ADAMTS1 Is a Unique Hypoxic Early Response Gene Expressed by Endothelial Cells*

Received for publication, March 6, 2009, and in revised form, March 31, 2009 Published, JBC Papers in Press, April 6, 2009, DOI 10.1074/jbc.M109.001313

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ADAMTS1 (a disintegrin and metalloproteinase with thrombospondin motifs) is a member of the matrix metalloproteinase family. We have previously reported that ADAMTS1 was strongly expressed in myocardial infarction. In this study, we investigated whether hypoxia induced ADAMTS1 and investigated its regulatory mechanism. In hypoxia, the expression level of ADAMTS1 mRNA and protein rapidly increased in endothelial cells, but not in other cell types. Interestingly, the induction of ADAMTS1 by hypoxia was transient, whereas vascular endothelial growth factor induction by hypoxia in human umbilical vein endothelial cells (HUVEC) increased in a time-dependent manner. CoCl2, a transition metal that mimics hypoxia, induced ADAMTS1 in HUVEC. The phosphatidylinositol 3-kinase inhibitor LY294002 dose-dependently inhibited the increase of ADAMTS1 mRNA expression in HUVEC. We characterized the promoter region of ADAMTS1, and the secreted luciferase assay system demonstrated that hypoxia induced luciferase secretion in the culture medium 4.6-fold in HUVEC. In the promoter region of ADAMTS1, we found at least three putative hypoxia-inducible factor (HIF) binding sites, and the chromatin immunoprecipitation assay revealed HIF-1 binding to HIF binding sites in the promoter region of ADAMTS1 under hypoxia. Recombinant ADAMTS1 protein promoted the migration of HUVEC under hypoxic conditions. In summary, we found that ADAMTS1 is transiently induced by hypoxia in endothelial cells, and its transcription is mediated by HIF-1 binding. Our data indicate that ADAMTS1 is a novel acute hypoxia-inducible gene.

ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) is a newly discovered matrix metalloproteinase with multiple domains, including propeptide, metalloproteinase, disintegrin, and spacer region domains, and domains containing thrombospondin type I motifs. The ADAMTS family now contains 19 members. One of the best characterized biological functions of ADAMTS is proteolytic activity against extracellular matrix proteins, including proteoglycans. ADAMTS1 is strongly expressed in myocardial infarction. In this study, we found that the exposure of endothelial cells to hypoxia up-regulated ADAMTS1 expression, and the transcriptional activation of ADAMTS1 was mediated by HIF-1, which binds to HREs in the promoter region of ADAMTS1 gene.

MATERIALS AND METHODS

Reagents and Cell Culture—All reagents were purchased from Sigma unless otherwise specified. HUVEC and human pulmonary artery endothelial cells (HPAEC) were purchased from Sanko Junyaku (Tokyo, Japan), and human microvascular arthritic cartilage, aggregan breakdown, mediated by members of ADAMTS, not matrix metalloproteinases, is one of the features of the progressive stage. ADAMTS5 (aggreganase-2) null mice show protection against experimental arthritis (4, 5).

ADAMTS1 was originally cloned from a murine colon carcinoma cell line (6). Recently, it has been reported that a polymorphism of ADAMTS1 is closely associated with the occurrence of ischemic heart disease (7). In the cardiovascular system, ADAMTS1 plays roles in atherosclerosis by degrading versican (8) and TFPI-2 (tissue factor pathway inhibitor-2) (9). We have previously reported that ADAMTS1 was induced in the infarcted heart (10); however, the regulatory mechanism of ADAMTS1 in ischemia is unknown.

Low oxygen tension (hypoxia) affects endothelial cells in a number of ways, including the transcriptionally regulated expression of vasoactive substances and matrix proteins (11). One mechanism of the regulation of gene expression in response to hypoxia is mediated by the transcriptional factor HIF-1 (hypoxia-inducible factor 1) (12). HIF-1 is a heterodimer composed of HIF-1α and HIF-1β/ARNT (aryl hydrocarbon receptor nuclear translocator) subunits. In normoxia, HIF-1α protein undergoes rapid ubiquitin-mediated proteolysis (13), thus enabling the modulation of HIF-1 activity in an oxygen-dependent manner. HIF-1 binds to the hypoxia response elements (HREs) in response to hypoxic stimuli. Genes transactivated by HIF-1 include EPO (erythropoietin), glucose transporter 1, VEGF (vascular endothelial growth factor), and ET-1 (endothelin-1) (14, 15).

In this study, we found that the exposure of endothelial cells to hypoxia up-regulated ADAMTS1 expression, and the transcriptional activation of ADAMTS1 was mediated by HIF-1, which binds to HREs in the promoter region of the ADAMTS1 gene.

* This work was supported in part by a grant-in-aid for scientific research from the Japan Society for the Promotion of Science (Grants 19659142 and 20390399 to S. H.).

1 The abbreviations used are: HRE, hypoxia response element; HUVEC, human umbilical vein endothelial cells; HPAEC, human pulmonary artery endothelial cells; FBS, fetal bovine serum; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) 2-(4-sulfophenyl)-2H-tetrazolium; RT, reverse transcription; PBS, phosphate-buffered saline; PIPES, 1,4-piperazinediethanesulfonic acid; ChiP, chromatin immunoprecipitation; IL, interleukin.
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endothelial cells were purchased from Kurabo (Osaka, Japan). Endothelial cells were cultured in EBM-2 (Cambrex Bioscience, Walkersville, MD) with a kit containing 2% fetal bovine serum (FBS), growth factors, 100 units/ml penicillin, and 100 μg/ml streptomycin, as previously described (16). ARPE-19 was purchased from the American Type Culture Collection (ATCC, Manassas, VA), and human skin fibroblasts were obtained as previously reported (16). Human skin fibroblasts and ARPE cells were cultured in Dulbecco’s modified Eagle’s medium with 10% FBS and antibiotics. Cells prior to passage 10 were used for analysis. Cells were cultured at 37 °C under 5% CO₂ and 20% O₂ in a humidified chamber. For hypoxic stimulation, cells were diluted to 2.0 × 10⁵ cells in a 12-well plastic plate and cultured for 48 h, and control cultures were continuously incubated in 5% CO₂ and 95% air at 37 °C. Hypoxic cultures were placed within an incubator chamber (ASTEC Co. Ltd., Fukuoka, Japan), filled with 1% O₂, 5% CO₂, and balance N₂ for the times indicated. For chemically mimicked hypoxia, cobalt chloride (CoCl₂) was used at a final concentration of 150 μM. We also tested inhibitors of RNA and protein synthesis under hypoxia. Actinomycin D (RNA synthesis inhibitor; 100 μg/ml) or cycloheximide (a protein synthesis inhibitor; 100 μg/ml) was added prior to stimulation. Purified recombinant human ADAMTS1 protein (amino residues 50–734) was purchased from R&D Systems (Minneapolis, MN). The phosphatidylinositol 3-kinase inhibitor (LY294002) was purchased from Cell Signaling (Danvers, MA), dissolved in DMSO, and used at the final concentrations indicated under hypoxic conditions.

Cell Proliferation Assay—The viability and proliferation of HUVEC were determined using a standard 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) viability test (CellTiter AQueous One Solution cell proliferation assay kit (Promega, Madison, WI)) according to the manufacturer’s instructions. Briefly, a suspension of transfected HUVEC (1.5 × 10⁵ cells/well) in 1000 μl of EBM2 with 2% FBS was added to 12-well plates for the chromatin immunoprecipitation (ChIP) assay. Cells were allowed to recover for 18 h, and the following day, cells were stimulated with hypoxia (1% O₂) for 24 h and then normoxia (20% O₂) for 24 h, respectively. After collecting culture medium at each point (Fig. 6B), cell viability was measured using a plate reader (17, 18). All samples were read in triplicate. For the functional assay, HUVEC (1 × 10⁵ cells/well) in 500 μl of EBM2 with 2% FBS were seeded into 24-well plates and stimulated with hypoxia (1% O₂) for 12 h with each concentration of recombinant ADAMTS1 protein. All samples were read in quadruplicate.

RNA Preparation 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 as previously reported (19). Quantitative real time RT-PCR was performed using a LightCycler rapid thermal cycler system (Roche Applied Science) following the previously reported protocol (20, 21). Each sample was amplified in triplicate or duplicate and independently repeated at least three times. The intra- and interassay coefficients of variations were <5% and were reasonably small compared with those in other reports. The primers used in this study are listed in Table 1. Regarding internal control, we used a human housekeeping gene primer set (Takara, Ohtsu, Japan) and determined the most stable housekeeping genes according to the manufacturer’s instructions. Briefly, 15 primers for housekeeping genes were examined, and we selected the three most stable genes (human ATP5F1 (ATP synthase, H⁺-transports, mitochondrial F0 complex, subunit B1), RPLP0 (ribosomal protein, large, P0), and RPLP2 (ribosomal protein, large, P2)) as suitable internal controls for our analysis (data not shown). Subsequently, the threshold cycle (Ct) (i.e. the number of cycles at which the amount of amplified gene of interest reached a fixed threshold) was determined. The amount of ADAMTS1 or VEGF mRNA under normoxic and hypoxic conditions was normalized to the internal control. The data obtained with RPLP0 and RPLP2 are not shown, because the results were identical to those obtained with ATP5F1 (data not shown). To verify the presence and the predicted size of amplified fragments, PCR products were separated by electrophoresis and visualized in 1.5% agarose gels with ethidium bromide.

Protein Extraction, Western Blot Analysis, and Antibodies—After stimulation, the cells were washed once with PBS and then scraped from the plates. Cells were solubilized in 250 μl of CellLytic™ M (Sigma) with a protease inhibitor mixture. After incubation in a rotator at 4 °C for 15 min, the samples were centrifuged, and the supernatants were collected. The protein concentration of the cell extracts was determined using a protein assay kit (Bio-Rad). Fifteen micrograms of total protein were used for Western blot analysis, as described previously (22, 23). Briefly, ~30 μl of each sample was mixed with 5× sample buffer and subjected to SDS-PAGE using a gradient gel (5–15%) (Bio-Rad). After electrophoresis, proteins were transferred to nitrate cellulose membranes (Advantech, Tokyo, Japan) overnight at 4 °C and then blocked for 1 h in 5% nonfat dried skim milk in PBS containing 0.05% Tween 20 (PBS-T). The membranes were hybridized at 4 °C overnight with anti-ADAMTS1 antibody (3C8F4; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or anti-β-actin antibody (Sigma). After stringent washing with PBS-T three times for 5 min each at room temperature, the membranes were incubated with the appropriate secondary antibodies (MP Biomedicals, Aurora, OH). Following three successive washes with PBS-T, immunoreactive bands were visualized using the enhanced chemiluminescence system (ECL plus; GE Healthcare). Signals were detected with LAS1000 (Fuji Film, Tokyo, Japan), and the densitometry was performed with Image J software (W. Rasband, Research Services Branch, NIMH, National Institutes of Health, Bethesda, MD) and normalized to the signal intensity of β-actin for equal protein loading control of each sample in each experiment. This quantification was performed with the linear range of the standard curve defined by the standard sample, β-actin, for all densitometry analysis.

Plasmid Constructs for Reporter Assays—We used the secreted luciferase vector system as previously reported (24). The structures of ADAMTS1 promoter-luciferase constructs are shown in Fig. 5. To generate the ADAMTS1 promoter reporter plasmid, a fragment containing bases −1638 through −342 of the ADAMTS1 gene 5′-flanking region was amplified by PCR with a proofreading enzyme (Takara LA taq; Takara,
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TABLE 1
Primers used for PCR analysis

| Primer | Sequence |
|--------|----------|
| ADAMTS1 Forward | 5’-GGACAGGTTGCGAAGCTTCATCTGTG-3’ |
| ADAMTS1 Reverse | 5’-CTCTACACCCTFFGGGCTGGAA-3’ |
| ChIP a Forward | 5’-GCCCCTCAGAGTAAGCCTGAGTGGT-3’ |
| ChIP a Reverse | 5’-GCTCCAGTATGATCTGGGTCTCCCT-3’ |
| ChIP b Forward | 5’-GGAGGACCTAGTGGACTCAATA-3’ |
| ChIP b Reverse | 5’-GCTNTATCCCGTTAAGAACCC-3’ |
| ChIP c Forward | 5’-GATAATACCTTTTCTCTCCT-3’ |
| ChIP c Reverse | 5’-TCAAGAAATGATCGCTCC-3’ |
| ChIP d Forward | 5’-TTTTTGTAGGAGACCCAGAAGCTG-3’ |
| ChIP d Reverse | 5’-CCCGGCTACCTCTTACC3’ |

RESULTS

ADAMTS1 Expression Is Increased in Hypoxic Endothelial Cells—We first examined whether the expression of the ADAMTS1 gene is induced in hypoxic HUVEC. As shown in Fig. 1A, the ADAMTS1 mRNA expression significantly increased as early as 1 h after hypoxic stimulation, as seen in quantitative real time RT-PCR analysis. Interestingly, the increase of ADAMTS1 mRNA by hypoxia peaked at 3 h, decreased at 6 h, and then returned to the normal level (normoxia) at 24 h. The production of ADAMTS1 at the protein level by hypoxia was confirmed to be transient by Western blot analyses. The ADAMTS1 promoter was functional in hypoxic HUVEC cells, as shown by the ChIP assays, indicating that the hypoxia-induced ADAMTS1 expression is regulated at the transcriptional level. The ChIP assays were performed using a Promoter ChIP kit (Clontech, Mountain View, CA) according to the manufacturer’s instructions. Briefly, 1.5 × 10⁶ HUVEC in a 10-cm dish were stimulated with either normoxia or hypoxia for 3 h. Chromatin was cross-linked with formaldehyde (final concentration at 1%) at room temperature for 15 min. The cells were then washed with PBS, centrifuged, lysed in 200 μl of lysis buffer (5 mM PIPES, 1 mM KCl, 10% Nonidet P-40) in the presence of protease inhibitors, and incubated for 10 min on ice. Cell lysates were sonicated for 5 s six times to shear DNA to a length of 200–800 bp. Lysates were centrifuged at 13,000 rpm for 10 min at 4 °C, and supernatants were transferred to new tubes. Supernatants were preclariied with a mixture of salmon sperm DNA/protein A-agarose 50% slurry for 30 min at 4 °C. Samples were centrifuged, agarose was then pelleted, and supernatants were transferred to new tubes. For immunoprecipitation, anti-HIF-1α monoclonal antibody (Novus Biologicals Antibodies, Littleton, CO) was added and incubated overnight at 4 °C with gentle rotation. Isotype-matched mouse IgG2b (Ancel Corp., Bayport, MN) served as a negative control. We also used anti-acetyl-histone H4 antibody (Upstate/Millipore) as a positive control. The next day, samples were washed with four supplied washing buffers for 15 min each at 4 °C with gentle rotation and then eluted twice with elution buffer for 15 min each. The eluted chromatin was dissolved in Tris-EDTA buffer, and agarose beads were spun down. Cross-links were reversed by the addition of 20 μl of 5 mM NaCl to a volume of 500 μl of chromatin-containing buffer and by heating the mixture to 65 °C for 4 h. EDTA and Tris–HCl buffers were added together with proteinase K. Sheared DNA was recovered using a DNA recovery kit (NucleoSpin Macherey-Nagel, Duren, Germany). To identify immunoprecipitated ADAMTS1 genomic DNA, PCR was performed using the primers listed in Table 1. PCR was performed at 96 °C for 4 min and then 96 °C for 30 s, 56 or 57 °C for 1 s, and 72 °C for 30 s for 30 cycles, as previously described (25). We used input DNA (genomic DNA after sonication) as a positive control and immunoprecipitated DNA with mouse IgG2b as a negative control for the experiments.

Cell Migration Assay—The endothelial cell migration assay was performed as previously described (26). In brief, HUVEC (passages 6–8) were seeded on a collagen-coated 24-well plate to 70% confluence. After an 18-h incubation, a single uniform scratch was made using a 1000-μl blue plastic pipette tip. The scratch resulted in a cell-free gap of ~1.0 mm between two adjoining areas of HUVEC. The medium was changed to with or without recombinant ADAMTS1 protein, and HUVEC were incubated under normoxia or hypoxia for 12 h. After incubation, cells were fixed with paraformaldehyde (w/v in PBS), and each scratch was photographed along the length of the scratch, starting proximally and ending distally (n = 6). Areas covered by migrating cells across wound edges were quantitatively measured using Win Roof software (Mitani Corp., Tokyo, Japan).

Statistical Analysis—Data are expressed as the mean ± S.D. unless specifically mentioned. Student’s t test was applied for statistical analysis, as appropriate. p values of <0.05 were considered significant.
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analysis and was quantitatively analyzed (Fig. 1B). The increased expression of ADAMTS1 protein was parallel to the mRNA expression increase in hypoxic HUVEC. CoCl$_2$, a chemically mimicked hypoxic condition, also increased the expression of ADAMTS1 mRNA (Fig. 1C). We then examined the hypoxic induction of ADAMTS1 mRNA expression in other endothelial cells (Fig. 2). Both human microvascular endothelial cells and HPAEC increased the ADAMTS1 mRNA expression by hypoxia, but the induction pattern and its increase differed somewhat depending on the cell line. The induction of ADAMTS1 mRNA by hypoxia was also confirmed in mice using mouse brain endothelial cells (data not shown).

**Actinomycin D Inhibits the Increased Expression of ADAMTS1 in Hypoxic HUVEC**—We then treated HUVEC with actinomycin D and cycloheximide and examined the effect of hypoxia on ADAMTS1 transcription. Although actinomycin D markedly blocked the expression of ADAMTS1 mRNA, cycloheximide had no effect on the ADAMTS1 mRNA expression, indicating that new protein synthesis under hypoxia is not required for ADAMTS1 mRNA induction (Fig. 3).

**ADAMTS1 Expression Is Not Increased in Hypoxic Nonendothelial Cells**—We then examined the hypoxic induction of ADAMTS1 mRNA in other types of cells (Fig. 4A). The cells examined in this study (fibroblasts and ARPE) did not show a significant increase of the ADAMTS1 mRNA expression by hypoxia. We then compared the pattern of ADAMTS1 and VEGF mRNA expressions in HUVEC and fibroblasts in hypoxia, respectively. In HUVEC, ADAMTS1 mRNA expression was increased by hypoxia, and then VEGF mRNA expression gradually increased (Fig. 4B). In fibroblasts, as shown in Fig. 4C, the VEGF mRNA expression was gradually increased by hypoxic stimulation, whereas ADAMTS1 mRNA was not significantly altered. These results indicate that the induction of ADAMTS1 mRNA by hypoxia is specific to certain types of cells.

**Phosphatidylinositol 3-Kinase Inhibitor Inhibits the Increased Expression of ADAMTS1 in Hypoxic HUVEC**—It is known that hypoxia increases the phosphorylation of AKT downstream of phosphatidylinositol 3-kinase activation (27). Treating cells with phosphatidylinositol 3-kinase inhibitor (LY294002) blocks the induction of ADAMTS1 mRNA expression in HUVEC under hypoxia in a dose-dependent manner (Fig. 5A). SB203580, a p38 inhibitor, attenuated ADAMTS1 mRNA expression by hypoxia in a dose-dependent manner (Fig. 5B). PD98059, an extracellular signal-regulated kinase inhibitor, moderately attenuated ADAMTS1 mRNA expression but did not reach statistical significance (Fig. 5C). On the
other hand, VEGF mRNA expression was attenuated by SB203580 but not by LY294002 in hypoxic HUVEC (Fig. 5D).

Human ADAMTS1 Promoter Is Activated by Hypoxia—To confirm that the increase in ADAMTS1 mRNA was related to an increase in gene transcription, we used a luciferase construct in which the ADAMTS1 promoter (∼1.8 kb) was inserted into the luciferase gene. We amplified the 5′-flanking region of the ADAMTS1 gene (−1628 to +207) to the upstream of the luciferase reporter gene with specific primers (Fig. 6A). Sequence analysis of the 1.8-kb promoter region revealed that the ADAMTS1 promoter contains a TATA box-like sequence. Promoter data base analysis revealed the presence of several conserved HIF (a major regulatory factor of hypoxia-induced gene expression) binding elements in the construct. To determine which region is responsible for hypoxic induction of ADAMTS1, we prepared seven different constructs by deleting the ADAMTS1 gene (Fig. 6A), and secreted luciferase activity was measured in HUVEC under hypoxia (Fig. 6B). In normoxia, luciferase activity by the ADAMTS1 promoter was low compared with the pMetLuc-Control vector (Fig. 6C). HUVEC were cultured for a further 24 h under hypoxia, and we measured the luciferase activity again (Fig. 6D). Luciferase activity was normalized to the change of that in the pMetLuc-Control vector as 1.0 (Fig. 6E). Luciferase activities driven by −1628, −1493, −1271, −960, −706, and −451 bp of ADAMTS1 promoters were induced 2.6–4.6-fold under hypoxia, but deletion of the sequence between −451 and −342 bp abolished the hypoxic responsiveness of ADAMTS1 promoter, resulting in a minimal promoter (−451-ADAMTS1) responding to hypoxia (Fig. 6E). These data suggest that HREs located between −1628 and −342 are responsible for the hypoxic induction of the ADAMTS1 promoter.

The ADAMTS1 Promoter Binds to HIF-1 under Hypoxia in HUVEC—We then determined where HIF-1 binds to the ADAMTS1 promoter under hypoxia. From the results of the luciferase assay, we chose four HREs in the promoter region for the

**FIGURE 3.** Effect of actinomycin D and cycloheximide on expression of ADAMTS1 mRNA in HUVEC under hypoxia. HUVEC were pretreated with actinomycin D or cycloheximide and cultured under hypoxia for 3 h (n = 4). The results are in triplicate. * p < 0.05 compared with the control.

**FIGURE 4.** A, expression level of ADAMTS1 mRNA in hypoxic nonendothelial cells (n = 4). The endogenous level of ADAMTS1 under normoxia was compared by real-time RT-PCR, and the change of the expression level of ADAMTS1 mRNA under hypoxia was examined. Note that no cells (fibroblast and a retinal pigment epithelial cell (ARPE)), except HUVEC, showed a significant change in the ADAMTS1 mRNA expression. B, differential induction pattern of two genes, ADAMTS1 and VEGF, in HUVEC (n = 4). The change in ADAMTS1 and VEGF mRNA expressions under hypoxia was examined. * p < 0.05 compared with the control. C, VEGF mRNA expression was induced, but that of ADAMTS1 was not induced by hypoxia in fibroblasts (n = 4). Fibroblasts were exposed to hypoxia and gene expressions of ADAMTS1 and VEGF mRNAs were examined. * p < 0.05 compared with the control.
Genomic DNA obtained from hypoxic HUVEC was immunoprecipitated with anti-HIF-1 antibody at at least two different HIF-1 binding sites (Fig. 7, bottom). Thus, the induction of ADAMTS1 mRNA expression in hypoxic HUVEC was mediated by HIF-1 binding to the ADAMTS1 promoter.

**ADAMTS1 Promotes Cell Proliferation and Migration of HUVEC under Hypoxia**—We finally examined the effect of ADAMTS1 on HUVEC under hypoxia. As shown in Fig. 8A, recombinant ADAMTS1 protein (1 and 6 nM) significantly increased the numbers of HUVEC after 12 h under hypoxic conditions. As shown in Fig. 8, B and C, recombinant ADAMTS1 protein enhanced the migration of HUVEC after 12 h under hypoxic conditions. Interestingly, this effect was negated at higher concentrations (12 nM) and rather tended to inhibit the migration of HUVEC (data not shown). Thus, a low concentration of recombinant ADAMTS1 protein promoted HUVEC migration under hypoxia.

**DISCUSSION**

This is the first report demonstrating that hypoxia induced ADAMTS1 transcription via the binding of HIF-1 in endothelial cells. Together with the observation that this hypoxic induction of ADAMTS1 is quick and selective for endothelial cells, our findings suggest the potential unique role of ADAMTS1 in acute hypoxic endothelium. Several conditions induced ADAMTS1 expression, with inflammation being a major factor (6). Ng et al. (28) reported that interleukin-1β (IL-1β) increased ADAMTS1 mRNA levels in human decidual stromal cells. Bevitt et al. (29) reported that ADAMTS1 was induced by TNFα stimulation in ARPE. Cross et al. (30) reported that ADAMTS1 mRNA was increased by IL-1β and TNF in cultured astrocytes; however, Kalinski et al. (31) reported that ADAMTS1 mRNA was decreased by IL-1β in the human chondrosarcoma cell line C3842. In fact, we have previously reported that IL-1β stimulation rather decreased ADAMTS1 mRNA in another chondrosarcoma cell line, OUMS-27 (32). Interestingly, Kalinski et al. (31) also reported that hypoxia did not change ADAMTS1 mRNA in another chondrosarcoma cell line, C3842, which was consistent with our data on nonendothelial cells. These results indicate that the transcriptional regulation of ADAMTS1 depends on stimulation and cell types.

The current study demonstrated that hypoxia induction of ADAMTS1 did not persist under longer hypoxia. The kinetics of ADAMTS1 induction by hypoxia was distinct compared with other hypoxia adaptive genes, such as EPO and VEGF. The regulatory mechanism of ADAMTS1 mRNA in hypoxia is not identical to that of VEGF in hypoxia, because the kinetics under hypoxic HUVEC are clearly different. One of the suggested mechanisms is the decay of ADAMTS1 mRNA. There are multiple AUUUA motifs in the 3′-untranslated region of ADAMTS1 mRNA, which plays a role in the early degradation of ADAMTS1 mRNA (33). Cross et al. (30) reported that ADAMTS1 mRNA increased after experimental cerebral ische-
The induction of \textit{ADAMTS1} peaked 24 h after occlusion and then declined (30), indicating transient induction of \textit{ADAMTS1} in ischemic disease, which was consistent with our previous results (10). These data and the current results suggest that \textit{ADAMTS1} is a rapid turnover protein in ischemic disease; therefore, we speculate that we can detect the acute phase of ischemia. The induction of \textit{ADAMTS1} peaked 24 h after occlusion and then declined (30), indicating transient induction of \textit{ADAMTS1} in ischemic disease, which was consistent with our previous results (10). These data and the current results suggest that \textit{ADAMTS1} is a rapid turnover protein in ischemic disease; therefore, we speculate that we can detect the acute phase of ischemia.
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ischemic disease by measuring the induced ADAMTS1 protein, and the serum ADAMTS1 level may be a unique biomarker for “acute hypoxia/ischemia.”

We used the secreted luciferase assay to determine hypoxic transcriptional activation in the ADAMTS1 promoter. To our knowledge, this is the first application of this system in hypoxia study. As expected, the control luciferase vector (pMetLuc-Control Vector) did not significantly change luciferase activity between normoxia and hypoxia, whereas ADAMTS1 promoter activation was observed as the secretion of luciferase under hypoxia. In our study, the −1271 construct was the most effective for hypoxic induction of secreted luciferase and this construct is now under investigation to identify whether it can be a novel tool for detecting acute ischemia.

Our study showed HIF-1 binding to the ADAMTS1 promoter. HIF plays a central role in the maintenance of oxygen (O₂) homeostasis, which is essential for cell survival (34). Under hypoxia, α and β subunits of HIF form a heterodimer complex, which binds to HRE in the promoter region of many hypoxia-inducible genes. As demonstrated by ChIP assay, HIF-1α binds to at least two different sites in the ADAMTS1 promoter under hypoxia. The most upstream of the HIF-1 binding sites was actually located in the F2 construct used for the luciferase assay, but F2 did not show the strongest luciferase activity. This discrepancy can be explained by the existence of a repressor for hypoxic luciferase induction in the F2 construct, but further investigation is needed for clarification. Thus, HIF-1 is considered a regulating mechanism of ADAMTS1 transcriptional activation under hypoxic conditions.

In our study, various endothelial cells expressed ADAMTS1 by hypoxic stimulation, indicating that this response to hypoxia is common in endothelial cells. Many reports have shown that hypoxia is closely related to angiogenesis. In addition, ADAMTS1 has been reported to inhibit angiogenesis in a cornea pocket assay and chorioallantoic membrane assay (35); however, Krampert et al. (36) reported that ADAMTS1 has dual functions (i.e. ADAMTS1 stimulated the migration of fibroblasts at low concentrations but inhibited this process at high concentrations). Our results using low concentrations of recombinant ADAMTS1 protein under hypoxia showed a promigratory effect on HUVEC. However, when a high concentration of recombinant ADAMTS1 (12 nm) was added to the hypoxic HUVEC, the promigratory effect by recombinant ADAMTS1 was absent (data not shown), indicating that the actual concentration of ADAMTS1 under the hypoxic endothelium is crucial for its biological function. We have previously reported that ADAMTS1 mRNA was expressed by endothelial cells in the border zone of infarction, where angiogenesis became prominent a few days later (10). Although we did not clarify the precise mechanism, either a low concentration or fall of a high concentration of hypoxia-induced ADAMTS1 may be linked to initiation of the angiogenic response in the acute phase of ischemic endothelial cells.

There are several limitations of this study. First, we did not clarify the role of ADAMTS1 in acute ischemia. Because ADAMTS1 is an active metalloproteinase and various substrates have been identified, there is possibly a substrate for ADAMTS1 in acute hypoxia. Second, the significance of ADAMTS1 induction in acute hypoxia has not been fully demonstrated. It was noted that the induction pattern of ADAMTS1 under hypoxia was dif-

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**FIGURE 7. HIF-1 binds to the ADAMTS1 promoter.** The position of the targeting HRE in the ADAMTS1 promoter is indicated as boxes (a–d), and the positions of the primers are shown as numbers (top). The results of the ChIP assay are shown at the bottom. Note that positive bands for HRE b and c were observed under hypoxia. Input, positive control; IgG, negative control; Nor, normoxia; Hyp, hypoxia.

**FIGURE 8. Effects of recombinant ADAMTS1 protein on HUVEC under hypoxia.** A, proliferation of HUVEC by recombinant ADAMTS1 protein under hypoxia. HUVEC were cultured under normoxia or hypoxia for 12 h, and cell proliferation was examined by MTS assay. Different concentrations of ADAMTS1 protein in medium containing 2% FBS were added to the cells. Medium containing only 2% FBS was used as a control. All counts were normalized to 100% of the control. *, p < 0.05 compared with the control. B, migration of HUVEC by recombinant ADAMTS1 under hypoxia. HUVEC were treated with each concentration of ADAMTS1 under hypoxia for 24 h. C, the newly covered area by migrating cells under normoxia or hypoxia was calculated and set as 100% for the control (n = 6). *, p < 0.05 compared with control in normoxia. **, p < 0.05 compared with the control in hypoxia. Data are expressed as the mean ± S.E.
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Acknowledgments—We thank Drs. Toshitaka Oohashi, Tomoko Yonezawa, Ryusuke Momota, Masahiro Murakami, Kursat O. Yaykasli, and members of our department for helpful discussions and advice. We thank Dr. Nobuhito Goda (Department of Life Science and Medical Bio-Science, Waseda University) for reviewing the manuscript.

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S. Hirohata, unpublished data.