Thrombospondin-1 Is Induced in Rat Myocardial Infarction and Its Induction Is Accelerated by Ischemia/Reperfusion

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Thrombospondin-1 (TSP-1) is a multifunctional, rapid-turnover matricellular protein. Recent studies demonstrated that TSP-1 has a role in regulating inflammatory reactions. Myocardial infarction (MI) is associated with an inflammatory response, ultimately leading to healing and scar formation. In particular, an enhanced inflammatory reaction and a massive accumulation of monocytes/macrophages is seen with reperfusion after MI. To examine the role of TSP-1 in MI, we isolated rat *TSP-1* complementary DNA (cDNA) and analyzed the level and distribution of the mRNA expression. In infarcted rat hearts, *TSP-1* mRNA increased markedly at 6 and 12 hrs after coronary artery ligation (27.97 ± 3.40-fold and 22.77 ± 1.83-fold, respectively, compared with sham-operated hearts). Western blot analysis revealed that TSP-1 protein was transiently induced in the infarcted heart. Using *in situ* hybridization analysis, *TSP-1* mRNA signals were observed in the infiltrating cells at the border area of infarction. We then examined the effect of ischemia/reperfusion (I/R) on *TSP-1* mRNA induction in the rats with infarcted hearts. Quantitative reverse transcriptase polymerase chain reaction (RT-PCR) demonstrated that I/R enhanced the *TSP-1* mRNA expression approximately 4-fold, as compared with the level in the permanently ligated heart. Finally, we examined the effect of TSP-1 on proinflammatory cytokine release in mononuclear cells. The releases of interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) from human mononuclear cells were enhanced by TSP-1 in a dose-dependent manner. Thus, the immediate and marked increase of *TSP-1* expression suggests that TSP-1 has an inflammatory-associated role in MI. Exp Biol Med 230:621–630, 2005

Key words: cytokine; extracellular matrix; gene expression; inflammation; ischemia

Introduction

Thrombospondin-1 (TSP-1) is a matricellular protein with multiple biological functions (1). Thrombospondin displays predominantly inflammatory-regulating activities *in vivo*, as shown by the inflammatory phenotype of TSP-null mice (2,3). Thrombospondin-1 is present in the platelet α-granules and is also expressed by endothelial cells and macrophages in a highly regulated manner (4). Its expression rapidly and dramatically increases in various situations, such as vascular injury (5) or exposure to growth factors (e.g., platelet-derived growth factor or basic fibroblast growth factor; Ref. 6). In ischemic heart disease, TSP-1 appears in the serum within 15 mins after the onset of myocardial infarction (MI) in dogs (7) and 24 hrs after the onset of MI in humans (8). However, there is no evidence that the infarcted heart directly expresses TSP-1 after MI, and, therefore, the role of TSP-1 in MI is not understood.

Recently, a new family related to TSP-1 was identified and designated ADAMTS (a disintegrin and metalloprotease with thrombospondin motifs), and shown to be involved in inflammatory diseases, such as arthritis. We recently reported that ADAMTS1 is transiently upregulated in MI in rats (9). The involvement of ADAMTS in MI led us to recognize the importance of the function of various domains of TSP-1,

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During the submission, review, and publication process, the following related paper was published by Frangogiannis NG, Ren G, Dewald O, Zymek P, Haudek S, Koerting A, Winkelmann K, Michael LH, Lawler J, Entman ML. Critical role of endogenous thrombospondin-1 in preventing expansion of healing myocardial infarcts. Circulation 111(22):2935–2942, 2005.

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which are partly conserved among these molecules (10). To analyze the function of these related molecules, TSP-1 and ADAMTS, and their contribution to cardiovascular disease, comparison of these molecules seems to be essential. A number of previous studies examined the role of TSP-1 in disease using animal models, such as *Rattus norvegicus* (11,12). However, surprisingly, none of the previous studies determined the complete sequence of rat TSP-1, and the sequence of rat TSP-1 complementary DNA (cDNA) was not deposited in the NCBI database (http://www.ncbi.nlm.nih.gov/). Accordingly, we cloned the entire coding region of rat TSP-1 and examined its homology with the human and mouse counterparts. Interestingly, TSP-1–deficient mice showed extensive acute and organizing pneumonia, with neutrophils and macrophages (3). Myocardial infarction after experimental ischemia and reperfusion (I/R) has been convincingly shown to be mediated by a number of inflammatory mediators and processes, including neutrophil accumulation and macrophage infiltration (13).

Thus, we hypothesized that TSP-1 expression would increase in the infarcted myocardium, and that TSP-1 expression would be altered by I/R. To test this hypothesis, we examined the TSP-1 expression after MI by means of Northern blotting, Western blotting, and *in situ* hybridization. We also investigated the effect of I/R on the *TSP-1* mRNA level in a rat MI model by quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) analysis. We further examined the effect of TSP-1 on the production of the proinflammatory cytokine, interleukin-6 (IL-6), and a chemokine, monocyte chemoattractant protein-1 (MCP-1), in human peripheral blood mononuclear cell (PBMC) cultures.

Materials and Methods

Molecular Cloning of Rat *TSP-1*. Screening of the database of expressed sequence tags (EST) (http://www.ncbi.nlm.nih.gov/BLAST/) indicated that the data for the coding region of rat *TSP-1* cDNA were incomplete (Fig. 1A). The missing region was amplified by RT-PCR using rat adult heart mRNA as a template, as previously described (14). Briefly, cDNA was synthesized from rat adult heart RNA by reverse transcription. Next, PCR was performed with this cDNA as the template and rat *TSP-1*–specific sense and antisense oligonucleotide primers 5′-TCAAGGCCTTC-CAGGTCCGACTCTC-3′ and 5′-GGGAGCCTGTTCTA-CAGCTGATCTC-3′, respectively, which were designed based on the rat EST sequence. The PCR products were cloned into a TA-cloning vector and sequenced using primers flanking both ends and an ABI BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA).

Animals and Experimental Protocols. All protocols involving experimental animals followed the local institutional guidelines for animal care, which are comparable to those in the "Guide for the Care and Use of Laboratory Animals" published by the Institute for Laboratory Animal Research (National Institutes of Health Publication No. 85–23, revised 1996). Myocardial infarction was induced as

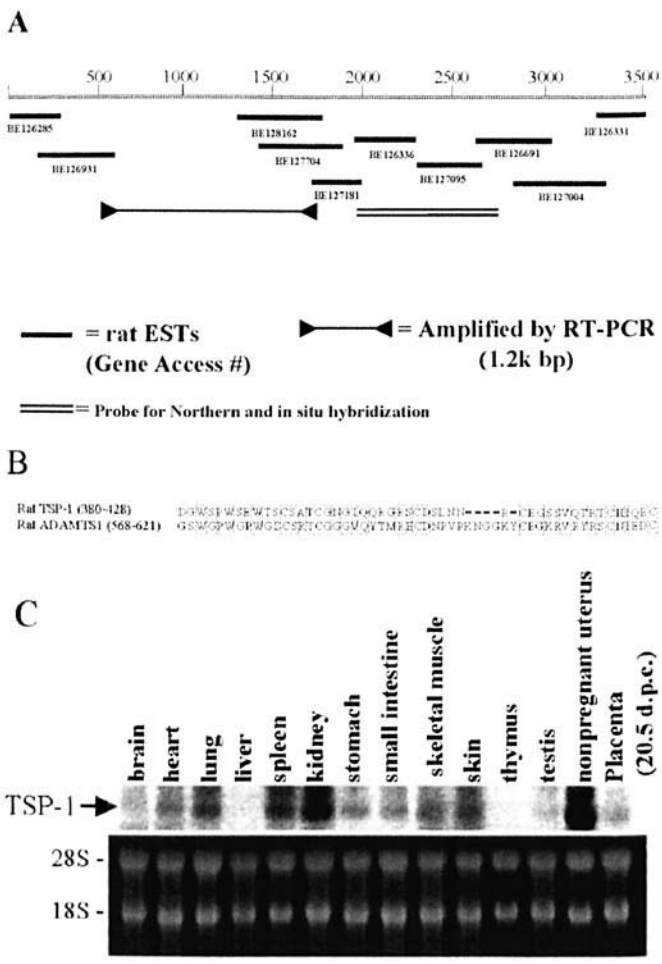

Figure 1. (A) The molecular cloning strategy for rat *TSP-1*. Single lines indicate each of the ESTs. The double line indicates the locus of the probe for Northern blotting and *in situ* hybridization. (B) Comparison of Type I repeat of *TSP-1* and *ADAMTS1* in rats. Identical amino acids are boxed. Note that all the important residues, such as cysteine, are conserved. (C) Northern blot analysis of *TSP-1* expression in various rat tissues. The size of *TSP-1* mRNA is indicated. Ethidium bromide-stained bands of 18S rRNA and 28S rRNA are shown below to indicate the amount of loaded RNA.

previously described (15,16). Briefly, adult Sprague-Dawley male rats weighing 200–250 g were anesthetized with intraperitoneal administration of 40 mg/kg of sodium pentobarbital, and the left coronary artery was ligated. For Northern blot analysis, rats were sacrificed using sodium pentobarbital at 6 hrs, 12 hrs, 24 hrs, 2 days, 7 days, 14 days, and 28 days after MI ($n = 9$ at each time point). For Western blot analysis, eight rats were sacrificed in groups of two each at 3 hrs, 6 hrs, 24 hrs, and 7 days after coronary occlusion. For *in situ* hybridization, three rats were sacrificed at 24 hrs after coronary occlusion. Sham-operated rats were also sacrificed on this schedule. With a slight modification, I/R was induced as we previously described (17). Briefly, reperfusion was carried out after 45 mins of coronary ligation, and confirmed by observation of the hyperemic color of the ischemic myocardium ($n = 4$). For quantitative RT-PCR analysis, rats were sacrificed at 24 hrs after coronary ligation or I/R.

Northern Blot Analysis. Northern blot analysis was

performed following the protocol previously reported (18,19). The infarct area was snap-frozen in liquid N_2, pulverized, and resuspended in RNAzolB (Tel-Test, Friendswood, TX). Electrophoresis was performed on aliquots of total RNA (40 μg from each tissue) on 1% agarose–formaldehyde gels, which was then transferred to nylon membranes. The cDNA probe used for Northern blotting is shown in Figure 1A. The membranes were hybridized with α^{32}P-labeled cDNA probes at 65°C for 3 hrs. After the radiolabeled filters were washed under stringent conditions, they were exposed to an imaging plate (Fuji Photo Film Inc., Tokyo, Japan), which was developed using an image analysis system (BAS-2000, Fuji Photo Film Inc.). The 28S ribosomal RNA (rRNA) bands stained with ethidium bromide served as an internal control for comparison. The densities of the hybridized bands for *TSP-1* were quantified using an image analysis program. A rat adult multiple-tissue Northern blot membrane was purchased from Seegene Inc. (Seoul, Korea) and was hybridized according to the manufacturer's protocol.

Western Blot Analysis. Western blot analysis was performed following a previously reported protocol (9,20). Proteins were extracted from the hearts using CelLytic MT (Sigma, St. Louis, MO). Protease inhibitor cocktail (Sigma) was added just before the addition of lysis buffer to the tissue samples. Protein concentrations were quantified using the DC protein assay (Bio-Rad, Hercules, CA). From each extract, 80 μg of total protein were separated on a 5–20% sodium dodecyl sulfate (SDS)-polyacrylamide gradient gel with a 4% stacking gel. After electrophoresis, proteins were transferred to a polyvinyl difluoride (PVDF) membrane using transfer buffer that contained 25 m*M* Tris-HCl and 200 m*M* glycine. The membrane was blocked in 5% milk dissolved in 1 × TTBS buffer (14 m*M* Tris-HCl, pH 7.5; 154 m*M* NaCl; and 0.5% Tween-20) overnight at 4°C. The membrane was incubated with the primary antibodies for 2 hrs at room temperature (RT) in 5% milk dissolved in 1 × TTBS. The primary antibody for TSP-1, Ab-11 (Labvision, Fremont, CA), was used at a 1:100 dilution. The membrane was washed three times for 15 mins each with 1 × TTBS at RT. Appropriate secondary antibody conjugated to horseradish peroxidase (Promega, Madison, WI) was incubated with the membrane for 1 hr at RT. After five successive washes with 1 × TTBS, the membrane was developed using an ECL+ kit (Amersham Pharmacia Biotech, Piscataway, NJ).

***In Situ* Hybridization.** Rat hearts were fixed with 4% paraformaldehyde and embedded in OCT medium (Sakura, Tokyo, Japan), then cut into 5-μm sections and placed on silane-coated slide glasses (DakoCytomation, Kyoto, Japan). Digoxigenin-UTP–labeled cRNA probes were synthesized by *in vitro* transcription as previously reported by our group (21,22). Hybridization was carried out overnight at 42°C in a humidified chamber. The immunologic detection of digoxigenin-labeled transcripts was performed according to the manufacturer's protocol (Boehringer-Mannheim, Mannheim, Germany). Finally, the sections were lightly counterstained with Mayer's hematoxylin and mounted with Crystal Mount (Biomeda, Foster, CA). For negative controls, *in situ* hybridization with a sense probe was performed. All *in situ* hybridization slides were photographed and converted to digital computer files with a Canoscan and Adobe Photoshop software.

Immunohistochemistry. Immunohistochemical analysis was performed as previously reported (15). Briefly, the excised hearts were immediately frozen and cut into 6-μm sections and fixed with ice-cold acetone for 10 mins. Monoclonal antibody against human vimentin was purchased from Nichirei (Tokyo, Japan). After the sections were washed with phosphate-buffered saline (PBS) with 0.01% Triton X-100, peroxidase-conjugated goat anti-mouse IgG (Nichirei) was used as the secondary antibody for Ab-11 and anti-vimentin for 60 mins at room temperature. Finally, the sections were washed three times in PBS with 0.01% Triton X-100 for 5 mins each time. The sections were then treated using an AEC kit (Dako, Carpinteria, CA).

Quantitative RT-PCR. The effect of I/R on TSP-1 mRNA expression in the infarcted hearts was analyzed by a quantitative real-time RT-PCR method using a LightCycler rapid thermal cycler system (Roche Diagnostics Inc., Mannheim, Germany) according to a recently reported protocol (9,23,24). Briefly, 1-μg aliquots of isolated total RNA were reverse transcribed and cDNA was added to the PCR reaction mix containing the following specific primers: 5′-CCGGTTTGATCAGAGTGGT-3′ and 5′-GGTTTCGGAAGGTGCAAT-3′ for *TSP-1* and 5′-CCACTGCCAACGTGTCAGTGG-3′ and 5′-AAGGTGGAGGAGTGGGTGTCG-3′ for glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)*. The amplification of a housekeeping gene, *GAPDH*, was used to normalize the amount of RNA applied, as previously reported (9,23). Each RT-PCR experiment was repeated at least three times to confirm reproducibility. The variability for triplicate measurements of each sample was <5%. Negative controls were checked with samples in which the cDNA templates were replaced by nuclease-free water in the reactions.

Isolation and Culture of PBMC. Human PBMC were isolated from heparinized venous blood of a healthy volunteer using a gradient of Lymphoprep (AXIX-SHIELD, Oslo, Norway), according to the manufacturer's protocol, as previously described (25,26). Briefly, the gradient was centrifuged at 800 *g* and the PBMC at the interface were removed, washed twice, and resuspended in RPMI-1640 medium (Sigma) containing 10% heat-inactivated fetal bovine serum and 100 IU/ml of penicillin and 100 μg/ml of streptomycin at 37°C in a humidified atmosphere of 5% CO_2 in air. The PBMC were plated at 5×10^5 cells/ml in 96-well plates. Purified human recombinant TSP-1 was purchased from Sigma and immobilized by adding 100 μl of protein solution (55 n*M*) in PBS to each well of the 96-well plates, which were then incubated overnight at 4°C before starting the cell culture. After 48 hrs, spent media were collected and centrifuged. After centrifugation, aliquots of the supernatants were frozen at −20°C until the assay.

IL-6 and MCP-1 Measurement by Enzyme-Linked Immunoabsorbant Assay (ELISA). IL-6 and MCP-1 in culture media were measured using a sandwich ELISA kit (BioSource, Camarillo, CA) according to the manufacturer's protocol. Briefly, the wells were incubated sequentially with culture media, biotinylated antibody for IL-6 and MCP-1, and horseradish peroxidase-conjugated avidin before color development. Standard curves were obtained with recombinant human IL-6 and MCP-1. Cultured medium from wells containing various concentrations of immobilized TSP-1 was compared with that from 1% bovine serum albumin (BSA)-coated plates ($n = 3$, respectively).

Statistical Analysis. Data were expressed as mean ± standard error (SE) for real-time RT-PCR analysis and as mean ± standard deviation (SD) for ELISA analysis. Statistical analysis of differences of real-time RT-PCR analysis was performed by analysis of variance (ANOVA) with Bonferroni's multiple-comparison correction and by the unpaired t test for ELISA. A value of $P < 0.05$ was considered statistically significant.

Results

Identification of Rat *TSP-1* cDNA. We scanned the GenBank rat nucleic acid database using the sequences of human *TSP-1* cDNA (accession number NM_003246) and mouse *TSP-1* cDNA (accession number M87276). Homology was found with several rat EST clones (accession numbers: BR126285, BE126931, BE127704, BE127181, BE126336, BE127095, BE126691, BE127004, and BE126331). However, it was not possible to generate a full-length rat *TSP-1* coding sequence from EST information alone. We amplified the missing part of the rat *TSP-1* cDNA, which was approximately 720 base pairs (bp) in size, by RT-PCR using rat heart cDNA (Fig. 1A). The resultant rat *TSP-1* cDNA contained 3746 nucleotides, with a 3522-nucleotide open reading frame coding for 1174 amino acids. The coding sequence of *TSP-1* has been deposited and is available with accession number AF309630. The sequence identity between the rat and mouse amino acid sequences was 96%. The rat *TSP-1* amino acid sequence was highly homologous to its human and mouse counterparts, especially in the *TSP*-repeat motifs. The Type-1 repeat of rat *TSP-1* was highly similar to that of rat *ADAMTS1* (accession number NM_024400), and all of the cysteine residues were conserved (Fig. 1B).

TSP-1 Is Expressed in the Infarcted Myocardium. Interestingly, the tissue distribution of *TSP-1* mRNA in the adult rat had not been investigated previously. Therefore, we examined the expression of rat *TSP-1* mRNA in normal adult rat tissues, including brain, heart, lung, liver, spleen, kidney, stomach, small intestine, skeletal muscle, skin, thymus, testis, nonpregnant uterus, and placenta (Fig. 1C). Rat *TSP-1* was abundantly expressed in the nonpregnant uterus, kidney, spleen, and lung, whereas it was only weakly expressed in the normal heart, stomach, and small intestine.

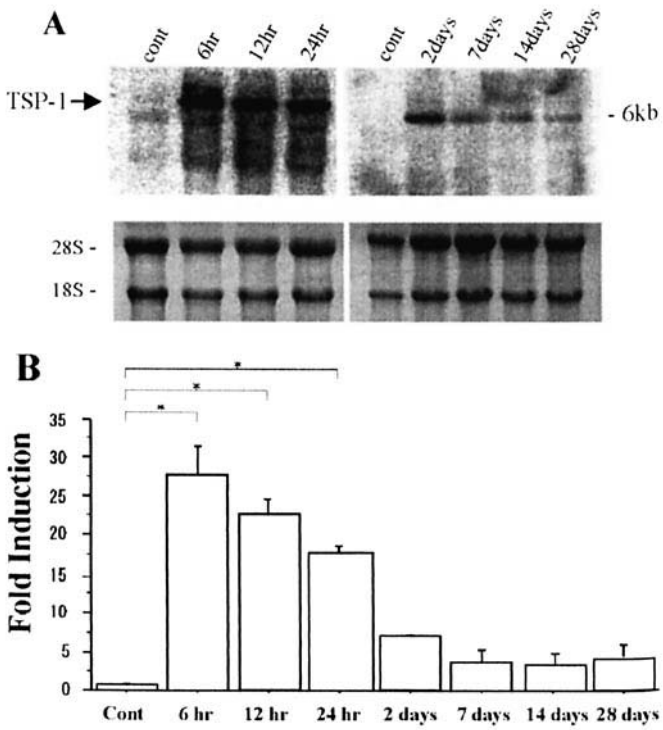

Figure 2. (A) Northern blot analysis of the time course of *TSP-1* expression in a rat experimental infarction model. The size of *TSP-1* mRNA is indicated. (B) The relative levels of expression of *TSP-1* mRNA in the infarcted heart were normalized to 28S rRNA and expressed as fold increase over the level in control hearts (mean ± SE). A significant increase in *TSP-1* mRNA levels was noted in the infarcted heart at 6 hrs, 12 hrs, and 24 hrs after artery ligation ($n = 9$, respectively). *, $P < 0.05$ compared with control hearts.

Figure 2A shows a typical analysis of *TSP-1* mRNA expression levels detected by Northern hybridization in experimental rat MI. When *TSP-1* mRNA expression was standardized relative to the level of 28S rRNA loaded, the average *TSP-1*/28S ratio in the infarcted heart increased quickly and markedly (6 and 12 hrs; 27.97 ± 3.40-fold and 22.77 ± 1.83-fold increase, respectively, compared with sham-operated hearts) and then declined (Days 1 and 2; 17.92 ± 0.65-fold, 7.09 ± 0.06-fold, respectively), as shown in Figure 2B. In the late phase (Days 7, 14, and 28; 3.81 ± 0.74-fold, 3.51 ± 0.75-fold, and 4.22 ± 1.06-fold, respectively), *TSP-1* continued to be expressed at detectable levels. This elevation was significant ($P < 0.01$), as revealed by ANOVA. The elevation of *TSP-1* mRNA in MI was further confirmed by quantitative real-time RT-PCR analysis, and the change detected in the level of the expression of *TSP-1* mRNA was almost the same as that estimated by Northern blot analysis (data not shown).

Western blot analysis showed that TSP-1 protein was produced in the infarcted heart (Fig. 3). An immunoreactive band (approximately 160 kDa) was clearly observed at 24 hrs after coronary artery ligation. Without primary antibody, the samples showed only nonspecific bands (approximately 50 kDa and approximately 70 kDa) that were similarly

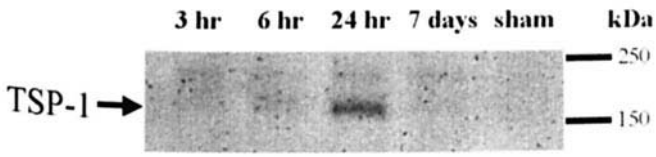

Figure 3. Transient induction of TSP-1 protein in the infarcted heart. A strongly positive band was observed at 24 hrs after infarction. The size of TSP-1 protein is indicated. The positions of the size markers are indicated at the right of the panel.

observed in all samples loaded and, thus, were considered to be bands that cross-reacted with the secondary antibody (data not shown). The induction of TSP-1 in the infarcted heart was transient, and TSP-1 protein was not detected at 7 days after MI.

***In Situ* Hybridization Analysis of TSP-1 mRNA.** Intense positive signals for *TSP-1* mRNA were observed around the infarct border area at 24 hrs (Fig. 4A and 4C). Signals from *TSP-1* mRNA were only detected in the mesenchymal cells in the border area of the MI in the early phase. Positive signals for *TSP-1* mRNA were rarely observed in the control hearts (sham-operated hearts; Fig. 4D). Two series of experiments with different rats showed identical signal distribution patterns. No positive signals for *TSP-1* mRNA were observed when hybridization was performed with the sense probe (Fig. 4B). In addition, no positive signals were observed in RNase-pretreated specimens (data not shown). In the area showing positive *TSP-1* mRNA staining, scattered vimentin-positive cells and CD68-positive cells were present. Mesenchymal infiltrating cells, including fibroblasts/myofibroblasts (vimentin-positive) and mononuclear cells (CD68-positive) expressed *TSP-1* mRNA (Fig. 5). Vimentin-positive cells that produce *TSP-1* mRNA included some myofibroblasts (i.e., both vimentin- and α-smooth muscle actin-positive cells) in the infarcted heart (data not shown).

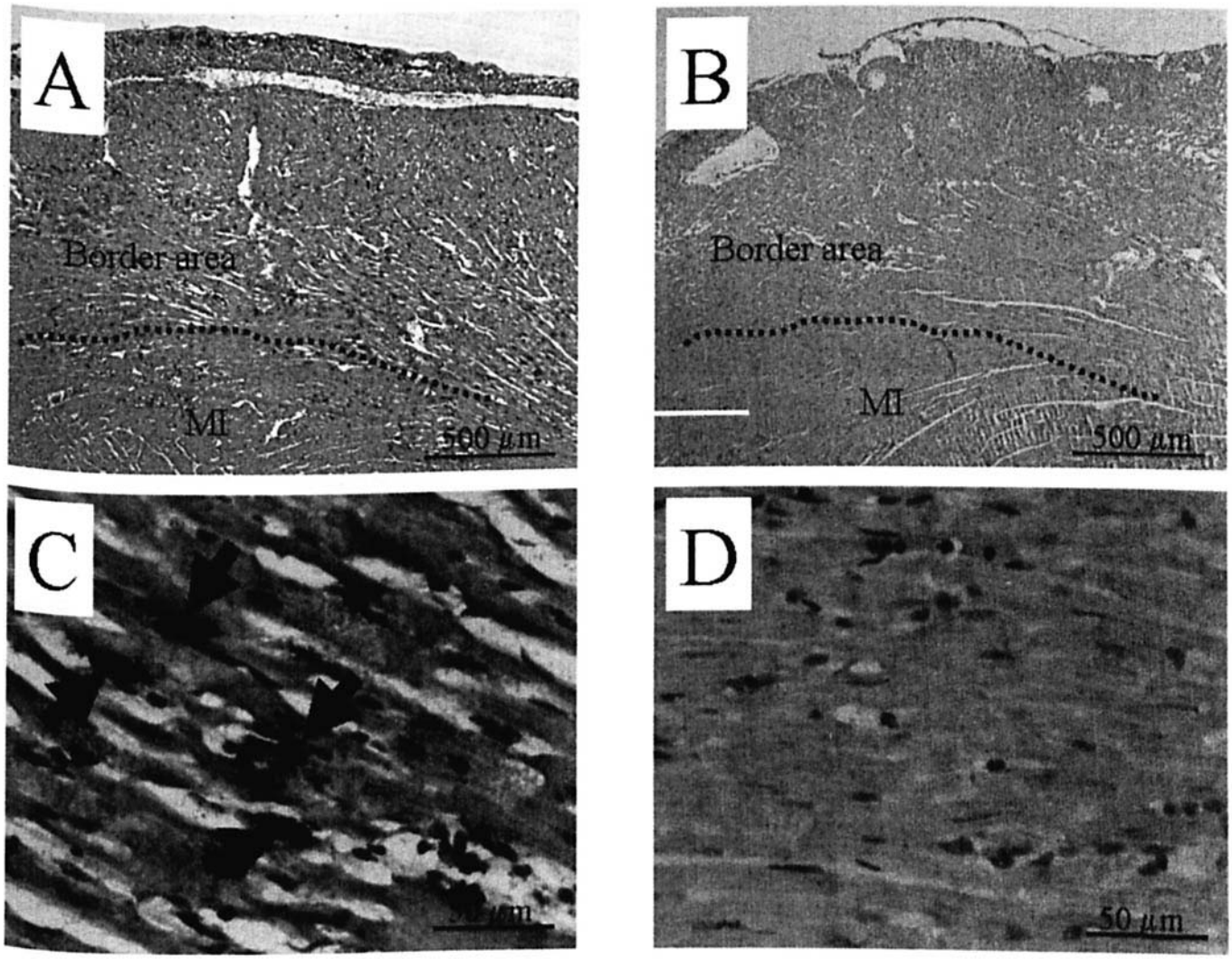

Figure 4. In the border area of the infarct, *TSP-1* mRNA is induced. MI indicates the infarct zone. The dotted line indicates the border area of infarction. Positive signals for *TSP-1* mRNA were observed in the border area after 24 hrs of coronary artery ligation. (A) Lower magnification. (B) Adjacent section hybridized with sense probe. (C) Higher magnification hybridized with anti–*TSP-1* probe in infarcted heart. Intense and distinct signals were observed in the mesenchymal cells (indicated by arrows). (D) Normal heart shows no distinct *TSP-1* signals. A scale bar is shown in each panel.

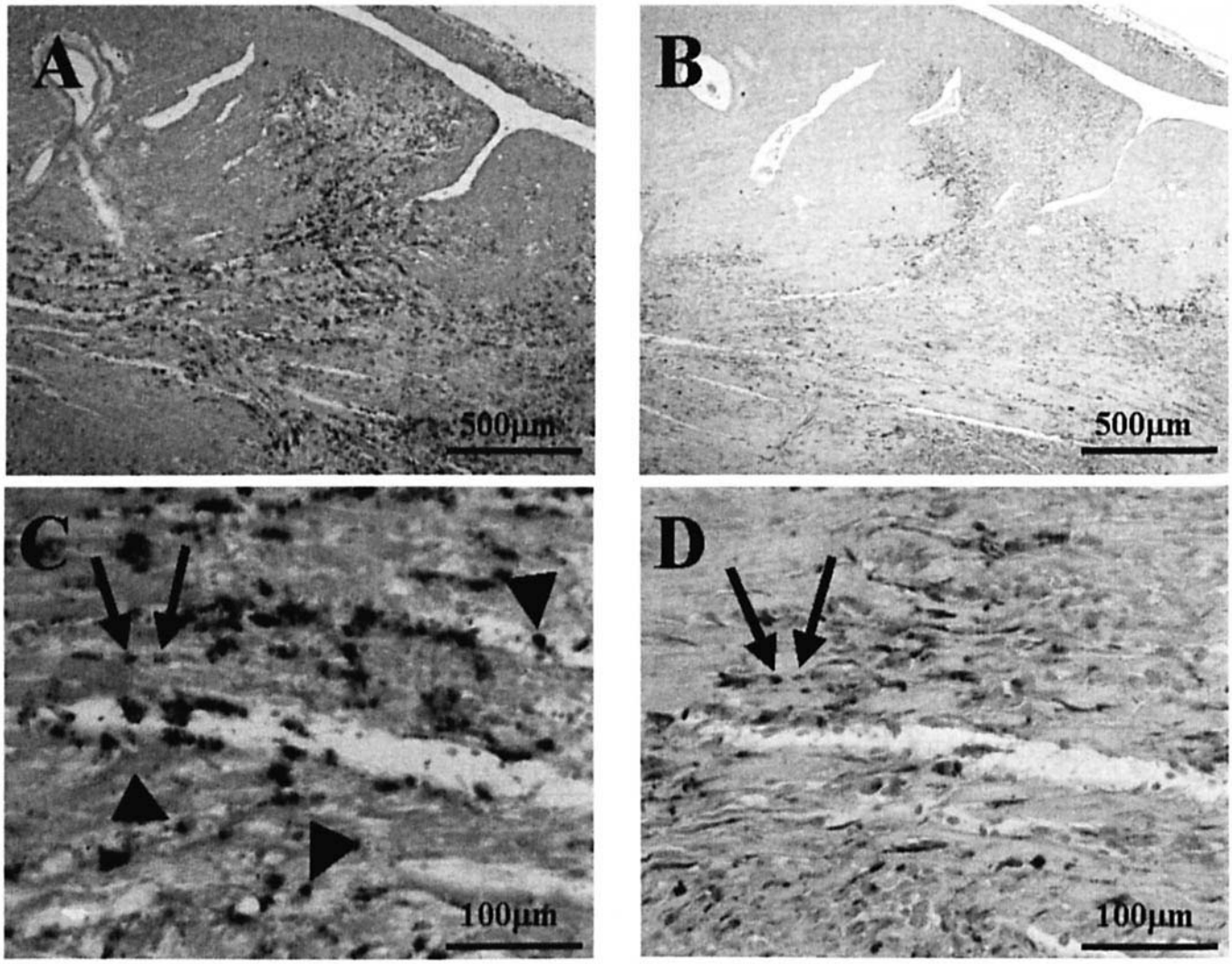

Figure 5. Infiltrating cells in the border area expressed *TSP-1* mRNA. (A) Expression of *TSP-1* mRNA was observed at the infarct border area by *in situ* hybridization analysis (blue). Lower magnification. (B) Adjacent section probed with a monoclonal anti-vimentin antibody. Note that scattered vimentin-positive cells infiltrated the infarct border area where *TSP-1* mRNA signals were observed. (C) Higher magnification of hybridization with an anti–*TSP-1* probe in the infarcted heart. Intense and distinct signals were observed in the infiltrating cells with morphologic characteristics of mononuclear cells (indicated by arrowheads) and fibroblasts (indicated by arrows). (D) Higher magnification of an area similar to that in (C), probed with an anti-vimentin antibody. Note that vimentin-positive cells were the source for *TSP-1* mRNA expression (indicated by arrows). A scale bar is shown in each panel.

Ischemia/Reperfusion Enhanced the Induction of *TSP-1* mRNA Expression in the Heart. Because *TSP-1* mRNA expression was localized at the infarct border area, where there were infiltrating CD68-positive cells (i.e., monocytes/macrophages; data not shown), we examined whether I/R altered the expression level of *TSP-1* mRNA. I/R is known to enhance inflammatory responses, such as IL-6 production from mononuclear cells (27,28). As shown in Figure 6, I/R enhanced the expression level of *TSP-1* mRNA approximately 4-fold compared with the permanent ligation group ($n = 4$, respectively).

TSP-1 Enhanced IL-6 and MCP-1 Release in Cultured PBMC Cells. The role of local inflammatory responses is well established in myocardial I/R (29). Among inflammatory mediators, a central role is played by proinflammatory cytokines, such as IL-6. Because *TSP-1* signals were enhanced by I/R and observed in the border area of the infarct, where CD68-positive cells infiltrate after artery ligation, we examined the effect of TSP-1 on inflammatory reactions of monocytes. We isolated PBMC and measured the release of the proinflammatory cytokine IL-6 and MCP-1 into the culture medium in the presence of either TSP-1 or BSA ($n = 3$, respectively). As shown in Figure 7, PBMC cultured on BSA-coated wells showed very low levels of IL-6 release (76.7 ± 45.48 pg/ml) and MCP-1 release (141.5 ± 59.26 pg/ml). When PBMC were cultured with immobilized TSP-1, the PBMC released a significantly higher level of IL-6 (5165.2 ± 760.5 pg/ml) and MCP-1 (5828.4 ± 756.6 pg/ml; Fig. 7). Immobilized TSP-1 induced both IL-6 and MCP-1 production in cultured PBMC in a dose-dependent manner (Fig. 8).

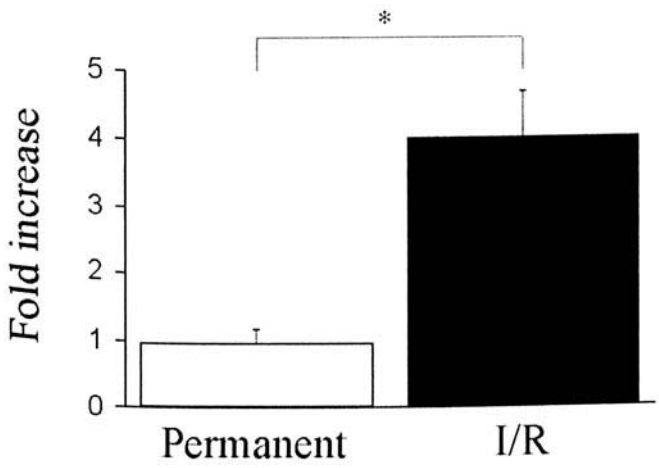

Figure 6. Enhancement of *TSP-1* mRNA induction by reperfusion of the infarcted heart. The RNA was extracted from the permanent ligation group (permanent) and the I/R group ($n=4$, each group). The *TSP-1* mRNA was measured by quantitative RT-PCR analysis, compared with the permanent group, and expressed as fold increase. Ischemia/reperfusion significantly enhanced the induction of *TSP-1* mRNA in the infarcted heart (mean ± SE). *, $P < 0.01$ compared with the permanent ligation group.

Discussion

The major novel finding that emerged from this study was that *TSP-1* mRNA was transiently induced in the infarcted heart, especially in the early phase after MI. Furthermore, *in vivo* I/R enhanced the induction of *TSP-1* mRNA in the infarcted heart. In cultured PBMC, TSP-1 facilitated proinflammatory cytokine release.

By molecular cloning, we isolated the coding sequence of rat *TSP-1*. Some unique domains, such as the Type I repeat, are shared by ADAMTS1 and TSP-1 (30). Important amino acids such as cysteine were conserved in their type I repeat. In fact, both TSP-1 and ADAMTS1 are reported to have antiangiogenic activity (31,32), and the Type I repeat is reported to be one of the responsible elements for this antiangiogenic activity (31). At the protein level, TSP-1 was transiently induced in the infarcted heart; ADAMTS-1 was also transiently induced (9). However, in the infarcted heart, the expression patterns of *TSP-1* mRNA and *ADAMTS1* mRNA were distinctly different (9). The localization patterns of their mRNAs were different, and ADAMTS1 was more rapidly expressed and disappeared earlier than TSP-1. This suggests that these two molecules have distinct roles in the infarcted heart.

In the present study, we showed that in rat MI, *TSP-1* mRNA was strongly expressed in the infarcted border area from 6 hrs to 24 hrs after coronary artery ligation. The rapid induction of *TSP-1* mRNA after MI occurred coincidently with a series of inflammatory reactions in the infarcted myocardium; for instance, with *IL-6* mRNA expression, which was reported to be induced at 6 hrs after artery ligation in rats (28,33). Signals from *TSP-1* mRNA were not detected by *in situ* hybridization in remote noninfarcted areas or in the normal heart. Sun and Weber (34), using immunohistochemistry with an anti–α-smooth muscle actin monoclonal antibody in rats, revealed that both myofibroblasts and fibroblasts infiltrated into the infarct zone. Similar to their findings, our *in situ* hybridization with immunohistochemistry for vimentin indicated that the infiltrated cells responsible for *TSP-1* mRNA production included fibroblasts/myofibroblasts in the infarct border area. Zhao and Eghbali-Webb revealed that cardiac fibroblasts produce TSP-1, and secreted TSP-1 was reported to affect endothelial cell proliferation under hypoxic conditions

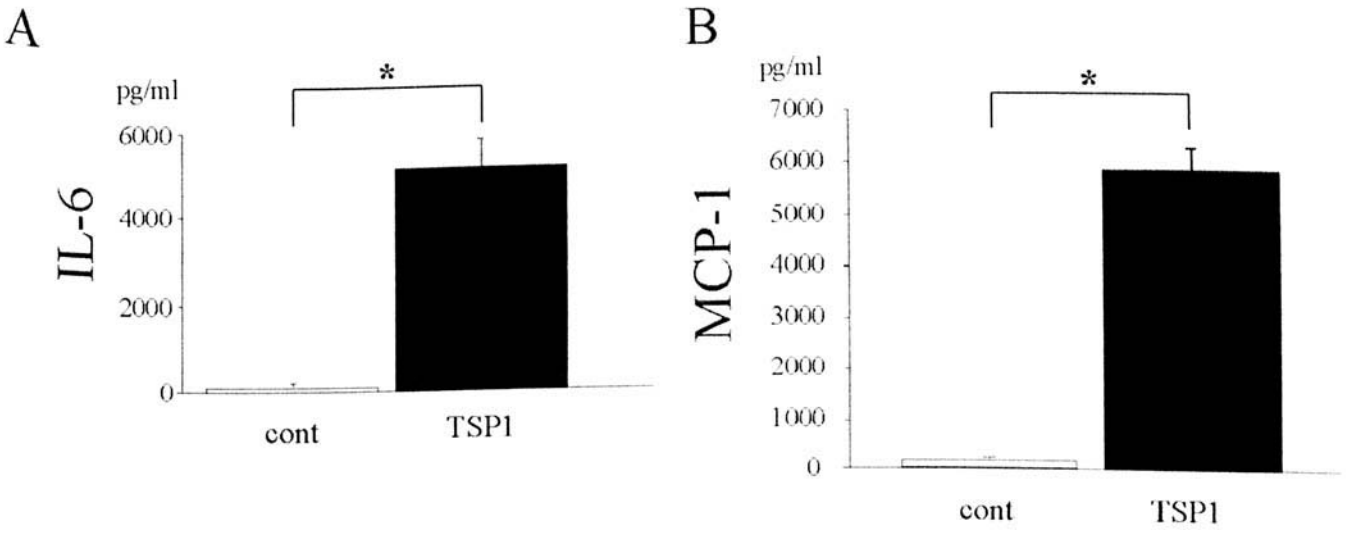

Figure 7. Enhancement of cytokine release in human PBMC by TSP-1. The production of IL-6 and MCP-1 in PBMC culture media was measured by ELISA assays ($n=3$, respectively). (A) Immobilized TSP-1 enhanced the release of IL-6 compared with the control (BSA-coated wells; mean ± SD). (B) Immobilized TSP-1 enhanced the release of MCP-1 compared with the control (BSA-coated wells; mean ± SD). "Cont" indicates that cells were incubated with BSA-coated wells (control) and "TSP-1" indicates that cells were incubated with immobilized TSP-1 wells. Note that immobilized TSP-1 induced the release of IL-6 as well as MCP-1 compared with the control. *, $P < 0.05$ compared with control (BSA-coated wells).

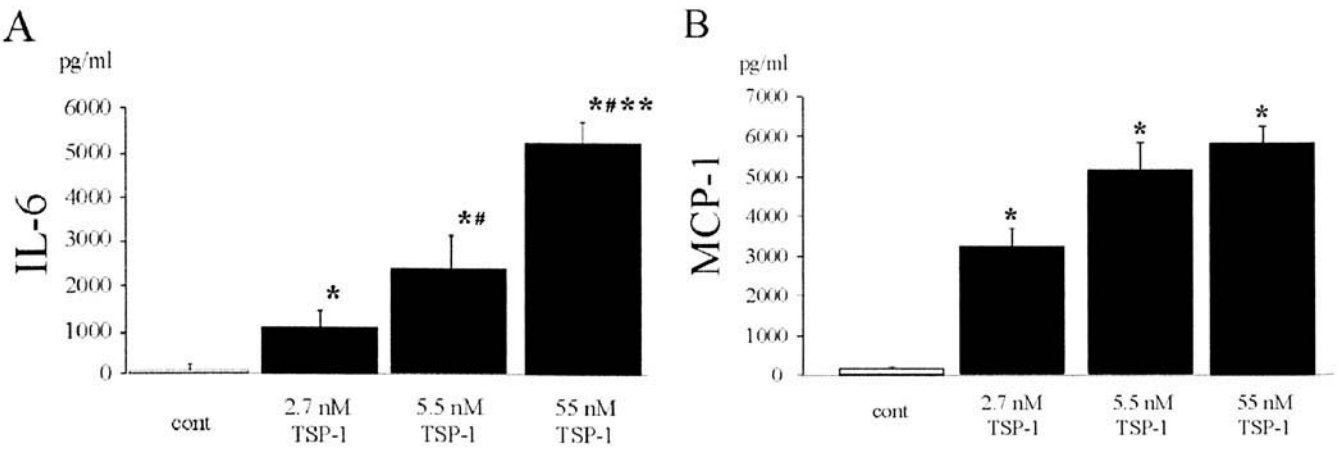

Figure 8. Dose-dependent induction of IL-6 and MCP-1 by TSP-1 in human PBMC. The production of IL-6 and MCP-1 in PBMC culture media was measured by ELISA assays ($n = 3$ in each group). Immobilized TSP-1 dose-dependently induced the release of both IL-6 and MCP-1 compared with the control (BSA-coated wells; mean ± SD). *, $P < 0.05$ compared with control (BSA-coated wells); #, $P < 0.05$ compared with 2.5 n*M* TSP-1; **, $P < 0.05$ compared with 5.5 n*M* TSP-1.

(35). One of the important pathologic conditions, inflammation, was also reported to induce *TSP-1* mRNA expression in various cells (36,37). The findings in those reports, together with our data, suggest that some physiologic conditions, such as inflammation, cause TSP-1 induction, primarily in the mesenchymal cells (including cardiac fibroblasts/myofibroblasts as well as infiltrating monocytes/macrophages) in the border area of the infarct myocardium.

Ischemia/reperfusion accelerated the induction of *TSP-1* mRNA expression. The importance of the inflammatory cascade in MI has been recognized and thoroughly investigated. The inflammatory response that is initiated in the ischemic and post-ischemic heart has the ultimate effect of ameliorating the outcome of the pathologic condition, thus, contributing to the remodeling process (38). Monocytes/macrophages create an environment rich in inflammatory cells, capable of regulating extracellular matrix metabolism, as well as cell proliferation, through the production of a variety of cytokines and growth factors. The role of TSP-1 in the process of wound healing remains controversial. Delayed healing is found in TSP-1–null animals, as indicated by the prolonged persistence of inflammation (2). Although our findings do not primarily deal with the direct biologic function of TSP-1 in I/R, the accelerated induction of *TSP-1* by I/R was concordant with increased inflammatory reaction in the I/R myocardium.

Immobilized TSP-1 increased the production of IL-6 and MCP-1 in human PBMC in a dose-dependent manner. Both IL-6 and MCP-1 were induced in the infarcted area (39). IL-6 is a major proinflammatory cytokine, which is reported to play a role in the infarct healing process (40). The production of IL-6 induced by I/R, which was characterized by increased monocyte infiltration (28), was concomitant with the induction of *TSP-1* mRNA expression. In addition, IL-6 production was significantly increased by immobilized TSP-1 *in vitro*. These results were in good agreement with those obtained in U937 cells, which are derived from a histiocytic lymphoma (41). Monocyte chemoattractant protein-1 is a cysteine–cysteine chemokine and the regulation of inflammatory responses by MCP-1 is important for the infarct healing process, as shown using MCP-1–null mice (42). We showed for the first time that immobilized TSP-1 induced MCP-1 production in a dose-dependent manner. PBMC were reported to intrinsically express TSP-1 under the regulation of soluble mediators (43,44). These observations and our results indicate that TSP-1 might have a role in regulating inflammatory reactions of monocytes/macrophages, and the effect on cytokine production seen with the immobilized TSP-1 may explain, at least in part, the increased degree of the inflammatory response in PBMC.

Although the mechanism of IL-6 induction has not been clarified, recent reports have suggested that cell-surface receptors for TSP-1 (e.g., CD36 and CD47) play roles in the induction of various cytokines, and a similar mechanism is also suggested in our system (45,46). These data taken together indicate that TSP-1 may affect cytokine release from PBMC, and that cytokine release regulates TSP-1 expression via a feedback mechanism.

In conclusion, we showed here that TSP-1 was quickly and locally produced in the infarcted heart, and its induction was accelerated by I/R. Our data suggest that the induction of TSP-1 in the infarcted heart may be associated with inflammatory responses.

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