

Radio Frequency Transmyocardial Revascularization Enhances Angiogenesis and Causes Myocardial Denervation in Canine Model

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Background and Objective: Transmyocardial revascularization (TMR) relieves angina and improves exercise tolerance in patients. Angiogenesis and myocardial denervation have been proposed as factors contributing to these benefits. To test whether radio frequency transmyocardial revascularization (RF-TMR) enhances angiogenesis and causes myocardial denervation.

Study Design/Materials and Methods: RF-TMR channels were created in 12 dogs which survived up to 4 weeks. Bromodeoxyuridine was administered subcutaneously to mark proliferating cells as an assay of angiogenesis. Western blot analysis of tyrosine hydroxylase and blood pressure response to topical bradykinin were used as indices of myocardial denervation.

Results: RF-TMR increased local vascularity by an average of 50%, whereas the rate of vascular cell proliferation was tripled over that of the untreated region. Changes in mean arterial pressure with bradykinin and tyrosine hydroxylase content were significantly decreased in RF-TMR regions as compared with normal myocardium in the same hearts.

Conclusion: RF-TMR enhances angiogenesis and causes myocardial denervation in canine myocardium as with laser TMR. *Lasers Surg. Med.* 27:18–28, 2000. © 2000 Wiley-Liss, Inc.

Key words: laser; proliferating cell; RF-TMR; TMR

INTRODUCTION

Laser transmyocardial revascularization (TMR) is a new therapy for patients with refractory angina not amenable to traditional therapies [1]. Clinical studies of laser TMR have shown an average two-class reduction in angina [2] and a mild improved myocardial perfusion in the laser-treated area 3–6 months after surgery [2,3]. Experimental studies have shown that laser TMR enhances angiogenesis above that normally seen in ischemic myocardium [4,5] and that laser TMR causes myocardial denervation [6], which may partly explain the relief of angina seen clinically. Both angiogenesis and myocardial denervation

have been proposed as factors contributing to the benefits of this therapy.

Both CO₂ and holmium:YAG lasers have been used to create the TMR channels in clinical and experimental studies and the clinical effects

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and histologic findings in the chronic setting are similar with these different lasers [5,7]. Therefore, it has been hypothesised that the inflammatory response incited after myocardial laser injury results in liberation of angiogenic cytokines and growth factors. Accordingly, it has been suggested that similar effects may also be achieved with other energy sources. Radio frequency (RF) energy is one possible alternative source for this procedure. The purpose of the present study is to test whether RF-TMR has similar effects as laser TMR with regard to inducing angiogenesis and myocardial denervation, two factors that may contribute to the benefits of laser TMR.

MATERIALS AND METHODS

All animals 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 (NIH publication 85-23, revised 1985).

Surgical Procedures

Twelve adult mongrel dogs, each weighing 21.5–26.5 kg, were anaesthetised with intravenous injection of thiopental sodium (15 mg/kg) and maintained with 0.5–2.5% inhaled isoflurane. A left thoracotomy was performed through the fifth intercostal space and transmural channels were created from the epicardial surface. A hollow RF electrode probe (17mm long, CardioGenesis Co., Sunnyvale, CA) connected to an Excalibur Plus II Electrosurgical RF Generator (ConMed) was used to create the channels. The firing of the RF energy was synchronised with the R wave of a surface electrocardiogram. Each energy pulse has 200 W power and was 80 ms in duration (16 J). Channels were made with a density of approximately 1 channel/cm² in the area of left anterior descending artery (LAD), and an average of 19 (range, 18–20) channels were made in each heart. Each channel required two to five bursts to create the transmural channel. An electrocardiogram was recorded during and at least 30 seconds after creation of each channel to determine whether there were any significant arrhythmias associated with the use of RF energy to create these transmural channels. Eight of the animals were allowed to recover from anaesthesia (four were survived for 2 weeks and four were survived for 4 weeks), whereas the remaining four

animals were killed after creating RF-TMR channels to provide acute tissue samples for Western blot analysis of tyrosine hydroxylase (TH) as detailed further below. An additional three healthy dogs without RF-TMR were killed to provide control samples for the TH analysis.

According to methods of Kwong et al. [6], a functional assessment of cardiac innervation was made by recording the blood pressure responses to topical applications of bradykinin (300 µg in 150 µl saline per stimulation). Each application of bradykinin was absorbed in a 1 × 1 cm gauze. The gauze was placed on one of two epicardial locations in the LAD territory: (1) anterior base (AB), or (2) midwall of the LAD (which is the area to receive RF-TMR treatment). Each application was for 2 minutes and at least 10 minutes was provided between different applications. Changes in mean arterial pressure (MAP) were recorded in response to these bradykinin applications.

To assess vascular cell proliferation after the surgery, 5-brom-2'-deoxyuridine (BrdU, 15 mg/kg, Sigma, St. Louis, MO) was administered subcutaneously on postoperative days 2, 4, 6, 8, 10, and 14 in four dogs that were survived for 4 weeks. Cardiac echocardiography was performed in these animals at baseline and 3 weeks after surgery to determine whether there was any detriment to normal cardiac function as a consequence of the treatment. At the time of terminal studies, dogs were anaesthetised, mechanically ventilated, and a thoracotomy was performed. Stimulation of cardiac visceral afferent nerves by using bradykinin was tested again by using the same procedures described above. Animals were killed (pentobarbital, 100 mg/kg) and the heart was explanted and cut into blocks from the various regions. Three LAD and two circumflex myocardial samples were submitted for immunohistochemical analysis in 4 weeks survival animals and samples for Western blot analysis were submitted from the 2-week survival animals.

Tissue Fixation and Preparation

Myocardial samples destined for histologic evaluations were fixed in 10% neutral buffered formalin overnight and routinely dehydrated and embedded in paraffin. Serial sections, 4–5 microns thick, were cut and stained with Masson's trichrome procedure to evaluate the general morphology of the lased and nonlased myocardium. Sister sections were stained by using standard immunohistochemical techniques with antibodies against BrdU; PC10 proliferating cell nuclear an-

tigen (PCNA); and alpha smooth muscle actin (SMA).

Assessment of Histology Samples

To quantify the degree of vascularity or proliferating vascular cell, three LAD and two circumflex myocardial samples in each animals were submitted for this analysis. Each samples was taken from the mid-myocardium, sliced horizontally between epicardium and endocardium. We determined vascular density (number of vessels with at least one layer of smooth muscle per cm^2) and proliferating vascular cell density (number of BrdU-positive vascular smooth muscle or endothelial cells per cm^2 and number of PCNA-positive smooth muscle or endothelial cells per cm^2) in different areas of each heart. Excluded from this quantification were vessels and cells within the channel remnants and in the vessels connected the remnant. We also analysed two different regions in the vicinity of the RF-TMR channels; the normal myocardium immediately surrounding the channel remnants (LAD_I , 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) and in the region neighbouring the channel remnant (LAD_N , 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). These oval shapes were chosen to match the generally elliptical shape of the channel remnants. Analysis was performed by an observer in a manner blind as to whether the samples came from a heart treated or not treated.

Western Blot Analysis

Biochemical evidence of changes in cardiac innervation was provided by immunoblot analysis to evaluate tissue TH content. More than 500 mg of myocardium was excised from the treated region (LAD territory) and nontreated region from animals in the acute study, from 2 weeks survival animals and from healthy animals without RF-TMR. TH was detected by autoradiograph film by using ECL detection reagents (Detection reagent 1 and reagent 2, Amersham Life Science).

Statistical Analysis

All data are presented as mean \pm SD. The statistical significance of differences among groups was determined by analysis of variance with Fisher post hoc test. A P value < 0.05 was considered to be significant.

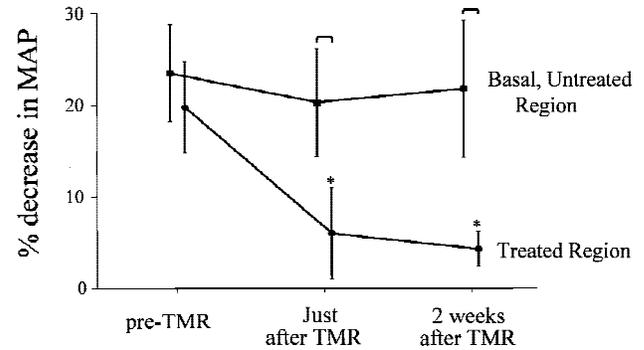


Fig. 1. Percentage decrease in mean arterial pressure (MAP) in response to topical stimulation of bradykinin shows that there is no change in the basal, untreated region, whereas in the left anterior descending artery (LAD)-treated region the value decreases just (1 hour) after RF-TMR and decreases in 2 weeks after RF-TMR. TMR, transmymocardial revascularization with radio frequency system. Each bar graph represents mean value \pm SD. $P < 0.05$ by analysis of variance with Fisher post hoc test; asterisks indicate $P < 0.05$ vs. pre-TMR.

RESULTS

Creation of RF-TMR channels by using the ECG-triggered system was performed without incident. Specifically, 81% of the channels had no arrhythmia during the 30-second period after their creation, whereas 11% exhibited a single premature ventricular contraction. All animals survived the intended period, and there was no occurrence of ventricular fibrillation after RF-TMR.

Response to Topical Bradykinin

Before RF-TMR treatment, mean arterial pressure (MAP) decreased by approximately 20% in response to topical bradykinin placed over the LAD distributions of the anterior basal and free wall regions of the heart (Fig. 1). One hour after treatment, this response was blunted in the treatment area, whereas it was preserved in the basal (untreated) region. This differential response was also observed 2 weeks after treatment.

Western Blot Analysis

In healthy hearts, TH was nearly evenly distributed throughout the heart (including little epicardial-to-endocardial variations), with the exception of the apex, which seemed to have less TH (Fig. 2A). TH content was significantly decreased in the RF-TMR region of the heart but was preserved in untreated regions (Fig. 2B). To quantitatively compare observations in the different regions, TH band intensities of the mid-anterior wall of LAD (treated) and other regions (anterior

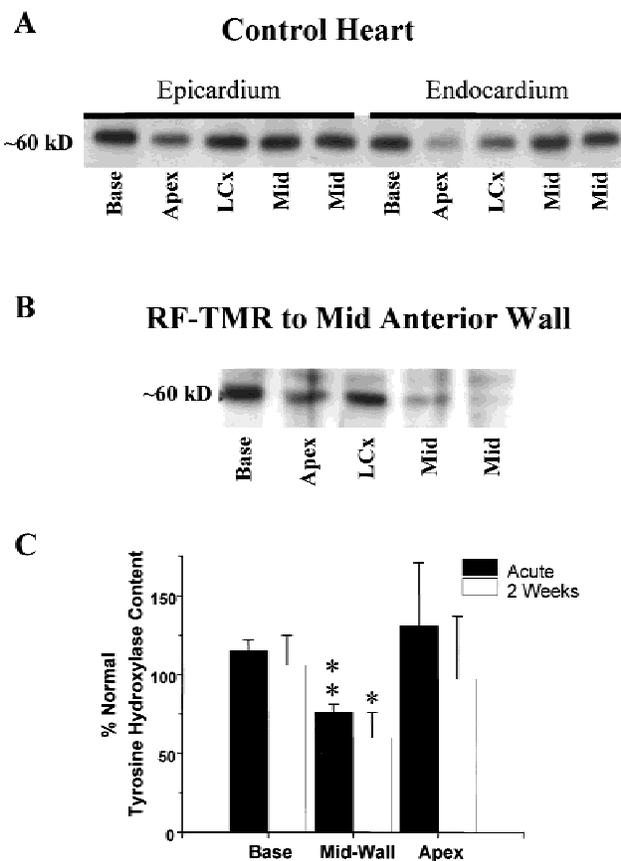


Fig. 2. **A:** A representative Western blot taken from a healthy dog. There is a relatively equal distribution of tyrosine hydroxylase (TH) in the different regions of the heart, with slightly less content in the apical region, as well as a relatively equal distribution in the epicardial and endocardial regions. **B:** A representative Western blot from an animal that received radio frequency transmural revascularization (RF-TMR) 2 weeks before killing. TH content was decreased in the treated mid-wall region (Mid), however, was preserved at both the base and apical regions. **C:** The graph summarizes the findings from all of the hearts. Relative contents of tissue TH are expressed as a percentage of content in the untreated circumflex territory. TH content in the untreated anterior base and apex of the heart did not change from normal, whereas TH content in the mid-wall of the left anterior descending artery territory was reduced just (1 hour) after RF-TMR (Acute) and continues to be low in 2 weeks after RF-TMR. Mid, treated with RF-TMR; LCx, nontreated with RF-TMR, left circumflex artery region; Base, anterior base. Each bar graph represents mean value \pm SD. $P < 0.05$ by analysis of variance with Fisher post hoc test: asterisks indicate $P < 0.05$ vs. Base; double asterisks indicate $P < 0.05$ vs. Base and Apex.

base and apex, untreated) were normalized to those of the distant left circumflex (LCx) region. As shown Figure 2C, TH content was significantly decreased in the treated region (LAD) compared with the nontreated regions (basal and apex region) in both acute and 2-week survival group.

Histologic and Immunohistochemical Findings

Histologic samples were prepared from tissue obtained from animal in the 4-week survival group. All RF-TMR channels were infiltrated with granulation tissue, which included a large amount of vascularity and BrdU-positive cells (typical trichrome stained examples of channel remnants cut transversely and longitudinally shown in Figure 3A and C, respectively). We have previously referred to these fibrotic regions as channel remnants. The channel remnants were generally elliptical and were longer in their fibre than cross-fibre direction. The average dimensions were 3.3 ± 0.7 mm long by 0.9 ± 0.3 mm wide with an average estimated channel remnant area of 2.1 ± 0.9 mm². Within the remnants, SMA staining revealed a high degree of vascularity (Fig. 3B,D, which show sections adjacent to those of panels A and C, respectively). Immunostained samples showed a high degree of BrdU incorporation not only in the interstitial spaces, but also in the endothelial and smooth muscle cells of vessels within the channel remnant (examples shown in Fig. 3E,F).

Surrounding the remnant, SMA staining revealed numerous vessels emerging from the channel remnants (Fig. 4A). BrdU incorporation was also detected not only in these vessels (Fig. 4B) but also in relatively large vessels clearly separate from the remnant (Fig. 4C,D). In addition, many of the smaller vessels and capillaries in myocardium surrounding the remnant had BrdU-positive cells (Fig. 4E,F). The number of PCNA-positive staining nuclei was much lower than that of BrdU-positive cells; nevertheless, some vessels did have PCNA-positive cell (not shown). For comparison, tissue samples obtained from the remote, nontreated myocardium in the LCx territory of each of these same hearts were also examined. SMA staining (not shown) revealed the normal relatively low density of arterial structures with one or more layers of smooth muscle. As shown in Figure 5, BrdU incorporation into smooth muscle or endothelial cells of vessels was very rare (e.g., Fig. 5A,B). Furthermore, even in the capillaries (Fig. 5C,D) there were rare BrdU-positive cells compared with the treated region (Fig. 4E,F). Similarly, there was rare PCNA-positive cells in the control sample.

We determined vascular density and proliferating vascular cell density in different areas of each heart. The results of this analysis are summarised in Figure 6. The vascular density in the

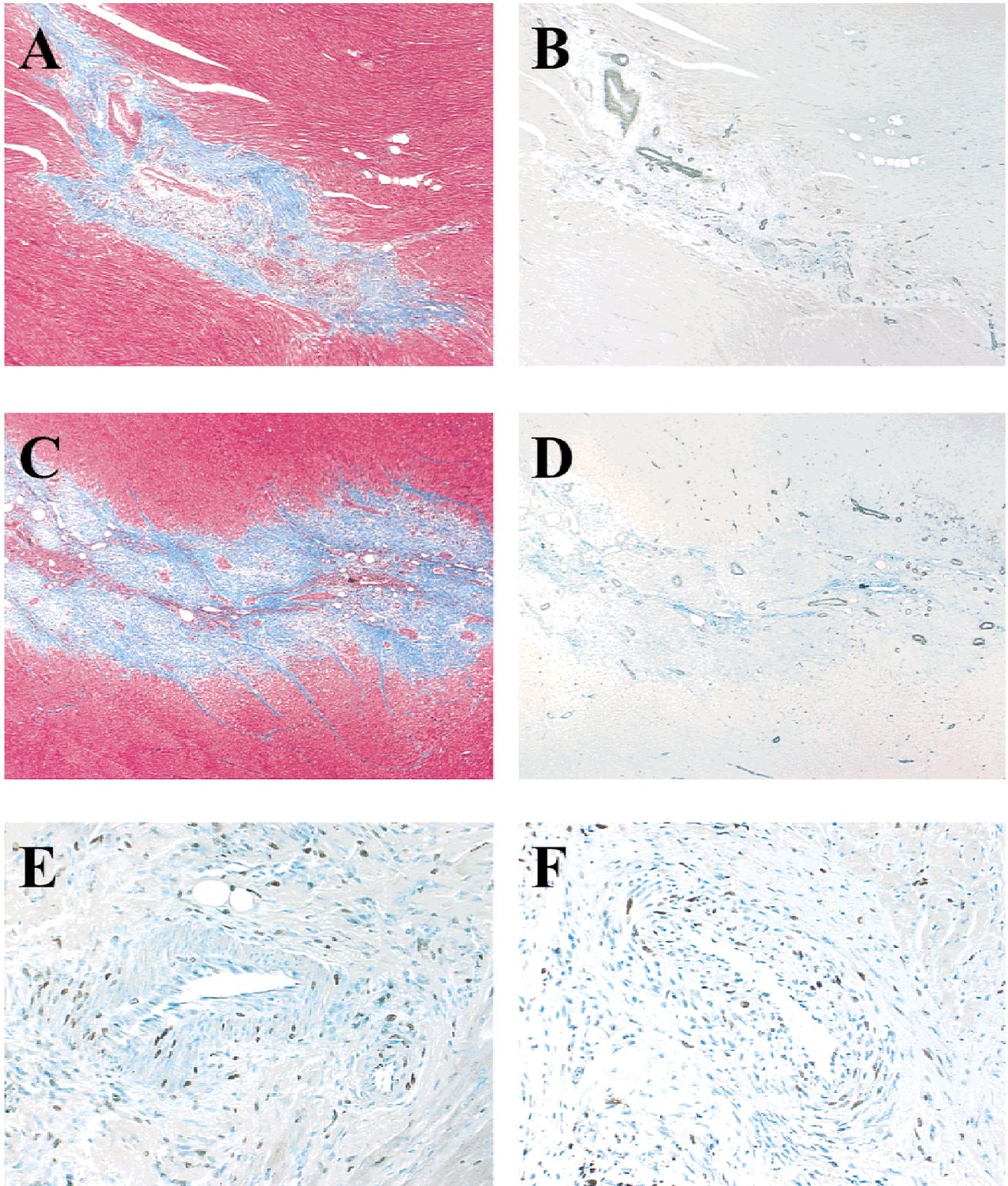


Fig. 3. Histologic appearance of channel remnants 4 weeks after radio frequency transmural revascularization (RF-TMR). **A,B,E:** Sections of a transverse cut through a channel remnant. **C,D,F:** Sections of a longitudinal cut. Trichrome stains (A,C: original magnification, $\times 20$) show the area to be infiltrated with granulation tissue. Smooth muscle actin immunostaining (B,D: original magnification, $\times 20$) highlights vessels within the remnants and shows that these are many and rather large vessels. Examples of bromodeoxyuridine incorporation into nuclei of smooth muscle and endothelial cells of vessels in the remnants (E,F: original magnification, $\times 100$) indicate that these vessels have undergone growth after RF-TMR. Scale bar = 1 mm in A-D; 0.2 mm in E,F.

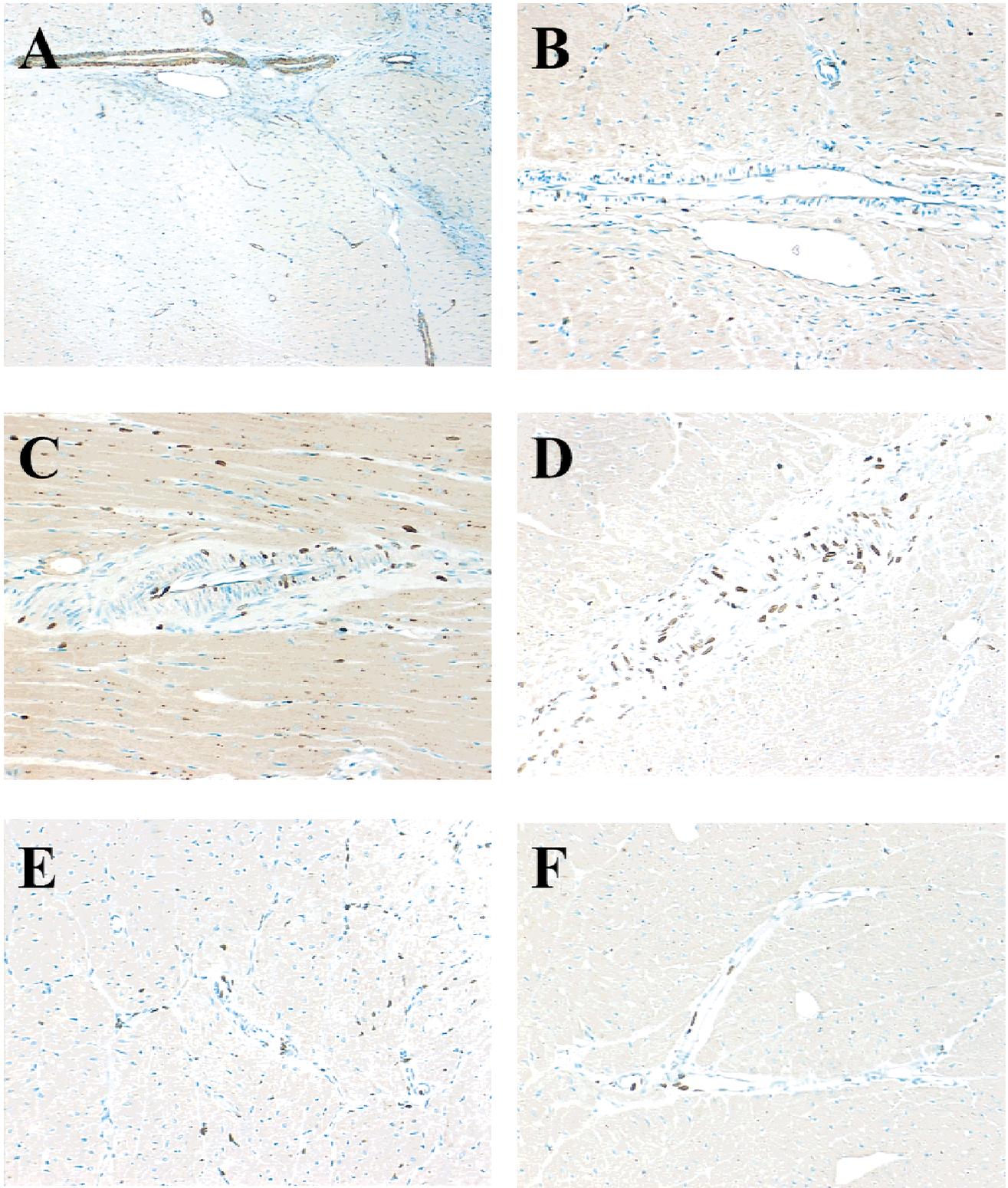


Fig. 4 Histologic appearance of myocardium surrounding the remnants 4 weeks after radio frequency transmural revascularization (RF-TMR). **A:** Smooth muscle actin stain (original magnification, $\times 40$) shows that this vessel is extending from the remnant. **B:** Bromodeoxyuridine (BrdU) incorporation in this vessel (original magnification, $\times 100$) indicates that this vessel has undergone growth. BrdU incorporation into rather large vessels are observed not only in the remnant or the region connected to the remnant such as panel B but also in the myocardium far from the remnant (**C,D:** original magnification, $\times 100$). **E,F:** Furthermore, there is heavy incorporation of BrdU into the capillary, which will grow into the vessels (original magnification, $\times 100$). Scale bar = 0.5 mm in A; 0.2 mm in B-F.

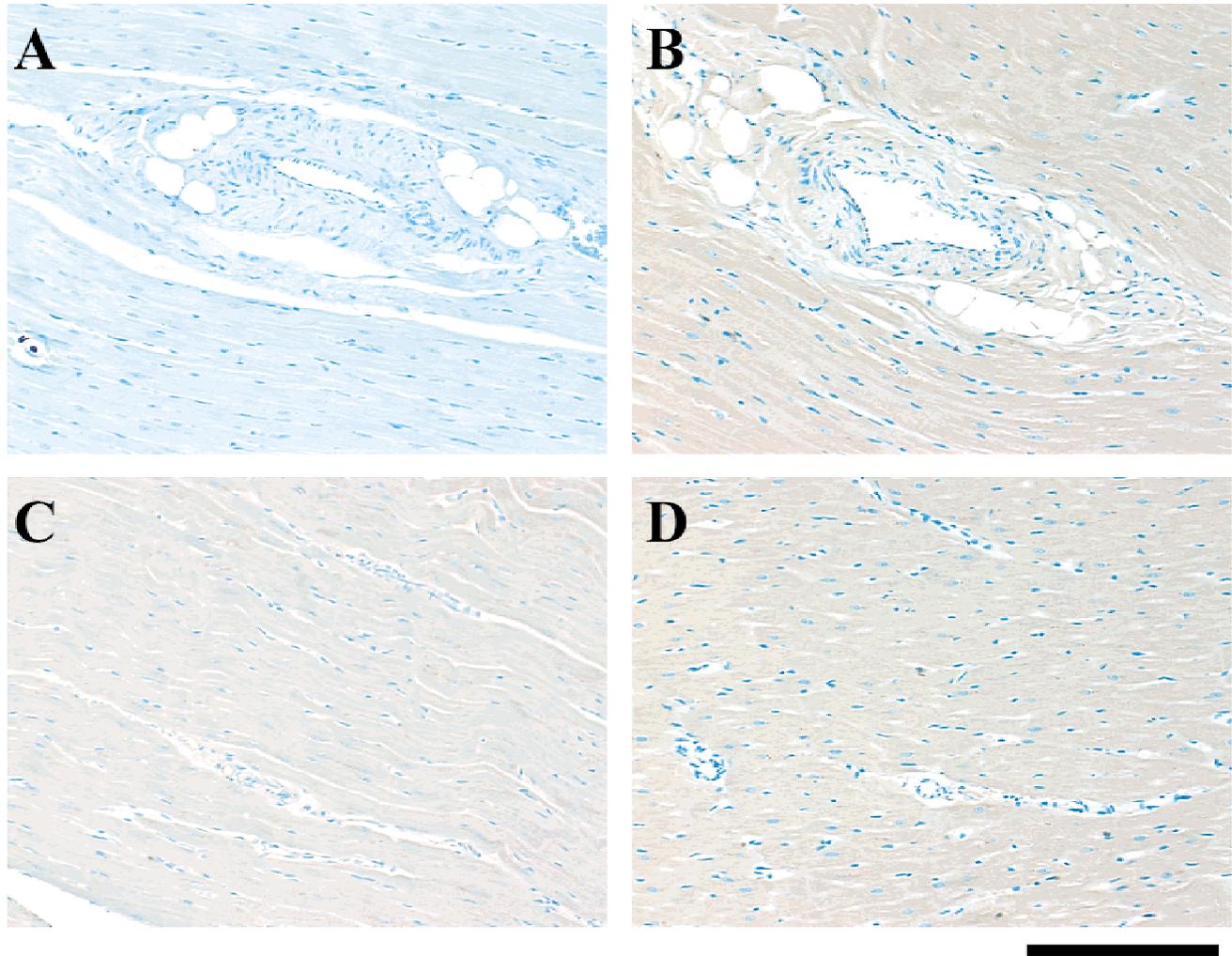


Fig. 5. Histologic appearance of myocardium in the nontreated left circumflex region 4 weeks after radio frequency transmural myocardial revascularization. All panels are bromodeoxyuridine (BrdU)-stained samples, and the original magnification is $\times 100$. **A,B**: BrdU incorporation into the vessels is rarely observed even into the relatively large vessels. **C,D**: Also, there is a little incorporation of BrdU into the capillary. Scale bar = 0.2 mm for all panels.

myocardium immediately surrounding the channel remnants (LAD_I) was significantly higher than that of the LCx (nontreated) territory. The number of PCNA-positive staining nuclei was increased in the LAD_I region and even in the region neighbouring the channel remnant (LAD_N) compared with LCx territory. Similarly, BrdU incorporation was significantly increased in the LAD_I and LAD_N regions compared with LCx territory.

Cardiac Echocardiography

Cardiac echocardiographic findings demonstrated that global contractile function and wall motion of the treated region was not reduced 3 weeks after RF-TMR surgery. Fractional shortening averaged $33 \pm 3\%$ before RF-TMR and $30 \pm 2\%$

3 weeks after the RF-TMR procedure. No regional wall motion abnormalities were noted.

DISCUSSION

TMR by using laser energy has been intensively investigated over the past 5 years in clinical and basic studies [2–10]. Despite lack of definitive proof of the underlying mechanisms, continued interest is fueled by the consistent finding among several clinical trials that TMR provides significant symptomatic relief in patients with severe, medically refractory angina who are not candidates for traditional therapies such as angioplasty or coronary bypass surgery. With regard to mechanism, a majority of experimental

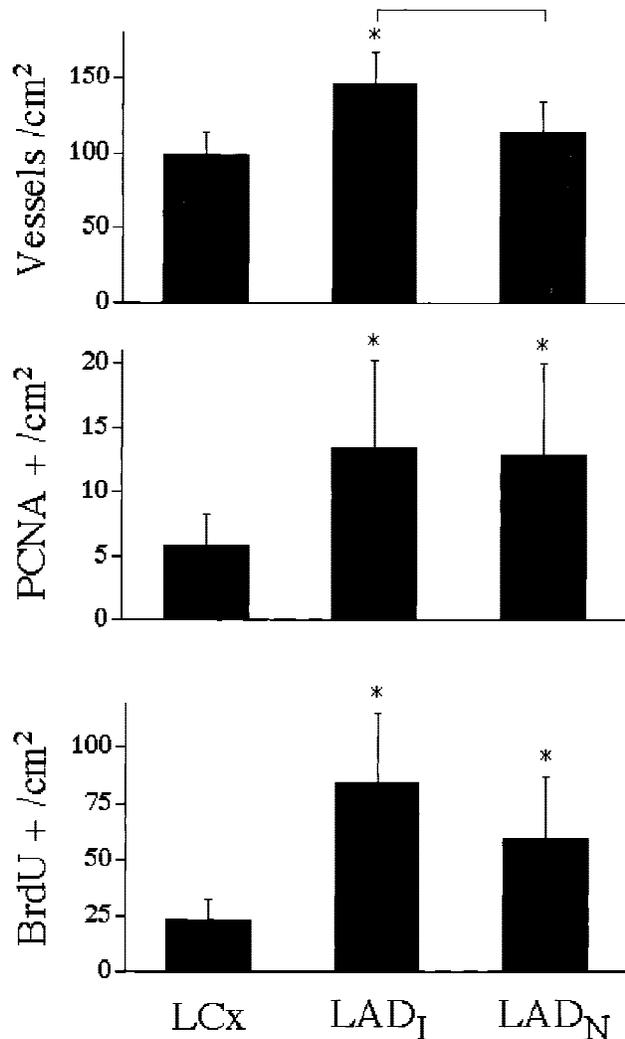


Fig. 6. Quantitative analysis of vascular density, proliferating cell nuclear antigen (PCNA), and bromodeoxyuridine (BrdU)-positive cells 4 weeks after radio frequency transmural revascularization (RF-TMR). The vascular density in the myocardium immediately surrounding the channel remnants is significantly higher than that of the untreated circumflex territory. The number of PCNA-positive staining nuclei is increased in both the immediately surrounding and neighbouring region. Similarly, BrdU incorporation is significantly increased in both the immediately surrounding and neighbouring regions. LCx, data from nontreated myocardium in the left circumflex territory; LAD_I, data from treated myocardium with RF-TMR immediately surrounding the 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 treated myocardium with RF-TMR neighbouring 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 \pm SD. $P < 0.05$ by analysis of variance with Fisher post hoc test; asterisks indicate $P < 0.05$ vs. LCx.

evidence now suggests that the original concept of nutritive blood flow by means of chronically patent channels does not occur. The two potential mechanistic factors that have been most actively pursued are neovascularization and cardiac denervation. The present study provides evidence that radio frequency TMR produces tissue effects that are very similar to, if not indistinguishable from, those observed with laser energy.

Results of recent studies in healthy animal hearts [5], ischemic animal hearts [4], and in human autopsy samples [11,12] seem to be converging on the notion that on a histologic basis, the TMR laser channels are infiltrated by granulation tissue which includes a large degree of neovascularization. A typical chronic laser channel made with a holmium:YAG laser in canine myocardium obtained in a 2-week survival study is shown for comparison in Figure 7. The histologic features of RF-TMR channel remnants, both in regard to their overall appearance on trichrome-stained sections and with regard to the degree of vascularity within the remnants that is evident on SMA stained sections, are very similar to those of this typical laser channel remnant. On a quantitative basis, the area of the channel remnants is also similar between laser TMR (1.9 ± 0.2 mm², [7]) and RF-TMR (2.1 ± 0.9 mm²). Although communications between these vessels within the remnant and the ventricular chamber have been identified, proof that nutritive blood flows from the chamber into the myocardium is not yet available.

In addition to vascular growth within channel remnants, laser TMR has been shown to induce vascular growth in the surrounding normal myocardium as evidenced by increased number of vessels and by demonstrating smooth muscle and endothelial cell BrdU incorporation. These findings have been obtained with different lasers and have been postulated to result from the inflammatory response after the myocardial injury [7,11,13]. By using these same histologic criteria, we have shown in the present study that RF-TMR also induces vascular growth around the channel remnants. On a quantitative basis, the 50% increase in vascular density in normal myocardium surrounding the RF-TMR channel remnants is similar to what has been observed with laser TMR [4,5].

In addition to improving blood flow to ischemic myocardium, an alternate means of decreasing angina could be through cardiac denervation. After cardiac denervation, patients may continue to have bouts of myocardial ischemia but the sen-

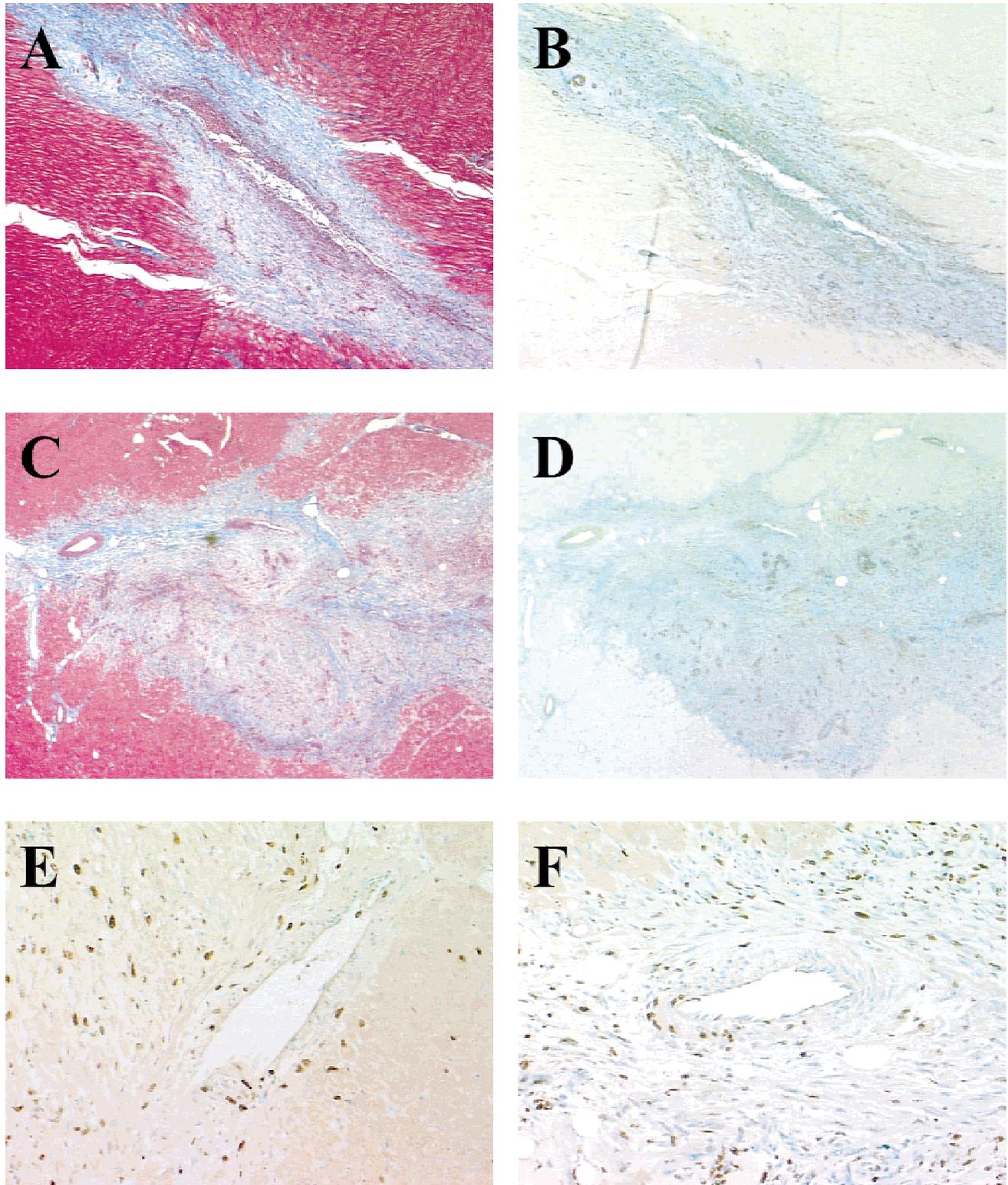


Fig. 7. Histologic appearance of channel remnants 2 weeks after transmyocardial revascularization (TMR) by using a holmium:YAG laser. **A,B,E:** Sections of a transverse cut through a channel remnant. **C,D,F:** Sections of a longitudinal cut. Trichrome stains (**A,C**, original magnification $\times 20$) show the area to be infiltrated with granulation tissue. Smooth muscle actin immunostaining (**B,D**, original magnification $\times 20$) highlights vessels within the remnants and shows that these are many and rather large vessels. Examples of bromodeoxyuridine (BrdU) incorporation into nuclei of smooth muscle and endothelial cells of vessels in the remnants (**E,F**, original magnification $\times 100$) indicate that these vessels have undergone growth after laser TMR. These findings are very similar to those of radio frequency-TMR (Fig. 3), with slightly smaller vessels in the channel remnants because of the 2-week survival animals. BrdU was administered on postoperative days 2, 4, 6, 8 and 10. Scale bar = 1 mm in **A-D**; 0.2 mm in **E,F**.

sation of angina, which is transmitted by the cardiac nerves, would be absent. There is debate as to whether cardiac denervation, though potentially decreasing patient suffering, could have detrimental consequences. The concern on the part of some investigators may stem in part from studies in the past showing increased mortality in patients with *silent ischemia*. However, controlled clinical studies, thus far, have suggested no change in mortality in patients after TMR [2,3,8]; therefore, concerns about potential deleterious consequences of denervation are being allayed. Cardiac denervation has been shown to occur in experimental animals after laser TMR [6] as evidenced by blunted blood pressure response to topical bradykinin and by decreased tissue content of TH. By using the same physiologic and biochemical evidence, we have shown in the present study that RF-TMR also causes acute and chronic cardiac denervation.

In addition to tissue effects, we also examined two important safety aspects of RF-TMR. It was demonstrated that there were no significant arrhythmias induced during or after the creation of channels when several brief (80 ms) pulses of RF energy gated to the cardiac electrocardiogram are delivered to the myocardium. Second, we showed by echocardiography that RF-TMR applied to normal myocardium did not reduce the global or regional cardiac function in the treated region.

The present study represents an initial step in the evaluation of the potential applicability of RF-TMR as an alternative to laser TMR in the clinical setting. On a histologic basis, the chronic tissue effects seem to be comparable. However, the present study has not encompassed a full physiologic evaluation of RF-TMR. Such an evaluation would include an assessment of whether RF-TMR is capable of improving blood flow to chronically ischemic myocardium. According to the results of the present study, such a full evaluation would seem to be justified.

In summary, the mechanisms by which laser TMR provides clinical benefit are not yet fully elucidated. However, evidence for two possible factors, vascular growth and cardiac denervation, has been accumulating. We have now demonstrated in normal canine myocardium that RF-TMR seems to produce similar chronic tissue effects as laser TMR with regard to these two mechanisms. Also, basic aspects of the safety of this energy source have been established. In view of the fact that RF generators are generally easier

to manufacture, need less maintenance, and may be more reliable than lasers, RF-TMR may be a viable alternative to laser TMR. In addition, because RF energy is transmitted by means of electrically conductive materials and is similar to the abrasion therapy, there may be the more advantages than lasers for implementation of a catheter-based, percutaneous RF-TMR system, especially in the aspect of avoiding the side effect such as cerebral microembolization [14], for example.

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