Role of ADAMTS-1 in Atherosclerosis
Remodeling of Carotid Artery, Immunohistochemistry, and Proteolysis of Versican

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Objective—We investigated the potential role of ADAMTS-1 (a disintegrin and metalloprotease with thrombospondin motif type I) in atherogenesis.

Methods and Results—ADAMTS-1 is expressed at the highest levels in the aorta when compared with other human tissues examined. Immunolocalization studies in human aorta and coronary artery indicate that ADAMTS-1 expression is mainly seen at low levels in the medial layer, but upregulated in the intima when plaque is present. We found that ADAMTS-1 mRNA levels are significantly higher in proliferating/migrating cultured primary aortic vascular smooth muscle cells (VSMCs) compared with resting/confluent cells. Using the mouse carotid artery flow cessation model, we show that there are differences in vessel remodeling in ADAMTS-1 transgenic/apoE-deficient mice compared with apoE deficiency alone, particularly a significant increase in intimal hyperplasia. We show that ADAMTS-1 can cleave the large versican containing proteoglycan population purified from cultured human aortic VSMCs. Finally, using versican peptide substrates, we show data suggesting that ADAMTS-1 cleaves versican at multiple sites.

Conclusion—We hypothesize that ADAMTS-1 may promote atherogenesis by cleaving extracellular matrix proteins such as versican and promoting VSMC migration. (Arterioscler Thromb Vasc Biol. 2005;25:180-185.)

Key Words: ADAMTS-1 ■ atherosclerosis ■ versican ■ neointima

Complications from atherosclerosis are the most common cause of death in Western societies. It is a form of chronic inflammation involving cells and proteins that normally reside in the artery and/or infiltrate the artery from the lumen. They include lipoproteins, macrophages, vascular smooth muscle cells (VSMCs), endothelial cells, and extracellular matrix proteins, such as proteoglycans, collagens, and elastins.2

Early in atherogenesis, VSMCs from the media are thought to migrate into the intima and contribute to the development of atherosclerotic lesions. Although what initially triggers these events is not known, it is thought that proteases released by VSMCs degrade the matrix proteins in the intima, particularly the main proteoglycan of the arterial intima versican, making the intima more permissive for invasion by VSMCs. One recently discovered family of metalloproteases, the ADAMTS family, might play a key role in atherogenesis by modulating the degradation of versican and possibly other proteoglycans.

The first member of this family to be identified is ADAMTS-1. It has been observed that ADAMTS-1 mRNA is upregulated substantially in human umbilical vein endothelial cells and cardiac microvascular endothelial cells under shear stress, suggesting regulation during flow-dependent vascular remodeling. ADAMTS-1 has been shown to cleave the proteoglycan versican, which is expressed by VSMCs. Versican can exist in 4 isoforms (V0, V1, V2, and V3), depending on alternative splicing of the chondroitin sulfate containing glycosaminoglycan domains. V0 versican contains all possible domains, whereas the glycosaminoglycan-alpha and glycosaminoglycan-beta domains are spliced out in the V1 and V2 versican isoforms, respectively. ADAMTS-1 and ADAMTS-4 have been shown to cleave V1/V0 versican at the Glu441-Ala442/Glu1428-Ala1429 bond and the product of this cleavage was shown to be present in human atherosclerotic plaques by immunohistochemistry using neo-epitope antibodies. ADAMTS-1 has also been shown to have a role in matrix remodeling during ovulation in mice, which involves dissolution of connective matrix and cellular layers.

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ADAMTS-1–deficient mice displayed impaired ovulation, and the authors proposed that this was at least partially caused by lack of versican degradation.9

In addition to its potential role in VSMC migration, versican is also thought to contribute to atherosclerosis by binding and protecting growth factors and cytokines from degradation. Versican can also bind apoB-containing lipoproteins, promote their modification, and enhance uptake by macrophages.10,11 Versican can interact directly with a number of matrix proteins, adhesion molecules, and membrane proteins such as CD44.12,13 Together, they can form a network of densely packed matrix proteins, which is expandable but resilient. Decreased versican levels are correlated with human abdominal aortic aneurysm, consistent with its role in maintaining visco-elasticity in the vessel wall.14

In the present study, we look at the potential contribution by ADAMTS-1 in atherogenesis by examining the expression pattern of ADAMTS-1 in human lesions, looking at changes in expression levels of ADAMTS-1 in primary human VSMCs under different proliferating conditions, investigating the effect of overexpression of ADAMTS-1 on intimal hyperplasia using the murine carotid artery ligation model, and characterizing possible cleavage sites using versican peptide substrates. Our results demonstrate that ADAMTS-1 may contribute to the development of atherosclerosis through degradation of versican and other potential proteins involved in regulating VSMC migration.

Methods

Real-Time Reverse-Transcription Polymerase Chain Reaction

Distribution of ADAMTS-1 in human tissues and parts of the cardiovascular system was analyzed using cDNA made from pooled human poly A+ RNA (Cat. #1420 to 1, 1421 to 1, and 1427 to 1; Clontech Laboratories, Inc). For both human and mouse samples, ribosomal protein 36B4 was used as an internal control.

For more information about RNA preparations, reaction conditions, and primer sequences, please see http://atvb.ahajournals.org.

Immunohistochemistry

The following paraffin-embedded human materials were used: fatty streak aortic lesion from an 18-year-old man (Addenbrooke’s Histopathology Tissue Bank, UK), a type III-IV/VII aortic lesion (Pathology Department, Sahlgrenska Hospital, Sweden), and a type III-IV/VII lesion from coronary artery of a 53-year-old man (Ullevål Hospital, Norway). For more information on staining conditions, please see online supplement.

Antibodies

Four different polyclonal anti-peptide antibodies against ADAMTS-1 were generated for immunohistochemistry and Western blotting (Agrisera). Please see online supplement for description of peptides used to generate the ADAMTS-1 antibodies/purification and of antibodies used to identify macrophages and VSMCs.

Cell Culture

Primary human aortic VSMCs (CC-2571; Clonetics) were cultured in SmGM-2 Bullet Kit (CC-3182; Clonetics) on ordinary or collagen-coated flasks and cultured according to supplier. Cells from passage 6 were seeded at a density of 5000 cells/cm² for each condition, migrating/proliferating (M/P), or resting/confluent (R/C) (n=7). M/P cells were harvested at ~50% confluence (3 to 4 days after plating) and the R/C cells were harvested 2 to 3 days after the cells had reached confluence (days 13 to 14).

For information about the culture conditions for DON cells overexpressing ADAMTS-1, please see online supplement.

Animal Model

ADAMTS-1 transgenic mice were generated using standard techniques at AstraZeneca. ADAMTS-1 transgenic (C57/BL6) and wild-type mice (C57/BL6) (Bommine M&B, Denmark) were euthanized at 7 weeks and tissues were taken out for real-time reverse-transcription polymerase chain reaction analysis (n=5).

Twenty-one male ADAMTS-1 transgenic/apoE-deficient mice (C57BL/6d) and 21 male apoE-deficient mice (C57BL/6d) for the carotid artery ligation experiments were fed a Western diet from age 10 to 14 weeks. Ligation of the left common carotid artery near the bifurcation was performed at age 14 weeks as described before.15 At 3, 7, and 14 days after ligation, the animals were perfusion-fixed and the carotid arteries were taken out for further study (n=7).

Morphometric Analysis

Each carotid artery was divided in 2 segments and marked with tissue touche at the distal part. Paraffin sections were collected at every 200-μm level throughout the whole length of the carotid artery for morphometric analysis, and additional sections were collected at every other 200-μm level for histological analysis.

Paraffin sections were counterstained with hematoxylin–eosin. Perimeters of the lumen, internal elastic lamina, and external elastic lamina were obtained by tracing the contours with eyepiece equipment Lucivid (BioMetricSystems GmBH) mounted to a Leica DM RBE microscope. Intima area (area between internal elastic lamina and lumen) and media area (area between external elastic lamina and internal elastic lamina) were provided by Microvid Image Access Analytic v1.0c (Bildanalyse-system AB) software program. These measurements were performed on all sectioned levels of the carotid to obtain a mean value for the media area, intima area, and lumen area along the whole length of the artery for each animal. All measurements were performed blind.

Statistical analysis between groups was performed by 1-way-ANOVA test using Astute version 2.

Construction and Transfection of ADAMTS-1 Expression Vector

Please see online supplement.

Purification of Human ADAMTS-1

DON cells expressing hADAMTS-1 were cultured on plastic until confluence. Media was collected for purification procedure.

Purification of Total Proteoglycan Secreted by Primary Aortic VSMCs

35S and 1H labeling and purification of total proteoglycan secreted from VSMCs were performed as described previously.16

Cleavage of Proteoglycan Isolated From Cultured Primary Human VSMCs and Versican Peptides

One hundred microliters of total proteoglycan preparation (10 000 to 15 000 cpm/35S) was cleaved with 3.8 μg ADAMTS-1 at 37°C overnight and analyzed by size-exclusion chromatography.

Freeze-dried peptide was dissolved and mixed with ADAMTS-1 at a 10:1 molar ratio, incubated overnight at 37°C, and analyzed by high-performance liquid chromatography and mass spectrometry/mass spectrometry.

Results

Real-Time Reverse-Transcription Polymerase Chain Reaction Analysis of ADAMTS-1

Expression in Human Tissues, Cultured Human Primary Aortic VSMCs, and Tissues From ADAMTS-1 Transgenic Mice

To determine the distribution of ADAMTS-1 mRNA in humans, real-time reverse-transcription polymerase chain
reaction was performed on pooled cDNAs from human tissues/organs and from different parts of the cardiovascular system. ADAMTS-1 was expressed at the highest levels in the heart, lung, adipose tissues, and brain (Figure 1A), and in the cardiovascular system in the aorta (Figure 1B).

To determine whether there is any correlation between the level of ADAMTS-1 mRNA and cell M/P, we compared the level of ADAMTS-1 mRNA between M/P and R/C primary aortic human VSMCs. In 3 separate experiments with a total of 7 samples for each condition, we observed significantly higher levels of ADAMTS-1 in M/P cells compared with R/C cells (**P<0.001; n=7). (Figure 1C).

In a separate experiment, BrdU incorporation and cell protein concentration were measured over time to measure cell proliferation and cell protein expression. Peak BrdU incorporation corresponded to when cells were ~50% confluent (days 3 to 4), with BrdU absorbance levels approaching 0 after cells reach confluency (after day 11), confirming that the cells were indeed M/P or R/C (Figure 1A, available online at http://atvb.ahajournals.org). Total cell protein content in each well, however, continued to increase, indicating that cells were healthy and were continuing to express proteins at high levels (Figure 1A). Similar pattern of BrdU incorporation and protein expression were also observed in uterine and coronary artery smooth muscle cells (Figure 1B and IC, respectively).

To confirm that ADAMTS-1 transgenic mice overexpressed ADAMTS-1 message, real-time reverse-transcription polymerase chain reaction was performed on various tissues from mice euthanized at 7 weeks of age (Figure 1D). Results show higher ADAMTS-1 expression in the transgenic mice compared with wild-type mice.

**ADAMTS-1 Localization in Atherosclerotic Lesions**

Immunolocalization studies were performed on a human aortic fatty streak lesion (type I) and on more advanced type III–IV/VII lesions from the coronary artery and aorta (Figure 2). Figure 2B shows the size and location of the fatty streak in relation to the medial layer, which is stained using antibodies against smooth muscle α-actin. In the fatty streak lesion, antibodies against ADAMTS-1 stained both the VSMCs and foam-like cells in the intima (Figure 2C, 2E, and 2F). ADAMTS-1 staining is also observed in the medial layer, but at much lower intensity compared with the intima (Figure 2C).

In the more advanced lesions with acellular scar tissue, ADAMTS-1 staining was mostly localized to the matrix-like core at the base of the lesions in both the coronary artery and the aorta, although weak staining of VSMCs is still present (see arrow in Figure 2H; 2J to 2L). Again, staining pattern for ADAMTS-1 and VSMCs overlap (see arrow in Figure 2H; 2I). Although not all sections contained macrophages and foam cells, we see weak staining pattern in many sections where they are present (Figure 2G; see arrow in Figure 2L). In sections in which an intact endothelial cell layer was observed, ADAMTS-1 staining was also observed in these cells (Figure II, available online at http://atvb.ahajournals.org). We have examined ADAMTS-1 staining using aortic and coronary samples from a total of 13 individuals, including 2 normal arteries, 4 with fatty streak lesions, and 6 with advanced lesions; we have observed consistent results in staining pattern.

Preabsorbed antibodies did not show staining, demonstrating specificity (Figure 2D). The ADAMTS-1 antibodies were pre-absorbed or pre-incubated with peptides that rabbits were immunized with to eliminate specific recognition of ADAMTS-1. Foam cells/macrophages and VSMCs were identified using antibodies against HAM-56 and α-actin, respectively (Figure 2B, 2E, 2F, 2G, and 2I).

**Vascular Remodeling in Murine Carotid Artery Flow Cessation Model**

To determine whether ADAMTS-1 has a role in smooth muscle migration and proliferation in an in vivo model, we used the mouse carotid artery ligation model to compare neointima formation in mice overexpressing ADAMTS-1 with apoE-deficient background and control apoE-deficient mice at days 3, 7, and 14 after ligation (n=7) (Figure 3).15,17 Because remodeling can vary throughout the ligated area, we took measurements of the neointima, media, and lumen from sections taken every 200 μm throughout the entire length of the ligated artery and averaged these values for each carotid artery. The data from each mouse were then combined with the data from the other mice in the same group and averaged to calculate the mean value. In the control group at day 14, one sample was excluded because the neointima formed was so complex that we could not distinguish different tissue boundaries. Total area was calculated as the sum of the area for the media, lumen, and neointima.

We found that in the ADAMTS-1 transgenic/apoE-deficient group there was a significant increase in the mean area of the neointima compared with the control mice at day 14 (21550±3733 versus 9905±1019 μm²; P<0.02) (Figure 3A). Figure 3A and 3B graphically show the changes occurring over.
time for the mean medial, neointimal, luminal, and total areas. In both the transgenic and control groups, lumen size was maintained even with neointimal growth by positive/expansive remodeling of the vessels at days 3 and 7. By day 14 in the control group, however, positive remodeling was followed by negative remodeling to return the vessel to its original size; because of the neointima, the mean area of the lumen is significantly smaller compared with that on day 3 ($P<0.01$). In the transgenic group, however, the total area remained significantly larger ($P<0.03$), and the size of the lumen was not significantly different between days 3 and 14, despite the larger neointima.

Figure 3C shows representative sections from a coronary artery taken from an ADAMTS-1 transgenic/apoE-deficient and ADAMTS-1 wild-type/apoE-deficient control mice at days 3, 7, and 21. Although the sections cannot be compared directly because they are only one of many sections used to calculate the average values for each time point, Figure 3C shows the kind of changes occurring during the growth of the neointima.

### ADAMTS-1 Can Cleave Versican Secreted by Primary Aortic VSMCs

To test whether ADAMTS-1 has a role in the degradation of versican, we tested whether proteoglycans secreted by primary aortic VSMCs are cleaved by ADAMTS-1 (Figure 4). Proteoglycans purified from these cells can be separated by size-exclusion chromatography into 2 populations: the large proteoglycan population that is composed primarily of versican and the small population that is composed of smaller proteoglycans.16 Incubation of total proteoglycan with ADAMTS-1 decreased the size of the larger peak and increased the size of the smaller peak (Figure 4A and 4B). In addition, incubation of ADAMTS-1 with only purified large proteoglycan population shows the disappearance of the large peak and appearance of a smaller peak (unpublished data, 2000).

Sandy et al demonstrated that ADAMTS-1 can cleave V1/V0 versican at the Glu$^{441}$-Ala$^{442}$/Glu$^{1428}$-Ala$^{1429}$ bond.8 However, it is likely that other cleavage sites exist. ADAMTS-4 and ADAMTS-5/11, for example, can cleave aggrecan at 5 sites.18 Using different synthetic 40 to 42 amino acid versican peptides, we were able to detect 2 additional cleavage sites by high-performance liquid chromatography and sequence analysis using MS/MS: Glu$^{950}$-Gly$^{951}$ bond in V0/V2 versican and Tyr$^{1411}$/Ile$^{1411}$/Tyr$^{1423}$/Ile$^{1424}$ bond in V0/V1 versican (Figure 5A to 5F).

### Discussion

The findings in this article suggest a role for ADAMTS-1 in atherosclerosis and possibly in vascular thrombosis. Although the expression of ADAMTS-1 is generally low in normal tissues and organs, it is possible that expression is induced by stimuli, as has been demonstrated in vitro by the inflammatory cytokine IL-1 and tumor necrosis factor-α, in vivo by lipopolysaccharide and both in vitro and in vivo by parathyroid hormone.3,19–20 In human fatty streak lesions, we observe ADAMTS-1 staining with stronger intensity in the VSMCs and foam cells in the lesion compared with the medial layer by immunohistochemistry. This is similar to the staining pattern we observe in ADAMTS-1 transgenic/apoE-deficient mice administered a high-fat diet (unpublished results, 2003). This is also consistent with our in vitro cell experiments. We observe significantly more ADAMTS-1 expression in migrating and proliferating VSMCs, as those presumably found in lesions, compared with R/C cells, as those found in the medial layer.

In more advanced type III/IV/VII human lesions, ADAMTS-1 staining is observed with strong intensity at the base of the lipid core containing matrix-like elements and adjacent to the medial layer in the aorta and coronary artery. The latter observation is consistent with in vitro studies using COS-7 cells, which showed association with the extracellular matrix.21 ADAMTS-1 staining was observed in some of the macrophages/foam cells when they were present, although they were not present in all sections (Figure 2L). In addition, mRNA for ADAMTS-1 is detected in THP-1 cells by real-time reverse-transcription polymerase chain reaction analysis, and expression is induced by the inflammatory cytokine interferon-γ, providing additional support that macrophages may also express ADAMTS-1 (unpublished data, 2000). These results
suggest that localization of ADAMTS-1 may change with progression of disease.

Using a mouse carotid artery ligation model to study the role of ADAMTS-1 in development of neointima in vivo, we found that overexpression of ADAMTS-1 in apoE-deficient mice significantly increased intimal hyperplasia compared with control apoE-deficient mice by day 14. Although the carotid artery ligation model is not a model for atherosclerosis, many of the changes taking place resemble those observed early in atherogenesis, such as VSMC migration and proliferation.15 The increased mass by the growth of the neointima is compensated by the expansion of the blood vessel to maintain lumen size and blood flow. Although the expansion of blood vessel may be a beneficial compensatory mechanism, this remodeling has been associated with vulnerable plaques.17 In the control group, the positive remodeling is followed by negative remodeling; these changes have been described for C57/BL/6J mice.17 In the transgenic group, however, the vessel remains significantly expanded. ADAMTS-1 transgenic mice appear to have reduced capacity for negative remodeling compared with controls, but they do not appear to be impaired in their capacity for positive remodeling. Although not significant, there appears to be even more positive remodeling in the ADAMTS-1 transgenic mice compared with control mice from days 3 to 7 (34% versus 20%, respectively).

Using the same carotid artery ligation model, Galis et al found that there was decreased intimal hyperplasia in mice deficient in matrix metalloproteinase-9 compared with control mice and less lumen reduction/negative remodeling.22 They attributed the latter to the decreased capacity for the VSMCs to contract and constrict vessels. Therefore, in terms of intimal hyperplasia, the results are consistent with metalloproteases promoting smooth muscle cell migration and proliferation. However, in terms of negative remodeling, matrix metalloproteinase-9 appears to contribute to negative remodeling, whereas ADAMTS-1 appears to inhibit it. This would suggest that different mechanisms are operating in formation of intimal hyperplasia and negative remodeling and that these 2 metalloproteases have overlapping and distinct functions.

As reported previously by Sandy et al, our data also show that ADAMTS-1 can cleave the proteoglycan versican, which is thought to have a role in the development of lesions by regulating aortic VSMC migration and in plaque stability by affecting the strength of the fibrous cap.8 To test potential cleavage sites, we used peptide substrates corresponding to the different sites, although it is not proof that versican is cleaved at these sites in vivo. In addition to the cleavage site described by Sandy et al, we also observed 2 additional potential cleavage sites.
The expression of ADAMTS-1 is regulated by cytokines and substrates was ADAMTS-1–specific (unpublished data, 2000). From other families and that cleavage of versican peptide that our protein preparation was not contaminated with proteases and aspartic proteases did not interfere with cleavage, indicating proteinase peptide substrate, collagen IV, elastin, and decorin way inhibitor-1) but does not cleave a general matrix metallo-protease associated with the extracellular matrix.

Based on our results, we hypothesize that ADAMTS-1 may be involved in atherogenesis by modulating VSMC migration. The expression of ADAMTS-1 is regulated by cytokines and nuclear hormone receptor agonists such as peroxisome proliferator-activated receptor (PPAR-γ) agonists thought to be important in atherogenesis (Figure IV, available online at http://atvb.ahajournals.org; unpublished results, 2001). Inhibitors of cytokine, serine, and aspartic proteases did not interfere with cleavage, indicating that our protein preparation was not contaminated with proteases from other families and that cleavage of versican peptide substrates was ADAMTS-1–specific (unpublished data, 2000).

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References

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A. Human Aortic Smooth Muscle Cells

B. Human Uterine Smooth Muscle Cells

C. Human Coronary Artery Smooth Muscle Cells
ADAMTS-1 mRNA levels in NIH 3T3-L1

- Control
- Rosiglitazone

Relative Expression Level

0.0000 0.0005 0.0010 0.0015 0.0020 0.0025 0.0030

Treatment
ONLINE DATA SUPPLEMENT

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Role of ADAMTS-1 in Atherosclerosis: Remodeling of Carotid Artery, Immunohistochemistry, and Proteolysis of Versican

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METHODS:

Immunohistochemistry
To study the presence of smooth muscle cells, a commercial antibody against α-actin was used at 1:100. Antibody HAM-56 was used at 1:50 to identify macrophages and foam cells. A polyclonal peptide antibody against the human ADAMTS-1 spacer domain of ADAMTS-1 was used at 1:400. For control staining, ADAMTS-1 antibodies were preabsorbed with the peptide used to immunize the rabbits. The immunohistochemistry was performed in a Techmate immunostainer from Daco, following manufacturers suggestions. The primary antibodies were incubated on the sections for 12 hours + 25 minutes. The secondary antibodies were either donkey-anti-rabbit-biotin conjugated antibodies diluted 1:2500 for ADAMTS-1 and donkey-anti-mouse-biotin conjugated antibodies diluted 1:1000 for HAM-56 and α-actin. Endogenous peroxidase activity was blocked with a kit from Daco for HP-blockage. Sections were incubated for 30 minutes with HRP, washed and color visualized using AEC chromogen kit (Daco). The sections were counterstained in hematoxylin, mounted in Kaisers gelatin glycerine, and examined by light microscopy.

Antibodies
For immunohistochemistry experiments, a peptide representing a sequence from the spacer domain of human ADAMTS-1 was used (amino acids 622-641). For Western blotting, a cocktail of two polyclonal anti-peptide antibodies recognizing sequences from the mouse spacer domain (amino acids 623-641) and the pro-domain (amino acids 194-211) were used. An antibody which specifically recognized human ADAMTS-1 (amino acids 523-546) was also generated and gave similar results as the cocktail of mouse antibodies in Western blots. All 4 antibodies were affinity purified on a peptide column and used at 5 mg/L. Antibodies against α-actin and HAM-56 were purchased from Cedarlane laboratories and Daco, respectively. Both donkey-anti-rabbit-biotin conjugated antibodies and donkey-anti-mouse-biotin conjugated antibodies were purchased from Jackson labs.

Cell culture
DON cells (ATCC: CCL-16) were cultured in Dulbecco’s Modified Eagle’s medium (DMEM)/F12 with glutamine and 15 mmol/L HEPES (#31330-038), 5% fetal calf serum, and PEST (#15070-089) (Life Technologies).

Real time RT-PCR
For real time RT-PCR analysis of human material, total RNA was isolated using RNA STAT-60 (Tel-Test, Inc.) or Trizol reagent (Life Technologies) according to the manufacturer’s instructions. The RNA was then treated with RNase treated with RQ1 DNase (Promega) or DNasefree™ (Ambion). cDNA was synthesized using SuperScript™ First Strand Synthesis System for RT-PCR (Life Technologies). The real time PCR reaction contained cDNA corresponding to 0.25 ng poly A+ RNA (figure 1A, B) or 2.5 and 25 ng total RNA (figure 1C) together with 0.4 µmol/L of each of the primers, 0.1 µmol/L of the probe and TaqMan Universal PCR Master Mix (PE Applied Biosystems) in a total volume of 25 µl and performed with an ABI Prism 7700 Sequence Detection System (PE Applied Biosystems).

For real time RT-PCR analysis of mouse tissues, RNA was prepared on a ABI Prism 6700 Automated Nucleic Acid Workstation from Applied Biosystems, homogenized in lysis solution (ABI) in Lysing matrix D tubes from BOB-101 using a FastPrep FP120 homogeniser from Bio-101/Savant. cDNA was synthesized using High Capacity cDNA Archive kit from Applied Biosystems and subjected to real-time PCR. The results are calculated in relation to the endogenous gene 36B4 in the same sample. The relative expression levels (REL) were calculated according to the formula 2^−ΔCT, where ΔCT is the difference in CT values between the target and the 36B4 control. Primers for human ADAMTS-1 were: 5′-AATGCCGAGACATTAATGGACA-3′ (forward) and 5′-AGGGATGGTCTGCACAAGGT-3′ (reverse). The probe sequence was 5′-CTTCCTTTGACACTCGGAAGCA-3′.

Primers for human internal control, h36B4 (acidic ribosomal phosphoprotein P0), were: 5′-CCATTCTATCATCAACGGGTACAA-3′ (forward) and 5′-AGCAAGTGGGAGGTGTAATCC-3′ (reverse). The probe sequence was 5′-TCTCCACAGACAACGGACTGT-3′.

Primers for mouse ADAMTS-1 were: 5′-AAGTGAAGCCAGCCAGTACCA-3′ (forward) and 5′-TCCCGCAAGTTTTGGAACA-3′ (reverse). The probe sequence was 5′-TGTCAGCATTCTCCTGTACAC-3′.

Primers for mouse internal control, m36B4 (acidic ribosomal phosphoprotein P0), were: 5′-GAGGAATCAGATGAGGATATGGGA-3′ (forward) and 5′-AAGCAGGCTGACTTGGTTGC-3′ (reverse). The probe sequence was 5′-TCGGTCTCTCGACTAA-3′.

Animal Model
ADAMTS-1 transgenic mice overexpressing human ADAMTS-1 under a metallothioneine promoter were produced using standard techniques. Treatment of the mice and isolation of the tissues from these mice were done in accordance with institutional guidelines.

Purification of human ADAMTS-1
2 days before media is collected, heparin is added to a final concentration of 5 µg/L. The media is collected, 2 tablet of Complete EDTA-free/L media (Roche) is added, the pH adjusted to pH 7.2 with glacial acetic acid, centrifuged for 15 minutes at 10000 x g, filtered through a 0.2 µm filter, and loaded on a heparin sepharose FF, XK 26 column pre-equilibrated with 15% Buffer B (Buffer B: 10 mmol/L Sodium phosphate, 1 mol/L NaCl, pH 7.2) (Amersham Pharmacia Biotech AB). The column was washed with 35% buffer B and eluted stepwise with 40%, then 80% buffer B. Fractions were collected and aliquots of each were analyzed by Western blot analysis and coomassie staining. Fractions containing ADAMTS-1 were pooled and dialyzed against cleavage buffer (20 mmol/L Tris, 0.2 mol/L NaCl, 10 µmol/L ZnCl2, 6 mmol/L CaCl2, 5 mmol/L n-Octyl β-d-Glucopyranoside (Sigma), and 10% glycerol, pH 8), concentrated with concentration cell Omegacell 30 K (Pall Filtron), and stored at −70°C until use.
Construction and Transfection of Expression vector
Expression vector for wild-type human ADAMTS-1, cloned from human skeletal muscle cDNA (Clontech), was constructed using pcDNA3.1/Hygro+ vector. DON cells were transfected with ADAMTS-1 expression vector by means of LipofectAMINE as described by manufacturer (Life Technologies).

Purification of total proteoglycan secreted by primary aortic VSMCs
Separation by size exclusion chromatography was performed using a Sephadex 200 HR 10/30 (Amersham Pharmacia Biotech AB). Total proteoglycan sample was dissolved in 50 mmol/L Tris, pH 7.5, 4 mol/L Guanidinium-HCl and loaded on a pre-equilibrated column with flow at 0.5 mL/min. 1 mL fractions were collected and the amount of $^{35}$S-label quantitated using a scintillation counter. The proteoglycans were pooled, dialyzed against water, lyophilized and stored at –20°C until use.

Cleavage of proteoglycan isolated from cultured primary human aortic vascular smooth muscle cells and versican peptides by ADAMTS-1
An aliquot of 100 µl of total proteoglycan preparation (10000 to 15000 cpm/$^{35}$S) was cleaved with 3.8 µg ADAMTS-1 in 50 mmol/L tris buffer, pH 7.5 and 6 mmol/L CaCl$_2$ in a final volume of 210 µL. The samples were incubated at 37°C overnight and analysed by size exclusion chromatography using a superdex 200 HR 10/30 (Amersham Pharmacia Biotech) equilibrated with 50 mmol/L Tris pH 7.5, 4 mol/L Guanidinium-HCl. Freeze-dried peptide was dissolved in cleavage buffer (20 mmol/L Tris pH 8, 10% glycerol, 0.2 mol/L NaCl, 10 µmol/L ZnCl$_2$, and 6 mmol/L CaCl$_2$) and mixed with ADAMTS-1 in a 10:1 molar ratio. Incubation was carried out at 37°C overnight and the cleavage product was analysed by HPLC using a vydac C18 column (4.6 x 250 mm) at 37°C with a linear gradient of 10-40 % Acetonitril/0.1 % trifluoroacetic acid. The cleavage products were also analyzed by mass spectrometry using a Mariner™ EIMS instrument.

ONLINE FIGURES

METHODS

Cell culture
For cell proliferation assay, VSMCs (primary aortic, uterine, and coronary artery from Clonetics, USA; CC-2571, CC-2562, and CC-2583, respectively) were plated at a density of 5000 cells/cm$^2$ on 96 well plates. 16 samples (wells) were extracted for each time point (days 1, 3, 4, 7, 11, and 14). To determine cellular protein content, 5000 cells/cm$^2$ were plated on 6 well plates. Total protein from each well was extracted for determination of protein concentration (days 1, 3, 4, 7, 11, and 14). Cells from 2 samples (2 wells) were extracted for each time point.

NIH 3T3-L1 cells (ATCC: CL-173), a murine pre-adipocyte cell line, were grown in 175 cm$^2$ flasks to near confluency in DMEM with 4 mmol/L L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose 90%, and 10% BCS. To induce differentiation into adipocytes, dexamethasone at 2 µg/L and methylisobutyl-xantine at 0.5 µmol/L were added to the media and incubated for 2 weeks. Dexamethasone/methylisobutyl-xantine containing media was removed and media containing 1 µmol/L Rosiglitazone was added and incubated for 24 hours. Control cells were treated with dexamethasone/methylisobutyl-xantine but with media
containing vehicle instead of Rosiglitazone. See under ‘Real time RT-PCR’ above for more detailed description of extraction conditions, reaction conditions and primers used to calculate relative expression levels. Endogenous gene m36B4 was used as an internal control.

**Immunohistochemistry**

Human aortic fatty streak lesion from an 18 year old male (Addenbrooke’s Histopathology Tissue Bank, UK) was stained with ADAMTS-1 antibodies. See ‘Immunohistochemistry’ and ‘Antibodies’ sections above for more detailed information about staining conditions (human) and detailed description about how ADAMTS-1 antibodies were generated.

**Cell proliferation assay**

BrdU Cell Proliferation ELISA (Roche, cat. No. 1 647 229) was used to measure cell proliferation, based on the measurement of BrdU incorporation during DNA synthesis, as recommened by manufactorer (n=16).

**Bradford protein assay**

Cells from 2 wells were dissolved in 0.2M NaOH (n=2). Total protein concentration from the cells in each well was determined using the Bradford assay (BioRad Protein Assay Cat No 500-0006) according to manufactorer’s suggestions. Bovine Serum Albumin was used as a standard (BioRad Cat No 500-0007).

**Cleavage of TFPI-1**

0.5 μg of recombinant human TFPI-1 (American Diagnostica Inc.) was incubated with 3 μg hADAMTS-1 in 30 μL cleavage buffer for 2 hours at 37°C and cleavage products were analyzed by Western blotting.

**Western Blotting**

Western blotting was performed using ECL detection system as suggested by manufacturers (Amersham Pharmacia Biotech). Polyclonal antibodies against purified human TFPI-1 were purchased from American Diagnostica Inc. (Cat. #4901).

**RESULTS**

**Quantification of BrdU incorporation in M/P and R/C in primary aortic, uterine, and coronary artery smooth muscle cells**

Cells were extracted 3-4 days after plating for M/P cells and 13-14 days after plating for R/C cells (figure I). For aortic VSMCs, BrdU incorporation is maximal at days 3-4 (Abs450-690= 1.3), suggesting cells are dividing quickly, and approaching 0 (Abs450-690=0.1) after day 11, suggesting few cells are dividing when they are resting/confluent (figure IA).

Protein concentration in aortic VSMCs, however, continued to increase during the 14 days of culture, indicating that the cells are healthy and protein continued to be synthesized (figure IA).

Similar results were obtained using smooth muscle cells from uterus and coronary artery (figure IB and IC, respectively).

**Immunohistochemistry**

Staining for ADAMTS-1 is observed in the endothelial cell layer, when it is intact as it is in this section (figure II). Preserum control does not show staining (unpublished results, 2003).
ADAMTS-1 can cleave an inhibitor of the tissue factor coagulation pathway, TFPI-1
Figure III shows that ADAMTS-1 can cleave TFPI-1; 2 smaller sized bands appear after incubation with ADAMTS-1. Western blot analysis of TFPI-1 with ADAMTS-1 without incubation show only the full length TFPI, indicating that the smaller sized bands are products from TFPI cleavage and not an impurity from the enzyme preparation (unpublished data, 2002).

ADAMTS-1 expression is sensitive to the PPARγ agonist Rosiglitazone in preadipocyte NIH 3T3-L1 cells
ADAMTS-1 mRNA was down-regulated by Rosiglitazone in murine NIH 3T3-L1 cells, showing that expression of ADAMTS-1 mRNA is sensitive to PPARγ agonist (figure IV).

FIGURES
Figure I. BrdU incorporation (solid line) and total protein content (dashed lines) in primary aortic (A), uterine (B), and coronary artery (C) VSMCs under culture for 14 days.
Figure II. Endothelium in human aortic fatty streak stained with ADAMT-1 antibodies. Scale bar is 20 µm.
Figure III. Western blot analysis of ADAMTS-1 cleavage of TFPI-1. A. TFPI-1 (0.5 µg) alone. B. TFPI-1 (0.5 µg) incubated with ADAMTS-1 (3 µg).
Figure IV. Comparison of relative ADAMTS-1 levels by real time RT-PCR in NIH 3T3-L1 cells treated with Rosiglitazone or vehicle control.