Increased ADAMTS-13 proteolytic activity in rat hepatic stellate cells upon activation *in vitro* and *in vivo*

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Summary

Introduction—ADAMTS-13 is a member of *A D*isintegrin *A*nd *M*etalloprotease with *T*hrombo*S*pondin type 1 repeats (ADAMTS) family, primarily synthesized in hepatic stellate cells (HSCs), one of the major cell types transdifferentiating into myofibroblasts during liver fibrosis. However, the association between ADAMTS-13 expression and HSC activation or liver fibrosis is not known.

Methods—In this study, we determined the *ADAMTS-13* mRNA, protein, and activity in isolated primary HSCs upon activation on a plastic dish and in liver after administration of carbon tetrachloride (CCl_4) in rats.

Results—We showed that ADAMTS-13 antigen and proteolytic activity in the activated rat HSCs were dramatically increased, whereas *ADAMTS-13* mRNA in these cells was only minimally altered. Similarly, the ADAMTS-13 antigen and proteolytic activity in rat liver after CCl_4 injury were also significantly increased, whereas the *ADAMTS-13* mRNAs in these liver tissues were only slightly increased compared with normal. Surprisingly, despite the dramatic up-regulation of ADAMTS-13 protein synthesis in the activated HSCs after CCl_4 administration, the plasma levels of ADAMTS-13 protease in rats did not increase concordantly.

Conclusion—We conclude that the up-regulation of ADAMTS-13 protein expression in rat HSCs during activation *in vitro* and *in vivo* suggests the possibility of ADAMTS-13 proteolysis, an important part of function of the activated HSCs, perhaps through modulation of liver regeneration or formation of liver fibrosis after various injuries. The data also suggest the minimal contribution of the activated HSCs in regulation of plasma levels of ADAMTS-13 protease.

Keywords

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Introduction

von Willebrand factor (VWF)-cleaving metalloprotease (ADAMTS-13) is a 195 kDa glycoprotein present in plasma at concentration of approximately 0.5–1.2 mg L^{-1} [1–4]. It cleaves VWF at the Tyr^{1605}-Met^{1606} bond [5]. Inability to cleave VWF multimers into smaller forms may result in an accumulation of hyperactive [6] or 'unusually large' VWF multimers [7–9], leading to platelet-rich thrombi in small arteries, a characteristic pathological feature of thrombotic thrombocytopenic purpura.

ADAMTS-13 biosynthesis appears to occur primarily in the liver [10–12], particularly in activated hepatic stellate cells (HSCs) located in the space of Disse, which normally store retinoids [13–15]. However, how such a big molecule of ADAMTS-13 protease synthesized and secreted from the activated HSCs gets into the blood stream is not known. We hypothesize that the ADAMTS-13 protease produced in the activated HSCs may function locally to regulate deposition of matrix proteins or to involve in the processes of liver regeneration or formation of liver fibrosis after various acute and chronic injuries. In the setting of chronic liver disease, HSCs transdifferentiate, adopting a myofibroblast-like phenotype characterized in part by proliferation and the deposition of abnormal matrix. This phenotypic change, termed 'activation', has been modeled *in vitro* by culturing HSCs on uncoated plastic. Freshly isolated HSCs appear small, round and undifferentiated, traditionally termed 'quiescent', whereas cells grown on uncoated plastic for 5–7 days become elongated and dendritic in shape, termed 'activated' [16]. In response to TGF-β, culture-activated cells produce extracellular matrix that is similar in composition with that seen in fibrotic liver [17–20]. In addition to type I collagen, activated HSCs produce type IV collagen, laminin, and proteoglycans, which accumulate in the fibrotic liver [21], despite increased expression of several matrix metalloproteases (MMPs) and tissue inhibitors of matrix metalloproteases (TIMPs). Studies have shown that MMP-2 and membrane type 1 (MT-1)-MMP, as well as TIMP-1 and TIMP-2 [22–25], are significantly up-regulated. These and perhaps many other proteases are involved in regulating the remodeling of both normal and fibrotic liver matrix [21].

To understand the biological roles of ADAMTS-13 proteolysis in liver fibrosis, we first determined the levels of *ADAMTS-13* mRNA, protein, and proteolytic activity in isolated rat primary HSCs during activation *in vitro*. We then determined the *ADAMTS-13* expression in rat livers after administration of carbon tetrachloride (CCl$_4$). Rats have been shown to be an excellent animal species for induction of liver fibrosis by CCl$_4$ [16,19,23,26–29]. In this study, we found that ADAMTS-13 protein and proteolytic activity, but not *ADAMTS-13* mRNA, were dramatically increased in rat primary HSCs during *in vitro* activation and after CCl$_4$-induced liver injury or fibrosis. However, the plasma levels of ADAMTS-13 in rats after CCl$_4$ injection did not increase concordantly. These data suggest that the dramatically increased ADAMTS-13 protein and proteolytic activity in the activated rat HSCs *in vitro* and *in vivo* may be important part of functions of activated HSCs, perhaps by modulating the processes of liver regeneration or formation of liver fibrosis after various insults.

Materials and methods

Isolation and culture of HSCs

Primary rat HSCs were isolated from Sprague–Dawley rats (Charles River) by *in situ* perfusion of pronase and collagenase, followed by a single-step density gradient centrifugation as described previously [16,29]. The purity of HSCs was determined microscopically by autofluorescence of the stored retinoids in HSC lipid droplets. The cell viability was determined by trypan blue staining (Invitrogen, Carlsbad, CA, USA). The isolated rat HSCs were consistently 95–99% pure and greater than 95% viable. The cells were grown on uncoated plastic dishes with M199 medium (Invitrogen) in the presence of 10% fetal bovine serum

(FBS). Under such culture condition, the HSCs start to change shape, lose their lipid droplets and become positive in α-smooth muscle actin (α-SMA). This process was termed activation.

CCl$_4$ treatment

Animal study protocol was approved by the University of Pennsylvania Institutional Animal Care and Use Committee. Liver fibrosis was induced in 250–300 g male Sprague–Dawley rats by twice weekly subcutaneous injection of CCl$_4$ (0.2 mL 100 g^{-1} of body weight for 2 weeks, then 0.1 mL 100 g^{-1} of body weight, thereafter). Animals were sacrificed at 10 and 70 days after the first injection. The animals prior to CCl$_4$ injection were used as normal control. The whole blood and liver tissues were collected. The liver tissues were used for isolation of total RNA, proteins, and activity. A fraction of tissue was frozen in Tissue-Tek$^{®}$ optimum cutting temperature (OCT) compound for cryostat sectioning for histological examination after being stained with hematoxylin and eosin.

RT-PCR

Total RNA was isolated from cultured cells or tissues by TRIzol reagents according to manufacturer's recommendation (Invitrogen). One microgram of purified total RNA was transcribed using random primers and SuperScriptFirst-Strand cDNA kit (Invitrogen). The routine PCR was performed with rat-specific *ADAMTS-13* primers (5′-cgaggacctccaggcta-agat-3′ and 5′-cagcatcccagtggtagaagg-3′) and rat-specific 18S ribosomal RNA primers (5′-cccagtaagtgcgg-gtcataa-3′ and 5′-gatccgagggcctcactaaac-3′) (Integrated DNA technology, Coralville, IA, USA). Serial log-dilutions of the first strand cDNA template were performed in order to reveal the difference in ADAMTS-13 transcript among various treated groups. The amplified fragments were determined by electrophoresis on 1.5% agarose gel with ethidium bromide staining (154 bp). The polymerase chain reaction (PCR) reaction was run for 34 cycles of 95 °C for 30 s, 60 °C for 50 s and 72 °C for 30 s.

For more accurate quantitation, a real-time PCR was performed with same sets of rat *ADAMTS-13* primers, rat-specific α-SMA primers (5′-gcttcctcttcttccctggag-3′ and 5′-agatggctggaagagggtctc-3′) and rat-specific 18S ribosomal RNA primers. The PCR reactions were performed in a total volume of 20 μL in the presence of 250 nM of primers, 30 nM diluted reference dye (Stratagene, La Jolla, CA, USA), and brilliant SYBR Green Q PCR master mix (Stratagene). The samples were incubated for 10 min at 95 °C initially, followed by 40 cycles of 95 °C for 30 s, 60 °C for 50 s and 72 °C for 30 s. The fluorescence signal from PCR reactions was monitored in real time on an M × 4000 instrument (Stratagene). The relative amount of *ADAMTS-13* was determined by a standard curve generated by a serial dilution of the single-strand cDNA and normalized to the signal of 18S ribosomal RNA.

ADAMTS-13 activity

The ADAMTS-13 activity in cell or tissue lysates or plasma was determined by its ability to cleave fluorescent resonance energy transfer (FRETS)-VWF73 (Peptide International, Lexington, KY, USA), a peptide substrate derived from the amino acid residues between D1595 and R1668 at the central A2 domain of VWF as described previously [30,31]. Briefly, cells or tissues were lyzed with 1% Triton X-100 in the presence of protease inhibitor cocktail (5 mM phenanthroline, 1 mM Pefablock, 10 μg mL^{-1} pepstatin A, 10 μg mL^{-1} leupeptin, 1 μg mL^{-1} aprotinin) (Sigma, St Louis, MO, USA). Ten microliters of the cleared supernatant of the cell lysates were added to FRETS-VWF73 (2 μM) in 5mM Bis-Tris, 25 mM CaCl$_2$, and 0.005% Tween 20, pH 6.0. Fluorescence was determined at 30 °C by a fluorescence spectrophotometer (Molecular Devices Corp., Sunnyvale, CA, USA) (λ_{ex} = 340 nm and λ_{em} = 450 nm) every 5 min for 1 h. The relative proteolytic activity in the samples was derived directly from the slope and compared with that of normal human plasma. The proteolytic activity in normal human plasma was arbitrarily defined as 1 U mL^{-1}.

Protein quantification

Bieinchoninic acid (BCA) reagents (Pierce, Iselin, NJ, USA) were used to quantify total protein concentration in the cell lysates with bovine serum albumin (BSA) as a standard.

Western blotting

Cell lysates or liver tissue lysates and serum-free 24-h conditioned medium were prepared for Western blotting. Briefly, a total of 30 μg protein from rat HSC lysates or a total of 300 μg protein from liver tissue lysates was electrophoretically separated on 8% sodium dodecyl sulfate (SDS)-gels and transferred onto polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). After being blocked by 2.5% non-fat milk (Pathmark, Philadelphia, PA, USA) in 20 mM Tris–HCl, pH 7.5, and 0.05% Tween 20, the membrane was incubated with rabbit antihuman ADAMTS-13 IgG (5 μg mL^{-1}) for 2 h, followed by antirabbit IgG, peroxidase-conjugated (1:5000) (DAKO, Carpinteria, CA, USA) and SuperSignal enhanced chemiluminescence (ECL) reagents (Pierce).

Immunohistochemistry

Frozen tissue sections (6 μm) of rat livers or cultured rat primary HSCs were fixed for 10 min with ethanol: acetic acid (9:1) at −20 °C. After being blocked with 2.5% BSA (Sigma) in phosphate buffered saline (PBS) (Invitrogen), the tissue sections or cells were incubated with rabbit antihuman ADAMTS-13 IgG (40 μg mL^{-1}) and mouse anti-α-SMA IgG (DAKO) in 0.5% BSA in PBS, 0.05% Tween 20 for 2 h, followed by Cy2-conjugated antirabbit IgG (1:100) or Cy3-conjugated antimouse IgG (1:100) (Jackson Immuno-Research Lab, West Grove, PA, USA) as previously described [2]. After being washed three times with PBS, 0.05% Tween 20, the nuclei were stained by 4,6-diamidino-2-phenylindole (DAPI) in Vectorshield™ mounting medium (Vector Lab, Burlingame, CA, USA). The control staining was performed without addition of primary antibody.

Results

ADAMTS-13 antigen and activity, but not the ADAMTS-13 mRNA levels were dramatically increased in rat HSCs upon *in vitro* activation

To determine the levels of *ADAMTS-13* mRNA in rat HSCs during activation *in vitro*, we performed both routine and real-time reverse transcriptase polymerase chain reaction (RT-PCR). We first generated a single strand cDNA by random primers. We then amplified *ADAMTS-13* cDNA by routine PCR after a serial log dilution of the single strand cDNA templates or by real-time PCR using the standard curve. In the real time PCR, the cDNA fragments corresponding to *ADAMTS-13*, *α-SMA* and 18*S* ribosomal RNA were concurrently amplified in the same reaction. We showed that upon activation of HSCs, the *α-SMA mRNA* was significantly up-regulated, indicating the activation of HSCs. The 18S ribosomal RNA was, however, not altered during HSCs activation, which was used as an internal control. *ADAMTS-13* mRNA (154-bp) was detected after approximately 25 cycles, but the amount of PCR product after 34 cycles of amplification appeared to be equivalent among HSCs at days 1, 4, and 7 after isolation. The serial log dilution of the single-strand cDNA templates did not reveal the difference in *ADAMTS-13* mRNA transcript in activated HSCs compared with the quiescent (Fig. 1A). Real time PCR showed that the levels of *ADAMTS-13 mRNA* in HSCs at days 4 and 7 were only increased by 30% and 50%, respectively, compared with day 1 (Fig. 1B). These data suggest that *ADAMTS-13* mRNA was minimally altered in rat HSCs upon activation *in vitro*.

To determine the levels of ADAMTS-13 protein and proteolytic activity in rat HSCs during activation, the total proteins were extracted from cultured rat primary HSCs at days 1, 4, and

7 by 1% Triton X-100, in the presence of 0.2% protease inhibitor cocktail. ADAMTS-13 proteolytic activity was determined by cleavage of FRETS-VWF73 [30,31]. We showed that ADAMTS-13 activity in the rat HSC lysate at day 1 was undetectable, increased to approximately 250 and 2500 mU mg^{-1} of total protein at days 4 and 7 after isolation, respectively (Fig. 2A). The proteolytic cleavage of FRETS-VWF73 by ADAMTS-13 was not inhibited nor augmented by addition of 1% Triton X-100 and 0.2% protease inhibitor cocktail into the reaction (data not shown). The cleavage of FRETS-VWF73 by rat HSC lysate was, however, completely inhibited by the addition of 10 mM ethyldiamine tetriceticacid (EDTA) or rabbit anti-ADAMTS-13 IgG (40 μg mL^{-1}) (Fig. 2A), suggesting the specific cleavage of the FRETS-VWF73 substrate by ADAMTS-13 protease, not by other non-specific proteases in the cell lysates. In addition, the proteolytic activity in 24 h conditioned medium of rat HSCs was also dramatically increased at days 4 and 7, respectively (Fig. 2B), compared with day 1, suggesting that the ADAMTS-13 is synthesized and secreted from activated rat HSCs.

The data above were consistent with those obtained from Western blotting (Fig. 3) and immunocytochemical staining of rat HSCs in culture (Fig. 4). We showed that, by Western blot analysis, a 190 kDa band was detected in rat HSC lysates at day 7, but not at days 4 and 1 after isolation (Fig. 3). Immunofluorescent staining with polyclonal antihuman ADAMTS-13 IgG, however, clearly detected very weak positivity of ADAMTS-13 antigen in rat HSCs at day 1 after isolation (Fig. 4A), but strong positivity of ADAMTS-13 antigen in rat HSCs at day 7 after isolation (Fig. 4E). These ADAMTS-13-positive rat HSCs also strongly expressed α-SMA (Fig. 4F), a marker specific for activated HSCs and transdifferentiated myofibroblasts that resemble those in the fibrotic lesions of liver.

ADAMTS-13 was significantly up-regulated in liver after CCl$_4$

Several studies have shown the up-regulation of MMPs (MMP-1, -2, -3, and -9) and TIMPs (TIMP-1, -2, and -3) in rat liver after CCl$_4$ [22,24,32] or bile duct ligation-induced liver fibrosis [33]. To determine whether ADAMTS-13 expression was also increased during the evolution of CCl$_4$-induced liver fibrosis, we measured *ADAMTS-13* mRNA, protein, and proteolytic activity in the liver tissues prior to injection (normal), 10 (mild fibrosis) and 70 days (severe fibrosis) after injection of CCl$_4$. We found that *ADAMTS-13* mRNA in the liver tissue lysates was only increased by 2.5- and 3.0-fold in the livers with mild fibrosis and severe fibrosis after administration of CCl$_4$, respectively, compared with the normal (Fig. 5A,B). However, as in the case of isolated rat HSCs, both ADAMTS-13 antigen (Fig. 6) and proteolytic activity (Fig. 7) were significantly increased in the rat liver after CCl$_4$ injury. The levels of ADAMTS-13 proteolytic activity in the liver were estimated to be approximately 600 and 4800 mU mg^{-1} of total protein at days 10 and 70, respectively (Fig. 7A). These results were consistent with those obtained by immunohistochemistry, showing that the number of ADAMTS-13-positive and α-SMA-positive cells was significantly higher in the liver tissues at day 10 (Fig. 6E,F) and day 70 (Fig. 6I,J) after CCl$_4$ administration than in the control tissues (Fig. 6A,B). The ADAMTS-13-positive cells were seen in the fibrotic strips that were also expressing α-SMA (Fig. 6I, J, L), but were not seen in the areas of normal liver architecture (Fig. 6A,B,D). All the control slides without incubation with the primary antibody were essentially negative. In no case was the red fluorescence in rat HSCs and liver tissues visible under a green filter or vice versa when ADAMTS-13 or α-SMA was stained separately.

In humans and mice, ADAMTS-13 synthesized and secreted from the activated HSCs were thought to be the major source of plasma ADAMTS-13 protease [14,15], although how such a big protein gets into the blood stream is not known at all. To our surprise, we found that despite the dramatic up-regulation of ADAMTS-13 antigen and proteolytic activity in the liver after CCl$_4$ injection, the plasma ADAMTS-13 activity in rats with mild liver fibrosis and severe liver fibrosis was even lower or slightly higher, although the differences were not statistically

significant ($P > 0.05$) when compared with that in normal rats (Fig. 7B). The reason for slight reduction of ADAMTS-13 activity at day 10 after CCl$_4$ is not known. The CCl$_4$ at concentration of 6.5 mM (similar to initially injected concentration) did not directly enhance or inhibit the cleavage of FRETS-VWF73 by ADAMTS-13 *in vitro* (data not shown). These data suggest that the activated HSCs may contribute little to regulation of plasma levels of ADAMTS-13 protease.

Discussion

The present study demonstrates for the first time a dramatic up-regulation of ADAMTS-13 antigen and proteolytic activity, but not *ADAMTS-13* mRNA in isolated rat primary HSCs upon activation *in vitro* by culturing them in uncoated plastic plates and in rat liver after CCl$_4$-induced liver fibrosis. We showed by both routine and real-time RT-PCR experiments that the levels of *ADAMTS-13* mRNA were only increased by 30% and 50% in rat primary HSC at days 4 and 7 after isolation, respectively, when compared with day 1 (Fig. 1). Similarly, the levels of *ADAMTS-13* mRNA in the liver tissues were only increased by 2.5- and 3.0-fold at days 10 (when liver exhibited mild fibrosis) and 70 (when liver exhibited severe fibrosis), respectively, after administration of CCl$_4$ (Fig. 5). These data suggest that regulation of ADAMTS-13 expression in rat HSCs during *in vitro* and *in vivo* activation does not occur to a significant degree at the transcriptional level.

Our data are consistent with the results reported by Claus *et al*. [34] showing that the alteration of *ADAMTS-13 mRNA* transcript in Hep3B cells is negligible after being stimulated by various pro-inflammatory stimuli such as endotoxin, tissue necrosis factor-α, interleukin-2 and -1β, and immunosuppressive agents such as cyclosporine and cortical steroids. However, the levels of ADAMTS-13 antigen and proteolytic activity in Hep3B cells and conditioned medium prior to or after various stimuli were not investigated in their study [34]. We showed, by FRETS-VWF73 and Western blotting, that the levels of ADAMTS-13 activity and antigen were dramatically increased in rat HSCs upon activation both *in vitro* (Figs 2 and 3) and *in vivo* (Fig. 7) after CCl$_4$-induced liver fibrosis. These results were corroborated by immunohistochemical staining demonstrating that ADAMTS-13 immunereactivity was also dramatically enhanced in activated rat HSCs (Fig. 4) and in liver tissues after CCl$_4$-induced liver injury and fibrosis (Fig. 6). Surprisingly, the plasma ADAMTS-13 proteolytic activity did not change significantly in rats with mild-and-severe fibrosis after CCl$_4$ injection despite of a dramatic up-regulation of ADAMTS-13 antigen and activity in activated rat HSCs (Fig. 7), suggesting that the activated HSCs may not be the primary source of plasma ADAMTS-13. This hypothesis is consistent with the phenomenon observed in humans in which plasma ADAMTS-13 activity is quite variable or frequently reduced in patients with liver cirrhosis [35]. Indeed, recent studies have suggested that ADAMTS-13 is also synthesized and secreted from arterial and venous vascular endothelial cells [36], megakaryocytes, and platelets [37,38]. The ADAMTS-13 mRNA is detected in brain, heart, lung, liver, kidney, adrenal glands, uterus, placenta, and prostate in humans by RT-PCR [11,39], suggesting a small amount of ADAMTS-13 protease may be produced in various organ tissues that contribute to the regulation of plasma levels of ADAMTS-13.

The mechanisms of the apparent discrepancy in the increment of ADAMTS-13 protein or activity and *ADAMTS-13* mRNA remain to be determined. One could speculate that the increased ADAMTS-13 protein upon rat HSC activation or CCl$_4$-induced liver injury or fibrosis may be resulted from an increase in protein translation efficiency and/or a reduction in protein degradation upon activation of HSCs *in vitro* and *in vivo*. It may also be possible that the increased ADAMTS-13 proteolytic activity in the activated rat HSCs is because of up-regulation of certain cofactors [40,41] such as heparin sulfate or other unknown factors for the ADAMTS-13 protease in the cells. However, the ADAMTS-13 activity in the conditioned

medium of cultured rat HSCs correlated well with the increased expression of ADAMTS-13 in the cells (Fig. 2B), suggesting that the ADAMTS-13 is readily secreted from activated rat HSCs in culture.

Several other MMPs [24,42] including MMP-3, -13, pro-MMP-2, and MT1-MMP and TIMPs, particularly TIMP-1 and TIMP-2 are up-regulated in rat livers upon CCl_4-injury [23,24,43], suggesting that the presence of active MMPs in acute and chronic liver injury may cleave extracellular matrix proteins, limit an excessive deposition of such matrix proteins and prevent liver fibrosis. This sequence of events may have a physiological function in liver regeneration. Considering that the only known substrate for ADAMTS-13, VWF [5,44,45], which is up-regulated in the microvascular endothelial cells in the area of necrosis and fiber-like structure during liver injury [46], one may speculate that the presence of the ADAMTS-13 protease at the time of tissue injury may also limit formation of microvascular thrombosis *in situ* or prevent excessive matrix protein deposition [46] and development of fibrosis in the liver [47,48]. However, there is no evidence thus far to demonstrate that ADAMTS-13 cleaves other matrix proteins except for VWF. The interaction between VWF and ADAMTS-13 protease do, however, occur intracellularly, particularly in the endoplasmic reticulum when both of them are expressed in the same cells [3]. The exact contribution of the enhanced ADAMTS-13 expression and proteolysis to liver fibrosis is, however, yet to be confirmed in future studies in animal models.

In summary, our data suggest that the dramatic up-regulation of ADAMTS-13 antigen and proteolytic activity during rat HSC activation *in vitro* and *in vivo* by CCl_4 injection may be crucial for the biological functions of the activated HSCs. ADMTS13 proteolysis may play a role in modulating liver regeneration or formation of fibrotic scar in injured liver after various acute and chronic insults. The data also suggest the sources of plasma ADAMTS-13 may be multiple. Finally, we believe that rat HSCs may be an excellent rat model for studying the regulation of ADAMTS-13 expression at posttranscriptional level.

Acknowledgements

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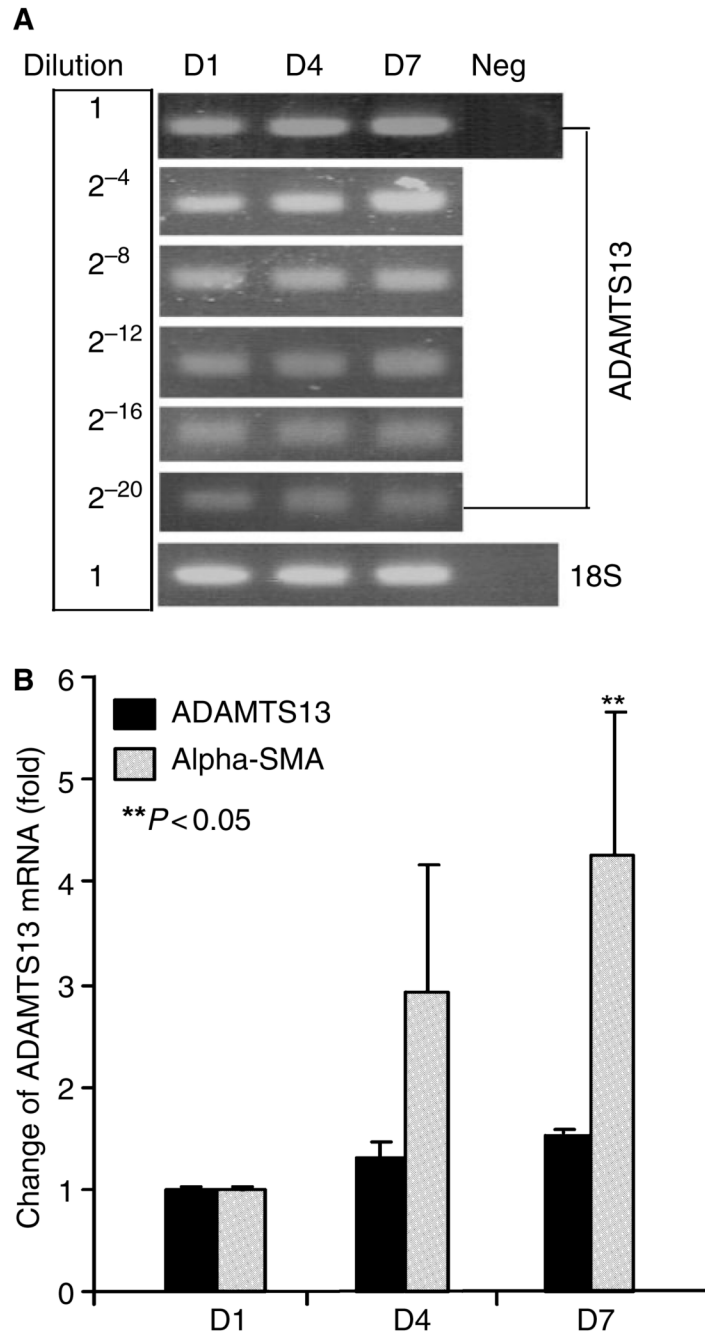
Fig. 1.
ADAMTS-13 mRNA in rat hepatic stellate cell (HSC) during *in vitro* activation. Total RNA was isolated from rat primary HSCs at days 1, 4 and 7 after isolation. *ADAMTS-13* mRNA was quantified by routine dilution RT-PCR (A) and real-time RT-PCR (B). The amplified *ADAMTS-13* fragments (154 bp) are detected by agarose-gel electrophoresis and stained by ethidium bromide after log dilutions of the single strain cDNA template (A). The relative amount of *ADAMTS-13* and *α-SMA* mRNA was determined by standard curve and normalized to the amount of 18S ribosomal RNA in each sample in real-time PCR. While the *α-SMA* mRNA in rat HSCs during activation *in vitro* was increased, the *ADAMTS-13* mRNA did not alter significantly (the entries are means $\pm$ SD, $n = 3$) (B).
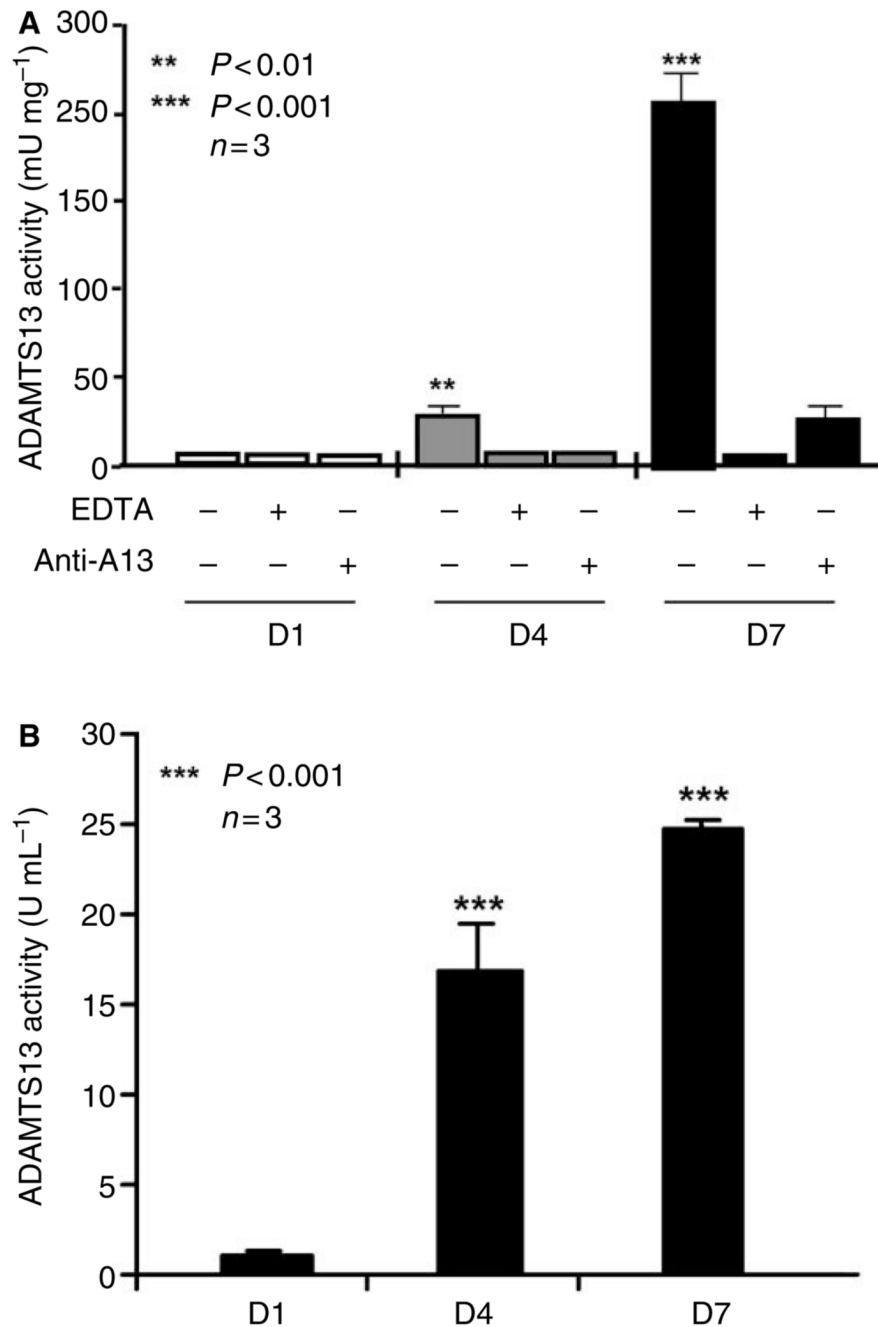
Fig. 2.
A disintegrin and metalloprotease with thrombospondin-13 activity in cell lysate and conditioned medium of rat hepatic stellate cells (HSCs). Fluorescent resonance energy transfer (FRETS)-VWF73 substrate at 2 μM was incubated with rat HSC lysate (total protein, 10 μg) and 5 μL of 24 h conditioned medium containing 10% fetal bovine serum in the absence (−) or presence (+) of 10 mM EDTA or rabbit anti-ADAMTS-13 IgG (40 μg mL^{-1}) (Anti-A13). A fluorescent ELISA reader detected the cleavage of FRETS-VWF73. The relative activity in the cell lysates and conditioned media was determined by comparison with that of normal human plasma (arbitrarily defined as 1 U or 1000 mU mL^{-1}). The proteolytic activity of

ADAMTS-13 in rat HSC lysate (A) and conditioned media (B) at days 4 and 7 was significantly increased compared with that at day 1. The entries are the means ± SEM ($n = 3$).
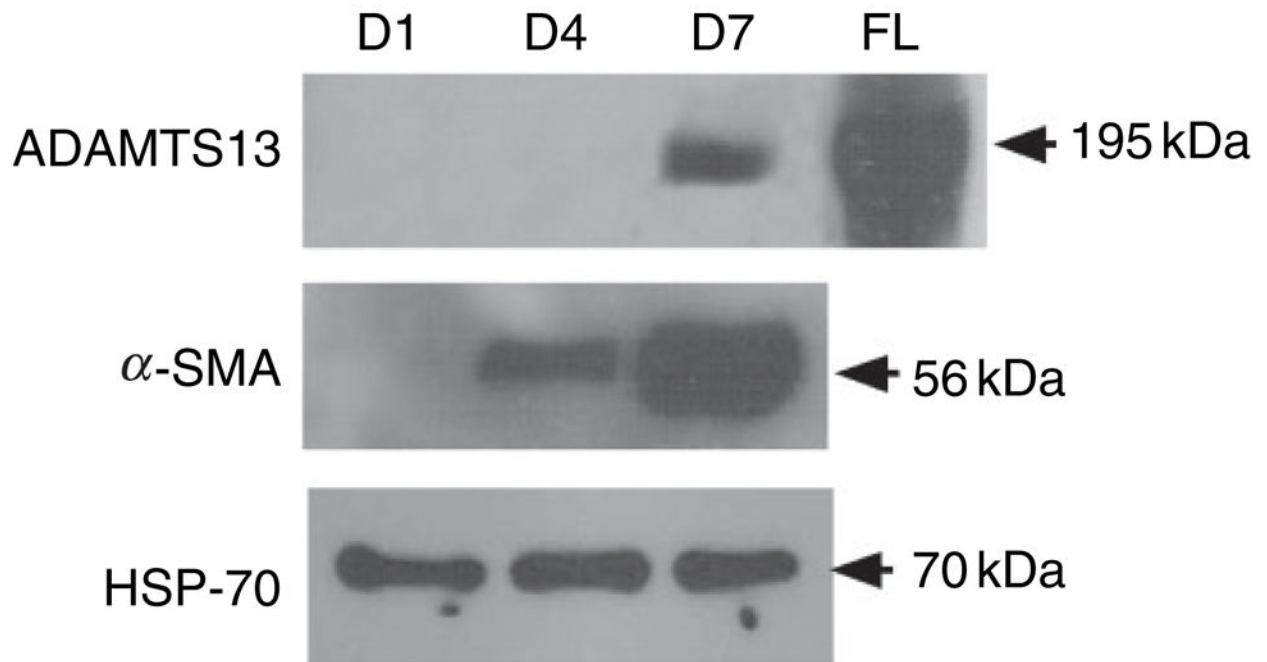
Fig. 3.
Western blotting analysis of ADAMTS-13 protein in rat hepatic stellate cells (HSCs) during activation *in vitro*. Total 10 μg of rat HSC cell lysate at days 1, 4 and 7 were used for Western blot. The ADAMTS-13, α-SMA, and heat shock protein-70 (HSP70) were detected by rabbit antihuman ADAMTS-13 IgG, mouse anti-α-SMA IgG, and mouse anti-HSP70 IgG, respectively. The recombinant full-length ADAMTS-13-V5-His was used as a positive control. The molecular weights of ADAMTS-13, α-SMA, and HSP70 are shown in the figure (195, 60, and 70 kDa, respectively).
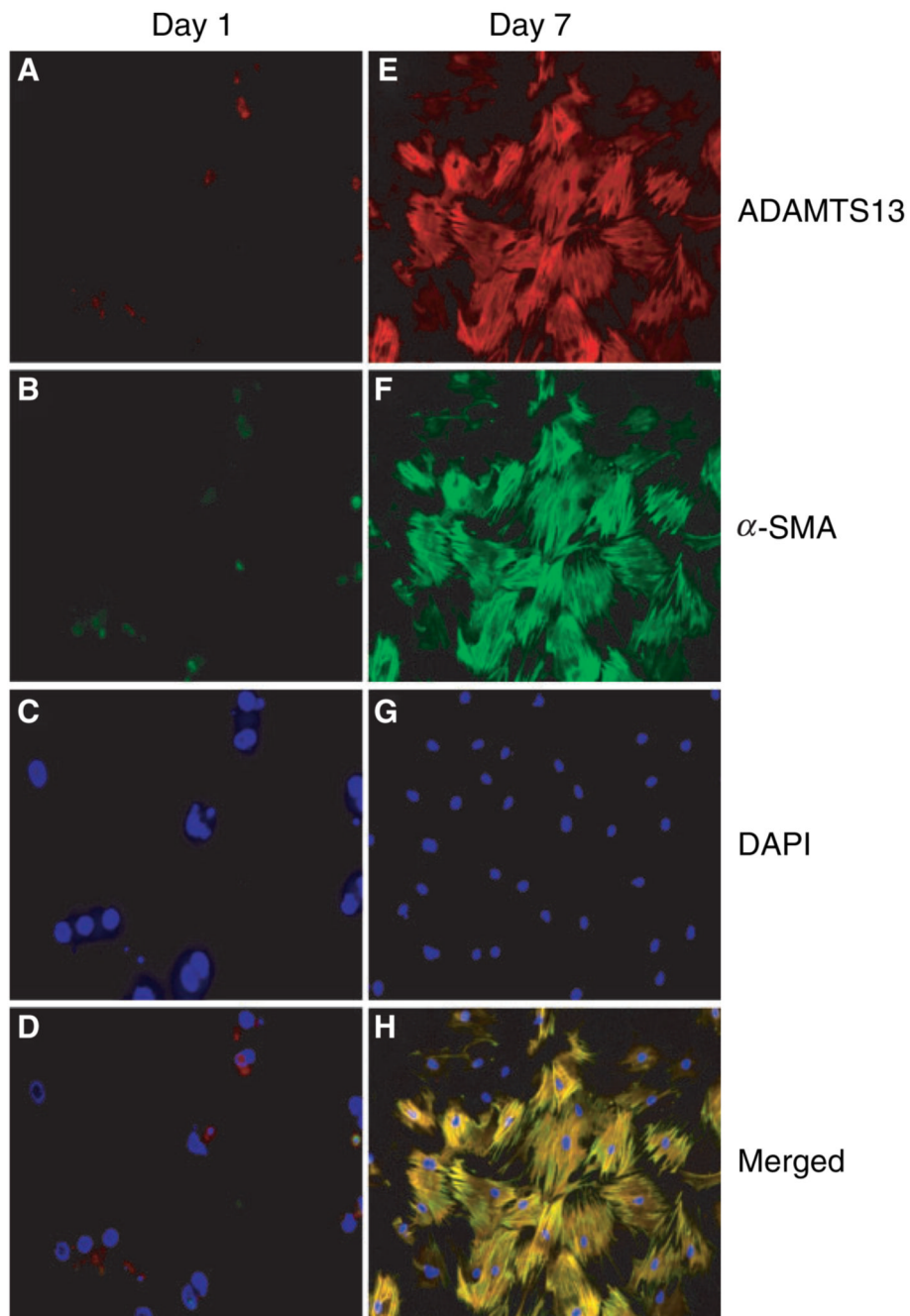
Fig. 4.
Immunocytochemical staining of ADAMTS-13 antigen in cultured rat hepatic stellate cells (HSCs) during *in vitro* activation. Isolated primary rat HSCs at days 1 (A–D) and 7 (E–H) after isolation were fixed and stained with rabbit antihuman ADAMTS-13 IgG (1:500) (A, E) and/ or mouse anti-α-SMA (1:200) (B, F), followed by a Cy3-conjugated antirabbit (red, A, E) or a Cy2-conjugated antimouse IgG (green, B, F) (1:100) as described in the Materials and methods. The nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) (blue, C, G, D, H). The merged images are shown in D and H. The original magnification of the digital image was 40×. The levels of ADAMTS-13 antigen in rat primary HSCs were dramatically increased upon *in vitro* activation.
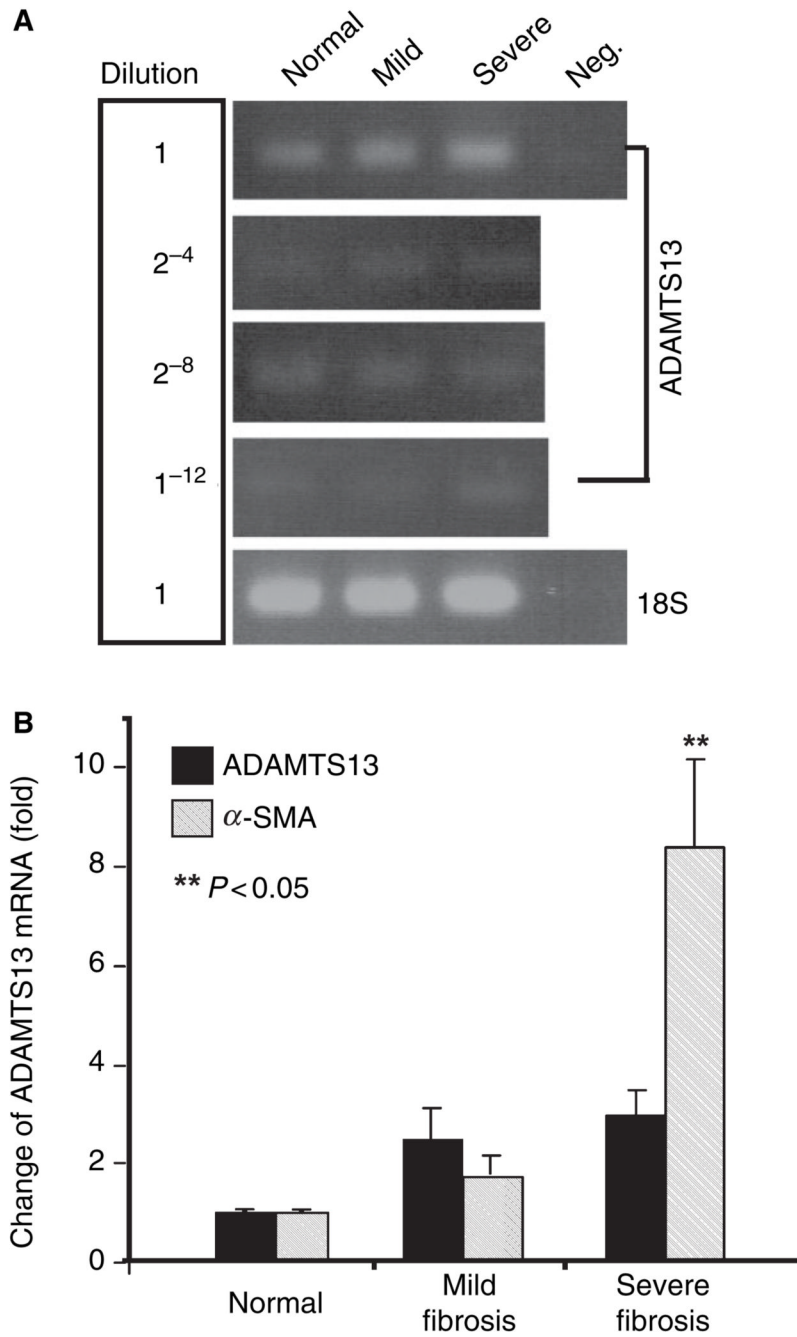
Fig. 5.
A disintegrin and metalloprotease with thrombospondin-13 (*ADAMTS-13*) mRNA in rat liver after CCl$_4$. *ADAMTS-13* and *α-SMA* mRNAs in rat livers prior to injection (normal), 10 (mild fibrosis) and 70 days (severe fibrosis) after administration of CCl$_4$, were determined by routine RT-PCR after log dilution of the first strand cDNA template (A) and real-time RT-PCR (B) as described in the Materials and methods. The relative amount of *ADAMTS-13* and *α-SMA* mRNAs was determined by a standard curve and normalized to 18S ribosomal RNA in the real-time PCR assay. The entries are the means ± SD (*n* = 3). While the levels of *α-SMA* mRNAs in rat HSCs after CCl$_4$ administration were increased dramatically, the *ADAMTS-13* mRNAs were only slightly increased.
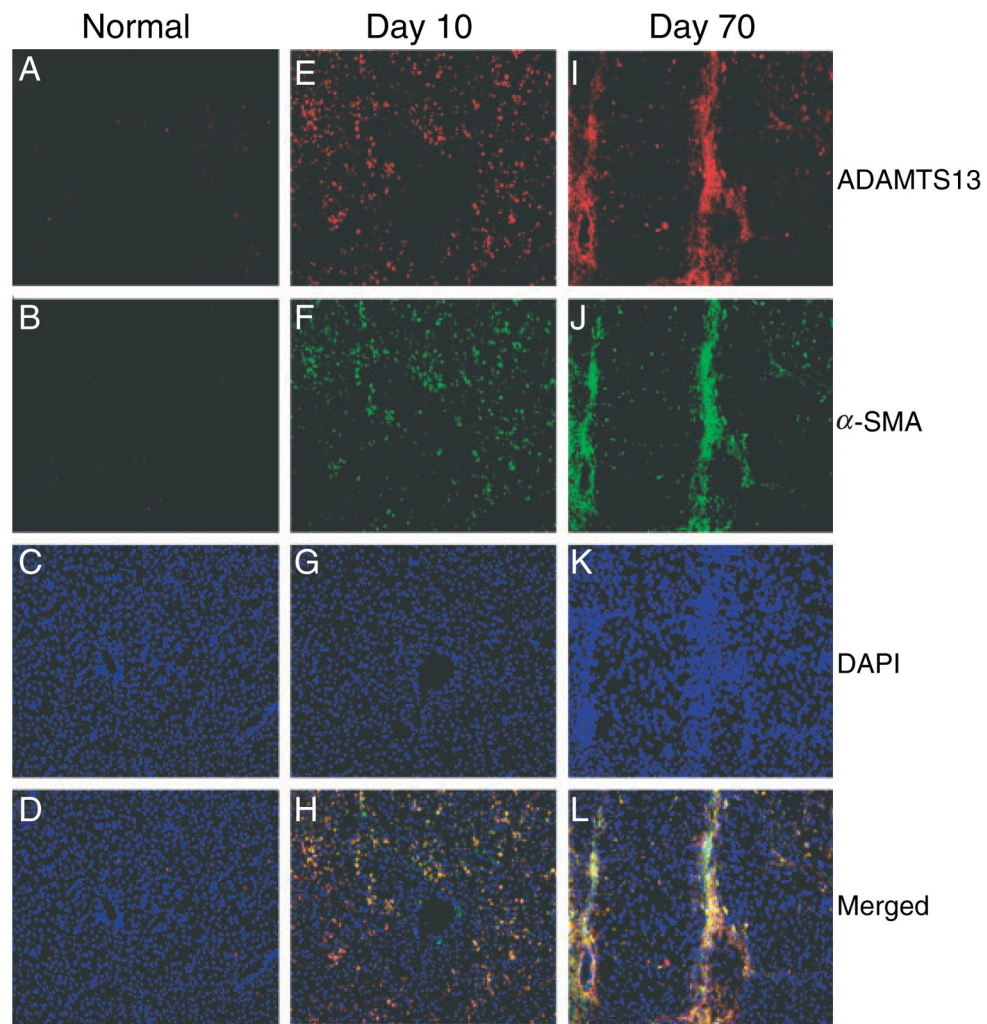
Fig. 6.
Immunohistochemical staining of ADAMTS-13 antigen in rat liver after CCl$_4$ injection.
Cryosections of rat liver obtained prior to (normal, A, B, C, D) 10 (E, F, G, H) and 70 days (I, J, K, L) after administration of CCl$_4$ were fixed and incubated with rabbit antihuman ADAMTS-13 IgG (1:500) (A, E, I) or mouse anti-α-SMA (B, F, J), followed by Cy3-conjugated antirabbit IgG (1:100) (A, E, I) or Cy2-conjugated antimouse IgG (1:100) (B, F, J). The nuclei were stained with DAPI in VECTORSHIELD™-mounting medium (C, G, K). The merged images are shown in D, H and L. The original digital images (magnification 10×) were obtained with a Nikon Inverse fluorescent microscope (Nikon, Melville, NY, USA) and processed by Canvas 7.0.
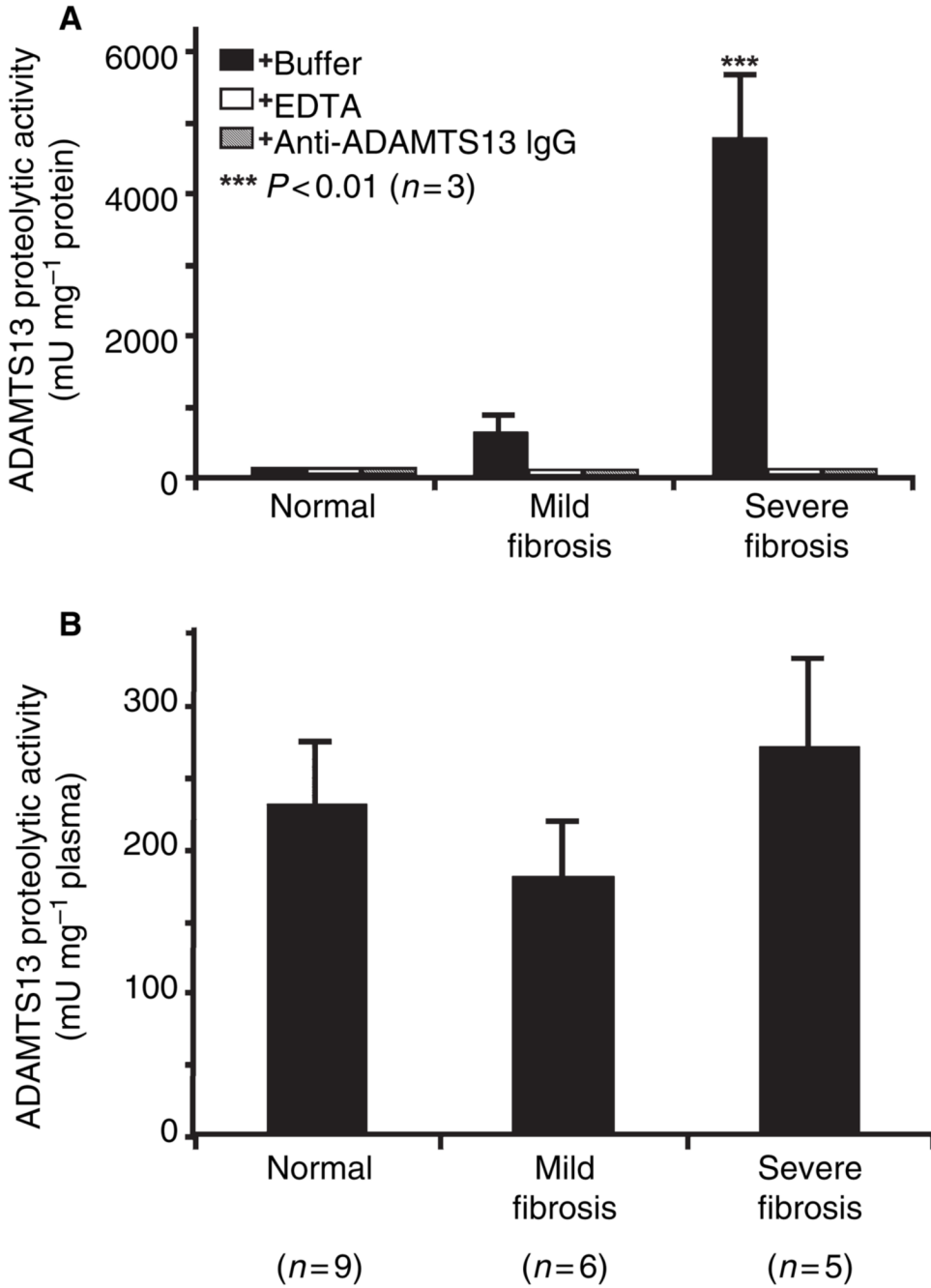
Fig. 7.
ADAMTS-13 activity in rat liver after CCl_4. The proteolytic cleavage of FRETS-VWF73 by rat liver tissue lysates (A) or plasma (B) prior to CCl_4 injection (normal), 10 (mild fibrosis) and 70 days (severe fibrosis) after administration of CCl_4 was determined as described in the Materials and methods. Although the levels of ADAMTS-13 activity in the liver was dramatically increased 10 and 70 days after CCl_4 injection (A), the levels of ADAMTS-13 activity in the plasma were not altered significantly compared with that of normal control (B).