed to be confined to the period of embryogenesis (21,23). However, stem cells capable of differentiating into endothelial cells have been shown to be circulating in the blood of adults and these migrate to ischemic tissue where they may assemble and form new vessels (23). Elucidation of the mechanisms underlying TMLR as they may relate to the role of myocardial injury, the subsequent inflammatory response and the induction of growth factors, may help devise schemes to optimize or even enhance the therapy.

Study limitations. Several potential limitations of the present study should be recognized. First, it is well known that the ameroid constrictor model of ischemia is not a faithful model of human ischemic heart disease in several respects. Therefore, the results may not be directly applicable to the clinical situation. Although perhaps not ideal, it must be recognized that despite these and other limitations, a great deal of useful information about myocardial angiogenesis has been obtained in this type of animals model (13,21,22). Second, we relied on the comparability of the acute flow data to indirectly substantiate that the degree of ischemia was truly comparable between the two groups of dogs. An alternative approach would have been to create a chronic ischemic model and then to randomize animals between the TMLR and control groups. That experimental paradigm would more closely mimic the clinical situation in which TMLR is applied.

Conclusions. With growing recognition that there are a large number of patients with one or more diffusely atherosclerotic epicardial coronary arteries in whom methods of direct coronary revascularization cannot be applied, there has been renewed interest in investigating alternate techniques to improve myocardial perfusion, such as TMLR. Reports of substantial and persistent reduction of angina in otherwise untreatable patients (2,3) and preliminary studies showing that over time, blood flow may increase in TMLR-treated myocardium (2,3) have stimulated a great deal of basic and clinical studies. The original idea behind the development of TMLR was to mimic the physiology of myocardial perfusion in reptiles, which derive a significant blood supply from an extensive network of large, branching channels emanating directly from the ventricular chamber. As revealed in a recent detailed study of alligator hearts (24), perfusion of a substantial amount of myocardium is possible because of the large surface area and short diffusion distance for nutrient and waste exchange created by this channel network. With increased recognition that TMLR channels do not conduct significant amounts of blood (6,7,25), that the amount of myocardium in close proximity to the channels is limited and that TMLR channels do not remain patent (6–11,18,19,26), it has become evident that TMLR does not mimic reptilian physiology and this hypothesis is falling out of favor (24,27). In its place, the angiogenesis hypothesis is gaining wider acceptance. Results of clinical studies showing that myocardial perfusion of treated regions increases over time also suggests involvement of a mechanism such as angiogenesis. Although several questions remain, the results of the present study provide the strongest, most comprehensive support to date that angiogenesis is enhanced by the TMLR procedure to an extent that may augment collateral blood flow into ischemic myocardium. This study represents a further step in the arduous task of unraveling how TMLR may provide clinical benefits. The findings of significant amounts of smooth muscle proliferation and an increased number of vessels with one or more layers of smooth muscle cells, neither of which were observed with any regularity in the control ischemic tissue, reveals a novel pattern of vascular growth induced by this form of therapy.

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