Role of MMPs and plasminogen activators in angiogenesis after transmyocardial laser revascularization in dogs

WEI LI, KUNIYOSHI TANAKA, YUKIO CHIBA, TETSUYA KIMURA, KOUICHI MORIOKA, TAKAHIKO USESAKA, AKIO IHAYA, MASATO SASAKI, TAKESHI TSUDA, AND NARIHISA YAMADA. Role of MMPs and plasminogen activators in angiogenesis after transmyocardial laser revascularization in dogs. Am J Physiol Heart Circ Physiol 284: H23–H30, 2003. First published September 5, 2002; 10.1152/ajpheart.00240.2002.—We examined the role of matrix metalloproteinases (MMPs), tissue inhibitors of MMP (TIMPs), and plasminogen activator (PA) in transmyocardial laser revascularization (TMLR)-induced angiogenesis. TMLR was accomplished with a carbon dioxide laser in seven dogs whose left anterior descending coronary artery (LAD) was ligated. Seven control dogs underwent only LAD ligation, and four dogs underwent a sham operation, consisting only of a left thoracotomy. Two weeks later, transmural myocardial samples were harvested from the distributions of the LAD and the left circumflex artery for substrate zymography, immunohistochemical staining, and in situ zymography. MMP-1, MMP-2, TIMP-1, TIMP-2, and urokinase-type PA levels in the distribution of the LAD were higher in the laser group than in the control or sham group. Counts of von Willebrand factor-positive microvessels and smooth muscle α-actin-positive arterioles demonstrated that the angiogenesis and arteriogenesis was promoted in the laser group and correlated directly with the number of MMP-stained microvessels. We conclude that TMLR induces the expression of MMPs, TIMPs, and urokinase-type PA and that these proteinases play an important role in angiogenesis after TMLR.

matrix metalloproteinase

transmyocardial laser revascularization (TMLR) has been shown to relieve angina in patients refractory to medical therapy who are not candidates for coronary artery bypass graft surgery or percutaneous transluminal coronary angioplasty. Although the clinical effectiveness of TMLR has been documented, the underlying mechanisms responsible for its success is not clear. The original premise on which transmyocardial revascularization was established depended on its ability to create channels that would carry blood directly from the ventricle into the ischemic myocardium. This theory, however, has not been substantiated, so other mechanisms have been postulated. One of the newly proposed mechanisms is that TMLR promotes angiogenesis (30), and a growing volume of experimental evidence suggests that TMLR induces the expression of vascular endothelial growth factor and platelet-derived endothelial cell growth factor (16, 19a). Both of these factors promote angiogenic activity.

The capacity of extracellular matrix macromolecules to affect angiogenesis and the formation of new microvessels from existing blood vessels has been well documented (17, 18). Destruction and compositional changes in the extracellular matrix are the key events in angiogenesis, and they depend on the actions of a range of proteases and their inhibitors (23). Evidence suggests that there are two systems that predominate and interact to achieve homeostasis within the vessel wall: the plasminogen activator (PA)-plasmin system and the matrix metalloproteinase (MMP) system (7). MMPs are a family of zinc-dependent endopeptidases that function in the turnover of extracellular matrix components. It has been shown that the secretion of MMPs by microvascular endothelial cells is an essential early step in the formation of new blood vessels (12). Interstitial collagenase (MMP-1), 72-kDa gelatinase A/type IV collagenase (MMP-2), and 92-kDa gelatinase B/type IV collagenase (MMP-9) dissolve the extracellular matrix and may initiate and promote angiogenesis (25). We designed this study to elucidate the changes in MMPs, their inhibitors (tissue inhibitors of MMP (TIMPs)), and PAs in a canine model of acute myocardial infarction. Revascularization was stimulated by carbon dioxide laser irradiation after left anterior descending coronary artery (LAD) occlusion.

METHODS

All procedures used in this experiment were approved by the Institutional Animal Care and Use Committee at Fukui Medical University and conform to the guidelines set by the American Physiological Society.

Experimental animal model. Eighteen adult beagle dogs without gender distinction (10.2–14.3 kg) were randomly assigned to one of three groups before the operation: the...
sham group (n = 4) underwent only thoracotomy, the control group (n = 7) underwent only LAD ligation, and the laser group (n = 7) underwent TMLR 1 h after LAD ligation. Dogs were anesthetized with pentobarbital sodium (25 mg/kg), intubated, and ventilated with room air. Under sterile conditions, the heart was exposed, and the LAD was ligated just distal to the first diagonal branch. In the laser group, 1-mm-diameter transmural channels were created in the beating heart with a carbon dioxide laser (Sharplan Lasers; Allendale, NJ) at 55 W with 0.3-s pulses. An average of 10 channels (1 channel/cm²; range, 8–11) were created in the region supplied by the LAD distal to the point of ligation. Dogs received routine postoperative care after the surgical procedure. Cefazolin sodium (25 mg/kg) was administered twice daily for 5 days, and pain medication was given as needed.

Sample procurement. Two weeks after the initial operation, animals were anesthetized and mechanically ventilated as before. A medium sternotomy was performed, and the hearts were isolated by careful dissection of adhesions. Animals were euthanized with an overdose of pentobarbital sodium. Hearts were removed immediately and washed with cold phosphate-buffered saline. The left ventricle was cut open along the interventricular septum. The areas of scar on the epicardial and endocardial surfaces were drawn on tracing paper, and the tracings were photographed and scanned under standard conditions. The size of the infarction was calculated using NIH Image (Program NIH; Bethesda, MD). The thickness of the infarcted region was measured at five points (four at the border between scar and normal myocardium and one in the center of the scar). The average was calculated and used as the wall thickness. An ~0.5-g transmural sample was cut from the center of the area of infarction and from the distribution of the left circumflex coronary artery (LCx). Myocardial tissue extraction was performed as reported previously (19a). The protein concentrations were determined using a standardized colorimetric assay, the Bio-Rad protein assay (2). Aliquots were stored at −80°C until use.

Gelatin zymography. Five micrograms of each sample were loaded onto a 7.5% SDS-polyacrylamide gel containing 1 mg/ml gelatin, electrophoresed, renatured, and developed as previously described (19a). A densitograph (ATTO Densitograph 4.0; Tokyo, Japan) was used to determine activity levels. A protein marker and control MMPs (Sigma-Aldrich; St. Louis, MO) were electrophoresed in parallel for molecular weight identification.

Plasminogen/casein zymography. Activities of PAs were measured using a 10% SDS-polyacrylamide gel containing 0.8 mg/ml casein (Research Organics; Cleveland, OH) and 20 μg of human plasminogen (Haemalogic Technologies; Essex Junction, VT) under nonreducing conditions. Extracts were diluted to a final protein concentration of 250 μg/ml, mixed with SDS sample buffer (pH 7.6, vol/vol, 1: 1.25; 50 mM Tris-HCl, 2% SDS, and 10% glycerol) and incubated at 37°C for 30 min. Twenty microliters of complex were loaded onto the gel. After electrophoresis, the gel was treated as described above.

In situ zymography. Frozen tissue samples from the distribution of the LAD were used for in situ zymography. Serial sections 4–5 μm thick were cut, and each section was attached to an MMP in situ Zymo-Film (Fuji Photo Film; Tokyo, Japan), which is a polyester film coated with a 7-μm layer of gelatin alone or gelatin plus 1,10-phenanthroline. The films were incubated for 14 h in a humid box at 37°C, air-dried at room temperature, and stained with Biebrich scarlet (Aldrich) solution containing 0.3% Biebrich scarlet, 3% trichloroacetic acid, and 50% methanol. The nuclei were counterstained with hematoxylin.

Histological evaluation. Tissue samples from the distribution of the LAD were fixed in 4% paraformaldehyde solution, dehydrated routinely, and embedded in paraffin. Serial sections 4–5 μm thick were cut. Standard immunohistochemical techniques were used to stain the tissue with antibody against MMP-1 (anti-human MMP-1, F-67, Daiichi Fine Chemical; Toyama, Japan), MMP-2 (anti-human MMP-2, PTG1), smooth muscle (SM) α-actin (N1584, mouse monocolonial antibody; Dako), von Willebrand factor (VWF, factor VIII-related antigen, polyclonal rabbit anti-human antibody, Dako), and myeloid/histiocyte antigen (Mac 387, monoclonal mouse anti-human, Dako). Bound antibody was detected with a LSAB2 kit (Dako), and complexes were visualized with 3′-diaminobenzidine tetrahydrochloride. The overall vascular density of positively stained VWF, SM α-actin, MMP-1, and MMP-2 microvessels was quantitated in a blinded fashion by two independent observers using previously described techniques (19a).

Light Cycler-based PCR assay and relative quantification of TIMP mRNA. The total RNA was extracted from the tissue specimens from the distribution of the LAD using the acid guanidium-phenol-chloroform method. The concentration of RNA was determined using the Bio-Rad protein assay (2). Aliquots of RNA were treated with DNase I (Sigma) and used for the first strand cDNA synthesis reaction. Reverse transcriptase reactions were carried out using the SuperScript premultiplication system (GIBCO-BRL, Life Technologies) as described by the manufacturer.

The Light Cycler PCR and detection system (Roche Diagnostics; Mannheim, Germany) was used for amplification and on-line quantification. The following sense and antisense primers were used (GeneBank Accession Nos. are in parentheses): canine TIMP-1 (AB016817), nucleotides 186–205 and 575–556; canine TIMP-2 (AF188489), nucleotides 38–58 and 384–364; and canine β-actin (Z70044), nucleotides 194–213 and 519–500. A Light Cycler-FastStart DNA Master SYBR Green I kit (Roche Diagnostics) was used for the PCR protocol. Samples were run in parallel by performing 40 cycles of repeated denaturation (15 s at 95°C), annealing (5 s at 60°C), and enzymatic chain extension (15 s at 72°C).

Relative quantification was performed by on-line monitoring for identification of the exact time point at which the logarithmic linear phase could be distinguished from the background (crossing point) (20). Serially diluted samples that expressed high TIMP activity (10– to 10,000-fold dilutions) were used as external standards in each run. The 10-fold dilution concentration of target DNA in the samples was calculated by comparing the cycle numbers of the logarithmic linear phase of the samples with the cycle numbers of the external standards. The values were standardized using amounts of canine β-actin mRNA.

Statistical analysis. The unpaired t-test or Mann-Whitney U-test was used for intergroup comparisons. The Friedman test was used for the analysis among all samples from the
two groups. Fisher’s exact test was used to analyze the correlation. Values are given as means ± SE. \( P < 0.05 \) was considered significant.

RESULTS

Two dogs (one from the laser group and one from the control group) died of perioperative arrhythmia and were excluded from this study. No postoperative death occurred in any group.

Gelatinase activity. Three bands were found in the gel, representing pro-MMP-9, pro-MMP-2, and active MMP-2 (Fig. 1A). MMP activity in the distribution of the LCx was similar in the three groups. Compared with the area perfused by the LCx, the pro-MMP-2 activity was 5.8-fold higher and pro-MMP-9 activity was 10-fold higher in the laser-treated area of infarction; the pro-MMP-2 activity was 5-fold higher and pro-MMP-9 activity was 2.8-fold higher in controls, whereas pro-MMP-2 and pro-MMP-9 activities did not change in the sham group. There was no detectable active MMP-9 in any samples of myocardial extract.

Fig. 1. A: representative gelatin zymogram of myocardial protein extract. The lytic band at 60 kDa corresponds to the active form of matrix metalloproteinase (MMP)-2, the lytic band at 67 kDa is consistent with pro-MMP-2, and the lytic band at 94 kDa corresponds to pro-MMP-9. B: densitography of zymographic gels. Values are means ± SE; \( n = 6 \) for the laser and control groups and \( 4 \) for the sham group.

Active MMP-2 was detectable only in the region of infarction. The pro-MMP-2, active MMP-2, and pro-MMP-9 activities in the distribution of the LAD were higher in the laser group than in the control or sham groups (Fig. 1B).

TIMP activity. After incubation, the gelatin in the gel was degraded completely by the addition of pro-MMP-2. However, in the zones where TIMP existed, pro-MMP-2 activity was inhibited, resulting in visible dark zones. Two bands were found in the gel with molecular masses of \( \sim 31 \) and 21 kDa, representing TIMP-1 and TIMP-2, respectively (Fig. 2A). TIMP-1 and TIMP-2 were not detected in the distribution of the LCx in any group or in the LAD distribution in the sham group. The activities of TIMP-1 and TIMP-2 in the distribution of the LAD in the laser group were higher than in the control group (Fig. 2B).

Fig. 2. A: representative reverse zymography. B: densitography of tissue inhibitor of MMP (TIMP) activity in the distribution of the left anterior descending coronary artery (LAD) in the laser and control groups. Values are means ± SE; \( n = 6 \) dogs/group.

TIMP mRNA expression. The TIMP mRNA level in samples of the LAD area (laser and control groups) was semiquantified using the real-time Light Cycler-based PCR technique. Results were standardized using amounts of dog \( \beta \)-actin mRNA. TIMP-1 and TIMP-2 mRNA levels were higher in the laser group than in the control group (ratio of TIMPs to \( \beta \)-actin: 0.143 ± 0.045 vs. 0.025 ± 0.01, \( P = 0.01 \), for TIMP-1; and 0.441 ± 0.16 vs. 0.133 ± 0.035, \( P = 0.025 \), for TIMP-2).

PA activity. In plasminogen/casein zymography, the added plasminogen was activated in the areas where PAs existed, resulting in visible lytic zones produced by the action of plasmin. Two lytic bands were found in the gel, with molecular masses of \( \sim 70 \) and 50 kDa, representing tissue-type PA (tPA) and urokinase-type PA (uPA) (Fig. 3A). There was no difference in the level of tPA between samples (\( P = 0.41 \)). uPA activity was
higher in the distribution of the LAD in the laser-treated group than in the control or sham groups (\( P < 0.05 \); Fig. 3B). However, uPA activities between samples from the distribution of the LCx was similar in each group and in the distribution of the LAD in the sham and control groups (\( P = 0.43 \); Fig. 3B).

Histology. The size of the scar area on the endocardium and epicardium was similar in the laser and control groups (data not shown). However, the left ventricle wall thickness in the infarcted area in the laser group was thicker than in the control group (7.2 ± 0.6 vs. 4.8 ± 0.4 mm, \( P = 0.009 \)).

Histologically, the laser-treated channels were filled with granulation tissue and fibrosis with scattered chronic inflammatory cells, consisting predominantly of lymphocytes, and histocytes with vascularity as reported elsewhere (19a). Immunohistochemical staining using Mac 387 antibody, which reacts with granulocyte, monocytes, reactive tissue microphages, and other inflammatory cells, demonstrated that this class of cells was abundantly present in the laser-channel remnant and scattered in the myocardium of the laser group and was less prominent in control and sham groups (data not show). Histological analysis of the region of LAD in animals undergoing sham thoracotomy was unremarkable, demonstrating normal histology. Angiogenic assessment was based on the number of blood vessels detected by vWF immunohistochemical staining. Results demonstrated that the density of microvessels in the laser group was higher than in the control or sham groups (Fig. 4A). SM \( \alpha \)-actin staining of arterioles, which is a measure of more mature blood vessels (15), demonstrated that the arteriolar density was higher in the laser group than in the control or sham groups (Fig. 4A). Immunohistochemical staining also showed that MMP-2 was expressed mainly by endothelial cells (Fig. 5, right), whereas MMP-1 was expressed in the microvessel wall and interstitial tissue (Fig. 5, middle). SM cells were stained with \( \alpha \)-actin (Fig. 5, left). Staining for MMP-1 and MMP-2 was stronger in the laser group than in the control group. Quantitative analysis of MMP-1- and MMP-2-positive blood vessels (<50 \( \mu \)m) demonstrated higher density in the laser group than in the control or sham groups (Fig. 4B). A positive correlation existed between MMP-1- and MMP-2-positive microvessels and vWF- or SM \( \alpha \)-actin-positive microvessels (Table 1).

In situ zymography. MMP activity was expressed around the microvessels in the laser-treated area of infarction (Fig. 6). This proteolysis is inhibited by 1,10-
phenanthroline, which demonstrates that the proteolysis was the result of MMP and not caused by trypsin or serine proteinase. MMPs activity was not detected in normal myocardial tissue by this technique.

**DISCUSSION**

TMLR mimics the reptilian heart, which has extensive, naturally occurring endocardial channels that provide significant myocardial perfusion, and is also based on knowledge of myocardial sinusoids and the thebesian system, whose communications were thought to allow direct perfusion of the myocardium by ventricular blood. Several studies have consistently demonstrated that channels created by a laser remain patent and therefore represent direct evidence of angiogenesis (5, 8, 19, 30). However, it also has been reported that the channels can become fibrotic and neither patent nor endothelialized (9). In other words, the mechanism and outcome of TMLR is uncertain, although clinical studies have shown that TMLR improves the quality of life and have provided objective evidence that TMLR improves functional capacity in otherwise untreatable patients.

It has been reported that TMLR influences vascular growth patterns, and TMLR-induced angiogenesis correlates with the expression of active MMP-2 and platelet-derived endothelial cell growth factor as determined by measurement of activities in myocardial extract ex vivo (19a). In this study, we used zymography of myocardial extraction ex vivo, in situ zymography, and immunohistochemical staining to demonstrate that MMP activity increases after laser treatment of areas of infarction. In situ labeling suggests that MMP-2 is located around microvessels, whereas MMP-1 is more prevalent in the interstitium. The main function of MMP-2 and MMP-9 is to degrade basement membrane components, such as laminin and type VI collagen, and MMP-1 is to degrade interstitial collagen for endothelial cell proliferation, migration, and angiogenesis. The increase in MMP activity is also important in the remodeling of uninfarcted cardiac stroma and collateral vessels (6, 27). The numbers of

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<th>vWF-Positive Microvessels</th>
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vWF, von Willebrand factor; SM α-actin, smooth muscle α-actin; MMP, matrix metalloproteinase.
vWF- and SM α-actin-positive microvessels correlated with the numbers of MMP-1- and MMP-2-positive microvessels. These data further confirmed that TMLR-induced angiogenesis depends on MMP activity.

The plasminogen system is composed of two PAs, tPA and uPA, and their PA inhibitor-1. This system may be involved in matrix remodelling after acute myocardial infarction. uPA is essential for capillary angiogenesis and growth of collateral arteries during infarct revascularization. The greater requirement for uPA in revascularization of the myocardium than in other tissues may relate to its greater interstitial collagen content (14). During revascularization, uPA is expressed by migrating endothelial and SM cells, but inflammatory cells, which produce angiogenic factors, also express uPA (3). Defective revascularization of the ischemic myocardium might provide an additional explanation for the increased incidence of reinfarction in patients suffering from impaired fibrinolysis. Vascular endothelial growth factor would be unable to improve infarct revascularization in the absence of uPA, possibly because its action depends on endothelial uPA expression (14). Recently, one study reported that inhibition of MMP and PA activity reduces left ventricular remodeling and depresses cardiac function after myocardial infarction due to impairment of therapeutic myocardial angiogenesis (14). Impaired PA/MMP proteolysis may also be involved in cardiac fibrosis (24) as well as coronary or myocardial remodeling after acute myocardial infarction (27). In this study, it was uPA, but not tPA, that significantly increased and was believed to promote angiogenesis after TMLR and be essential for cardiac functional recovery and to activate pro-MMPs (27).

The rise in TIMPs in this study was not unexpected, although the effect of increased TIMPs activity was not clear because MMP upregulation supersedes the increase in TIMP-1 and TIMP-2. TIMPs bind to active sites of the MMPs in a stoichiometric 1:1 molar ratio and form an important endogenous system for regulation of MMP activity in vivo. Disruption of the balance of the MMP-to-TIMP ratio can result in serious diseases such as arthritis, tumor growth, and metastasis. In addition, binding of specific TIMPs to the COOH-terminal hemopexin-like domain of some MMPs regulates activation of the proforms. TIMP-1 binds and shows activation of the latent proform of MMP-9, whereas TIMP-2 binds and regulates activation of pro-MMP-2 (8a, 11, 16a, 28). Baker et al. (1) reported that TIMP-2 and TIMP-3 can reduce SM cell proliferation and promote SM cell death by apoptosis independent of MMP inhibition. Hayakawa et al. (13) have shown that TIMP-1 promotes cell survival in serum-free conditions for a wide variety of cell lines sensitive to serum deprivation, including lymphoid, myeloid, endothelial, fibroblasts, hepatoma, breast carcinoma, and chondrocyte cell lines. It is known that these cells undergo apoptosis under conditions of serum starvation. The addition of TIMP-1 was sufficient to maintain cell growth and viability, suggesting that TIMP-1 may have inhibited serum starvation-induced apoptosis in this lines representing multiple lineages. Therefore, increased TIMP levels in this study may be responsible for 1) regulation of MMP activity, or 2) activation of MMPs or prevention of SM cell proliferation and migration, which leads to neointima formation after vascular injury.

Interestingly, although MMP activity increased in the laser-treated group compared with the control
group, the left ventricle wall did not become thinner but actually became thicker than in the control group. This phenomenon can be explained as follows. First, increases in MMP activity promote the migration of myofibroblast-like cells into the area of the infarct, the myocardium by inducing therapeutic angiogenesis. Second, we did not measure the activity of angiogenic growth factors, including tumor necrosis factor-α, transforming growth factor-β, and interleukin-1β, which leads to myocardial remodelling after TMLR. However, the hypertrophic myocardium has its own problems with oxygen supply and delivery due to a relative reduction in capillary density that is directly connected to angina. Although there is no literature concerning the effect of TMLR on hypertrophied myocardium, our data suggest that TMLR may have a beneficial effect on hypertrophic myocardium by inducing therapeutic angiogenesis. Second, we did not present any data on cardiac function. A further study using gene transfer technology should be done to clarify the correlation among MMP, TIMP, and PA with improved myocardial function improvement after TMLR.

In summary, we demonstrated that TMLR induces the expression of MMP-1, MMP-2, and MMP-9, their inhibitors TIMP-1, and TIMP-2, and uPA. Although the effect of the increased TIMPs is not yet clear, the expression of MMPs, especially MMP-2 and MMP-1, and uPA should play an important role in angiogenesis, which leads to myocardial remodelling after TMLR.

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