Only transient increase of vascular growth factors and microvascular density after percutaneous myocardial laser
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Objective We tested the hypothesis that percutaneous myocardial laser may stimulate microvascular growth in areas surrounding the laser channels.

Methods We conducted a study of 24 domestic pigs, which underwent percutaneous myocardial laser to left ventricular myocardium using holmium:YAG laser. The pigs were sacrificed in groups of four after one day, 3–4 days, one week, three weeks and six weeks. Frozen sections from both normal and treated myocardium were prepared for immunofluorescence microscopy and stained with antibodies against von Willebrand factor, vascular endothelial growth factor (VEGF) and Extra Domain-A cellular fibronectin (ED-AcFN). Microvascular density (MVD) and vascular area (VA) were determined in sections stained with antibodies against von Willebrand factor VIII using a digitised image analysis system. When determined in laser treated areas, channel core remnants were excluded from analysis.

Results Within the laser channel remnants and in the tissue closely surrounding these, expression of VEGF and ED-AcFN increased significantly after treatment at one, 3–4, and seven days and decreased to normal at three and six weeks. Expression of ED-AcFN was detected adjacent to endothelial cells of microvessels. The original laser channels were rapidly invaded by granulation tissue. There was no sign of recanalization at any stage during the six weeks. Morphometric analysis showed no increase in MVD and VA in the myocardium surrounding the laser channels.

Conclusion An increase of VEGF and ED-AcFN after myocardial laser is transient and is not associated with increase of MVD or VA in myocardium not involving laser channel remnants. Coron Artery Dis 15:441–448 © 2004 Lippincott Williams & Wilkins.

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Introduction
The increasing number of patients with refractory angina and coronary artery disease not amenable to traditional methods of revascularization has led to the development of new therapeutic approaches. Current data indicate that transmyocardial laser revascularization (TMR) via thoracotomy or the more recent percutaneous myocardial laser revascularization may provide these patients with improvements in angina symptoms and there is some evidence also of improved myocardial perfusion [1–6]. The mechanisms by which these techniques reduce anginal symptoms are not fully understood. Two blinded clinical studies using different laser sources have excluded [7] or not excluded [8] a placebo effect as the only mechanism. Conflicting results have also been reported for a denervation effect [9,10]. The initial hypothesis that myocardial perfusion should be established via direct connections from the left ventricular cavity to myocardial sinusoids through patent channels [11,12] has not been completely refuted [13–19], although sequential temporal studies were not performed. Previous studies have shown that transmyocardial revascularization using either laser or mechanical puncture was associated with significantly elevated vascular endothelial growth factor (VEGF) expression and microvascular densities in treated areas [20,21]. It is well known that during the inflammatory and proliferating phases of wound healing, there is a significant up-regulation of several growth factors in order to promote angiogenesis. Growth factors promote the proliferation of endothelial cells engaged in angiogenesis and induce the formation of capillary-like tubes of microvascular endothelial cells [21]. Extra Domain-A cellular fibronectin (ED-AcFN) is a high molecular weight glycoprotein, which serves as a bridge between cells and interstitial collagen meshwork, and influences diverse processes including cell growth, adhesion, migration and wound repair [22]. Extra Domain-A cellular fibronectin, when

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found adjacent to endothelial cells, could therefore be a useful marker of vascular growth processes after myocardial laser [23]. However, increases of growth factors and blood vessels within the scar tissue of laser channel remnants may not contribute meaningfully to myocardial perfusion.

In this study we assessed the maximum and time-related expression of VEGF and ED-AcFN in treated and non-treated myocardium after percutaneous myocardial Holmium:YAG laser. Microvascular density was quantitated in the myocardium surrounding the laser channel remnants and compared with non-treated areas.

Methods

Animals

Twenty-four healthy domestic pigs of either sex, weighing 40 kg on average were included in the study. All animals were transported to the laboratory at least one week prior to inclusion in the study. They were fed on a standard diet and kept under controlled environmental conditions. The local ethical committee for animal care and use at our hospital approved the study protocol, and the study was performed according to the guidelines on the care of laboratory animals and their use for scientific purposes. Animals were randomised to six equal groups (four animals in each group) according to the time of sacrifice after percutaneous myocardial laser. The first group was sacrificed one day after percutaneous laser, the second after three days, the third after four days, the fourth after seven days, the fifth after three weeks and the last group was sacrificed six weeks after the procedure.

Percutaneous myocardial laser

Acetyl salicylic acid 300 mg orally was administered in the morning before the procedure. Sedation was induced by a mixture of ketamine (10 mg/kg), methomeline (0.1 mg/kg) and atropine (1 mg). General anaesthesia was achieved by a gas stream of 5% isoflurane (Forene, Abbot) in 40% oxygen and 60% nitrous oxide. The depth of anaesthesia was evaluated in each animal and electrodes were placed over the chest for continuous ECG monitoring. Ketamine and diazepam administered intravenously supplemented anaesthesia. Haemodynamic parameters were continuously evaluated through the entire procedure and post-procedure. The right femoral artery was palpated manually and 5 ml xylocain was injected subcutaneously for infiltration anaesthesia. A 5 cm incision was done and the femoral artery was exposed via a 5 cm incision and canulated with a 9 Fr sheath. A bolus of heparin 100 μ/kg was injected and arterial blood pressure was monitored invasively during the procedure. A 6 Fr pigtail catheter was then advanced via the sheath under fluoroscopic screening to the left ventricular cavity. Left ventriculography was performed in two orthogonal views (RAO 30° and LAO 60°). Two transparent films were fixed over the monitors and the left ventricular wall was traced in both views to determine the target wall and to guide proper positioning of laser fibre to obtain 1 cm distance between the laser channels. The pigtail catheter was then exchanged for a laser-guiding catheter (CardioGenesis Corporation, Foothill Ranch, CA, USA) over a 0.038 inch curved coated extra stiff guide wire shaped to conform the left ventricular wall. The guide wire was then removed and an Axcis™ PMR™ System (CardioGenesis Corporation, Foothill Ranch, CA, USA) was advanced through the guiding catheter into the left ventricular cavity under fluoroscopy. The fibre-optic laser catheter is approximately 6 Fr with a 1.75 mm focusing lens at its tip. Both the laser catheter and the fibre were connected to an infusion system with heparinized saline to flush the entire system during the procedure. The catheter and fibre were aligned together, advanced to the apex and deflected against the anterior wall (target wall). Laser fibre was connected to the cardiogenesis laser system which was calibrated and adjusted to deliver one pulse of two joules of Holmium:YAG laser (pulse duration 250 microsecond, wave length 2.14 μm) for each laser channel at late systole with a density of 1 channel/cm and a total of 10 channels/animal. After the procedure the whole system was removed, the femoral artery was repaired, and the incision closed. Anaesthesia was then reversed and the animals allowed to recover. Analgesics were given post-procedure.

Sample fixation and processing for immunohistochemistry

On the day of animal sacrifice, median sternotomy was done under general anaesthesia. The pericardium was incised and the heart isolated carefully. Animals were sacrificed by injecting 20 ml potassium chloride. Vessels were clamped and transected. The hearts was incubated in saline solution and cut into 5–10 mm cross-sectional slices from laser treated areas in addition to slices from the normal myocardium obtained from the right coronary territory. All the slices were embedded in Tissue-Tek II compound, shock-frozen in liquid nitrogen-chilled isopentane and stored at – 80°C. Cryostat sections (10 μm thick) were chemically stabilized in a 3.7% neutral buffered formaldehyde solution, made permeable with Triton X-100, incubated in a 0.2 M glycine solution and incubated first with normal serum for 30 minutes and thereafter with primary antibodies. For the visualization of capillary vessels an anti-endothelial polyclonal antibody, the von Willebrand factor VIII (DAKO A/S, Denmark; Code No. A0082) was used for one hour at room temperature. Sections were thereafter incubated in fluoresceine isothiocyanate (FITC)-F(ab′)2 fragment conjugated sheep anti-rabbit IgG (Boeheringer, Mannheim, Germany). The number of microvessels were counted in six separate optical fields (each of
Statistical analysis
Data are presented as mean ± standard deviation (SD). We used one-way analysis of variance (ANOVA) to test for the differences between the six groups. Subsequent comparisons were made by the use of Tukey’s test. Statistical differences were considered significant with two-sided p-value < 0.05. Analysis was done by use of SPSS version 10.0.

Results

Channel patency
The original channel region became invaded by granulation tissue that included fibrosis and a large amount of vascularity starting at day one. The result of the laser impact on myocardial tissue was a highly localized tissue removal. None of these channels had a widely patent form, and only channel remnants were detected. There was no sign of recanalization of channels at any interval up to six weeks.

Microvascular density
Endothelial cells in the biopsies showed a strong staining for von Willebrand factor VIII (Fig. 1) that enabled the morphometric analysis in the digitised images. Quantification of the total MVD showed a non-significant increase of microvascular growth at day 3–4 after laser, compared to the normal myocardium (217 ± 33.3 no/a versus 210.7 ± 66.4 no/a) (p > 0.05). At six weeks, no significant increase of capillaries had been detected (Fig. 2). Although we selected the sections with a high degree of accuracy as regards the direction of blood vessels, capillaries were not completely running in a parallel form but at a mutual angle with each other. This resulted in a shift in the estimated vascular area between the stages, which was not attributed to the difference in the vascular cross section. To avoid this interference, we limited the roundness factor to between 1 (full circle) and 1.5. Repeated measurements after that showed a mean roundness between 1.27 ± 0.01 and 1.29 ± 0.001 in all stages. With this modulation we excluded the longitudinally oriented profiles. The total number of the registered capillaries decreased in relation to the total MVD. However, this resulted in a more homogenous material and more correct selection as regards the vessel diameter. Under this condition the mean vascular area (VA) of all stages was between 21.9 ± 4.6 μm² and 27.3 ± 2.9 μm² (Fig. 3) and the mean values of MVD became homogenous through all stages.

Angiogenic growth factors
All tissue components of the normal myocardium lacked immunoreactivity to ED-AcFN and VEGF with the exception of the endothelia of some of the larger blood vessels (arterioles). The granulation tissue of laser wound sites was filled with ED-AcFN (Fig. 4) immunoreactive materials at three days and seven days. After seven days a number of ED-AcFN immunoreactive endothelial vessel profiles were detected. The extent of VEGF labelling was variable, and in many tissue sections confined to the outer areas of the channel remnants towards the sub-endocardial layers (Fig. 5). Expression of VEGF was found in the extracellular matrix of the area immediately surrounding the channel remnant and in the neighbouring area on the von Willebrand factor stained sections. Only vascular structures cut in cross sections were submitted for quantitative analysis. Vessels within the core of the channel remnants or within fibrous tissue related to the channel remnants were excluded. Microscopic images of each area were recorded by a video camera and digitised by a computer equipped with an image analysis system. Quantification on the digital images was realized by using a QuantaWin Colour (Cambridge, UK) software. Single endothelial cells or clusters of endothelial cells positive for von Willebrand factor were considered individual vessels. The total count of microvessels per optical field was defined as total microvascular density. The size of the counted vessels was restricted to between 1 μm² and 70 μm² and the roundness of vascular profiles to between 1 (full circle) and 1.5. The number of countable microvessels per field was defined as microvascular density (MVD). In each of the microvessels the outline was traced and the cytoplasmic area was separately measured and finally added to total vascular area, the mean of which was defined as vascular area (VA). Myocardial capillaries have diameters between 3 μm and 10 μm. However, we found that 90% of vessel profiles had internal diameter less than 5 μm, which corresponds to vascular area of less than 70 μm². This last mentioned vascular area was considered as the upper limit for small and medium sized capillaries. To avoid detection of megakaryocytes and thrombocytes, we considered 1 μm² vascular area as the lowest limit. Immune reaction with anti-alpha-actin showed that blood vessels which had one or more layers of smooth muscle, represented only 1.5% of the total number of the quantified vessels. Analysis of 432 optical fields from 72 biopsies entered the final statistical analysis.

For double labelling of vascular endothelium and ED-AcFN, sections were immunolabelled for von Willebrand Factor VIII as described above and were then exposed to monoclonal mouse anti-c-FN, MabDH1 (Sigma), followed by incubation in rhodamine-conjugated rabbit anti-mouse IgG (DAKO A/S). For labelling of VEGF, sections were exposed to polyclonal anti-VEGF (147): sc-507 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by incubation in FITC-F(ab’)2 fragment conjugated sheep anti-rabbit IgG (Boehringer, Mannheim, Germany).

Omitting the specific primary antibodies or substituting them with non-immune serum constituted the control sections.
surrounding the channel remnant and in the neighbouring area at one day (11559 ± 7320 μm² versus 230 ± 749 μm², p = 0.02), 3–4 days (7570 ± 7486 μm² versus 230 ± 749 μm², p = 0.04), and seven days (21671 ± 4455 μm² versus 230 ± 749 μm², p < 0.0001), compared to the normal myocardium (Fig. 6). The immunoreactivity material of these areas was localized...
predominantly on cardiac myocytes and on the endothelia of microvessels. However there was no observed immunoreactivity to ED-AcFN and VEGF in the sections at three weeks and six weeks after laser. The distribution of ED-AcFN and VEGF had therefore subsided to normal during the interval of one week and three weeks.

**Discussion**

We have recently suggested that the symptomatic effect of myocardial laser is not due to placebo. Channel patency has been discussed as one of the mechanisms behind myocardial laser. Mirhoseini and colleagues [24] suggested that transmyocardial laser has specific physical characteristics promoting long-term channel patency. Several studies have demonstrated channel patency weeks to months after they were made both in animals [18] and humans [19] TMR studies. However, several recent histological studies revealed that laser channels did not remain patent shortly after laser [13–17]. Our study is the first to assess channel patency in a sequential temporal manner. There was no sign of channel patency or recanalization between one day and six weeks after percutaneous laser.

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**Fig. 4**

Double immunostained section from pig myocardium three days after PMLR. The section was stained with monoclonal antibodies against ED-AcFN conjugated with rhodamine and with polyclonal antibodies against von Willebrand Factor VIII conjugated with FITC. Remnants of the laser channel (upper right) show strong expression of ED-AcFN (red colour), which extends as septa into the intact myocardium (lower left). Note several von Willebrand Factor VIII positive microvessels surrounded by rhodamine staining, indicating formation of new vessels. The diameter of some of the vessels within the laser channel remnants are larger than in the myocardial tissue. Bar = 100 μm.

**Fig. 5**

Representative image of normal pig ventricular myocardium (A), and of PMLR-treated ventricular myocardium (B), illustrating the increased number of small vessels with VEGF-immunostained endothelium four days after laser treatment. Using digitised images, FITC-stained areas in six separate optical fields inside, as well as surrounding the channel remnants, were selected to give quantitative measures of VEGF expression (Fig. 6). Bar = 100 μm.
Many experimental studies have revealed histological evidence of neovascularization (angiogenesis) and up-regulation of angiogenic growth factors in laser treated areas that may play a role in increasing myocardial perfusion to non-perfused ischaemic areas [14,25–29]. However, it is not clear whether these new vessels persist for a long time and are able to supply the ischaemic myocardium with sufficient nutrition. The results of the present study show that percutaneous myocardial laser did not stimulate the microvascular growth in the areas surrounding the channel remnants. The maximal number of microvessels was achieved at 3–4 days after laser, however it was not a statistically significant change. Furthermore, there was no evidence of any microvascular growth at three and six weeks. We also found transient up-regulation of both ED-AcFN and VEGF in the lased area at seven days, which returned back to normal levels in the sections obtained at three and six weeks after myocardial laser.

Results of the previous studies in this field are conflicting. Whittaker et al., [30] studied myocardial channels induced by either laser or needle puncture in normal rat myocardium after allowing the animals to survive for two months. After that time acute ischaemia was induced to assess the effects of the treatment procedures. Needle channels introduced some myocardial protection during acute ischaemia; this was not seen after laser. However, they did not find any evidence of increased capillary density in either type. In contrast, Pelletier et al., [21] revealed significant growth of new blood vessels and up-regulation of growth factors in a model of myocardial ischaemia with peak growth at one week after mechanical puncture. They did not report specifically on microvascular growth within or outside areas of scar tissue. Kohmoto et al., [31] found a two-fold increase of vascular growth as early as two weeks after TMR in normal canine myocardium. Furthermore, Yamamoto et al., [32] and Chu et al., [33] have shown significant vascular growth in areas treated with either TMR or mechanical puncture in models of chronic myocardial ischaemia induced by implantation of an ameroid constrictor to the coronary arteries.

A difference between our and Whittaker’s findings and those of Kohmoto’s is that the latter did not quantify capillary density but selectively measured the density of arteries and arterioles. Furthermore, our alpha actin studies showed marked growth of blood vessels with one or more layers of smooth muscles inside the laser channels as early as 3–7 days after myocardial laser. It is possible that these vessels inside the channels extended to the surrounding areas, which gave the picture of increased vascular density in the above-mentioned studies [30–33]. This was avoided in our study by the exclusion of the channel related fibrotic areas. Some of the studies had short follow-up, and therefore only may have detected the first transient rise of growth factors. The conflicting results of growth factors or microvascular growth is probably not caused by any difference between TMR and percutaneous myocardial laser, or between CO₂ and Holmium:YAG lasers—this is neither supported by any difference in improvement of angina in the clinical studies [3–7] nor experimentally [27].

Less than 2% of the vessels included in our assessment of microvascular density were arterioles. Neither the total number of microvascular density changed over time nor was there a relative shift of the percentage of arterioles or capillaries at any time interval during the six weeks. Thus there was no sign of development of some important forms of increased vascularity, such as budding and growth of small vessels from pre-existent vessel, the formation of new vessel from stem cells, or remodelling of pre-existing vessel by endothelial and smooth muscle proliferation. However, our study may not have excluded development of collaterals. Increased capillary density reduces vascular resistance and enhances blood flow, but collateral development is probably more important as it carries a bulk of blood to the ischaemic area [34]. One previous study showed an increased blood flow capacity during stress two months after TMR [32].

It is likely that the multifunctional glycoprotein ED-AcFN plays an important role in promoting cell adhesion, proliferation, and migration [22]. We do not know why it
showed a similar temporal rise as VEGF. It is tempting to speculate that like VEGF it has a function for promoting growth of vasculature. It could also be a result of the general cellular changes seen with laser injury and inflammation but such processes are likely to go on beyond the temporal rise that we detected for VEGF and ED-AcFN in our study. To our knowledge we are the first to report on ED-AcFN after myocardial laser.

Study limitations
The absence of myocardial ischaemia in our model is a limitation factor similar to several of the above-mentioned positive or negative studies. Hypoxia stimulates the up-regulation of hypoxia-inducible factor (HIF-1) and certain proteins that promote the up-regulation of vascular endothelium growth factor (VEGF), bind to VEGF mRNA and prevent its degradation, thereby possibly prolonging the increment of VEGF [35]. This could promote microvessel proliferation but would also increase inflammation and thereby increase fibrosis around the original channels. Furthermore, the ameroid constrictor model is by far not a perfect mimic of human ischaemic heart disease. A possible limitation is that myocardium distant from the target area was assumed to be normal. It is known that for instance, myocardial infarction results in an increase in collagen content in regions away from the heart.

Conclusion
Percutaneous myocardial laser induced only transient increase of vascular growth factors and microvascular density in myocardial tissue surrounding laser channels.

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References
32 Yamamoto N, Kohmoto T, Gu A, DeRosa C, Smith CR, Burkhoff D. Angiogenesis is enhanced in ischemic canine myocardium by...

