ADAMTS1 Proteinase Is Up-regulated in Wounded Skin and Regulates Migration of Fibroblasts and Endothelial Cells*

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The metalloproteinase ADAMTS1 (a disintegrin and metalloproteinase with thrombospondin motifs) is induced under inflammatory conditions, and it is also a potent inhibitor of angiogenesis. Due to these properties, we speculated about the role of ADAMTS1 in cutaneous wound repair. Here we have shown up-regulation of ADAMTS1 expression in wounds of normal and particularly of healing-impaired genetically diabetic mice. Immunofluorescence staining identified macrophages as the source of ADAMTS1 in early wounds, whereas keratinocytes and fibroblasts produce this protein at later stages of wound healing. The distribution of ADAMTS1 in the normal and wounded epidermis, its regulation in cultured keratinocytes, as well as the skin phenotype of ADAMTS1 knock-out mice suggests a role of this metalloproteinase in keratinocyte differentiation. Furthermore, we provide evidence for a novel dual function of ADAMTS1 in fibroblast migration; although low concentrations of this protein stimulate fibroblast migration via its proteolytic activity, high concentrations inhibit this process because of binding to fibroblast growth factor-2 and subsequent inhibition of its promotogenic activity. Similar effects were also observed with endothelial cells. Taken together, our results suggest a role of ADAMTS1 in keratinocyte differentiation and migration of fibroblasts and endothelial cells in healing skin wounds.

Repair of wounded tissue is a complex process that requires temporal and spatial coordination of different events, including inflammation, formation of new tissue, angiogenesis, and matrix remodeling. These processes are regulated by growth factors and cytokines but also by cell-cell and cell-matrix interactions (1–5). Proteolytic processes are required during all phases of cutaneous wound healing. Proteolysis is necessary to allow inflammatory cells to enter the wound site and to degrade the provisional fibrin clot. Proteinases are involved in cell migration, in wound contraction, and in scar remodeling, and the role of serine proteinases, such as plasmin and leukocyte elastase and of matrix metalloproteinases, in wound repair is well established (4–6). In addition, another proteinase family has recently been identified, whose members are of potential interest for the wound-healing process based on their biological activities. These proteinases, which are designated ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) comprise a family of Zn2+-dependent metalloproteinases closely related to the matrix metalloproteinases and ADAM family of proteinases. They are structurally characterized by a metalloproteinase domain, a disintegrin-like domain, and several thrombospondin motifs (7). The adams1 gene was originally cloned from a colon carcinoma cell line (8). Some of the ADAMTS1 substrates have been identified, and they include mainly proteoglycans, such as aggrecan (9, 10) and versican (11). adams1 knock-out mice are characterized by growth retardation, changes in kidney structure, and impaired female fertility, demonstrating an important role of the protein in organogenesis (12, 13). ADAMTS1 has been shown to be a potent inhibitor of angiogenesis (14). It reduces the proliferative response induced by fibroblast growth factor 2 (FGF-2) and vascular endothelial growth factor (VEGF) in endothelial cells. For VEGF, this effect is achieved through direct binding of ADAMTS1 to the growth factor, resulting in inhibition of VEGF binding to its transmembrane receptor (14, 15). A role in inflammatory processes has also been suggested for ADAMTS1, because its expression is induced by interleukin-1α in colon carcinoma cells and because it was found to be up-regulated in the heart, liver, and kidney after systemic treatment of mice with lipopolysaccharides (8, 16). Recently, adams1 mRNA levels were found to increase after surgical nerve injury in rats (17) and also in kainate-induced lesions in rat brain (18). These findings make ADAMTS1 an interesting candidate for a possible role in various tissue repair processes. However, the expression and function of ADAMTS1 in the skin has not been investigated. In this study, we determined the temporal and spatial expression pattern of ADAMTS1 in normal and wounded mouse skin. We showed that ADAMTS1 is a novel player in the wound-healing process, which regulates keratinocyte differentiation as well as migration of fibroblasts and endothelial cells.

EXPERIMENTAL PROCEDURES

Wounding and Preparation of Wound Tissue—Mice (2–4 months old) were anesthetized by intraperitoneal injection of ketamine (75 mg/kg)/xylazine (5 mg/kg). Two full-thickness excisional wounds, 5 mm in diameter, were made on either side of the dorsal midline by excising the

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‡ The abbreviations used are: FGF-2, fibroblast growth factor 2; VEGF, vascular endothelial growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; TGF, transforming growth factor; HA, hemagglutinin; HEK, human embryonic kidney.
skin and the rodent-specific subcutaneous muscle panniculus carnosus, as described previously (19). Wounds were left uncovered and harvested at different time points after injury. For expression analyses, the complete wounds, including 2 mm of the epithelial margins, were excised and immediately frozen in liquid nitrogen. Non-wounded back skin served as a control. For each expression study, two independent wound-healing experiments were performed using 4 mice (16 wounds/time point. For immunofluorescence, the complete wounds were isolated, bisected, and directly embedded in tissue-freezing medium without prior fixation. Sections from at least 5 mice/time point were analyzed. BALB/c mice were obtained from Charles River (Sulzfeld, Germany); genetically diabetic (db/db) mice (C57BL/KsOlaHsd-Lepr db/db or db/db) were from Harlan (Horst, The Netherlands). Adams1 knock-out mice in a C57BL/6 background were generated by targeting exons 2–8 and replacement by a neomycin resistance gene. All animal experiments were performed with permission from the local veterinary authorities (Kantonales Veterinäramt, Zurich, Switzerland).

RNA Isolation and RNase Protection Assay—Isolation of total cellular RNA and RNase protection assays were performed as described previously (20, 21). A template for mouse adams1 was described previously (16). Templates for human adams1 (nucleotides 2990–3160, GenBank™ accession number AF170084.1) and human keratin 10 (nucleotides 721–960, GenBank™ accession number BC034697) were synthesized with a DNA transcription-PCDN vector pBluescript KSII(−) (Stratagene, La Jolla, CA). A loading control, the RNA was hybridized with a probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (nucleotides 566–685 of the murine cDNA, GenBank™ accession number NM_008084; and nucleotides 580–685 of the human cDNA, GenBank™ accession number BC010681) or β-actin (nucleotides 422–550 of the human cDNA, accession number NM_002393). All protection assays were performed at least in duplicate with different sets of RNA from independent experiments. Quantification of the mRNA levels was performed with the National Institutes of Health Image program.

Preparation of Protein Lynates and Western Blot Analysis—Cultured cells were lysed in urea buffer (10 mM Tris, pH 8.0, 5.0 mM urea, 2 mM EDTA, 0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride), and cell culture supernatants were precipitated with 4 volumes of acetone. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions and transferred to nitrocellulose filters. Antibody incubations were performed in 5% nonfat dry milk in phosphate-buffered saline (PBS) at room temperature. Secondary antibodies were diluted in PBS containing 0.05% Tween 20. For mouse samples, a goat polyclonal antibody directed against the catalytic domain of mouse adams1 (diluted 1:100) (Santa Cruz Biotechnology, La Jolla, CA) was used. For human samples, we used a mouse monoclonal antibody directed against the catalytic domain of human ADAMTS1 (22).

Immunofluorescence—Method—fixed sections (7 μm) from the middle of the wound were incubated overnight at 4 °C with the primary antibodies diluted in phosphate-buffered saline containing 3% bovine serum albumin and 0.025% Nonidet P-40. After three washes in PBS, primary antibody was incubated overnight with the secondary antibodies, washed again, mounted with Mowiol (Hoechst, Frankfurt, Germany), and photographed with a Zeiss Axioplan fluorescence microscope. The following antibodies were used: the anti-ADAMTS1 antibodies described above; rat monoclonal antibodies directed against the platelet endothelial cell adhesion molecule (diluted 1:100) (PharMingen); Ly-6G (diluted 1:100) (BD Biosciences); CD11b (diluted 1:50) (BD Biosciences); mouse monoclonal antibodies directed against α smooth muscle actin (fluorescein isothiocyanate–coupled) (Sigma); keratin 10 (Dako, Glostrup, Denmark); rabbit polyclonal antibodies directed against keratin 14 (Babo, Richmond, CA); and keratin 6 (Babo). All secondary antibodies (coupled to Cy2 or Cy3) were from Jackson ImmunoResearch. The images were analyzed with Adobe Photoshop (Mountain View, CA) to prove the specificity of the goat-anti-mouse ADAMTS1 antibody (Santa Cruz Biotechnology), which was incubated overnight at 4 °C with a 100-fold molar excess of the immunization peptide before performing immunofluorescence.

Histology—Tail skin was separated from the bone, fixed overnight in 95% ethanol/1% acetic acid, and embedded in paraffin. Sections (8 μm) were stained with hematoxylin/eosin. Only animals of the same age and sex were used for histological comparison.

Growth Factor Treatment of HaCaT Keratinocytes—The human keratinocyte cell line HaCaT (23) was cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were grown to confluence, rendered quiescent by a 24-h incubation in serum-free DMEM, and subsequently incubated in fresh DMEM containing 2 μg/ml transferrin stimulating growth factor (TGF)-β1 or -β2. The cells were harvested before and at different time points after growth factor treatment and used for RNA isolation. For preparation of protein lysates, 1 unit/ml heparin was added to the medium together with the growth factor. TGF-β1 and -β2 were purchased from R&D Systems (Minneapolis, MN). DMEM and heparin were purchased from Sigma, and FCS was from Amimed-BioConcept (Allschwil, Switzerland).

In Vitro Differentiation of Primary Mouse Keratinocytes—Primary mouse keratinocytes were prepared as described previously (25) and cultured in keratinocyte serum-free medium (Invitrogen) supplemented with 0.1 ng/ml epidermal growth factor and 25 μg/ml bovine pituitary extract. For in vitro differentiation, cells were grown to 70% confluence. The medium was subsequently replaced by keratinocyte basal medium (Gibco, Germany) supplemented with hydrocortisone and gentamycin, according to the manufacturer’s recommendations, but without any growth supplements. The cells were kept under differentiation-promoting conditions up to 13 days, and the medium was changed every second day. The cells were harvested at different time points after induction of differentiation and used for RNA isolation.

In Vivo Wound Assays—Primary mouse skin fibroblasts were obtained by outgrowth from biopsies of neonatal mouse skin in DMEM supplemented with 20% FCS, 100 units/ml of penicillin, and 100 μg/ml streptomycin. Cells were further cultured in DMEM/10% serum and used for experiments from passage 2 to 6. Human microvascular endothelial cells were isolated from foreskin as previously described (26) and cultured in medium containing 20% fetal calf serum, endothelial cell growth supplement (5 μg/ml) (Gibco), and heparin (90 μg/ml) (Sigma). Cells were used from passage 2 to 4.

Cells were grown to confluence and treated with 10 μg/ml mitomycin C (Sigma) for 2 h. A scratch was made within the cell layer with a sterile pipette tip. Cells were washed with phosphate-buffered saline and kept in medium containing various concentrations of ADAMTS1 protein, FGF-2 (Roche Applied Science), and/or VEGF (R&D Systems). The wounds were visualized by light microscopy at different time points after scratching, 20–24 h later for fibroblasts, and 36 h later for endothelial cells. Areas that were not covered by cells were measured using the Openlab software. Cloning of adams1 constructs, generation of a catalytically inactive mutant, and purification of recombinant proteins have been described previously (10, 22).

Immunoprecipitation—In Eukaryotic Cells—A PCR fragment containing the open reading frame of ADAMTS1 without the stop codons (nucleotides 243–3143, GenBank™ accession number AF170084.1) was amplified from the full-length cDNA (14) using the following primers: adf5′-CCG TCT AGA ATG CAG CGA GCT GTG CCC GA-3′ and adf3′-GGG GCA TTC TGC CAT TGT GCA A-3′. The fragment was cloned into the XbaI/SmaI restriction sites of the pcDNA mammalian expression vector, thus allowing the expression of full-length ADAMTS1 with an in-frame carboxy-terminal hemagglutinin (HA) epitope under the control of the cytomegalovirus promoter.

Transient Transfection of HEK293 Cells—HEK293 cells (American Type Culture Collection, Manassas, VA) were transiently transfected with the pcDNA-adams1-HA expression construct or the empty vector using the calcium phosphate transfection method (27). Briefly, 1 × 106 cells in 6-cm dishes were transfected with 4 μg of the plasmid in a calcium phosphate precipitate. After a 5-h incubation, the cells were washed with HEPES buffer (142 mM NaCl, 10 mM HEPES, pH 7.3, 6.7 mM KCl) and kept in serum-free DMEM for 48 h. Conditioned media were collected and concentrated 10× with AMICON filter devices (Millipore, Bedford, MA).

Urea precipitation—100 μl of concentrated conditioned media were mixed 1:2 with ureaprecipitation buffer (50 mM Tris/HC1, pH 7.5, 250 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF, 1 mM MgCl2, 1 mM CaCl2, 0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.15 units/ml aprotinin) and incubated with 2 μg of a rabbit polyclonal antibody (Santa Cruz Biotechnology) for 90 min at 4 °C. Antibody-bound proteins were precipitated with protein A-Sepharose, and beads were washed three times with immunoprecipitation buffer. For subse-

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RESULTS

Enhanced Expression of ADAMTS1 after Skin Injury—To gain insight into a possible role of ADAMTS1 in skin repair, we first determined its expression during cutaneous wound healing in mice. RNase protection assays with RNAs from intact skin and from full-thickness excisional wounds taken at different time points post-wounding revealed that *adamts1* expression was indeed up-regulated by 30 min after injury. Maximal mRNA levels were found 6 h post-wounding (Fig. 1A). Expression subsequently declined; however, it did not return to basal levels within 13 days. In 7- and 13-day wounds, we also observed a second small peak of *adamts1* expression.

To further determine the role of ADAMTS1 for normal wound repair, we analyzed its expression in genetically diabetic (db/db) mice, which are a well established model for impaired wound healing (28, 29). As shown in Fig. 1B, *adamts1* mRNA expression was significantly higher in diabetic mice compared with the heterozygous control animals, in particular during the early phase of wound healing (6 h until 1 day post-wounding). All results were repeated with different sets of RNAs from independent wound-healing experiments.

Expression of ADAMTS1 in Inflammatory Cells—ADAMTS1 has originally been identified as the product of an inflammation-associated gene (8). The early inflammatory phase of wound healing comprises mainly days 1–3 post-wounding, although neutrophils and, in particular, macrophages are still found in 5-day wounds (Fig. 2A). To find out whether inflammatory cells are a source of ADAMTS1 during early wound healing, we performed immunofluorescence co-stainings with an anti-Ly6G antibody as a marker for neutrophils and an anti-CD11b antibody as a marker for macrophages on sections from 1-day and 5-day wounds. In 1-day wounds, ADAMTS1 was mainly expressed in a subset of CD11b-positive cells within the clot (Fig. 2, B–D) but not in Ly6G-positive cells (Fig. 2, E–G). In the granulation tissue of 5-day wounds, only very few macrophages still expressed ADAMTS1 (Fig. 2, H–J), and the majority of the ADAMTS1-positive cells were no longer macrophages (CD11b-negative). Again, no expression of ADAMTS1 was found in neutrophils at this time point (Fig. 2, K–M). A similar expression pattern was also found in wound sections from diabetic mice (data not shown).

ADAMTS1 Is Expressed in Fibroblasts of the Granulation Tissue and in the Hyperproliferative Epithelium—To determine the cellular origin of ADAMTS1 at later stages of wound healing, we performed immunofluorescence staining on frozen sections from 5-day wounds. Fig. 3A shows that the ADAMTS1 protein is mainly present in the granulation tissue and, to a lesser extent, in the keratinocytes of the hyperproliferative epithelium. To confirm the specificity of the antibody, it was pre-incubated with an excess of the immunization peptide, leading to disappearance of the observed signal (Fig. 3C). Incubation with the secondary antibody alone was performed as an additional negative control and is shown in Fig. 3B.

As seen by co-staining with 4',6-diamidino-2-phenylindole as a nuclear marker, ADAMTS1 partially localized to cells within the granulation tissue, which appear to be fibroblasts according to their morphology (Fig. 3, D and E, open arrowheads).
addition, extracellular deposits of ADAMTS1 were also detected (Fig. 3, D and E, filled white arrowheads). No expression of ADAMTS1 protein was found in blood vessels, as determined by co-staining with an antibody against the endothelial marker protein platelet endothelial cell adhesion molecule (Fig. 3, F and G).

A subpopulation of fibroblasts within a wound differentiates into myofibroblasts, which are responsible for wound contraction (30). Using α-smooth muscle actin as a marker protein for myofibroblasts, we did not find expression of ADAMTS1 in these cells. As shown in Fig. 3, H and I, ADAMTS1 was mainly found in normal fibroblasts in the middle of the wounds but not in the myofibroblasts at the wound edge.

In 10- and 13-day wounds, which were already completely closed, ADAMTS1 expression was more prominent in the new epidermis compared with the granulation tissue (Fig. 3, J and K). Within the latter, we still found some ADAMTS1-positive cells at day 10 after injury, whereas in 13-day wounds, almost no more ADAMTS1-expressing fibroblasts were detected.

To confirm the expression of ADAMTS1 in keratinocytes and fibroblasts, we performed Western blots with lysates and supernatants from cultured primary fibroblasts and keratinocytes derived from neonatal mouse skin. Because heparin has been shown to protect the 87-kDa isoform of ADAMTS1 from proteolytic degradation in culture, we used lysates from cells that had been cultured in the presence or absence of heparin (22, 31). We found the active 87-kDa variant of ADAMTS1 in the supernatant of both fibroblasts and keratinocytes, whereas only low levels of the 110-kDa pro-form were detected in the cell lysates. Bands of lower molecular mass most likely result from partial proteolytic degradation of ADAMTS1 (Fig. 4). The specificity of the antibody was confirmed by blocking with the immunization peptide (data not shown). No expression of ad-
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**Fig. 4. Processing and secretion of ADAMTS1 in cultured fibroblasts and keratinocytes.** Western blot analysis of primary fibroblasts and keratinocytes derived from mouse skin. Total lysates (50 μg, labeled C) and acetone-precipitated conditioned medium (labeled CM) from cells that had been cultured in the presence (+) or absence (−) of heparin (1 unit/ml) were analyzed for the presence of ADAMTS1. The pro- and mature forms of ADAMTS1 are marked with arrows.

adams1 mRNA or protein was found in primary macrophages isolated from the peritoneum of both control and genetically diabetic mice (data not shown), indicating that macrophages induce the expression of this protein upon invasion into the wound.

**Adams1 mRNA Expression in Keratinocytes Depends on the Differentiation State of the Cells**—The high expression of ADAMTS1 in keratinocytes of 13-day wounds indicated a role of this protein in the redifferentiation process of the wound epidermis. To determine whether ADAMTS1 expression is also dependent on the differentiation state in intact skin, we performed immunofluorescence stainings on tail skin, which is characterized by a thicker epidermis compared with back skin, thus allowing a better analysis of differentiation-specific gene expression. ADAMTS1 expression was much more prominent in the epidermis than in the dermis (Fig. 5A). In the epidermis, the strongest ADAMTS1 signals were found at the basal side of basal keratinocytes, as well as in the uppermost living cells. This finding indicates that adams1 expression is influenced by the differentiation state of keratinocytes. To further address this question, we performed in vitro differentiation assays with primary human foreskin keratinocytes. Differentiation was induced by serum withdrawal, and RNAs isolated from the cells at different time points were analyzed for the expression of adams1 by RNase protection assay. As shown in Fig. 5B, adams1 was expressed in proliferation-competent keratinocytes and down-regulated when differentiation was initiated. Keratin 10 expression was used as a marker for the onset of differentiation. Interestingly, adams1 mRNA levels increased again strongly at late stages of differentiation (days 9–11), thus reflecting the situation in mouse epidermis.

To further explore the contribution of ADAMTS1 to keratinocyte differentiation in vivo, we analyzed the tail skin of adams1 knock-out mice (several areas from the tail of two knock-out and two heterozygous control animals). Interestingly, the epidermis of these mice (Fig. 5, D and E) appeared irregular compared with control animals (Fig. 5C), with hyper-thickening and parakeratosis (persistent nuclei in the cornified layer) being observed to a variable extent (Fig. 5, D and E). To determine the differentiation status of the keratinocytes, we stained the sections with antibodies against differentiation-specific keratins. In normal epidermis, keratin 14 is expressed at high levels in the basal layer, whereas synthesis of keratin 10 starts when keratinocytes are committed to terminal differentiation and move to the suprabasal layers (32). Expression of keratin 14 was detected in basal cells of both control and knock-out mice (Fig. 5, F and G), although the staining in knock-out mice appeared more irregular. Keratin 10 expression started in the first suprabasal layers of both mice (Fig. 5, H and I), demonstrating that the additional cell layers seen in the knock-out mice consist of at least partially differentiated cells. Finally, we stained the skin sections with an antibody against keratin 6. This keratin is restricted to the hair follicles in normal skin, but is up-regulated in hyperplastic, neoplastic, and psoriatic epidermis and represents a marker for abnormal differentiation (32). Indeed, keratin 6 expression was found in the interfollicular epidermis of adams1-deficient mice (Fig. 5K), further supporting the role of ADAMTS1 in keratinocyte differentiation.

**TGF-β Strongly Induces ADAMTS1 Expression in Cultured Keratinocytes**—Because the balance between proliferation and differentiation in keratinocytes is regulated by growth factors and cytokines, we used the immortalized keratinocyte cell line HaCaT to investigate the influence of different growth factors on the expression of adams1. TGF-β1 and -β3 strongly induced adams1 mRNA levels (Fig. 6A), whereas activin A, keratinocyte growth factor, epidermal growth factor (EGF), as well as the pro-inflammatory cytokines tumor necrosis factor α and interleukin-6, did not influence adams1 mRNA levels (data not shown). The effect of TGF-β1 and -3 was confirmed by Western blotting, where highest levels of ADAMTS1 protein were detected in the supernatant of HaCaT cells 48 h after the addition of these factors (Fig. 6B). Ponceau S staining of the blot was performed as a loading control (data not shown). These results were reproduced in at least two independent experiments.

**ADAMTS1 Affects Fibroblast Migration**—As shown above, fibroblasts are the major source of ADAMTS1 in 5-day skin wounds. Because this time point is characterized by massive migration of fibroblasts into the blood clot/granulation tissue, we speculated about a possible role of ADAMTS1 in the regulation of fibroblast migration. For this purpose, we performed in vitro migration assays with the purified 87-kDa protein variant of ADAMTS1. Using scratch wounding assays with primary mouse fibroblasts, we found that low doses of exogenously applied ADAMTS1 protein (1–2 nM) significantly increased migration of the cells into the wounded area (Fig. 7A). This effect required the presence of low amounts of serum or growth factors (0.1% FCS), indicating that ADAMTS1 can enhance fibroblast migration but does not induce migration of quiescent cells. A catalytically inactive mutant of ADAMTS1 had no influence on migration (Fig. 7B), indicating that proteolytic activity is required for this effect. These results were reproduced with several batches of primary fibroblasts from different mice.

**High Doses of ADAMTS1 Inhibit the Pro-migratory Effect of FGF-2**—Interestingly, the pro-migratory effect of ADAMTS1 was no longer observed at higher concentrations (4–10 nM) of the protein (Fig. 7A). To find out whether this was because of excessive proteolysis or to the ability of ADAMTS1 to block growth factor function (15), we applied ADAMTS1 (12 nM) together with FGF-2 (1 ng/ml). FGF-2 is the only pro-migratory factor known to act on fibroblasts in an autocrine manner (33). Indeed, after the addition of ADAMTS1, we no longer observed a significant pro-migratory effect of FGF-2 (Fig. 7C). The same result was obtained with the mutant ADAMTS11, demonstrating that this effect of ADAMTS1 is independent of its catalytic activity. To determine whether the inhibition is mediated by direct interaction of ADAMTS1 with FGF-2, we performed co-immunoprecipitation experiments. Supernatant from ADAMTS1-transfected cells was immunoprecipitated, and immobilized ADAMTS1 was incubated with recombinant FGF-2. Western blot analysis with an anti-FGF-2 antibody showed direct binding of ADAMTS1 to FGF-2 (Fig. 7D, upper panel). Precipitation of ADAMTS1 was confirmed by Western blotting with an anti-HA antibody (Fig. 7D, lower panel). Binding as-
says required the presence of heparin to prevent nonspecific binding of FGF-2 to protein A-Sepharose (data not shown). The specificity of the ADAMTS1/FGF-2 interaction was also confirmed by performing the binding assay with the non-heparin-binding growth factor TGF-β3. No interaction was observed between ADAMTS1 and TGF-β3 (data not shown).

**ADAMTS1 Modulates Endothelial Cell Migration**—As ADAMTS1 is known to affect angiogenesis and endothelial cell proliferation (14), we investigated whether this protein also affects migration of this cell type. Indeed, ADAMTS1 affected endothelial cell migration in a concentration-dependent manner (Fig. 8A). Lower amounts of the protein were required to enhance migration of endothelial cells compared with fibroblasts. Surprisingly, only the catalytically active form of ADAMTS1 was able to inhibit FGF-2/VEGF-induced migration at higher concentrations (Fig. 8B), suggesting that different mechanisms are responsible for the inhibitory effect in endothelial cells and fibroblasts.

**DISCUSSION**

ADAMTS1 belongs to a recently identified family of metalloproteinases that is characterized by the presence of additional disintegrin- and thrombospondin-like domains. Therefore, it has multiple possibilities for interaction with cells and the extracellular matrix. ADAMTS1 is involved in tissue remodeling, for example, during ovulation (13, 34), in inflammation (8), and in angiogenesis (14). Because inflammation and
angiogenesis are important for the repair of injured tissues, we analyzed the expression and potential function of ADAMTS1 during cutaneous wound healing. ADAMTS1 expression was strongly up-regulated early after injury, and a second peak was observed at the late stage of healing, indicating that ADAMTS1 might have various functions during the wound-healing process. This induction was even more pronounced in healing-impaired diabetic mice, suggesting that ADAMTS1 may contribute to the excessive proteolysis seen in these animals (35).

Adams1 has originally been cloned as an inflammation-associated gene, and its expression was induced after systemic lipopolysaccharide administration (8). However, the cellular origin of ADAMTS1 during inflammatory processes has not yet been determined. Here we have shown expression of ADAMTS1 in macrophages of 1-day wounds. Interestingly, although macrophages were still present at later stages of wound healing, the majority of them did no longer express the protein at day 5. In addition, ADAMTS1 was not found in macrophages isolated from the peritoneum (data not shown), suggesting that ADAMTS1 is transiently induced during the invasion of macrophages into injured tissue.

Both in 5- and 13-day wounds, ADAMTS1 was expressed in fibroblasts and keratinocytes. Interestingly, the major site of expression of the protein changed during the wound-healing process. Keratinocytes stained much stronger for ADAMTS1 in 13-day wounds compared with 5-day wounds, suggesting that this proteinase might be involved in the process of keratinocyte redifferentiation, which occurs in late skin wounds. Indeed, our results revealed that ADAMTS1 expression is strongly dependent on the differentiation state of the keratinocytes both in vivo and in vitro; ADAMTS1 was highly expressed in non-differentiated cells, down-regulated in partially differentiated keratinocytes, and induced again at a late stage of differentiation. Because the differentiation process is accompanied by extensive morphological and biochemical changes that also require proteolytic activity (36–38), a function of ADAMTS1 in these events seemed likely, and this hypothesis is strongly supported by the differentiation abnormalities seen in the epidermis of adams1 knockout mice. ADAMTS1 processes several proteoglycans (9–11), some of which are expressed in proliferation-competent and/or differentiated keratinocytes (39–41). Preliminary experiments showed enhanced staining for carbohydrates in the epidermis of the knock-out mice (data not shown), indicating that glycoproteins are indeed in vivo substrates of ADAMTS1. Proteoglycans are important co-receptors for a variety of growth factors (42, 43). Therefore, one might

FIG. 7. Effects of recombinant ADAMTS1 protein on fibroblast migration. A–C, primary fibroblasts isolated from newborn mouse skin were grown to confluency, treated with mitomycin C to block proliferation, and the monolayer was subsequently wounded with a pipette tip. The wounded fibroblast layer was photographed immediately after injury and 24 h later. Each experiment was performed in triplicate, and pictures of two spots/dish were taken (n = 6). The clear area at the beginning and after 24 h was measured using the Openlab software. The area, which was newly covered with cells, was calculated and set as 1 for the control (ctrl) dishes. A, low doses of ADAMTS1 protein have a pro-migratory effect on fibroblasts. Different concentrations of ADAMTS1 protein in medium containing 0.1% FCS were added to the cells after scratch wounding. Medium containing only 0.1% FCS was used as a control (ctrl). Statistical analysis was performed using the GraphPad Prism4 software (p = 0.0001 for comparison of control with both 1 and 2 nM ADAMTS1, according to Student’s t test). B, a catalytically inactive ADAMTS1 mutant has no effect on fibroblast migration. Experiments were performed as described in A using 1 nM of both wild-type (wt) and mutant ADAMTS1 (p = 0.0028 for comparison of control with ADAMTS1 wild type; p = 0.0008 for comparison of ADAMTS1 wild-type with mutant). C, high doses of ADAMTS1 protein inhibit the pro-migratory effect of FGF-2. Migration assays, as described in A, were performed in the presence of 1 ng/ml FGF-2 and/or 12 nM ADAMTS1 protein (wild-type or mutant) in serum-free medium (p = 0.0089 for comparison of control with FGF-2; p = 0.031 and p = 0.015 for comparison of FGF-2 with ADAMTS1 wild type and mutant, respectively). D, ADAMTS1 binds to FGF-2. Supernatants from ADAMTS1-HA transfected HEK293 cells (lane 3) or vector-transfected cells (ctrl, lane 2) were immunoprecipitated with an anti-HA antibody, and immunocomplexes were incubated with recombinant FGF-2 (50 ng) in the presence of heparin (150 ng). Binding was analyzed by Western blotting using an anti-FGF-2 antibody (upper panel). Precipitation of ADAMTS1 was confirmed by Western blotting using an anti-HA antibody (lower panel). *p < 0.05; **p < 0.01; ***p < 0.001.
speculate that the altered differentiation pattern in adams1−/− mice is at least partially because of an imbalance in growth factor signaling. In addition, it is also possible that ADAMTS1 can act directly on growth factors or cytokines that are involved in the regulation of keratinocyte differentiation. Consistent with a potential role in differentiation, ADAMTS1 expression was induced in cultured keratinocytes by TGF-β, a factor that inhibits keratinocyte proliferation (44) but not by the mitogens keratinocyte growth factor and EGF or by pro-inflammatory cytokines.

The high expression of ADAMTS1 in fibroblasts of 5-day wounds tempted us to speculate on, as yet, unknown effects of ADAMTS1 on this cell type. At this stage of wound healing, fibroblasts migrate into the provisional matrix of the clot/granulation tissue. This process requires extensive ECM turnover and also cell surface proteolysis, because contacts to the surrounding matrix have to be formed and rebroken continuously. In vitro migration assays indeed showed the pro-migratory effect of ADAMTS1 on primary mouse fibroblasts. This effect was dependent on the catalytic activity of ADAMTS1. Surprisingly, the pro-migratory action was limited to a very narrow concentration range. This could be explained by the fact that cultured fibroblasts already express rather high levels of ADAMTS1 (Fig. 4). Thus, there might be only a small window where additional recombinant protein can still increase the positive effect. In this scenario, higher concentrations of ADAMTS1 would lead to excessive proteolysis, for example, in the degradation of proteoglycans that serve as growth factor co-receptors (as discussed in the previous paragraph), which is no longer advantageous to the cells. Alternatively, it seemed possible that ADAMTS1 is able to directly bind growth factors that enhance fibroblast migration, similar to the already reported interaction with VEGF (15), a potent endothelial cell growth factor. In this case, higher concentrations of ADAMTS1 would inhibit the function of these factors, thereby overriding its own pro-migratory effect. Here we show that ADAMTS1 can indeed bind to FGF-2, a major mitogen for fibroblasts. Furthermore, higher concentrations of this enzyme interfered with the effect of exogenous FGF-2 on fibroblast migration. This high dosage effect of ADAMTS-1 was independent of its proteolytic activity, suggesting that it is indeed mediated by the observed interaction of the two proteins and most likely by a subsequent inhibition of FGF-2 binding to its receptors.

Finally, ADAMTS1 might play a role in the regulation of wound angiogenesis. Here we have shown that low doses of this enzyme enhanced endothelial cell migration, similar to its action on fibroblasts, whereas higher doses had an inhibitory effect. Furthermore, ADAMTS1 has recently been shown to inhibit VEGF and FGF-2-mediated endothelial cell proliferation and angiogenesis (14, 15). Macrophages and keratinocytes are the most important source of VEGF during wound healing (3), and fibroblasts are in close proximity to the newly forming blood vessels. Therefore, the expression of ADAMTS1 in these cells might also serve as a mechanism to regulate the bioavailability of VEGF and FGF-2 and possibly also of other growth factors and their diffusion into the granulation tissue. In this context, the overexpression of ADAMTS1 in diabetic mice is particularly interesting. Besides the already mentioned excess in proteolysis, severely reduced angiogenesis is another important feature of the wound-healing defect in diabetic mice. Together with the aberrant expression of pro-angiogenic growth factors, such as VEGF (45), the up-regulation of ADAMTS1 may underlie the impaired angiogenesis and the generally reduced growth factor activity in these animals (35).

In summary, we found a dual function of ADAMTS1 in the context of fibroblast and endothelial cell migration in addition to its known anti-angiogenic role. In a process as complex as wound healing, the actual local concentrations of the proteinases, together with the differential binding to ECM and cell surface molecules, will most likely decide how these different activities work together or which effect is predominant at a certain state of the wound-healing process.

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