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Histologic evidence that basic fibroblast growth factor enhances the angiogenic effects of transmyocardial laser revascularization

Received: 18 June 1998
Returned for revision: 10 September 1998
Revision received: 2 August 1999
Accepted: 11 August 1999

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Abstract Objectives. To determine whether addition of basic fibroblast growth factor (bFGF), an angiogenic growth factor, enhances the angiogenic effects of transmyocardial laser revascularization (TMR).

Background. TMR is an investigational therapy for treating patients with medically refractory angina not amenable to traditional therapies. Histologic and blood flow studies in animals have suggested that TMR enhances angiogenesis above that normally seen in ischemic myocardium. We tested the hypothesis that bFGF administered into TMR channels further enhance the angiogenic effects of TMR.

Methods. Chronic ischemia was created in 3 groups of dogs using an ameroid constrictor on the proximal LAD. In the bFGF group (n = 5) non-transmyocardial channels were created in the LAD territory and bFGF, (100 ng/ml) dissolved in pluronic gel was injected into the each channel. In the TMR group (n = 7), transmyocardial channels were created without bFGF. A control group (n = 7) had ischemia without TMR of bFGF. 5-bromo-2'-deoxyuridine (BrdU) was administered to mark proliferating cells. After 8 weeks survival, colored microspheres were injected to assess the regional myocardial blood flow.

Results. TMR and TMR+bFGF increased total vascular density by ~ 40 % over that observed in the control group. However, the number of large vessels (internal diameter $\geq 50 \mu\text{m}$) was doubled by the addition of bFGF, and this correlated with a 50 % increase in the density of proliferating vascular cells and a tripling of the total estimated vascular cross sectional area. Blood flow to the LAD territory was increased by TMR compared to controls, with no further benefit observed in the bFGF group.

Conclusions. On a histologic basis, basic fibroblast growth factor further enhances angiogenesis following TMR in ischemic myocardium mainly by increasing the size but not the total number of vessels.

Key words Transmyocardial laser revascularization – angiogenesis – basic fibroblast growth factor (bFGF) – blood flow – ischemic heart disease

Introduction

Transmyocardial laser revascularization (TMR) is a new therapy being evaluated to treat patients with refractory angina not amenable to traditional therapies (1). Clinical studies of TMR have shown an average two-class reduction in angina (2–4). In addition, results of some nuclear flow studies have suggested that myocardial perfusion is improved to a mild degree in laser-treated areas 3-to-6 months after surgery (2, 5). Experimental results have cast doubt on the original hypothesis underlying TMR which proposed that myocardial perfusion could be achieved through chronically patent channels communicating with the LV chamber (6–10) such as achieved in reptilian hearts (11). On the other hand histologic and blood flow studies in animal models have shown that TMR enhances vascular growth (*angiogenesis*) above that normally seen in ischemic myocardium (12, 13). Although the mechanisms by which TMR enhances angiogenesis have not been identified, it has been hypothesized that the inflammatory response incited by the laser injury results in liberation of angiogenic cytokines and growth factors (14). Angiogenic growth factors such as basic fibroblast growth factor (bFGF) have also been shown to enhance angiogenesis in experimental models of ischemia when administered into the myocardium or when injected into the coronary arteries (15–17).

The purpose of the present study is to test the hypothesis that bFGF enhances TMR induced angiogenesis. An ameroid constrictor model of chronic myocardial ischemia was used in which bFGF was placed into the TMR channel.

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

Effects of bFGF in combination with TMR were studied in 5 mongrel dogs with chronic myocardial ischemia; results were compared to those previously obtained from a group of dogs with chronic ischemia alone and another group with ischemia and TMR (14). In brief, each dog was anesthetized with intravenous injection of thiopental sodium (15 mg/kg) and maintained with 0.5–2.0 % inhaled isoflurane. A left thoracotomy was performed through the fifth intercostal space using sterile technique. Non-transmural laser channels were created from

the epicardial surface over the anterior and anteroapical region of the heart supplied by the LAD. A fiber optic cable with a 1.75 mm focusing lens (CardioGenesis Co., Sunnyvale, CA) connected to a holmium: YAG laser (CardioGenesis Co., Sunnyvale, CA) was used to create the channels. The firing of the laser was synchronized with the R wave of a surface electrocardiogram, with each firing of the laser consisting of three pulses of energy (2J/pulse). Two such laser bursts were performed to create each non-transmural channel. Channels were made with a density of approximately 1 channel/cm², and an average of 15 (range 13–17) channels were made in each heart. bFGF (100 ng/ml, Scios Inc., Sunnyvale, CA) with 20 % gel (Pluronic F127NF, Poloxamer 407NF, BASF Corporation, NJ) was injected into each channel (0.15 ml/channel) and a shallow epicardial stitch (4-0 polypropylene suture) was placed to retain the bFGF inside the channel.

All visible epicardial collaterals from the circumflex and right coronary arteries were ligated with a 4-0 stitch to minimize collateral flow to the LAD territory. An ameroid constrictor was placed on the LAD proximal to the first diagonal branch. The chest was closed in layers and the animal was allowed to recover from anesthesia.

In order to assess vascular cell proliferation following the surgery, 5-bromo-2'-deoxyuridine (BrdU, 25 mg/kg, Sigma, St. Louis, MO) was administered subcutaneously on post-operative days 7, 14 and 28.

Animals were allowed to survive for 8 weeks, at which time a terminal (the second) surgery was performed. The dogs were anesthetized, mechanically ventilated, and a median sternotomy was performed. In order to measure regional myocardial blood flow, colored microspheres (15 µm diameter, ~ 3 x 10⁶ spheres/ml in a saline suspension with 0.01 % Tween 80 and thimerosal, Dye-Trak, Triton Technology, Inc. San Diego, CA) were injected into the left atrium through a left atrial catheter. A second microsphere injection was performed after inducing a vasodilatory stress by infusing adenosine into the left atrium at a dose titrated to cause a 20 % fall of mean arterial pressure. Blood microsphere content during each of these microsphere infusions was determined by withdrawing arterial blood samples from the femoral artery using a syringe pump set at a constant rate of 7 ml/min; these samples were used to calibrate tissue microsphere contents into units of absolute blood flow as detailed below.

Animals were euthanized (pentobarbital, 100 mg/kg) after these blood flow measurements and the hearts were explanted. Every ameroid constrictor was found to be completely occluded at autopsy. After euthanasia, the heart was explanted and cut into blocks from the various regions. Three LAD and two circumflex myocardial samples were submitted for immunohistochemical analysis; remaining samples were submitted for microsphere analysis.

Histologic and physiologic results obtained from these 5 animals were compared to historic controls (14). Chronic

ischemia alone was created in one group of control animals ($n = 7$) and a second group of animals had chronic ischemia plus TMR without bFGF in the anterior wall ($n = 7$). The methods employed to study these control and TMR groups were identical to those used in the present study and these historic data therefore provide an appropriate data base for comparing the effects of bFGF in combination with TMR.

Preliminary studies

In a preliminary study, pluronic gel without bFGF was injected into TMR channels to test whether the gel itself had any influence on the TMR channels. We found that there was no histologic difference between TMR alone and TMR with the pluronic gel; the results will not be discussed further.

Tissue fixation and preparation

Myocardial samples destined for histologic evaluations were fixed in 10 % neutral buffered formalin over night and routinely dehydrated and embedded in paraffin. Serial sections, 4–5 microns thick, were cut and stained with Masson's trichrome procedure or hematoxylin-eosin to evaluate the general morphology of the lased and non-lased myocardium. Sister sections were stained using standard immunohistochemical techniques with antibodies against BrdU; PC10 proliferating cell nuclear antigen (PCNA); and alpha smooth muscle actin (SMA).

Assessment of histology samples

In order to quantify the degree of vascularity or proliferating vascular cell, three LAD and two circumflex myocardial samples in each animal were submitted for this analysis. Each sample 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. We also analyzed two different regions in the vicinity of the TMR channels; the normal myocardium immediately surrounding the TMR channel remnants (contained between the edge of the channel remnant and an ellipse with minor axis 6 mm and major axis 10 mm, LAD_I) and in the region neighboring the channel remnants (with boundaries defined by the first ellipse and a second concentric ellipse with minor axis 10 mm and major axis 14 mm, LAD_N). These oval shapes were chosen to match the generally elliptical shape of the channel

remnants. Analysis was performed by an observer who was blinded to whether the samples came from a heart treated with bFGF or not.

Microspheres analysis

Retrieval and quantitative analysis of the microspheres were performed as described previously. Regional myocardial blood flow (RMBF, ml/min/g) was calculated using the following equation:

$$\text{RMBF} = F_{\text{ref}} / M_{\text{sample}} \times \text{OD}_{\text{sample}} / \text{OD}_{\text{ref}}$$

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

Statistical analysis

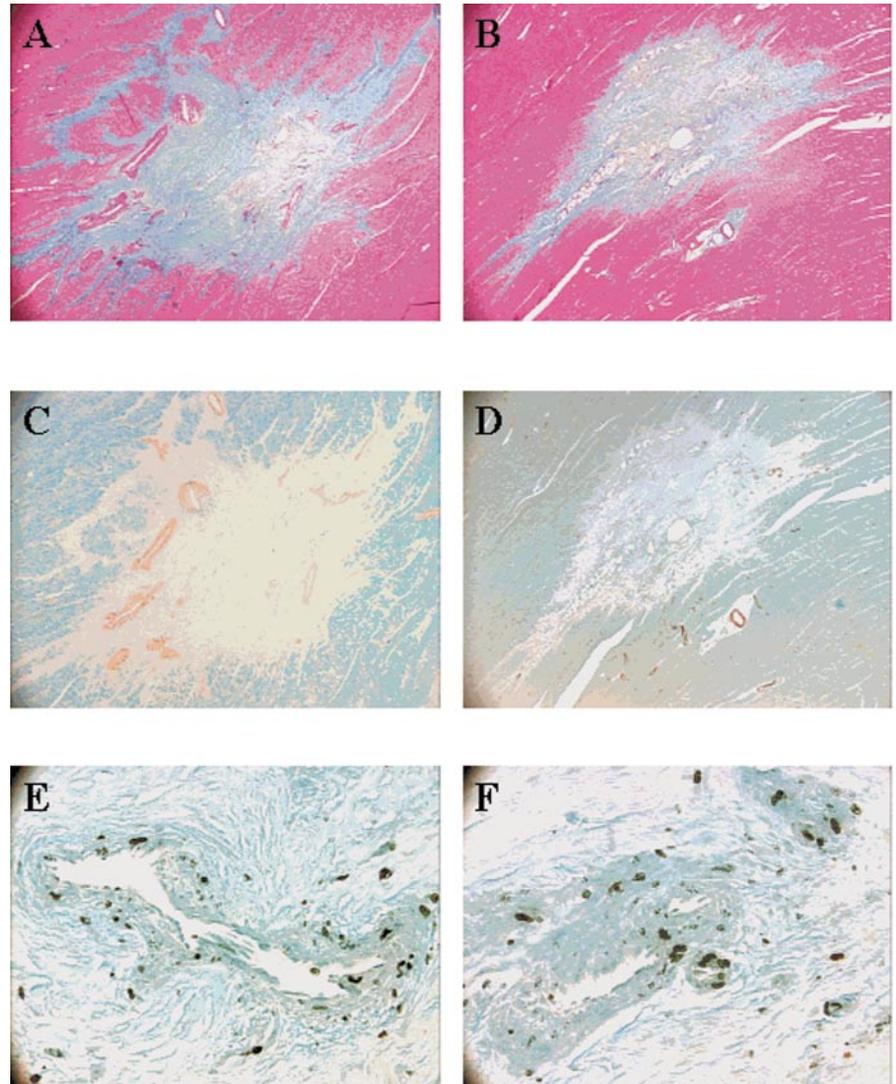
All data are presented as mean \pm SD. The statistical significance of differences between groups was determined by Student t-test or analysis of variance with a Bonferroni post-hoc test in cases when multiple groups were being compared. $p < 0.05$ was considered to be significant.

Results

Histologic and immunohistochemical findings

All TMR channels were infiltrated with granulation tissue independent of whether they did or did not have bFGF, as shown in the representative examples of Fig. 1 (A, TMR+ bFGF; B, TMR alone). We have referred to these fibrotic regions as *channel remnants*. As is typical of any granulation response, the channel remnants included significant amounts of vascularity. The channel remnants were generally elliptical and were slightly longer in the fiber direction. The average dimensions were 4.2 ± 0.5 mm in the long axis and 2.2 ± 0.6 mm in the short axis in the bFGF group versus 3.3 ± 0.7 mm by 1.3 ± 0.3 mm, respectively, in the TMR group. Accordingly, the channel remnant areas were significantly larger in the bFGF group than in the TMR alone group: 5.2 ± 1.3 mm^2 versus 2.5 ± 0.6 mm^2 ($p < 0.05$ by student t-test). This difference likely reflects increased fibroblast proliferation induced by the growth factor.

Fig. 1 Histologic appearance of channel remnants 2 months after treatment. All panels are sections of transverse cuts through the channel remnants. Trichrome stains (A, TMR+bFGF; B, TMR, original magnification 20x) show that the remnant is larger with TMR+bFGF than with TMR alone. Smooth muscle actin immunostaining (C, TMR+bFGF; D, TMR, original magnification 20x) highlights vessels within the remnants in the TMR+bFGF and TMR group. Examples of BrdU incorporation into nuclei of smooth muscle and endothelial cells of vessels in the channel remnants (E and F, TMR+bFGF, original magnification 200x) indicate that these are new vessels. Calibration bar shows 1 mm for panels A through D; 0.1 mm for E and F.

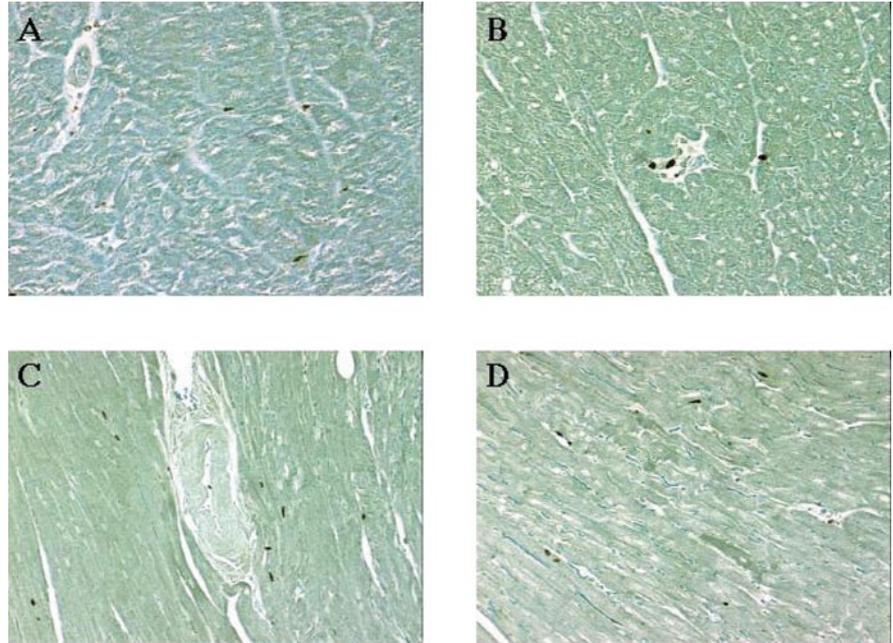


SMA staining revealed many vessels within the channel remnants of bFGF (Fig. 1C) non-bFGF (Fig. 1D) treated channels. In both cases, many of the smooth muscle and endothelial cells of these vessels within the remnants incorporated BrdU (Figs. 1E and 1F, both taken from the bFGF treated animal), indicating that these were actively growing vessels. For comparison, examples obtained from the circumflex territory of these same hearts (Fig. 2A and B) and from ischemic myocardium from the control (non-TMR) group (Fig. 2C and D) showed very rare BrdU incorporation; when present in non-treated ischemic myocardium, BrdU incorporation was observed only in capillaries but never in larger vessels. PCNA positive nuclear staining was rare in all tissues of this study (pictures not shown); all positive control (tonsil) samples showed abundant staining, confirming of the adequacy of the PCNA staining technique. This latter finding

indicates that the phase of active vascular growth revealed by the BrdU staining was completed at some time before the 2 month sacrifice time point.

In both TMR and TMR+bFGF groups, vascular density and cellular proliferation revealed by BrdU incorporation was markedly increased in the normal myocardium surrounding the channel remnants compared to the control group. However, addition of bFGF to the channels was associated with evidence of vascular proliferation in much larger arteries and at greater distances from the channel remnants. Figure 3 demonstrates large intramyocardial arteries up to 3.0 mm away from the edge of channel remnants; BrdU staining reveals a high density of positive cells in these arteries. It is rare to see vessels of this size deep in the myocardium; furthermore, we have never observed this degree of BrdU incorporation in an artery of this size in control ischemic hearts or even following TMR.

Fig. 2 Histologic appearance of myocardium in the circumflex myocardium from the TMR + bFGF group (A and B, original magnification 200x) and from ischemic LAD myocardium from the control group (C and D, original magnification 100x). Nuclear BrdU incorporation was rare in both cases and, when present, was observed only in small vessels and capillaries. Calibration bar shows 0.1 mm for panels A and B; 0.2 mm for C and D.



In addition to the increased number of larger vessels, we also observed other effects of bFGF. In some channels, there was extensive fibrosis and intense apparently unorganized vascular proliferation as shown in the examples of Fig. 4A and B. In other examples, we sometimes observed highly organized stimulation of conduit vessel growth. Figure 4C shows an example of such a large conduit vessel within the core of

the channel remnant. This vessel, shown in higher magnification in panel D, is seen reaching the edge of the channel remnant near the endocardial surface of the heart (seen at the bottom of panel C).

In order to compare, quantitatively, observations in the different treatment groups, we determined vascular density and proliferating vascular cell density in different areas of each

Fig. 3 Histologic appearance of myocardium surrounding the channel remnants in TMR+bFGF group. A high density of nuclear BrdU incorporation are observed in large arteries in the normal myocardium surrounding the TMR+bFGF injection sites. Original magnification 40x in A, 100x in B through D. Calibration bar shows 0.5 mm for panels A; 0.2 mm for B through D.

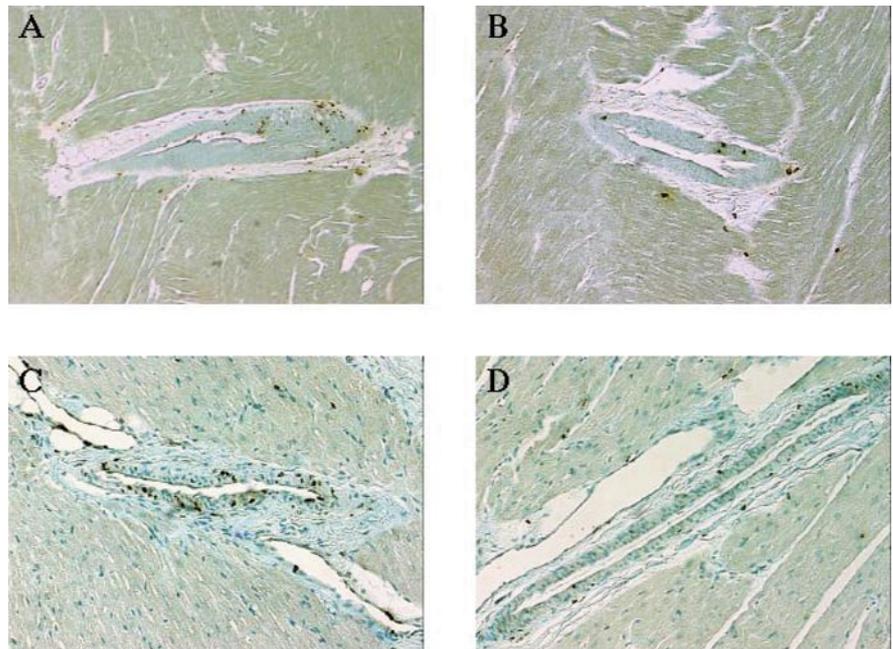
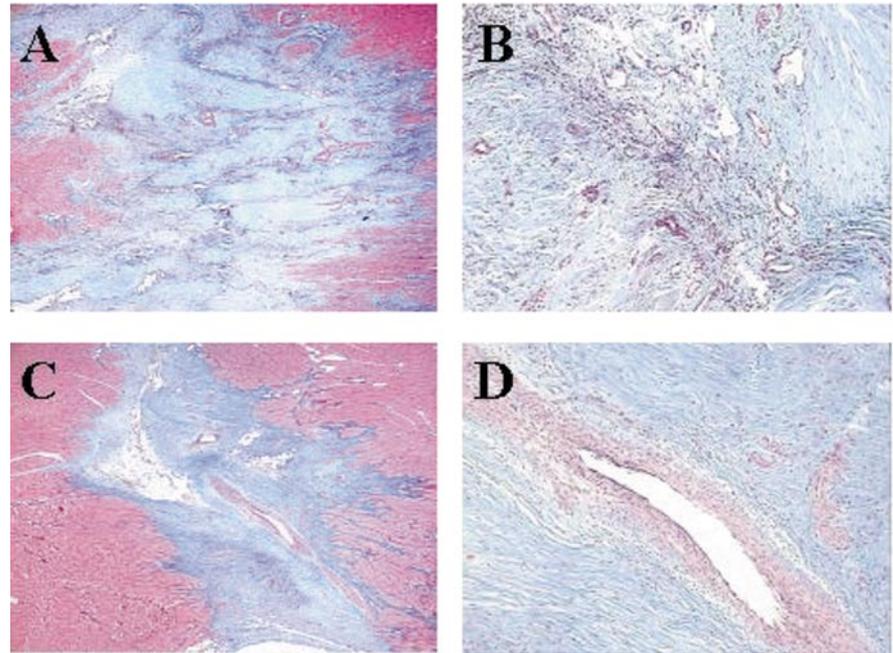


Fig. 4 Histologic appearance of channel remnants in TMR+bFGF group 2 months after treatment. Extensive fibrosis and intense, apparently unorganized vascular proliferation was observed in some channel remnants (A and B). In other examples, very large mature vessels were observed traversing the channel remnant (C and D) with much less unorganized angiogenesis. Trichrome stained samples. Original magnification 40x in A, 100x in B, 20x in C and 200x in D. Calibration bar shows 1.25 mm for panel A; 0.5 mm for B; 2.5 mm for C and 0.25 mm for D.



heart. The results are summarized in Table 1. Vascular density in the normal myocardium immediately surrounding the TMR channel remnants (LAD_1) and in the region neighboring the channel remnants (LAD_N) were comparable in the TMR and TMR+bFGF groups. These were each greater than the vascular

density in the LAD territory of the ischemic control group and of the non-treated, non-ischemic circumflex region in each group. However, the number of BrdU positive vascular cells in the LAD_1 area in TMR+bFGF group was ~ 6 times greater than that in controls and about 50 % greater than that in the TMR group ($p < 0.05$ for each comparison). In the LAD_N area, BrdU positivity was ~ 4 times greater in the TMR+bFGF

Table 1 Histologic measurement

	Vessels in SMA staining	BrdU positive cells	PCNA positive cells
TMR+bFGF group			
LAD_1	144±31*§	70±12*†#§	5.3±2.0
LAD_N	122±26	47±7*#§	2.4±2.2
LCx territory	87±10	8.4±2.2	2.8±2.3
TMR group			
LAD_1	136±26*§	44±13*†§	7.3±2.0*†§
LAD_N	113±19*	17±7	2.7±1.4
LCx territory	77±14	6.0±1.4	2.1±1.6
Control group			
LAD territory	99±18	1.2±0.6	1.7±1.2
LCx territory	82±16	4.9±3.0	1.3±0.7

Unit, /cm²; TMR, transmural laser revascularization; LAD_1 , immediately surrounding the channel remnants in the area of left anterior descending artery; LAD_N , neighboring the channel remnants in the area of left anterior descending artery; LCx, left circumflex artery.

* $P < 0.05$ vs LCx territory, † $P < 0.05$ vs LAD_N , # $P < 0.05$ vs TMR, § $P < 0.05$ vs Control.

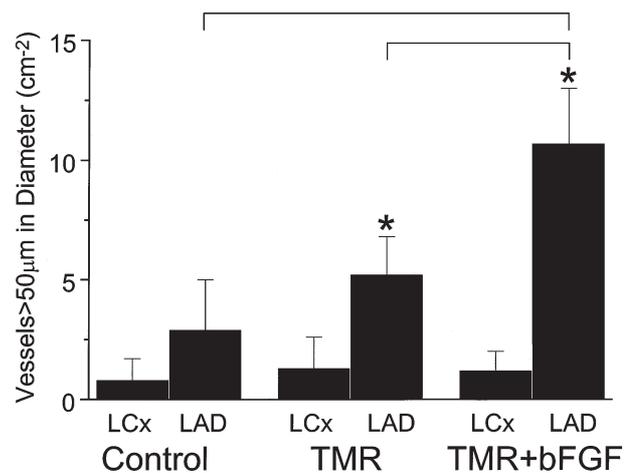


Fig. 5 The density of large vessels exhibiting more than one BrdU positive cell whose shortest diameter is more than 50 μm in the normal myocardium surrounding the channel remnants. LCx, data from non-ischemic, normally perfused left circumflex territory; LAD, data from ischemic anterior wall of treated heart. $p < 0.05$ by ANOVA with Bonferroni post hoc test. (* $p < 0.05$ vs LCx).

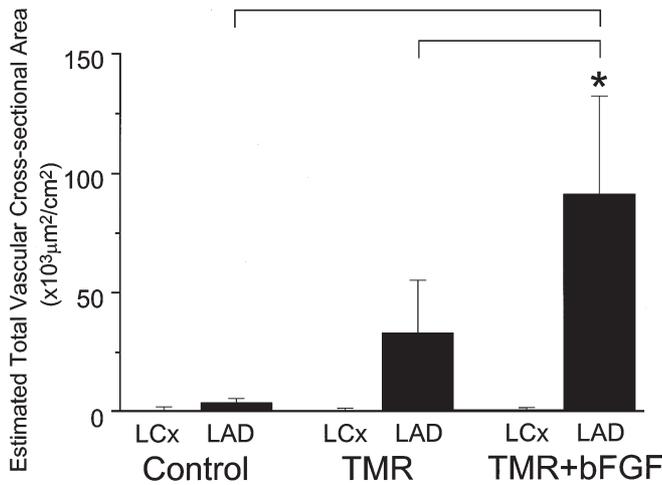


Fig. 6 Estimated total vascular cross sectional area of proliferating vessels in the ischemic territory. LCx, data from non-ischemic, normally perfused left circumflex territory; LAD, data from ischemic anterior wall. $p < 0.05$ by ANOVA with Bonferroni post hoc test. (* $p < 0.05$ vs LCx).

group than that in the control group and ~ 3 times greater than that in the TMR group.

The density of large vessels exhibiting BrdU positive cells whose internal diameter (measured in the shortest direction) was at least 50 μm in the area surrounding the channel remnants (LAD_I and LAD_N) was twice as great in the TMR+bFGF group as in the TMR group and this was about 3 times greater than in the ischemic territory of the control hearts (Fig. 5).

Table 2 Regional myocardial blood flow (ml/min) and ratios

	Resting	Stress
TMR+bFGF group		
LCx territory	0.82±0.11	1.19±0.74
LAD territory	0.67±0.07	0.84±0.37
LAD/LCx (flow rate)	0.83±0.11	0.77±0.19*
TMR group		
LCx territory	1.14±0.49	1.67±0.87
LAD territory	0.99±0.41	1.21±0.60
LAD/LCx (flow rate)	0.90±0.14	0.73±0.08*
Control group		
LCx territory	0.98±0.36	2.05±1.30
LAD territory	0.76±0.30	0.96±0.39
LAD/LCx (flow rate)	0.77±0.16	0.53±0.16

Flow unit, ml/min/g; TMR, transmural laser revascularization; LCx, left circumflex artery; LAD, left anterior descending artery.

* $P < 0.05$ vs Control.

† We use the term patent to signify a channel which retains its original diameter at the time it was created.

In addition, we estimated the total vascular cross sectional area of proliferating vessels [i.e., $\sum \pi (1/2D)^2$ of all vessels with BrdU incorporation] in the LAD_I and LAD_N regions. This index was approximately 3 times greater in the TMR+bFGF group than in the TMR group and about 20 times greater than in the ischemic territory of the control hearts (Fig. 6).

Regional myocardial blood flow

Coronary blood flow measurements in the ischemic LAD territory measured at rest and during vasodilatory stress measured 2 months after surgery in each group are summarized in Table 2; values are normalized to blood flow measured in the non-ischemic circumflex territory. Resting blood flow to the ischemic territory was comparable in the three groups. Vasodilatory stress accentuated the ischemia in all groups, but revealed that blood flow potential was significantly increased by TMR or bFGF. However, addition of bFGF did not statistically significantly increase blood flow during vasodilatory stress compared to TMR alone.

Discussion

The results of the present study show that addition of bFGF enhances the angiogenic process induced by TMR in ischemic myocardium on a histologic basis. Although the total density of smooth muscle lined vessels is not increased, bFGF increased the number of larger vessels and, consequently, markedly increased the next cross-sectional area of proliferating vessels. Furthermore, bFGF also increased the distance from the treatment site over which proliferation was observed. The area of fibrosis comprising the channel remnants was larger when bFGF was added, thereby reflecting fibroblast proliferation.

Most clinical studies have consistently shown that TMR is effective in relieving angina in patients with otherwise untreatable disease (1–3). Results of some clinical studies have shown that myocardial blood flow can be increased in the treated region (2, 5), but this has not been a consistent finding (3). It has been suggested that different lasers may result in channels which have different blood flow conducting capacities. However, results of our previous studies have suggested that different lasers create nearly indistinguishable long-term myocardial effects, and in all cases, the channels do not remain patent† and do not conduct blood from the chamber (18). On the other hand, we have previously shown that TMR increases vascular density and active smooth muscle and endothelial proliferation in normal and ischemic canine myocardium (12). As summarized above, we have also shown that TMR can

increase blood flow in ischemic myocardium during vasodilatory stress.

In addition to apparently beneficial effects of bFGF, we also observed a few examples where bFGF induced unorganized angiogenesis and excessive fibrosis. It is unlikely that vascular growth of this nature could provide nutritive blood flow to myocardium. Angiographically, it is possible that these regions would appear as the 'blushes' of contrast agent but may not reflect nutritive blood flow potential.

There is currently a great deal of interest in the use of angiogenic growth factors such as bFGF and vascular endothelial growth factor (VEGF) in treating ischemic heart disease (16, 19–22). The clinical effects of combining TMR with growth factors may be synergistic. TMR appears effective in relieving symptoms but at best, incompletely restores myocardial blood flow in patients with severe coronary artery disease. Supplemental growth factors may ultimately enhance the angiogenic process enough to beneficially impact on blood flow.

We obtained histologic evidence indicating that bFGF enhanced angiogenic response following TMR. However, we did not observe a statistically significant increase in blood flow when bFGF was added to TMR channels over that observed due to TMR alone. In order to substantially increase nutritive blood flow in response to an angiogenic process, new conduit vessels need to connect with vessels in neighboring regions with more normal blood flow. It may be that the spacing between injection sites, the distance of the injection site to a region of normal perfusion, the dose of bFGF, and other factors may have to be optimized in order to observe a substantial increase in nutritive blood flow.

Another important point concerning blood flow relates to the fact that in the TMR plus growth factor group, the TMR channels were non-transmural and did not communicate with the LV chamber. Yet, we observed increases in blood flow which were slightly greater than what was observed in the TMR alone group. The original idea underlying TMR was to create a chronically patent pathway for blood to flow from the ventricular chamber directly into the myocardium. Our previous studies have indicated that blood does not flow through TMR channels, even in the acute setting (6, 7). Furthermore, we recognized early in the course of our research that the channels did not remain patent (6, 7, 9, 10, 18). Accordingly, we did not believe that making non-transmural channels or filling the channels with a pluronic gel would interfere with the ability of the blood flow to increase in response to TMR.

In conclusion, bFGF enhances the angiogenic response induced by TMR. It will be of interest to determine whether different growth factors have similar effects. Strategies for optimizing and controlling the process will result in means of enhancing blood flow while minimizing secondary effects such as increased fibrosis. In addition, it will be important to determine whether growth factors used as sole therapy in the clinical setting will be as effective or more effective than TMR or TMR in combination with growth factors. The principle tests will be the determination of which form of therapy provides the greatest relief of symptoms, the greatest increase in blood flow, and the strongest impact on survival.

Acknowledgments This study was supported by a research grant from CardioGenesis Corp. The authors are grateful to Scios Inc., Sunnyvale for supplying bFGF.

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