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## Abstract

ADAMTS1 (a disintegrin and metalloproteinase with thrombospondin motifs 1) is an inflammatory-induced gene. We have previously reported that ADAMTS1 was strongly but transiently expressed in the infarcted heart. In this study, we investigated whether a 3'-untranslated region (UTR) affects the mRNA stability of this gene. When stimulated with tissue necrosis factor (TNF)-alpha, the expression level of ADAMTS1 mRNA rapidly increased, but the induction of ADAMTS1 mRNA peaked at 6h after stimulation, after which the expression levels of ADAMTS1 mRNA decreased. The 3'-UTR ADAMTS1 mRNA contains multiple adenine and uridine-rich elements, suggesting that the 3'-UTR may regulate gene stability. The addition of actinomycin D, an RNA synthesis inhibitor, demonstrated the decay of induced ADAMTS1 mRNA by TNF-alpha. Furthermore, a region containing multiple AUUUA motifs within the ADAMTS1 3'-UTR destabilized transfected Enhanced Green Fluorescence Protein (EGFP) mRNA expression. These results demonstrated that the ADAMTS1 3'-UTR may regulate the expression of ADAMTS1 mRNA.

**KEYWORDS:** ADAMTS1, gene regulation, metalloproteinase

Original Article

## The 3'-untranslated Region of ADAMTS 1 Regulates Its mRNA Stability

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ADAMTS1 (a disintegrin and metalloproteinase with thrombospondin motifs 1) is an inflammatory-induced gene. We have previously reported that ADAMTS1 was strongly but transiently expressed in the infarcted heart. In this study, we investigated whether a 3'-untranslated region (UTR) affects the mRNA stability of this gene. When stimulated with tissue necrosis factor (TNF)- $\alpha$ , the expression level of ADAMTS1 mRNA rapidly increased, but the induction of ADAMTS1 mRNA peaked at 6h after stimulation, after which the expression levels of ADAMTS1 mRNA decreased. The 3'-UTR ADAMTS1 mRNA contains multiple adenine and uridine-rich elements, suggesting that the 3'-UTR may regulate gene stability. The addition of actinomycin D, an RNA synthesis inhibitor, demonstrated the decay of induced ADAMTS1 mRNA by TNF- $\alpha$ . Furthermore, a region containing multiple AUUUA motifs within the ADAMTS1 3'-UTR destabilized transfected Enhanced Green Fluorescence Protein (EGFP) mRNA expression. These results demonstrated that the ADAMTS1 3'-UTR may regulate the expression of ADAMTS1 mRNA.

**Key words:** ADAMTS1, gene regulation, metalloproteinase

A disintegrin and metalloproteinase with thrombospondin motifs 1 (ADAMTS1) is a member of the matrix metalloproteinase (MMP) family [1]. ADAMTS1 was originally reported to be induced by inflammatory stimulation [2], and we have previously reported that ADAMTS1 was induced in the infarcted heart [3]. Interestingly, the induction of ADAMTS1 mRNA in the infarcted heart peaked at 6h and then declined to the normal level [3], although the subsequent inflammatory reaction persists in the infarcted

healing heart.

The mRNA level was not only determined by the ADAMTS1 induction rate but also by the rate of its disappearance. Differential mRNA turnover may contribute to rapid changes in the pattern of cellular gene expression in various responses, such as inflammation. Several decay pathways have been characterized, and the adenylate and uridylate (AU)-rich elements (AREs) present within the 3'-untranslated region (UTR) of mRNAs represent the most characterized and well conserved pathway [4-6].

A recent report has shown that ADAMTS1 was an early response gene in endothelial cells stimulated with leukotriene [7]; accordingly, we hypothesized

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that ADAMTS1 is a rapid turnover gene. To investigate this hypothesis, we examined the expression kinetics of ADAMTS1 mRNA in tissue necrosis factor (TNF)- $\alpha$ -stimulated endothelial cells and the half-life of its RNA. We then examined the effects of AREs in the 3'-UTR of ADAMTS1 mRNA.

## Materials and Methods

**Reagent and cell culture.** COS-7 cells were purchased from ECACC (Dainippon Sumitomo Pharmaceutical Co., Osaka, Japan), as previously reported [8]. COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% Fetal Bovine Serum (FBS), 100U/ml penicillin, and 100 $\mu$ g/ml streptomycin. Cells were cultured at 37°C under 5% CO<sub>2</sub> and 20% O<sub>2</sub> in a humidified chamber. For cytokine stimulation, cells were diluted to 2.0 $\times$ 10<sup>5</sup> cells/well in a 12-well plastic plate and cultured for 24h. Tissue necrosis factor (TNF)- $\alpha$  was purchased from R&D Systems (Minneapolis, MN, USA), and actinomycin D was purchased from Sigma (St. Louis, MO, USA). For the mRNA stability assay, cells were stimulated with TNF- $\alpha$  for 3h, actinomycin-D was added to the medium (100 $\mu$ g/ml), and the mRNA was extracted as indicated.

**RNA preparation, RT-PCR, and quantitative real-time RT-PCR.** Following stimulation, the cells were washed once with ice-cold phosphate-buffered saline (PBS) and total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions as previously reported [9, 10]. Two micrograms of total RNA were reverse-transcribed to cDNA with ReverTra Ace (Toyobo, Osaka, Japan) according to the manufacturer's protocol. Reverse transcription polymerase chain reaction (RT-PCR) was performed as previously described [11]. Quantitative real-time RT-PCR analysis was performed using a LightCycler rapid thermal cycler system (Roche Diagnostics, Ltd. Mannheim, Germany) following the protocol previously reported [12, 13]. The PCR mixture consisted of 2 $\times$ SYBR Green PCR Master Mix (Toyobo, Osaka, Japan), which includes DNA polymerase, SYBR Green I Dye, dNTPs, including dUTP, PCR buffer, forward and reverse primers, and cDNA of samples in a total volume of 20 $\mu$ l. The amplification of a housekeeping gene,  $\beta$ -actin, was used to normalize the

efficiency of cDNA synthesis and the amount of RNA applied.

The primers for ADAMTS1 were 5'-GGAC-AGGTGCAAGCTCATCTG-3' and 5'-GAGC-TCCTAGTAACATATATTGTAT-3' and for  $\beta$ -actin were 5'-TTCCTGGGCATGGAGTCCT-3' and 5'-AGGAGGAGCAATGATCTTGATC-3', respectively. There was rarely significant primer dimer formation within the cycles required for quantification from a range of experimental samples. To validate the specificity of amplification of ADAMTS1 and  $\beta$ -actin, we analyzed each PCR product by agarose gel electrophoresis after real-time detection. Experiments were repeated with cDNA from at least 3 different experiments, using a minimum of triplicates for each cell. Negative controls were performed with samples in which RNA templates were replaced by nuclease-free water in the reactions. The intra- and interassay coefficients of variations were <5% and were reasonably small compared with those in other reports. Subsequently, the threshold cycle (Ct), *i.e.*, the number of cycles at which the amount of the amplified gene of interest reached a fixed threshold, was determined.

**Expression construct with the ADAMTS1 3'-UTR.** To examine the effect of the 3'-UTR on the decay of RNA, we amplified the 3'-UTR of human ADAMTS1 mRNA by PCR. Proofreading polymerase (LA taq; Takara, Ohtsu, Japan) was used for amplification. The primers used for amplification were 5'-AGATCTGGTTTAAGTGGTGTAGC-3' (3'-UTR sense; italics indicate the added sequence of restriction enzyme Bgl II), 5'-GAGCTCGACTG-AGATGGTCC-3' (3'-UTR anti-sense short; italics indicate the added sequence of restriction enzyme Sac I), and 5'-GAGCTCCTAGTAACATATATTGTAT-3' (3'-UTR anti-sense long; italics indicate the added sequence of restriction enzyme Sac I). The locations of each primer are shown in Fig 1. We amplified the ADAMTS1 mRNA 3'-UTR using human umbilical vein endothelial cells (HUVEC) cDNA as templates and cloned into TA-ligation vector (PCR 2.1, Invitrogen). The constructs were confirmed by sequencing. After restriction enzyme digestion and DNA elution, each insert was ligated to the EGFP carrying vector (pmaxGFP; Amaxa Gaithersburg, MD, USA).

**Transfection assays.** Gene transduction was

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taagtgtttaaagtggtttagctttgagggcaaggcaaagtgaggaagggctggtgcagggaaagcaaga
aggctggaggatccagcgtatcttgccagtaaccagtgagggtatcagtaagggtggattatgggggtag
atagaaaaggagttgaatcatcagagtaaactgccagttgcaaattgataggatagttagtgaggattataa
cctctgagcagtgatagcataataaagccccgggcattattattattctttgttacatctattacaagtttag
aaaaaacaagcaattgtcaaaaaaagttgaactattacaaccctgtttcctggacttatacaataacttagt
atcatgggggtgggaaatgaaaagtaggagaaaagtgagatttactaagacctgttttactttacctactaa
caatgggggggagaaaggagtacaaataggatctttgaccagcactgtttatggctgctatggttcagagaat
gtttatacattattctaccgagaattaaaactcagattgtcaacatgagagaaaggctcagcaacgtgaaat
aacgcaaatggcttcctcttctttttggaccatctcagctcttattgtgtaattcatttggagaaaaacaactc
catgtttttatcaagtgcataaagttcacaatggaaaaaagcagtgaaagcattagatgctgtaaaaagcta
gaggagacacaatgagcttagtacctccaactccttcttctaccatgtaaccctgcttgggaaatggatgt
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gagggtgtgtagaacaggggtcccacaggttggggacattgagatcactgtctgtggtggggaggctgtgga
ggggtagcaggtccatctccagcagctgtccaacagctgctatcctgggtaagtctgttcagctctctgtgag
aatatgatttttccatagtatatagtataatgttactataaattacatgtactttataagattggttgggtgtcc
ttccaagaaggactatagttagtaataatgcctataataacatattttttatacatttttctaatgaaaaaaa
ctttaaattatcgccttttggagtgcatataaataagattttttacaatatatgttactagaaataaaaaga
acacttttgaaaaaaaaaaaaaaaaaaaaaa

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**Fig. 1** The 3'-UTR sequence of human ADAMTS1 mRNA. The 3'-UTR sequence of human ADAMTS1 mRNA is shown. The stop codon is indicated in italics, and the primers used for each construct are indicated by arrows. The forward primer was common for the GFP-ADAMTS1-3'-UTR-short construct (pmax-S) and GFP-ADAMTS1-3'-UTR-long construct (pmax-L). The reverse primer for pmax-S was designed upstream of the first AUUUA motif in the human ADAMTS1 mRNA 3'-UTR. The reverse primer for pmax-L was designed downstream of triple AUUUA motifs in the ADAMTS1 mRNA 3'-UTR. Multiple AUUUA sequences in the ADAMTS1 3'-UTR are boxed.

performed as previously described [14, 15]. In brief, COS-7 cells were plated at a density of  $2 \times 10^5$  cells/well in 12-well plates (Falcon, Franklin Lakes, NJ, USA). After 18h, cells were transfected with each construct (1.0  $\mu$ g of DNA/well) by Lipofectamine 2000 (3  $\mu$ l; Invitrogen), according to the manufacturer's instructions. After 6h of transfection, the DNA-containing medium was changed and cells were grown for an additional 24h in serum-supplemented medium. As a control, cells were transfected with pmaxGFP or the vector alone without GFP and ADAMTS1-3'-UTR. For the mRNA stability assay, cells were transfected with each construct, and after 48h of transfection, actinomycin-D was added to the medium (100  $\mu$ g/ml) and the mRNA was extracted as indicated.

**Statistical analysis.** All data are expressed as the mean value  $\pm$  SD unless otherwise specifically mentioned. Student's *t*-tests were applied for statistical analysis, as appropriate. *P* values  $< 0.05$  were considered significant.

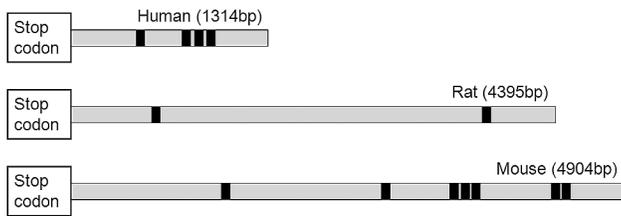
## Results

### *Unique sequence located in the 3'-UTR of*

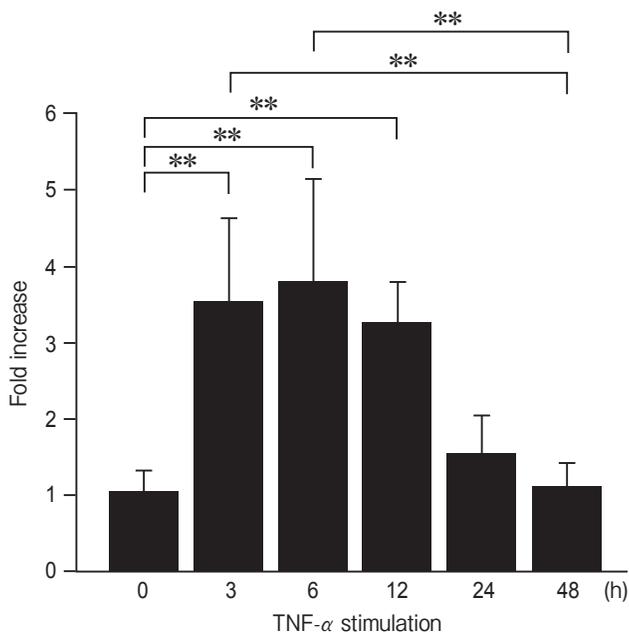
**ADAMTS1 mRNA.** The AUUUA sequence located in the 3'-UTR in various genes binds to another molecule, which degrades RNA by making a complex [16, 17]. As shown in Fig. 1, the ADAMTS1 mRNA sequence obtained from the NCBI database and amplified mRNA extracted from HUVEC contained the unique 3'-UTR sequence that involved multiple AUUUA sequences. A database search revealed that these unique multiple repeats within ADAMTS1 3'-UTR were conserved within humans, mice, and rats (Fig. 2).

**ADAMTS1 expression is increased in TNF- $\alpha$ -stimulated cells.** We examined the expression of ADAMTS1 mRNA induction in COS-7 cells stimulated with TNF- $\alpha$  (20 ng/ml) by quantitative real-time RT-PCR analysis. As shown in Fig. 3, the ADAMTS1 mRNA expression level was increased as early as 3h by TNF- $\alpha$  stimulation. Interestingly, the increase of ADAMTS1 mRNA by TNF- $\alpha$  peaked at 6h, and then the level decreased.

**ADAMTS1 expression is increased in TNF- $\alpha$ -stimulated cells.** We next examined the stability of ADAMTS1 mRNA in stimulated cells. COS-7 cells were stimulated with TNF- $\alpha$  (20 ng/ml) for 3h, and then actinomycin D was added. As shown in Fig. 4,



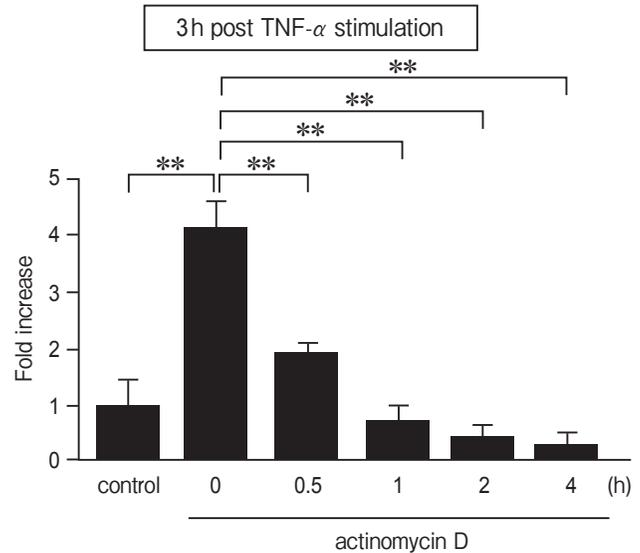
**Fig. 2** Comparison of the 3'-UTR AUUUA sequence location of each ADAMTS1 mRNA. Human, mouse, and rat ADAMTS1 3'-UTR sequences were compared. The locations of each AUUUA repeat are indicated by black bars.



**Fig. 3** Expression of ADAMTS1 mRNA in COS-7 cells stimulated by TNF- $\alpha$ . COS-7 cells were stimulated with TNF- $\alpha$  for the indicated time ( $n=5$ ). The relative expression level of ADAMTS1 was compared with that of unstimulated cells. Data are the mean  $\pm$  S.D. \*\* indicates  $p < 0.01$  compared to the control.

the level of ADAMTS1 mRNA induced by TNF- $\alpha$  was significantly decreased at 30 min when actinomycin D was added.

**The effect of the ADAMTS1 mRNA 3'-UTR on the stability of GFP mRNA.** We amplified the 2 kinds of 3'-UTR of human ADAMTS1 mRNA, that is, the short 3'-UTR and long 3'-UTR, and connected them to the pmaxGFP vector after the stop codon. The connected GFP-ADAMTS1-3'-UTR constructs were transfected to COS-7 cells. The transfection efficiency was 46.2%. After 24h, 48h, and



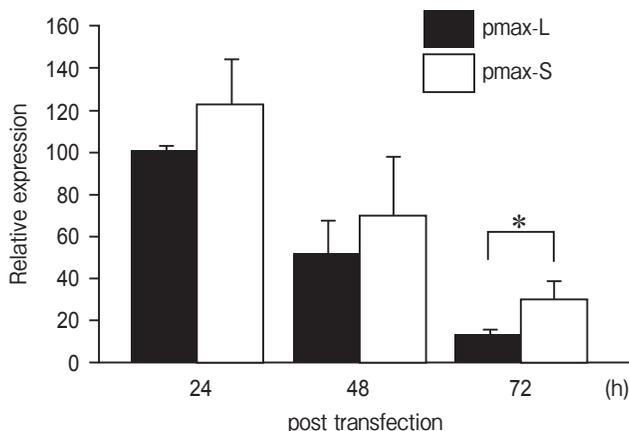
**Fig. 4** Stability of ADAMTS1 mRNA induced by TNF- $\alpha$ . COS-7 cells were stimulated with TNF- $\alpha$  for 3h, and then actinomycin D was added. RNA was extracted at the indicated time post-actinomycin D treatment, and the mRNA level was examined by real-time RT-PCR ( $n=4$ ). Data are the mean  $\pm$  S.D. \*\* indicates  $p < 0.01$  compared to the control.

72h of transfection, mRNA was extracted and the expression of GFP mRNA was examined by real-time RT-PCR analysis. As shown in Fig. 5, the GFP mRNA expression level was significantly lower in GFP-ADAMTS1-3'-UTR-long (pmax-L) than in GFP-ADAMTS1-3'-UTR-short (pmax-S) at 72h post-transfection. We then determined the stability of GFP mRNA with each construct by adding actinomycin D. As shown in Fig. 6, the induced GFP mRNA level by pmax-L was significantly decreased at 2h after actinomycin D treatment, and its magnitude was greater than that in pmax-S.

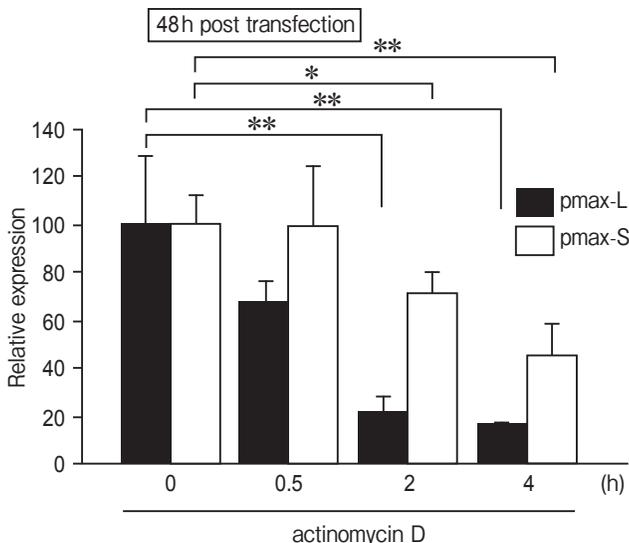
## Discussion

In this study we demonstrated the posttranscriptional regulation of ADAMTS1. Together with the observation of the decay of ADAMTS1-3'-UTR-driving GFP mRNA, our findings suggest a role of the ADAMTS1-3'-UTR in regulating its mRNA stability.

ADAMTS1 mRNA expression was induced in this study by various stimulations described previously [2, 18]. We have previously reported that ADAMTS1 is rapidly induced in myocardial infarction and



**Fig. 5** Comparison of GFP mRNA level transfected with two constructs. COS-7 cells were transfected with two constructs. GFP mRNA was connected with either ADAMTS 1-3'-UTR-long (pmax-L; black bar) or ADAMTS 1-3'-UTR-short (pmax-S; white bar). The constructs were transfected, and mRNA expression levels were compared ( $n=3$ ). Data are the mean  $\pm$  S.D. \* indicates  $p < 0.05$  compared to the control.



**Fig. 6** GFP-ADAMTS 1-3'-UTR-long mRNA was decreased by actinomycin D treatment. After 0.5h, 2h, or 4h incubation with actinomycin D, total RNA was subjected to real-time RT-PCR. GFP mRNA levels were compared. Mean  $\pm$  S.D.,  $n=3$ . (black bar, pmax-L; white bar, pmax-S). \* indicates  $p < 0.05$  and \*\* indicates  $p < 0.01$  compared to the control, respectively.

ADAMTS 1 mRNA was expressed by endothelial cells in the border zone of infarction [3]. Interestingly, the induced expression of ADAMTS1 mRNA peaked as early as 3h after coronary ligation. Cross *et al.*

reported that ADAMTS1 mRNA was induced in cerebral ischemia, but the induction peaked at 24h after infarction, although the subsequent inflammatory reaction continued thereafter [19]. These observations led us to hypothesize that ADAMTS1 mRNA may be unstable. To test our hypothesis, we examined mRNA stability by adding actinomycin D and measured its mRNA half-life [16].

The effect of actinomycin D on ADAMTS1 mRNA stability was examined by real-time RT-PCR, as previously reported [20]. The posttranscriptional regulation of RNA is widely distributed and has been observed in various genes [21, 22]. One motif that plays key roles in its regulation is the conserved AU-rich sequence in the 3'-UTR. Observation of the instability demonstrated the involvement of AUUUA elements of the human ADAMTS1-3'-UTR. Malter reported identification of the AU-binding factor (AUBF) that binds to four AUUUA repeats [23]. Bohjanen identified 3 factors (*i.e.*, AU-A, AU-B, and AU-C) that bind AUUA multimers [24, 25]. These factors bind to the 3'-UTR of the transcript, form a complex, and modulate RNA metabolism. We did not primarily deal with the identification of factors that bind to ADAMTS1 mRNA, but the cluster of AUUUA sequences in the ADAMTS1 3'-UTR suggested the involvement of these RNA-binding factors in its posttranscriptional regulation. To confirm the regulating role of AUUUA sequences in the ADAMTS1 3'-UTR, further experiments using either specific deletion or mutation of AUUUA are needed.

In this study, we constructed the ADAMTS1-3'-UTR with and without AUUUA motifs located at the 3'-end of ADAMTS 1 mRNA (pmax-L and pmax-S). Using the transfection of constructs with GFP, we examined the effects of the ADAMTS1-3'-UTR by examining the GFP mRNA level in transfected COS-7 cells. When COS-7 cells were transfected with the long 3'-UTR-GFP, which contains multiple AUUUA sequences, the mRNA expression level was not significantly different from that of other constructs with the short 3'-UTR (*i.e.*, without AUUUA repeats). This may be due to strong induction of the GFP mRNA level as compared with the decay of mRNA. We therefore added actinomycin D to the transfection medium and examined the decay of induced GFP mRNA. Actinomycin D treatment demonstrated the short half-life of GFP-ADAMTS 1-3'-UTR-long, indi-

cating that the 3'-UTR-long of ADAMTS1 mRNA is responsible for its short half-life and stability.

On the basis of the results of this study, it is reasonable to propose that changes in protein levels of ADAMTS1 are generally due to changes of ADAMTS1 mRNA. Our group has previously reported that ADAMTS1 mRNA was induced in myocardial infarction as early as 3h after the onset of infarction, but its protein level quickly decreased 2 days after coronary ligation [3]. ADAMTS1 is a secreted protein, and after secretion, ADAMTS1 is further degraded by either MMP or itself; however, the change of the ADAMTS1 protein level in the infarcted heart is almost parallel to that of the ADAMTS1 mRNA level in myocardial infarction [3, 26]. Therefore, we speculate that degradation of ADAMTS1 protein is not a major factor for this change.

There are several limitations of this study; for instance, the biological significance of the short half-life of ADAMTS1 mRNA remains unknown. We did not primarily deal with the biological role of ADAMTS1 stability. In fact, ADAMTS1 is recognized as an early response gene against stimulation from the outside. Because ADAMTS1 is an active metalloproteinase, it makes sense that there is a substrate for ADAMTS1 in acute inflammation and that this proteolytic activity is required only in the acute phase. Screening the substrates in acute inflammation may elucidate the biological role of ADAMTS1 in inflammation. Next, whether the AUUUA motif on the ADAMTS1-3'-UTR is essential for its mRNA regulation has still not been completely clarified. Either specific deletion or mutation of the AUUUA motif on ADAMTS1-3'-UTR will explain this issue.

In summary, our study results demonstrated that the ADAMTS1-3'-UTR contains multiple AUUUA motifs and may regulate the stability of ADAMTS1 mRNA.

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