ADAM-9 belongs to a family of transmembrane, disintegrin-containing metalloproteinases involved in protein ectodomain shedding and cell-cell and cell-matrix interactions. The aim of this study was to analyze the expression of ADAM-9 in skin and to assess the role of this proteolytic/adhesive protein in skin physiology. In normal skin, ADAM-9 expression was detected in both the epidermis and dermis and in vitro in keratinocytes and fibroblasts. Here we report that ADAM-9 functions as a cell adhesion molecule via its disintegrin-cysteine-rich domain. Using solid phase binding assays and antibody inhibition experiments, we demonstrated that the recombinant disintegrin-cysteine-rich domain of ADAM-9 specifically interacts with the β1 integrin subunit on keratinocytes. This was corroborated by co-immunoprecipitation. In addition, engagement of integrin receptors by the disintegrin-cysteine-rich domain resulted in the disintegrin-like sequence ECD(6–8), where ADAM-15 can interact with integrins on adjacent cells as observed with their disintegrin-like sequence ECD(6–8), whereas ADAM-15 can interact with integrins on adjacent cells as observed with αvβ3 and α5β1 integrins on hematopoietic cells (9). Its activity seems to be mediated by the presence of an RGD motif in the disintegrin domain (10). However, recently, Takeda and coworkers (11) solved the crystal structure of a snake venom metalloproteinase, a homologue of mammalian ADAMs, and excluded this motif from binding activities due to its inaccessibility for protein binding.

Degradation of the extracellular matrix is a prerequisite for tissue repair but also for cell migration and for release of bound factors and bioactive peptides. Different proteases have been implicated in these processes, such as the matrix metalloproteinase (MMP),2 serine, cysteine, and aspartic protease families. In recent years, the family of proteases (a disintegrin and metalloproteinase (ADAM)) has drawn attention because the manifold proteolytic and adhesive activities of the different ADAM family members were attributed a pivotal role in physiological and pathological situations.

The ADAM family includes ~30 members of proteins containing disintegrin- and metalloprotease-like domains. Most of the family members share a common well conserved domain structure, including a prodomain, metalloprotease, disintegrin-like, cysteine-rich, EGF-like, and a short cytoplasmic domain (reviewed in Refs. 1 and 2).

Structurally, the ADAMs are most closely related to the P-III snake venom metalloproteases. However, in contrast to snake venom metalloproteases, most ADAMs possess EGF-like, transmembrane, and cytoplasmic domains. Half of the ADAM proteins are predicted to be active metalloproteinases, although the identification of specific substrates is still lacking for most of them. Various cell surface proteins are shed by ADAMs, such as IL-6 receptor, FAS-ligand, transforming growth factor-α, tumor necrosis factor-α, heparin-binding EGF, and L-selectin. The release of soluble forms of these proteins might lead to autocrine and distal paracrine effects that are usually restricted to a limited environment when cell surface-bound (1).

The cell-adhesive function of ADAM proteins has been attributed to the presence of both the disintegrin and cysteine-rich domains. These domains are involved in binding to integrins, the heterodimeric cell surface receptors involved not only in the interactions of cells with the surrounding matrix but also with neighboring cells (3). Most of the known ADAMs contain the integrin-binding amino acid sequence RGD (4) or instead ECD or DCD, which can compete with integrin-ligand interactions (4, 5). ADAM-2 and ADAM-9 bind to -2 integrin through their disintegrin-like sequence ECD (6–8), whereas ADAM-15 can interact with integrins on adjacent cells as observed with αvβ3 and α5β1 integrins on hematopoietic cells (9). Its activity seems to be mediated by the presence of an RGD motif in the disintegrin domain (10). However, recently, Takeda and coworkers (11) solved the crystal structure of a snake venom metalloproteinase, a homologue of mammalian ADAMs, and excluded this motif from binding activities due to its inaccessibility for protein binding.

Thodeti et al. (12) have also shown association of ADAM-12 to syndecan-4, leading to cellular spreading, suggesting that homology 3; ERK, extracellular signal-regulated kinase; PBS, phosphate-buffered saline; BSA, bovine serum albumin.
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additional receptors might be involved in the interaction with ADAM proteins. Recent studies suggest that the cytoplasmic domain of ADAMs may be involved in intracellular signaling leading to activation of proteolytic processes. The cytoplasmic domains of a significant number of ADAM proteins contain proline-rich SH3-ligand motifs and a consensus sequence for phosphorylation by protein kinase C that may transmit signals between the interior and exterior of the cells. In vitro binding assays have shown that ADAM-9 and ADAM-15 interact with two SH3-containing proteins, endophilin and SH3PX1, which may have a role in regulating the function of both proteases by influencing their intracellular processing, transport, and final localization (13). Similarly, PACSIN 2, another SH3-binding protein, was found to bind ADAM-13, thereby regulating its function during embryonic development (14). Further studies showed that protein kinase Cδ induces heparin-binding EGF-like growth factor shedding by binding to and phosphorylating the cytoplasmic domain of ADAM-9 (15).

ADAM-9 distribution is quite broad, and in human skin it is localized in epidermal keratinocytes, where it may be involved in the constitutive shedding of collagen XVII, thereby modulating migration of keratinocytes (16). However, it is still unclear whether ADAM-9 may also be involved in cell-cell interactions once exposed on the cell surface and, if so, which cellular receptor would be involved in these interactions. Whether its function is primarily enzymatic or adhesive is not certain. In the present work, we have analyzed the cell-adhesive function of ADAM-9 in keratinocytes and the signals elicited by these interactions.

EXPERIMENTAL PROCEDURES

Antibodies—For immunodetection analysis, goat polyclonal antibodies raised against human ADAM-9 were purchased from R&D Systems (Wiesbaden, Germany), and rabbit anti-filaggrin antibodies were from Covance (Biozol, Eching, Germany). Actin was detected using a mouse monoclonal antibody (MP Biomedicals, Irvine, CA). Anti-His tag antibodies were from Qiagen (penta-His horseradish peroxidase-conjugated mouse monoclonal antibody, Qiagen, Hilden, Germany). Detection of phosphorylated and unphosphorylated p38, ERK, and c-Jun N-terminal kinase proteins was performed using antibodies that specifically recognize the phosphorylated and unphosphorylated forms (Santa Cruz Biotechnology, Heidelberg, Germany). The mouse anti-B1 integrin antibodies used for Western blot analysis and immunofluorescence were from Biomol (Hamburg, Germany). The blocking monoclonal mouse antibody 4B4 directed against the human β1 integrin chain was obtained from Coulter Corp. (Hialeah, FL), whereas antibodies to the α integrin subunits were from Chemicon (Beta1 Integrin Partners Kit; Chemicon, Hofheim, Germany). Mouse anti-MMP-9 antibodies were from Calbiochem (Merck). Purified control IgG was purchased from Dako (Hamburg, Germany).

Cells and Cell Culture—HaCaT cells were kindly provided by N. Fusenig (German Cancer Research Center, Heidelberg, Germany). Cells were routinely cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 100 units/ml each of penicillin and streptomycin. Human epidermal keratinocytes were isolated from adult skin as previously described (17). Keratinocytes were cultured on collagen-coated dishes in FAD medium (DMEM/Ham’s F-12: 3:1; Invitrogen) containing 100 units/ml penicillin, 100 μg/ml streptomycin, 10% fetal calf serum, 5 μg/ml insulin, 1 ng/ml epidermal growth factor, 10−10 mol/liter cholera toxin, and 24 ng/ml adenine. Epidermal-dermal split skin was prepared by thermolysin treatment of human skin specimens overnight at 4°C (thermolysin bacillus type X, 1 mg/ml; Sigma). After washing twice with PBS, separated epidermis and dermis were directly processed for RNA preparation as described below.

To obtain undifferentiated/differentiated HaCaT cells, the cells were cultured for 2 weeks in low calcium medium (0.05 mM) and switched to high calcium medium (1.8 mM) for 4 days. After incubation, lysates were prepared as described below.

RNA Isolation and Reverse Transcription-PCR—Total RNA from human skin was prepared after fine mincing of the tissue using RNeasy according to the manufacturer’s instructions (QIAGEN, Hilden, Germany). Reverse transcription-PCR was performed following the manufacturer’s instructions (REVerseTaq™ ReadyMix™ PCR reaction mix; Sigma). Briefly, 1 μg of RNA was reverse transcribed using oligo(dT) as primer in a total volume of 25 μl. 5 μl of the cDNA was used to amplify specific transcripts by PCR. The following primers were used: for amplification of human ADAM-9, 5′-CTCGGGGACCTTCCGTG-3′ and 5′-ATCCATACTCGCATCTCATAAA (18); for murine ADAM-9, 5′-TCTGACCATCCAAACGTACA and 5′-GCTGTTGTG-CAGAGATTCC (19). Amplification of S26 was used for normalization (20). PCRs were performed on 1 μl of cDNA for 35 cycles (within the linear range of amplification): denaturation (94°C, 1 min), annealing (60°C, 1 min), and extension (72°C, 1 min). The products were then analyzed on 2% agarose gels in TBE.

Expression and Purification of the Disintegrin-Cysteine-rich Domain of ADAM-9—Total RNA from human dermal fibroblasts was used as template for reverse transcription. Reverse transcription followed by PCR was carried out using the primers 5′-ttttgctagttgctccctctgttt-3′ and 5′-ttttgccgccagcagctatact-3′. The amplified DNA fragments were digested and cloned into the NheI/NotI-digested expression vector pCEP-pu BM40-cHis (21). This gave rise to a fusion protein with a His6 tag placed in frame with the disintegrin-cysteine-rich coding regions of ADAM-9. After transfection of this plasmid into 293-EBNA cells by FuGENE (Roche Applied Science), the cells were subsequently selected for puromycin resistance (0.5 μg/ml). Serum-free supernatants were tested for expression of the soluble disintegrin-cysteine rich domain (DC-9-his) by SDS-PAGE on a 10% polyacrylamide gel followed by immunoblotting using antibodies specific for the His tag. For purification, supernatants were loaded on an immobilized metal affinity chromatographic column (Talon metal affinity resin; Clontech) with a flow rate of 0.5 ml/min. After washing with 5 column volumes of a buffer containing 20 mM Hepes, 100 mM NaCl, pH 8.0, and 2.5 mM imidazol, the proteins were eluted with 250 mM imidazol and dialyzed.
against PBS overnight at 4 °C. Purified proteins were stored at −80 °C.

Transient transfections of HaCaT cells were performed using Lipofectamine (Invitrogen) on 70% confluent cell monolayers. After 6 h, medium was replaced, and expression was analyzed after a further 48 h of culture. The full-length cDNA for ADAM-9 was kindly provided by C. Blobel (Hospital for Special Surgery, New York).

Cell Adhesion Assays—Semiconfluent monolayer cultures of HaCaT cells or human primary keratinocytes were detached by incubation with 0.05% EDTA after three washes with 0.02% EDTA. The cells were then washed with PBS and resuspended in Hepes buffer containing 0.5% BSA and 1 mM each of CaCl₂ and MnCl₂. Adhesion assays were performed as described before (22). Briefly, 96-well tissue culture plates were coated with recombinant DC-9-his (20 μg/ml corresponding to ~0.6 μM), His₅ peptides (0.6 μM), bovine monomeric collagen type I (40 μg/ml), and human plasma fibronectin (30 μg/ml) at 4°C overnight. BSA coating and blocking of nonspecific binding sites were performed with heat-denatured BSA (1% BSA in Ca²⁺/Mg²⁺-free PBS) for 1 h at room temperature. After washing the wells twice, cells (2 × 10⁴ cells/well) were seeded and incubated for 1 h at 37°C. For competition assays, antibodies (10 μg/ml) or peptides (0.6 μM) were added to the cell suspension before plating. Nonadherent cells were removed by washing twice with PBS. Adherent cells were fixed with 3% formaldehyde in PBS, pH 7.6, and stained with 0.5% crystal violet in 20% (v/v) methanol. The dye was released from the cells by the addition of 0.1 m sodium citrate in 50% (v/v) ethanol. The optical density of the released dye solution was determined at 595 nm. Adhesion was either expressed directly as A₅₉₅ nm, or in percentage relative to untreated controls, which were set arbitrarily as 100%. Statistical analysis was performed with Student’s t test.

Zymographic Analysis—Serum-free conditioned media were analyzed by gelatin zymography as previously described (20). To analyze whether MMP-9 is secreted as active or latent form of the enzyme, samples were activated with 1 mM 4-aminophenylmercuric acetate in substrate buffer (see below) for 1 h at 37°C and directly analyzed by gelatin zymography. Brieﬂy, media were fractionated on 10% SDS-polyacrylamide gels containing 1 mg/ml gelatin (bovine; Sigma). After electrophoresis, the gels were washed in 2.5% Triton X-100 for 30 min before overnight incubation in metalloproteinase substrate buffer (50 mM Tris-HCl, pH 8.0, 5 mM CaCl₂). Thereafter, the gels were stained with Coomassie Blue R-250, and the bands corresponding to gelatinase activities appeared white against the blue background.

Immunoprecipitation and Western Blot Analysis—Lysates were prepared by washing twice the cells in PBS and directly scraping them off on ice in radioimmuneprecipitation buffer containing the protease inhibitors aprotinin (10 μg/ml), peflubloc (0.25 mg/ml), and leupeptin (1 μg/ml). For analysis of phosphorylated proteins sodium vanadate (50 mM) was additionally included. After overnight incubation at 4 °C, lysates were clarified by centrifugation at 16,000 × g and 4°C for 20 min, and the supernatant was collected and stored at −20 °C until use. Protein concentration was determined using a commercial assay (Micro-BCA; Perbio Science, Bonn, Germany). For immunoprecipitations, equal amounts of lysates were precleared for 2 h on protein-G-Sepharose (Amersham Biosciences). After centrifugation at 1,000 × g for 10 min, precleared lysates were either applied to mouse IgG or mouse anti-human β1 integrin antibodies bound to protein-G-Sepharose (2 μg of antibodies preincubated for 2 h with protein-G-Sepharose at 4 °C) and incubated overnight at 4°C. After incubation, protein-G-Sepharose-bound proteins were washed three times with radioimmune precipitation buffer, and bound proteins were eluted in sample buffer containing 0.7 M β-mercaptoethanol. Eluted protein was further analyzed by Western blotting. For Western blotting, proteins were fractionated by SDS-PAGE on 10% polyacrylamide gels under reducing conditions and transferred onto Hybond-C Super™ (Amersham Biosciences). After blockage of nonspecific binding sites with 5% skimmed milk (w/v) in PBS containing 0.5% Tween (v/v), for analysis of phosphorylated proteins, 5 mM sodium fluoride was included, and the blots were incubated with the primary antibodies overnight at 4°C. Bound primary antibodies were detected using a horse-radish peroxidase-conjugated secondary antibody (1:2000; Dako, Hamburg, Germany) and visualized with the ECL™ system (Amersham Biosciences).

Cell Migration Assays—Cell migration assays were performed in 24-well tissue culture plates. Wells were coated with DC-9-his (20 μg/ml) and human plasma fibronectin (30 μg/ml) overnight at 4°C. BSA blockage of nonspecific binding sites was performed by a 1-h incubation with heat-denatured BSA (1% BSA in Ca²⁺/Mg²⁺-free PBS) at room temperature. HaCaT cells were treated with mitomycin-C (1.6 μg/ml) for 2 h to arrest cell growth and then washed and detached with 0.05% EDTA. After washing twice with PBS, cells were resuspended in Hepes buffer containing 0.5% BSA and 1 mM of each CaCl₂ and MnCl₂. The cells (5 × 10⁴ cells/well) were seeded in cloning rings (0.5-mm diameter) and incubated for 1 h at 37°C. After removing the cloning rings and three washes with PBS to remove unbound cells, plates were placed on a microscope stage heated to 37°C in a humidified atmosphere. For inhibition experiments, cells were incubated, after washing, either in the presence of purified mouse IgG, used as control, or mouse anti-MMP-9 antibodies (10 μg/ml). Images were collected every hour for 48 h. Areas covered by cells at these time points were calculated using CellR™ software (Olympus Biosystems, Munich, Germany).

Immunolocalization—To detect proteins in monolayer cultures, cells were cultured on tissue culture slides for 48 h and then fixed for 10 min with cold acetone. Stainings were performed, incubating the cell monolayers or tissue with the first antibodies described under “Antibodies” for 16 h at 4°C in PBS containing 2% BSA and 0.05% Tween. After extensive washes, primary bound antibodies were detected using donkey anti-goat 594, goat anti-rabbit 594, and, for the colocalization studies, rabbit anti-mouse fluorescein isothiocyanate (all diluted in PBS with BSA/Tween) for 1 h at room temperature. Nuclei were counterstained with 1 μg/ml 4′,6-diamidino-2-phenylindole (Roche Applied Science). Negative controls were performed using control IgG as primary antibodies.
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For colocalization studies, fluorescence images were recorded using a Zeiss (Thornwood, NY) Axiovert M-200 inverted epifluorescence microscope with Apotome slider confocal attachment. Images captured at *×*630 magnification were analyzed using the three-dimensional analysis imaging software of the Axiovision LE Rel. 4.5 and Adobe Photoshop version 7.0.

RESULTS

Expression of ADAM-9 in Human Skin—Analysis of protein expression by immunofluorescence showed ADAM-9 expression throughout the whole epidermis with a stronger staining in all suprabasal layers when compared with the basal layer. Expression was also observed in spindle-shaped cells (indicated by white arrows; Fig. 1a). To assess ADAM-9 mRNA expression in human skin, we performed reverse transcription-PCR analysis of RNA preparations from thermolysin-dissociated epidermis and dermis and from total skin (Fig. 1b). ADAM-9 transcripts were detected in both epidermis and dermis with a significantly higher expression in the epidermis. In agreement with the described expression pattern, specific ADAM-9 transcripts were detected by Northern blot analysis of total RNA preparations in cultured keratinocytes, endothelial cells, and fibroblasts but not in melanocytes (data not shown).

To confirm the protein expression pattern observed in the epidermis, we analyzed cellular extracts from undifferentiated (low Ca\(^{2+}\)) or differentiated (switched to high Ca\(^{2+}\)) HaCaT cells. The HaCaT cells are spontaneously immortalized keratinocytes, which closely resemble normal keratinocytes in their growth and differentiation characteristics. This has made the HaCaT cell line a widely used model of normal human keratinocytes (17, 18).

Cells undergoing differentiation, as indicated by the expression of the late differentiation marker filaggrin (25), produced increased amounts of ADAM-9 protein. Both pro (115 kDa) and active forms (80 kDa) of ADAM-9 were detected by Western blot analysis of total cell lysates (Fig. 1c). By immunofluorescence staining of monolayer cultures, an increase in expression throughout the whole epidermis with a stronger expression by immunofluorescence showed ADAM-9 expression in human skin. (Fig. 1d). ADAM-9 expression was observed upon differentiation and paralleled the enhanced in filaggrin staining induced by the culture conditions (Fig. 1d). These data corroborate the expression pattern observed in human skin (Fig. 1a).

Recombinant Production of the Disintegrin-Cysteine-rich Domain of ADAM-9 and Analysis of Its Interaction with Epidermal Cells—The proteolytic functions of ADAM-9 have been analyzed in several studies (26, 27). However, its binding activity and putative functional role as an adhesive cell receptor in human skin has not been investigated. Therefore, to elucidate whether ADAM-9 possesses an adhesive function on keratinocytes and to identify ADAM-9 ligands, we produced a recombinant protein consisting of the disintegrin-like cysteine-rich domain of ADAM-9 fused to a His\(_6\) tag in a eukaryotic expression system. The protein was purified from serum-free supernatants of stably transfected 293-EBNA cells using immobilized metal affinity chromatography.

The purified recombinant disintegrin-like cysteine-rich His-tagged domain of ADAM-9 (DC-9-his) was secreted as a 35-kDa protein as shown by SDS-PAGE and subsequent Coomassie staining and by immunoblotting with anti-His antibodies (Fig. 2). The identity of the protein was further confirmed by peptide mass fingerprint analysis of tryptic fragments of the recombinant protein (data not shown); the lower band was identified as a smaller degradation product of the disintegrin-cysteine-rich domain.

To examine whether the recombinant DC-9-his could support cell adhesion of human keratinocytes, we performed cell adhesion assays with immobilized recombinant protein. HaCaT cells specifically adhered to the DC-9-his domain but very poorly to histidine peptides and BSA used as negative controls (Fig. 3A). The adhesion observed to DC-9-his was 60% of that measured on fibronectin and 35% of that measured on collagen type I, both used as positive controls. At low coating concentrations of DC-9-his, cells appeared rounded and dis-
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played small filopodia projecting out of the cells. With increasing coating concentrations, HaCaT spread on the immobilized ligand (Fig. 3B). An adhesion pattern comparable with that of DC-9-his was observed using human primary keratinocytes (data not shown).

Interaction of Epidermal Cells with the Recombinant Disintegrin-Cysteine-rich Domain Requires β1 Integrin Receptors—To identify the integrin subunits potentially responsible for cell adhesion to DC-9-his, inhibitory anti-integrin antibodies were used to analyze inhibition of keratinocyte adhesion to the DC-9-his protein. Antibodies directed against the β1 but not the β3 integrin subunit efficiently inhibited adhesion by 60% (Fig. 4A). Furthermore, antibodies against the α3 subunit reduced adhesion by 70%, whereas inhibitory antibodies directed against the α2, α5, and α6 subunits had no significant effect when compared with the IgG control antibodies. Interaction of integrin receptors or disintegrins with their substrates is mediated by RGD-containing amino acid sequences. In the case of ADAMs that do not contain such a motif (e.g. ADAM-9), the ECD sequence takes over the activity of the RGD amino acid sequence (28). Both RGD and ECD cyclic peptides as well as their respective control peptides containing the RAD and ECD motifs did not inhibit adhesion to immobilized DC-9-his (Fig. 4A). The association of the endogenous β1 integrin receptor subunit with ADAM-9 protein was further corroborated by co-immunoprecipitation studies.

Immunoprecipitation of β1 integrin from HaCaT cell lysates led to co-precipitation of ADAM-9, thus suggesting a direct interaction between these two proteins (Fig. 4B). This observation was further substantiated by immunolocalization of ADAM-9 and β1 integrin in cell monolayers, where both proteins co-localized at sites of cell-cell contacts, as indicated by the white arrow and shown in the detail of the optical section, whereas in cellular protrusions, only β1 integrin was detected (Fig. 4C).

The Recombinant Disintegrin-Cysteine-rich Domain Induces Migration of Keratinocytes—One of the cellular processes resulting from integrin receptor engagement by extracellular matrices is migration. In keratinocytes, this event is of particular importance during physiological wound repair, where different integrins are expressed at specific time points to facilitate wound re-epithelialization (29). To analyze whether the adhesive domain of ADAM-9 influences cellular migration, HaCaT cells were plated on plastic or on DC-9-his-coated surfaces, and their migration was monitored every hour by time lapse video microscopy (Fig. 5). The mean migration areas of HaCaT cells on plastic were 0.07 and 0.1 mm² at 24 and 48 h, respectively. In contrast, plating HaCaT cells on DC-9-his resulted in increased cellular migration of 0.2 and 0.33 mm² at 24 and 48 h, respectively (p < 0.0001).

Interaction of Cells with DC-9-his Leads to Changes in Cell Signaling—We have previously shown that activation of αβ1 integrin receptors on cells by anti-integrin activating antibodies or by snake venom metalloproteinases (whose human homologues are ADAM proteins) is followed by enhanced MMP activities (22, 24). To investigate the role of the adhesive property of ADAM-9 in inducing cellular signaling leading to alteration of proteolytic activities, either directly or indirectly by engagement of integrin receptors, we stimulated HaCaT cells with the soluble recombinant DC-9-his domain. The addition of DC-9-his to HaCaT cells resulted in a dose-dependent increase of pro-MMP-9 secretion (Fig. 6A), whereas synthesis of other MMPs was unchanged (data not shown). Interestingly, transient transfection of full-length ADAM-9 cDNA in HaCaT cells leading to increased ADAM-9 mRNA levels was also associated with increased MMP-9 secretion (Fig. 6B).
Analysis of the intracellular signaling events that are activated upon HaCaT interaction with the DC-9-his domain indicates that activation/phosphorylation of ERK1/2 (5 min after stimulation) but not of c-Jun N-terminal kinase or p38 is involved in the regulation of MMP-9 expression, whereas no phosphorylation events were elicited by stimulation with histidine control peptides (Fig. 7A). In addition, a significant inhibition of MMP-9 secretion was observed only upon inhibition of ERK activation by PD98059 but not by other inhibitors, including the p38 inhibitor SB203580, the phosphatidylinositol 3-kinase inhibitor wortmannin, and the tyrosine kinase inhibitor genistein (Fig. 7B). No inducing effects were observed upon cell stimulation with either histidine peptides or Me2SO, which was used as solvent for both the SB203580 and PD98059 inhibitors.

To explore whether the increase in cell migration on DC-9-his is dependent on MMP-9 secretion induced by this ADAM-9 domain, we performed migration experiments with HaCaT cells on immobilized DC-9-his in the absence or presence of anti-MMP-9 neutralizing antibodies. As shown in Fig. 8, migration of HaCaT cells on DC-9-his was inhibited in the presence of anti-MMP-9 antibodies at 24 and 48 h, thus suggesting that MMP-9 secretion induced by cell interaction with DC-9-his contributes to the increased migration capacity of keratinocytes on immobilized DC-9-his.

**DISCUSSION**

ADAMs present on the cell surface have emerged as key regulators of numerous cellular processes. They are localized at the cell surface or are themselves integral membrane proteins, which after activation can act in close proximity to the cell membrane. They influence cell-cell interactions by shedding ligands and receptors involved in cell-cell contact and signaling but also modulate cell-matrix interactions by directly cleaving and remodeling ECM proteins and by interacting with matrix adhesion molecules.

In skin, we found ADAM-9 protein and transcripts in both the epidermal and dermal compartments, and its function in this tissue has been proposed to be the constitutive shedding of collagen XVII, which in turn may modulate keratinocyte migration (16). However, whereas proteolytic and adhesive functions of ADAM-9 in cancer have been investigated in many studies (30), little is known about the adhesive function in skin physiology.

In this report, we could demonstrate that keratinocytes interact with the recombinant disintegrin-cysteine-rich domain of ADAM-9. Adhesion required the presence of Mn2+ but not Ca2+ ions, suggestive of an integrin-mediated ion-dependent interaction (data not shown). In addition, this interaction was found to be dependent on the β1 integrin receptor subunit but not on the ECD motif, present in the disintegrin domain of ADAM-9. In contrast, Nath et al. (7) as well as Mazzocca et al. (31) have shown that interaction of the ADAM-9 recombinant ectodomain with fibrosarcoma and liver stromal cells could be efficiently inhibited by ECD-containing peptides.

However, a recent analysis of the crystal structure of VAP (vascular apoptosis-inducing protein-1), a snake venom homologue of mammalian ADAMs, identified the high variable region of the cysteine-rich domain, present in this protein, as the responsible region for substrate interaction but not the disintegrin loop, which is packed and inaccessible for protein binding (11). This latter finding would agree with the lack of inhibition of DC-9-his-mediated cell adhesion by ECD peptides we observed in our experimental system.

Two main integrin receptors have been shown to interact with the ADAM-9 ectodomain, namely α6β1 and α2β1 (7, 31). In human skin and in vitro, keratinocytes express mainly α2 and
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α3 integrin subunits. Additionally, in vitro keratinocytes also express the α3 integrin subunit in association with the β1 subunit, and the expression profile is influenced by the cellular differentiation status (32). Whereas expression of α2 and α6 integrins is limited to the basal layer, α3 integrin is expressed at low levels also in cells of the suprabasal layers. Furthermore, differentiation of keratinocytes by Ca2+ switch leads to general reduced expression of α3 but more importantly, to relocation of the α3 subunit from the intracellular pool to the cell membrane (33).

We could show that binding of keratinocytes to the DC-9-his domain was primarily mediated by the α3β1 integrin receptor, thereby suggesting that interaction of integrin receptors with ADAM-9 depends on the integrin repertoire expressed by different cell types. This is, for instance, the case for ADAM-12, where the main co-receptor in myogenic cells is represented by α9β1 (34); however, carcinoma cells that do not express this integrin can utilize other receptors of the β1 integrin family (12).

We also demonstrate a direct interaction between the β1 subunit and ADAM-9, but we failed to co-precipitate the α subunit (data not shown). One possibility to explain this finding is that an additional unidentified receptor located in the close vicinity of either the ADAM-9 or the integrin receptor might contribute to ADAM-9 binding. A similar hypothesis has been formulated for ADAM-10, whose association with heparin-binding EGF is followed by interaction with CD9, known to complex with the integrin α3β1 (35, 36). Interaction between ADAM-9 and β1 integrin might similarly involve an additional unknown interacting partner, an interesting question that will be explored in future work.

The reports dealing with the functional interplay between ADAMs and integrins indicate that ADAMs can modulate, by inhibiting or supporting, cell migration mediated by integrins independently of their metalloprotease activity (37). These interactions may play an important role in promoting cell migration in physiological processes, such as during embryogenesis, as well as in cancer cell invasion. In the case of ADAM-9, Nath et al. (7) have shown in fibrosarcoma cells not only that the recombinant soluble ectodomain of ADAM-9 binds to α6β1 but that this interaction leads to enhanced migration. Furthermore, an alternatively spliced form of ADAM-9, lacking the transmembrane and cytoplasmic domains, is secreted by hepatic stromal cells and binds to α6β4 and α2β1 integrins on colon carcinoma cells, thereby inducing an invasive phenotype (31).

We also found that adhesion of keratinocytes to the disintegrin-cys-
tein-rich domain of ADAM-9 leads to increased cell migration. In vivo migration of keratinocytes is important during re-epithelialization of epithelial wounds, starting as early as 2 days after injury and continuing until the wound bed is completely covered (38). This process is known to be accompanied by cellular reprogramming, leading to an altered integrin expression pattern as well as to an increased secretion of proteolytic enzymes (39, 40). For instance, pericellular proteolysis is thought to be essential for the detachment of keratinocytes from the basement membrane and for their migration into the wound bed (40). Increased expression of ADAM-9 transcript levels, which we have observed at the early time points of our wound healing study, might indeed be required not only for establishing cellular contacts to increase migration but also for the induction of proteolytic enzymes, such as MMP-9, which was found to be induced in the same phase of wound healing studies in mouse.3

We have previously shown that jararhagin, a snake venom proteinase homologue of human ADAM proteins, can function as an agonist of collagen type I in fibroblast by inducing cellular signaling, leading to an up-regulation of MMP expression (24). However, the generation of cellular signals, leading to up-regulation of promigratory activities through the interaction of ADAM-9 and β1 integrin, has not yet been investigated. In this report, we could show that the adhesive domains of ADAM-9 indeed induce cellular signaling, leading to modulation of proteolytic enzymes.

Stimulation of HaCaT cells with soluble DC-9-his enhanced MMP-9 secretion, which we also observed upon transient over-expression of the full-length ADAM-9 cDNA. In addition, this interaction involves phosphorylation of ERK kinases.

Importantly, Holvoet and co-workers (41) have shown that in HaCaT cells, induction of MMP-9 by tumor necrosis factor requires the mitogen-activated protein kinase pathway. However, in our studies, induction of MMP-9 by the DC-9-his domain did not occur via release of cytokines/growth factors, since we could not detect changes in these factors (e.g. tumor necrosis factor-α) (data not shown). However, activation of integrins has also been shown to induce MMP-9 synthesis. Using antibodies raised against the β1 and α3 integrin subunits, expression of MMP-9 in cultured keratinocytes was induced in a dose-dependent manner (42). Thus, we speculate that upon binding of ADAM-9 to the integrin receptor, phosphorylation events take place, leading to up-regulation of MMP-9, which in turn contributes to the increased migratory activity.

Indeed, we could demonstrate partial inhibition of migration on DC-9-his by incubation of the cells with anti-MMP-9 neutralizing antibodies. This observation indicates that MMP-9 is directly involved in the change of the migratory capacity of keratinocytes in response to the disintegrin-cysteine-rich domain of ADAM-9. Future studies will be necessary to examine the role of ADAM-9 in vivo and the functional importance of its adhesive domains not only for modulation of protease expression but also for modulation of cell-cell interactions.

3 P. Zigrino, unpublished data.
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