Biology

Biology fields

Okayama University *Year* 2005

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Kenichi Toeda[*] Keigo Nakamura[†] Satoshi Hirohata[‡]
Omer F. Hatipoglu[**] Kadir Demircan[††] Hitoshi Yamawaki[‡‡]
Hiroko Ogawa[§] Shozo Kusachi[¶] Yasushi Shiratori[‖]

[*]Okayama University
[†]Okayama University
[‡]Okayama University, hirohas@cc.okayama-u.ac.jp
[**]Okayama University
[††]Okayama University
[‡‡]Okayama University
[§]Okayama University
[¶]Okayama University
[‖]Okayama University

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Kenichi Toeda[*], Keigo Nakamura[*], Satoshi Hirohata[*§], Omer F. Hatipoglu[§], Kadir Demircan[§], Hitoshi Yamawaki[*], Hiroko Ogawa[§], Shozo Kusachi[*¶], Yasushi Shiratori[*], Yoshifumi Ninomiya[§]

*Dept. of Medicine and Medical Science, [§] Dept. of Molecular Biology and Biochemistry, Okayama University Graduate School of Medicine and Dentistry, [¶]Dept. of Medical Technology, Faculty of Health Science, Okayama University Medical School.

Running Title: Versican expression in myocardial infarction

Address correspondence and reprint requests to:

Satoshi Hirohata

Department of Molecular Biology and Biochemistry and

Department of Medicine and Medical Science

Okayama University Graduate School of Medicine and Dentistry

2-5-1, Shikata-cho, Okayama, Okayama 700-8558, Japan.

Telephone: 81 86 235 7129 Fax: 81 86 222 7768

e-mail: hirohas@cc.okayama-u.ac.jp

Abbreviations: bp, base pairs; hr, hours; kbp, kilo base pairs; min, minutes; RNA, ribonucleic acid; sec, seconds

Abstract

Versican, a large chondroitin sulfate proteoglycan, plays a role in conditions such as wound healing and tissue remodeling. To test the hypothesis that versican is transiently expressed and plays a role in the infarcted heart, we examined its expression in a rat model of myocardial infarction. Northern blot analysis demonstrated increased expression of versican mRNA. Quantitative real-time RT-PCR analysis revealed that versican mRNA began to increase as early as 6 hours and reached its maximal level 2 days after coronary artery ligation. Versican mRNA then gradually decreased, while the mRNA of decorin, another small proteoglycan, increased thereafter. Versican mRNA was localized in monocytes, as indicated by CD68-positive staining, around the infarct tissue. The induction of versican mRNA was accelerated by ischemia/reperfusion (I/R), which was characterized by massive cell infiltration and enhanced inflammatory response. To examine the alteration of versican expression in monocytes/macrophages, we isolated human peripheral blood mononuclear cells and stimulated them with granulocyte/macrophage colony-stimulating factor (GM-CSF). Stimulation of mononuclear cells with GM-CSF increased the expression of versican mRNA as well as cytokine induction. The production of versican by monocytes in the infarct area represents a novel finding of the expression of an extracellular matrix gene by monocytes in the infarcted heart. We suggest that upregulation of versican in the infarcted myocardium may have a role in the inflammatory reaction, which mediates subsequent chemotaxis in the infarcted heart.

Key Words: coronary artery disease, cytokine, extracellular matrix, GM-CSF, monocyte

Introduction:

The extracellular matrix (ECM) has recently been recognized not only to play a part in contributing to the shape and biomechanical strength of organs and tissues, but also to function in the modulation of cell-matrix interactions resulting in cell-signaling [1]. During the healing of the infarcted heart, the sequential changes that occur in the infarct area in the myocardium initially involve the processes of cell infiltration and chemotaxis, which then affect subsequent ventricular remodeling [2]. During inflammation, activated monocytes migrate into tissues, where they interact with extracellular matrix components, such as hyaluronan (HA), produced in high amounts at inflammatory sites. During inflammatory immune responses, HA as well as HA-related molecules are thought to have roles in monocyte attachment.

Versican is a member of the chondroitin sulfate proteoglycan (CSPG) family, also referred to as the hyalectan family because of the capacity of these molecules to bind to HA. Hyalectans consist of versican, aggrecan, neurocan, and brevican [3]. Although aggrecan is most abundant in cartilage, while both neurocan and brevican are restricted to nerve tissues, versican is rather widely distributed in tissues, including the heart [4-5]. Versican has roles in modulating cell-cell and cell-matrix interactions during cell migration and proliferation processes [6]. Furthermore, the accumulation of a versican- and HA-enriched ECM has been suggested to have a role in inflammation [7]. For example, HA can serve as an attachment ligand for macrophages [8] and lymphocytes through CD44-mediated interactions [9]. Accordingly, we hypothesized that versican is involved in the inflammatory reaction after myocardial infarction (MI).

Granulocyte/macrophage colony-stimulating factor (GM-CSF) is a cytokine that stimulates the growth and differentiation of granulocyte and macrophage precursor cells *in vitro* [10]. Recently, GM-CSF was reported to facilitate infarct expansion in association with the promotion of monocyte recruitment [11]. However, the molecular mechanism by which this cytokine affects the infarcted heart is not fully understood. Thus, we examined the effect of GM-CSF on versican mRNA expression in human peripheral blood mononuclear cell (PBMC) cultures.

MATERIALS AND METHODS

Experimental myocardial infarction

All protocols involving experimental animals followed our local institutional guidelines for animal care, which are comparable to 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). The method for induction of MI has been reported by our group [12-17]. Adult Sprague-Dawley male rats weighing 200 to 250 g were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally) and the left coronary artery was ligated permanently. Echocardiographic studies were performed before the surgical procedure and immediately before sacrificing animals, as previously described [18]. LV wall motion asynergy due to MI was confirmed using ultrasound sonography (ProSound SSD- 4000; Aloka CO., LTD., Tokyo, Japan). Rats were allowed to recover and then killed at each indicated time as described below. In each analysis, sham-operated rats were also killed on the schedule noted below to serve as a control group. All tissues were weighed and rapidly frozen in liquid nitrogen and stored at -70°C. For RT-PCR analysis, pats were sacrificed at 3 hours, 6 hours, 12 hours, 1 day, 2 days, 7 days, 14 days, or 28 days post-MI and sham-operated rats served as controls (n=6 at each time point). *In vivo* ischemia and reperfusion (I/R) was induced as we previously described [2-19]. Briefly, reperfusion was carried out after 45 min of coronary ligation and it was confirmed by observation of hyperemic color of the ischemic myocardium (n=4). Rats were sacrificed at 2 days after coronary ligation or I/R for quantitative RT-PCR analysis. For the *in situ* hybridization and immunohistochemical staining, 3 rats were sacrificed 2 days after coronary obstruction.

Splicing variant analysis

The primers used in the initial semi-quantitative RT-PCR reaction were designed according to our previous report (Table I) [20]. Splicing variant-specific primers were designed using the sequence of mouse versican. Rat brain cDNA and nuclease-free water were used for positive-

and negative-controls, respectively. The number of PCR cycles was determined as previously reported [21]. Briefly, PCR was performed for 20 cycles, and the numbers of cycles was then increased by 2 cycles up to 30. Densities of the bands of PCR products were compared on the same agarose gel after electrophoresis and the number of cycles was determined as the number that showed a linear trajectory before it reached the maximum plateau.

Northern blotting

Northern blot analysis was performed following the protocol previously reported [22]. Aliquots of total RNA (40 μg from each tissue) were electrophoresed on 1% agarose-formaldehyde gels and then transferred to nylon membranes. The probe for versican cDNA used for Northern blotting was the same as recently reported [23]. The membranes were hybridized with α^{32}P-labelled cDNA probes at 65 °C for 3 hr. After the radiolabelled 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.

Quantitative RT-PCR analysis

To examine the changes in the level of versican mRNA in more detail, samples were analyzed by quantitative real-time RT-PCR as previously described [17]. The primers used for quantitative RT-PCR were designed in the α-domain of versican, and thus detected the net level of the expression of V0 and V1 mRNAs (Table I). Briefly, quantification of the mRNAs coding for versican, decorin and GAPDH was performed using the LightCycler rapid thermal cycler system (Roche Diagnostics Ltd, Lewes, UK) according to the manufacturer's instructions. A typical protocol took approximately 45 min to complete and included a 10-min denaturation step followed by 40 cycles of a 95 °C denaturation for 10 seconds, 65 °C annealing for 5 seconds, and 72 °C extension for 20 seconds. There was rarely significant primer dimer formation within the

number of cycles required for quantification for a range of experimental samples. Each RT-PCR was repeated at least 3 times to ascertain reproducibility, and data were analyzed using the absolute standard curve method [17]. The scores of “relative versican”, which indicated the expression of versican relative to that of GAPDH, were obtained by dividing the number of copies of versican transcript in a sample by the corresponding correction factor as previously described [17].

***In situ* hybridization**

In situ hybridization was performed as previously reported [24]. The hearts were fixed with 4 % paraformaldehyde. The specimens were then cut and placed on silane-coated glass slides (DakoCytomation, Kyoto, Japan). Probes were synthesized by RT-PCR by using gene-specific primers, which were identical to those used for real-time RT-PCR analysis, and ligated into TA cloning vector. Digoxigenin-UTP-labelled sense and antisense cRNA probes were synthesized by *in vitro* transcription with the relevant RNA polymerases. Hybridization was carried out overnight at 42 °C in a humidified chamber with digoxigenin-UTP-labelled antisense or sense probe. Immunological detection of digoxigenin-labelled transcripts was performed according to the manufacturer's protocol (Boehringer-Mannheim). Finally, the sections were lightly counterstained with nuclear fast red (Merck, Darmstadt, Germany).

Immunohistochemistry

Immunohistochemical analysis was performed as previously reported [12-25]. Briefly, rat hearts were fixed with 10% buffered formalin and embedded in paraffin, and then cut into 5 to 6-μm sections and placed on silane-coated glass slides (DakoCytomation, Kyoto, Japan). Monoclonal antibody against human CD68 was purchased from Serotec Ltd (Oxford, UK). After the sections were washed with PBS with 0.01% Triton X-100, they were treated with the second antibody (peroxidase-conjugated goat anti-mouse IgG (Nichirei, Tokyo, Japan) was used as the secondary antibody for CD68) for 60 min at room temperature. Finally, the sections were washed 3 times in PBS with 0.01 % Triton X-100 for 5 min each time. The sections were

then treated using an AEC kit (DAKO, Carpinteria, CA, USA). All other chemicals were purchased from Sigma Chemical Co. Ltd. (St. Louis, MO, USA.).

PBMC isolation and culture

Human PBMC were isolated from heparinized venous blood of healthy volunteers using a gradient of Lymphoprep (AXIX-SHIELD, Oslo, Norway) according to the manufacturer's protocol as previously described [26]. Briefly, the gradient was centrifuged at 800 X *g* and the PBMC at the interface were removed, washed twice and resuspended in RPMI medium-1640 (Sigma) containing 10 % heat-inactivated fetal bovine serum (FBS) and 100 IU/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5 % CO_2 in air. PBMC were plated at 5 X 10^5 / ml in 6-well plates and stimulated with GM-CSF (R&D system, Minneapolis, MN, USA) at various concentrations. After 4 hr, cells were collected and centrifuged. After centrifugation, spent media were collected and cells were lysed and RNA was extracted. The effect of GM-CSF on versican mRNA expression in PBMC was analyzed by quantitative real-time RT-PCR.

Cytokine measurements by ELISA

Interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) in spent culture media were muasured using a sandwich enzyme-linked immunoabsorbant assay (ELISA) kit (BioSource, Camarillo, CA, USA) according to the manufacturer's protocol. Briefly, the wells were incubated sequentially with spent 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. The culture medium from wells containing various concentration of GM-CSF was compared with that from unstimulated control (n=3, respectively).

Statistical analysis

Data were expressed as mean ± SE for the infarcted heart analysis and as mean ± SD for PBMC 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 PBMC analysis. A value of $P<0.05$ was considered statistically significant.

RESULTS

Versican expression in the infarcted heart

To examine which splicing variants of versican are present after MI in rats, we performed versican splicing variant expression analysis by semi-quantitative RT-PCR with primers specific for each isoform. The semi-quantitative RT-PCR results demonstrated that the V0 and V1 isoforms of versican mRNA were expressed in rat MI, while V2 mRNA was not detected and V3 mRNA was only faintly detected (Fig. 1A). Sham-operated hearts did not show any detectable versican mRNA expression with the employed numbers of cycles of amplification for semi-quantification (26 cycles for V0 and 28 cycles for other isoforms) (Fig. 1B). However, when PCR was performed at 40 cycles, all the splicing variants were observed using the same template (Fig. 1C). In contrast, mRNA obtained from rat brain showed all of the splicing variants with the employed numbers of cycles of amplification for semi-quantification (Fig. 1D). Northern blot analysis also demonstrated that the V1 isoform was the predominantly expressed isoform in the infarcted heart (Fig. 2).

Quantitative RT-PCR analysis

To examine the changes in the level of versican mRNA after MI in rats, we performed quantitative RT-PCR analysis. Figure 3A shows the time-dependent changes in versican mRNA expression levels. When the level of versican mRNA expression was standardized relative to that of GAPDH mRNA, the relative versican mRNA expression levels in the infarcted heart at 3, 6, 12, and 24 hours and 2, 7, 14, and 28 days were 9.27, 26.06, 46.96, 47.93, 75.94, 49.62, 23.52 and 15.16, respectively (Fig. 3A). ANOVA showed that the changes of versican expression were significant. On the other hand, the relative decorin mRNA expression ratios in the infarcted heart at 2 days, 7 days, 14 days, and 28 days were 4.55-, 4.89-, 7.84- and 18.2-fold higher, respectively, than those on day 1 (Fig. 3B). The expression level of versican mRNA reached its peak 2 days after MI and gradually decreased through 28 days, while the expression level of decorin mRNA increased gradually until 28 days.

In situ hybridization

Since the level of expression of versican mRNA in the infarcted heart reached its peak on day 2, we then assessed the localization of versican mRNA in the infarcted heart on day 2. Intense signals for versican mRNA were observed around the infarct zone on day 2 (Fig. 4A and 4C). The infiltrating cells were also positive for CD68 staining, as shown by using contiguous sections (indicated by arrows in Fig. 4D). Three series of experiments with different rats showed the identical signal distribution pattern. In contrast, signals for α1 (III) collagen were observed in spindle-shaped cells (indicated by arrows in Fig. 4F), but not in monocytes (Fig. 4E). The results of hematoxylin-eosin and azan-mallory staining accorded well with the findings of Fishbein et al. [27].

Ischemia/reperfusion accelerated the induction of versican mRNA expression in heart

Since versican mRNA was expressed by CD68-positive cells (i.e., monocytes/macrophages), we then examined whether I/R altered the expression levels of versican mRNA. I/R is known to enhance inflammatory responses such as IL-6 production from mononuclear cells [28-29]. As shown in Figure 5, I/R enhance the expression level of versican mRNA approximately 43-fold compared to that in the permanent ligation group (n=4, respectively).

Induction of versican mRNA expression in human PBMC by GM-CSF

Because the expression of versican mRNA was enhanced by I/R, which is characterized by enhancement of the inflammatory reaction, and CD68-positive cells were shown to express versican mRNA, we then examined whether the expression of versican mRNA was related to monocyte-to-macrophage differentiation, which is one of the important events in inflammatory reactions. PBMC were stimulated with GM-CSF at various concentrations for 4 hours. The expression level of versican mRNA was dose-dependently increased by GM-CSF stimulation (Fig. 6). The induction of versican mRNA expression by GM-CSF occurred transiently during the differentiation of monocytes to macrophages (data not shown).

GM-CSF induced cytokine production in human PBMC

To test that GM-CSF induced inflammatory cytokine release in PBMC, we finally examined the production of inflammatory cytokines, IL-6 and MCP-1, in GM-CSF-stimulated PBMC by ELISA. GM-CSF induced both IL-6 and MCP-1 in PBMC in a dose-dependent manner (Fig. 7A and 7B). However, stimulation of PBMC with human recombinant IL-6 (50 ng/ml) did not induce versican mRNA expression (data not shown).

DISCUSSION

In this study, we demonstrated that versican was transiently expressed in the infarcted heart, and showed that infiltrating monocytes were a source of versican mRNA. Stimulation of monocytes with GM-CSF was shown to induce versican mRNA expression.

Versican has been reported to show a wide distribution in adult tissues, including the heart, where it has functions in pathological conditions of the vessels such as atherosclerosis and vascular injury [20-23-30]. Versican mRNA was transiently induced after coronary artery ligation, while the mRNA of decorin, one of the major PGs in the infarcted heart, was gradually increased during the healing process after MI (e.g., 14-28 days after coronary artery ligation), which is in line with previous reports, and similar to the findings for another PG, biglycan [25-31-32]. The mRNA of another multifunctional PG, syndecan, was reported to increase on day 2 and to reach its peak on day 3 in rats [33], and was thus induced slightly later than versican mRNA. In the infarcted heart after 2 days of artery ligation, inflammatory response in the area surrounding the infarct tissues was observed [27]. Together with these data, our results demonstrated that the induction of versican mRNA expression precedes the induction of other PGs (i.e., syndecan, biglycan and decorin) and collagens, which are major components of the ECM in the infarcted heart. The time-course of versican induction indicated that versican plays a characteristic role that may be different from that of other matrix proteins in the healing process after MI.

Our *in situ* hybridization analysis using the V0/V1-specific probe demonstrated that monocytes/macrophages are the source of versican mRNA in the infarcted heart 2 days after artery ligation. Previous reports revealed that versican was inducible in various cells, including vascular smooth muscle cells and endothelial cells under stimulation with growth factors [20-34]. The significance of the V0/V1 splicing form in the infarcted heart will be investigated in future studies. Recently, THP-1 cells, which are a human leukemic monocytic cell line, were reported to express versican mRNA [35]. Our results are in line with that report, and demonstrated, in addition, that infiltrating monocytes/macrophages express versican in an *in vivo* tissue injury model. The

differentiation of monocytes to macrophages is another important factor in inflammation [36]. Monocyte-to-macrophage differentiation is associated with collagen deposition in the healing heart after infarction. Recently, Frangogiannis et al. reported that mature macrophages are found in an area of collagen deposition in the infarct area [37]. Because versican expression was shown to precede collagen expression, as we reported previously [2], the fact that monocytes express versican may indicate that versican is involved in monocyte-to-macrophage differentiation in the infarcted heart.

Ischemia/reperfusion induced versican mRNA expression. The importance of inflammation in myocardial infarction has been recognized [38]. The infiltrating cells including monocytes play roles in the cardiac repair process through a complex cascade involving cytokines and growth factors [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. Wight et al. reported that versican-HA-enriched matrix may influence the retention of inflammatory cells [34]. Although our findings do not primarily deal with the direct biological function of versican in I/R, the induction of versican by I/R was in accord with the increased inflammatory reaction in the ischemic-reperfused myocardium. Taking into account the previous findings as well as our result, it can be postulated that versican-expressing monocytes contribute to increasing the extracellular matrix environment under inflammatory conditions, along with fibroblasts, which are known to secrete HA.

GM-CSF is a cytokine that stimulates the growth and differentiation of granulocyte and macrophage precursor cells [10]. GM-CSF was reported to increase the infiltration of monocyte-derived macrophages in the infarcted heart [11]. GM-CSF was also reported to increase after MI and to be related to LV dysfunction after MI [39], although Woldbaek et al. reported that the mRNA of GM-CSF was not detected in the infarcted heart in mice [40]. These data indicate that GM-CSF is produced in other organs and circulates throughout the body,

including the healing infarcted heart. Many studies have demonstrated that GM-CSF promotes the infiltration of monocytes and macrophages after tissue injury. However, GM-CSF itself has no effect on monocyte transmigration or adhesion [41]. Under certain conditions in which chemoattractants such as MCP-1 are produced in response to injury, GM-CSF promotes the effective recruitment of monocytes to local inflammatory areas [42-43]. GM-CSF was reported to activate Akt and extracellular signal-regulated kinase (ERK) in lipopolysaccharide (LPS)-stimulated lung, and nuclear factor-kappaB (NFκB) and activator protein-1 (AP-1) were involved in this effect in the LPS-stimulated lung [44]. Versican is known to be induced by platelet-derived growth factor (PDGF) in smooth muscle cells and ERK was reported to be involved in this induction [45]. Thus, a similar cellular mechanism is proposed in our system, but this possibility remains to be tested.

One limitation of this study is that the biological function of versican expressed by monocyte/macrophages in the infarcted heart is obscure. One of the complexities of clarifying its biological function is due to the existence of isoforms of versican but, from a different point of view, isoforms may be interesting alternative tools. Versican has four isoforms and Cattaruzza et al. reported that angiogenic endothelial cells produce de novo the V3 isoform (which lacks the large size and high charge density of other isoforms, but retains the HA-binding domain of the larger isoforms), which could not be identified with the probe used for Northern analysis in the present study [20]. Recently, Lemire et al. reported that treatment with the V3 isoform affects the cell functions of arterial smooth muscle [46]. From the therapeutic standpoint, those findings may be useful information.

In conclusion, the present study demonstrated the transient induction of versican in the infarcted heart, indicating that versican may contribute to the inflammatory reaction and/or differentiation of monocytes in the infarct area. This is the first report showing that infiltrating cells express an extracellular matrix gene in the inflammatory phase after infarction, which may be associated with subsequent chemotaxis in the infarcted heart.

Acknowledgements

The authors would like to thank Dr. Toshitaka Oohashi, Dr. Tomoko Yonezawa, Ms. Yoko Bekku, Dr. Aiji Ohtsuka, and other members of our Department for stimulating discussions and help. The authors are also grateful to Mr. Tomoaki Okamoto, Ms. Tomoko Maeda and Ms. Kahori Tanaka for technical help. The 12C5 developed by Dr. Richard A. Asher was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. This work was supported in part by funding from grants-in-aid for Scientific Research from the Japan Society for the Promotion of Science (grants 15390459 to S.H. and 16591755 to S.K.).

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Table I. The primers used for the quantitative real-time RT-PCR analysis.

versican for real-time PCR	**Forward**	**5'-TGGTTGTAAATGGTCATCC-3'**
	Reverse	**5'-CCATCTTCATCTTCCTCACT-3'**
versican V0	**Forward**	**5'-GATCACGAGTATCACATGAC-3'**
	Reverse	**5'-CAGTGATGTACTGCACTGAC-3'**
versican V1	**Forward**	**5'-GCTTTGACCAGTGCGATTAC-3'**
	Reverse	**5'-CAGTGATGTACTGCACTGAC-3'**
versican V2	**Forward**	**5'-GATCACGAGTATCACATGAC-3'**
	Reverse	**5'-AGAGGCATCTAAATGTATTCAG-3'**
versican V3	**Forward**	**5'-GCTTTGACCAGTGCGATTAC-3'**
	Reverse	**5'-AGAGGCATCTAAATGTATTCAG-3'**
GAPDH	**Forward**	**5'-AACACAGTCCATGCCATCAC-3'**
	Reverse	**5'-TCCACCACCCTGTTGCTGTA-3'**
decorin	**Forward**	**5'-TGGACTGAACCGTATGATTG-3'**
	Reverse	**5'-GCTGGCTGCATCAACTTT-3'**

Figure legends

Figure 1. Splicing variant analysis of versican mRNA in myocardial infarction. Splicing variant-specific primers (for V0, V1, V2 and V3, respectively) were used for RT-PCR analysis. An equal amount of template was used for each RT-PCR analysis. A molecular size marker, 100 bp ladder, was loaded in the light lane in each panel. **A**. Infarcted heart expressed versican V0 and V1 forms after artery ligation, while V2 mRNA was not detected and V3 mRNA was only faintly detected. **B**. Sham-operated heart showed undetectable expression of versican mRNA at the same number of cycles of RT-PCR as used in A. **C**. The same templates as used in B were used for 40 cycles in RT- PCR analysis. Note that sham-operated heart expressed all the splicing variants at low levels. **D**. Brain cDNA was used as a control for all splicing variant forms.

Figure 2. Representative results of Northern blot analysis in the infarcted heart. Northern blot analysis revealed the transient induction of versican mRNA in rat infarction (cont, normal heart; 2 d, 2 days after artery ligation; 7 d, 7 days after artery ligation; 14 d, 14 days after artery ligation; 28 d, 28 days after artery ligation). The hybridized band corresponded to the V1 isoform, as indicated by its size. Note that versican mRNA expression was upregulated 2 days after artery ligation, and then decreased.

Figure 3. A: Quantitative real-time RT-PCR analysis of versican mRNA in the infarcted heart. 3 hr, 3 hours after artery ligation; 6 hr, 6 hours after artery ligation; 12 hr, 12 hours after artery ligation; 1 d, 1 day after artery ligation; 2 d, 2 days after artery ligation; 1 d, 1 day after artery ligation; 7 d, 7 days after artery ligation; 14 d, 14 days after artery ligation; 28 d, 28 days after artery ligation (n=6, mean ± SE). Closed circles indicate infarcted heart and open-circles indicate sham-operated heart. Note that the relative versican mRNA level (the ratio of the expression level of versican/GAPDH mRNA) was increased as early as 6 hours, and the transient induction of versican reached its peak at 2 days after coronary artery ligation. * Indicates $P < 0.05$ compared to compared to 3 hr. Sequential changes in versican mRNA expression are also noted. **B: Quantitative real-time RT-PCR analysis of decorin mRNA in the infarcted**

heart. In the infarct zone, decorin reached its peak 28 days after ligation, exhibiting a pattern of gradual increase different from that of versican mRNA (n=6, mean ± SE). The expression level of decorin mRNA is shown as fold-increase compared with that 1 day after ligation. All the quantitative real-time PCR analyses were repeated three times.

Figure 4. *In situ* hybridization analysis of versican mRNA in the infarcted heart. Versican mRNA signals were observed in the infarct peripheral zone. 'MI-center' indicates the infarct central zone. **A:** Versican mRNA detected in the infarct peripheral zone 2 days after MI. The boxed area shows the area observed at higher magnification in C. **B:** Hybridization with sense-probe in the infarcted heart 2 days after MI. There were no signals detected. The boxed area shows the area observed at higher magnification in E. **C:** Higher magnification of the boxed region in A. Versican mRNA was detected in the mononuclear cells (blue) in the area surrounding the infarct zone 2 days after MI. **D:** CD68 immunostaining observed in a contiguous section is shown for comparison. Numerous infiltrating monocytes were observed in the area surrounding the infarct zone 2 days after MI (brown). Note that versican mRNA was detected in CD68-positive cells (arrows), as shown in C. **E:** Higher magnification of the boxed region in B (with sense-probe). **F:** α1 (III) mRNA expression (blue) in the infarcted heart (contiguous sections to 3E). There was no detectable α1 (III) mRNA expression in the presumed monocytes (arrows) using contiguous sections. Spindle-shaped mesenchymal cells (presumably fibroblasts) expressed α1 (III) mRNA. Note that the distribution pattern of versican mRNA was distinct from that of collagen mRNA. A scale bar is shown in each panel.

Figure 5. Enhancement of versican mRNA induction by reperfusion in the infarcted heart. RNA was extracted from the permanent ligation group (permanent) and ischemia/reperfusion (I/R) group (n=4, respectively). The amount of versican mRNA was determined by real-time RT-PCR analysis and compared with that in the permanent ligation group as fold-increase. Ischemia/reperfusion significantly induced versican mRNA (43.35±23.75) in the infarcted heart (mean ± SE). * Indicates $P < 0.01$ compared to the permanent ligation group.

Figure 6. Effect of GM-CSF on versican mRNA expression in PBMC. PBMC were stimulated with various concentrations of GM-CSF for 4 hr. The levels of versican mRNA expression were normalized with that of GAPDH and compared with the levels in unstimulated cells and expressed as fold-increase. The expression level of versican mRNA was dose-dependently increased (n=3, mean ± SD). * Indicates $P < 0.05$ compared to unstimulated cells.

Figure 7. GM-CSF induced IL-6 and TNFα production in PBMC. Human PBMC were isolated as described in *Material and Methods*. PBMC were stimulated with various concentrations of GM-CSF for 4 hr. The induction of IlL-6 and MCP-1 were measured by ELISA. The induction of cytokines was dose-dependently increased (n=3, mean ± SD). * Indicates $P < 0.05$ compared to unstimulated cells.

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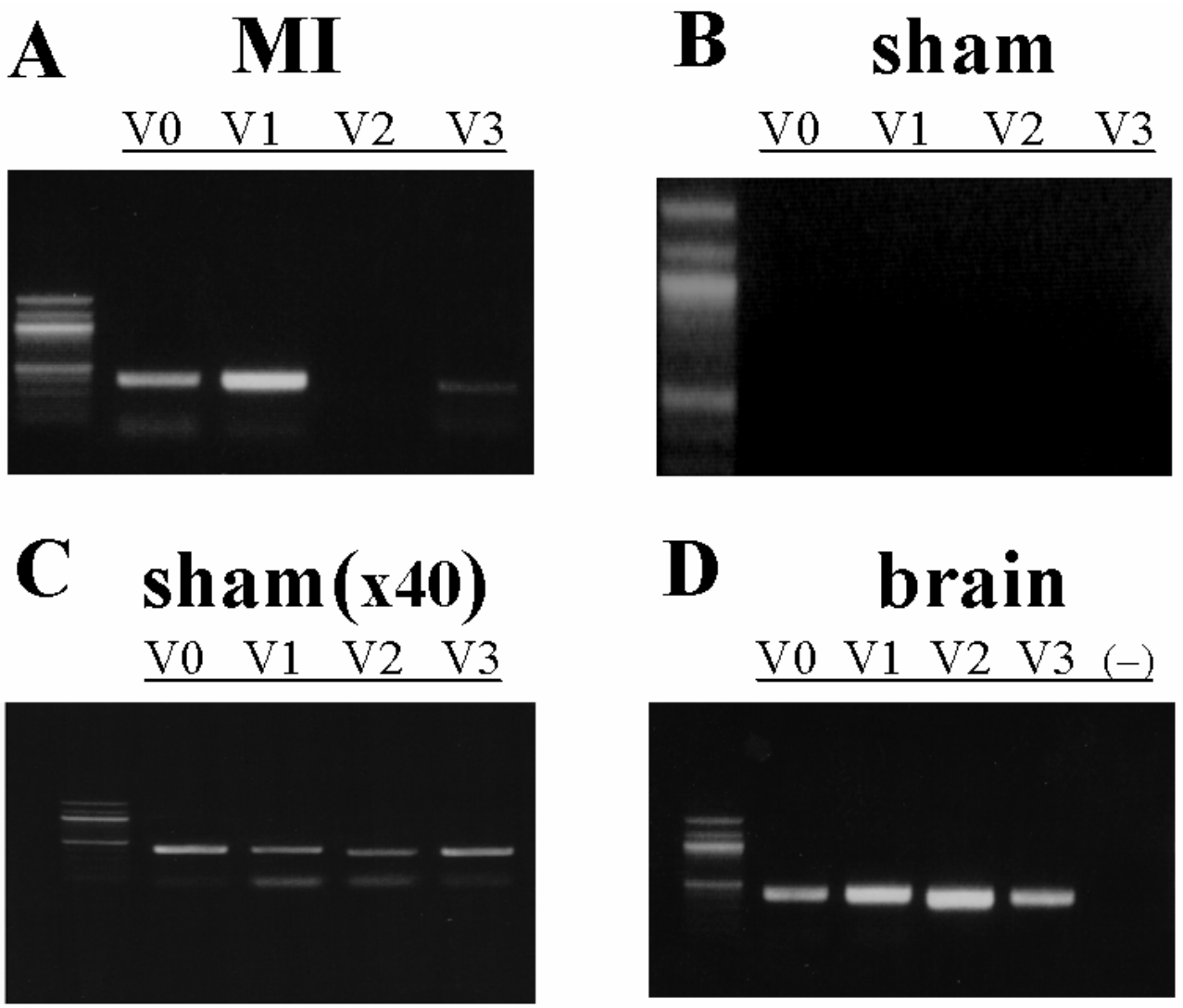

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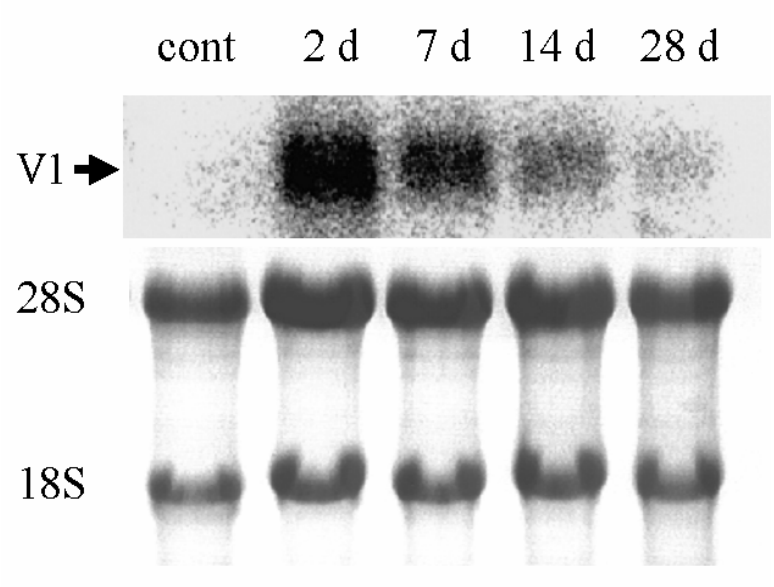

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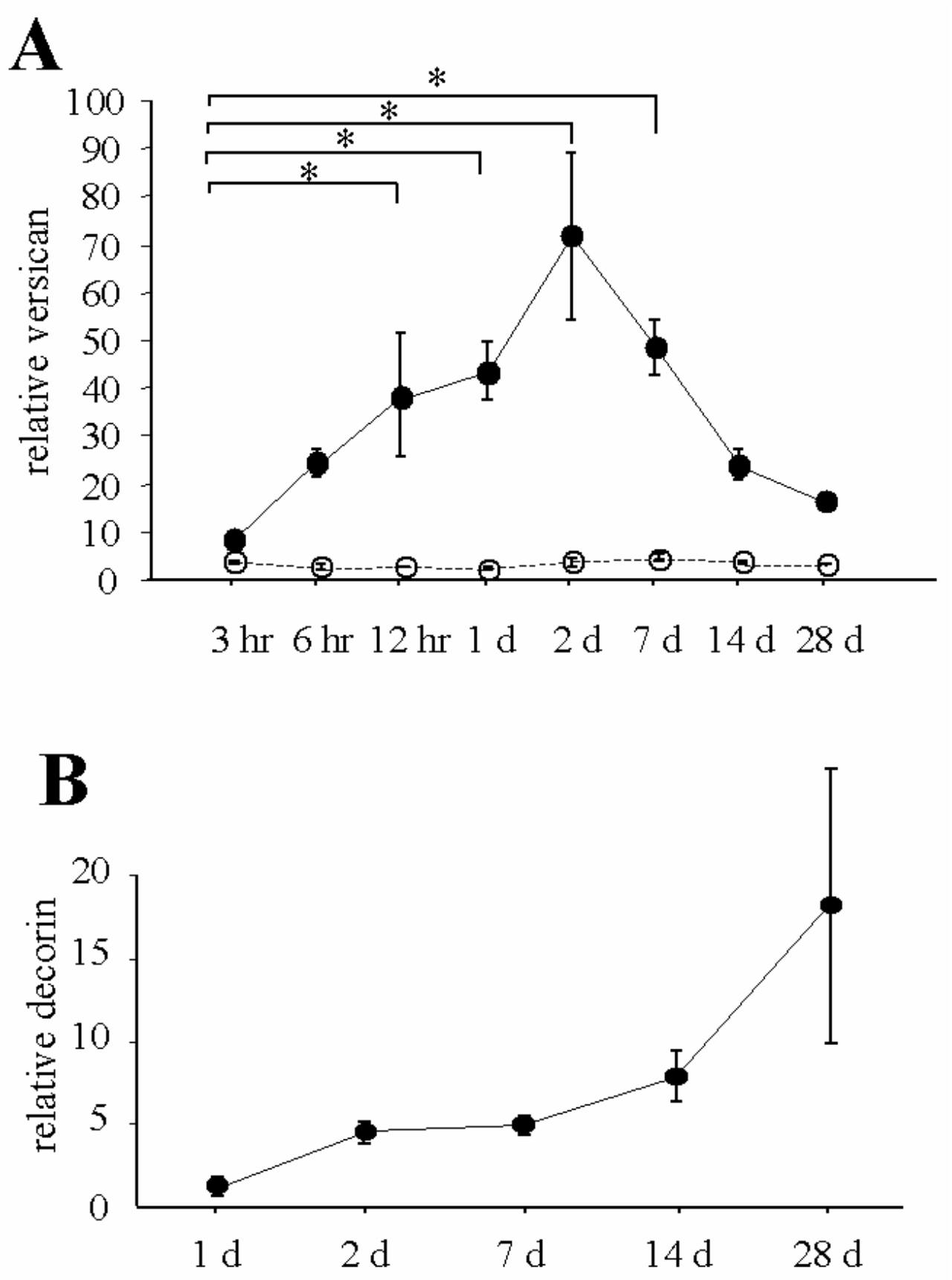

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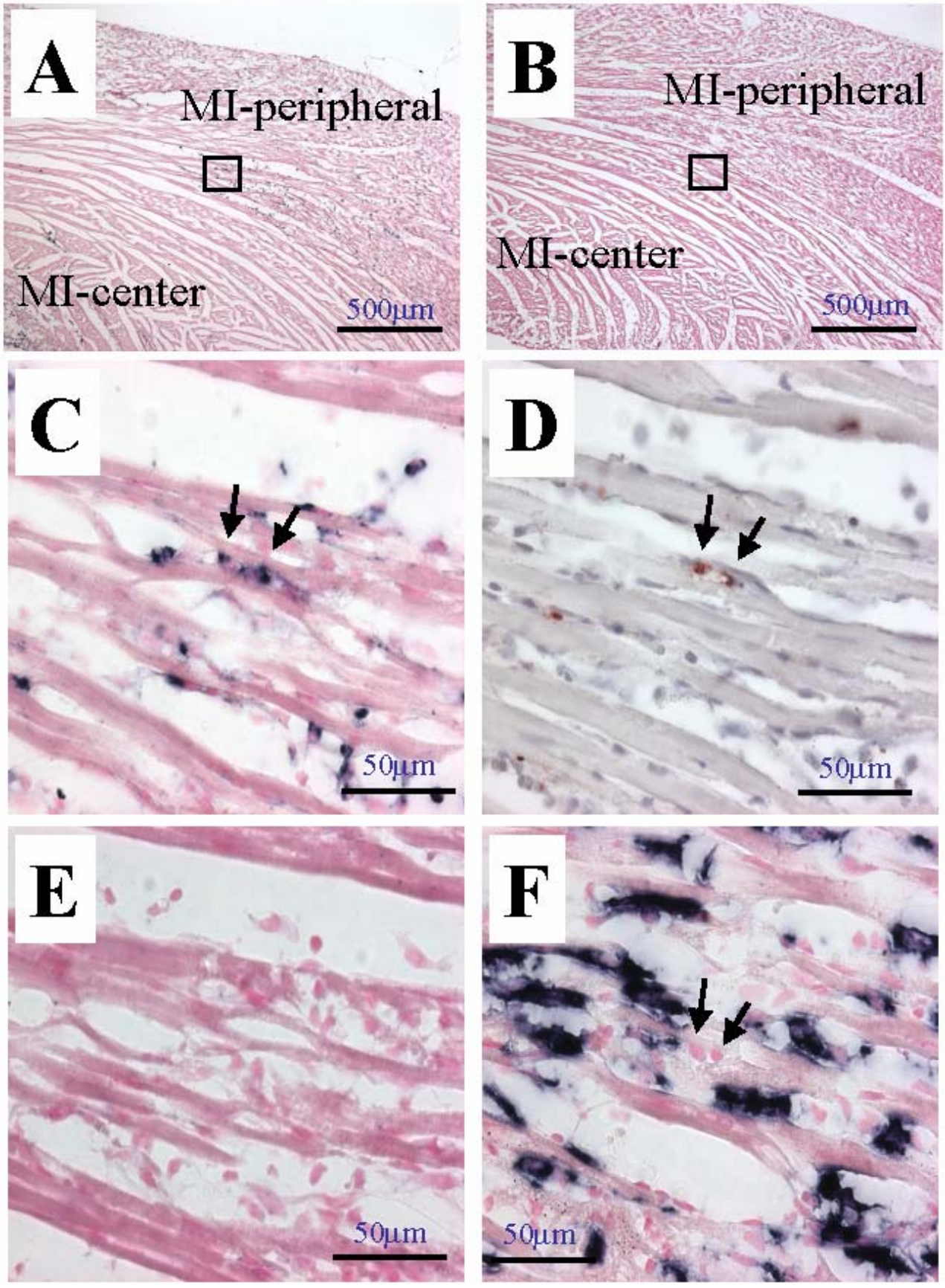

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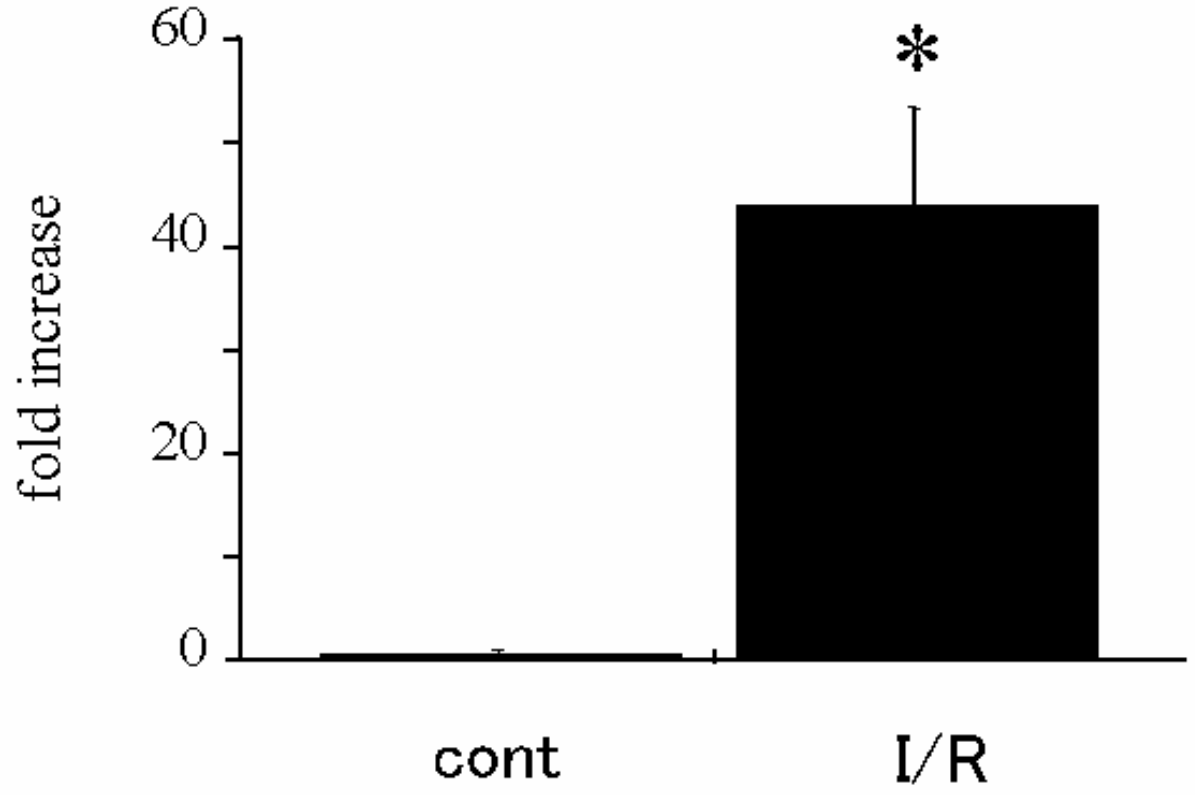

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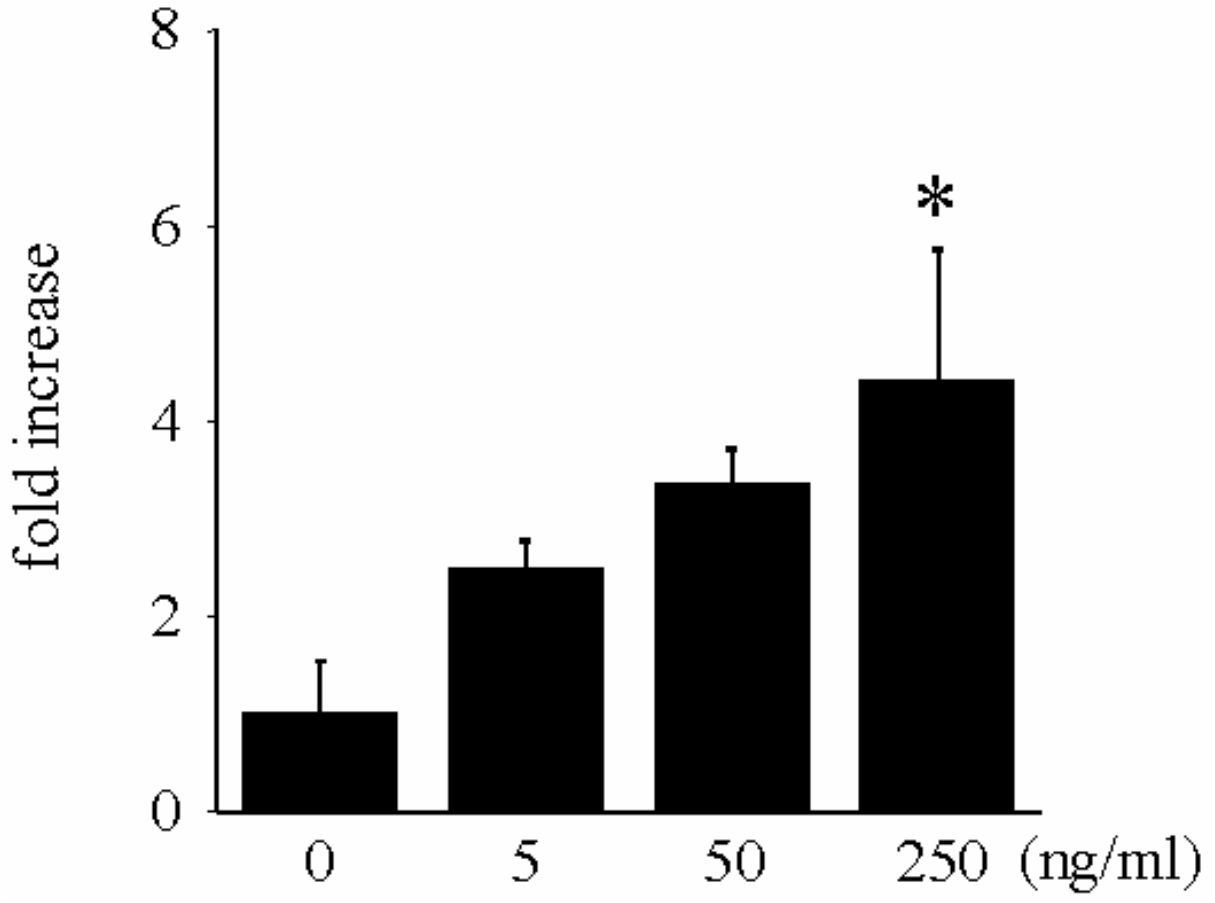

Figure 7. Toeda et al,