Cysteine-Rich Domain of Human ADAM 12 (Meltrin α) Supports Tumor Cell Adhesion

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The ADAMs (A disintegrin and metalloprotease) comprise a family of membrane-anchored cell surface proteins with a putative role in cell-cell and/or cell-matrix interactions. By immunostaining, ADAM 12 (meltrin α) was up-regulated in several human carcinomas and could be detected along the tumor cell membranes. Because of this intriguing staining pattern, we investigated whether human ADAM 12 supports tumor cell adhesion. Using an *in vitro* assay using recombinant polypeptides expressed in *Escherichia coli*, we examined the ability of individual domains of human ADAM 12 and ADAM 15 to support tumor cell adhesion. We found that the disintegrin-like domain of human ADAM 15 supported adhesion of $\alpha v \beta 3$ -expressing A375 melanoma cells. In the case of human ADAM 12, however, recombinant polypeptides of the cysteine-rich domain but not the disintegrin-like domain supported cell adhesion of a panel of carcinoma cell lines. On attachment to recombinant polypeptides from the cysteine-rich domain of human ADAM 12, most tumor cell lines, such as MDA-MB-231 breast carcinoma cells, were rounded and associated with numerous actin-containing filopodia and used a cell surface heparan sulfate proteoglycan to attach. Finally, we demonstrated that authentic full-length human ADAM 12 could bind to heparin Sepharose. Together these results suggest a novel role of the cysteine-rich domain of ADAM 12 — that of supporting tumor cell adhesion. (Am J Pathol 1999, 154:1489–1501)

Cell-cell and cell-matrix interactions are known to critically determine cell adhesion, cell migration, cell survival, and proliferation, processes that play key roles in cancer invasion and metastasis.^{1–5} Several major classes of membrane-bound cell adhesion molecules have been described: immunoglobulin superfamily members, cadherins, integrins, selectins, and cell-surface proteoglycans. Recently the ADAMs (A disintegrin and metalloprotease), a large family of type-I membrane-bound cell

surface proteins, have been added to the list.^{6–8} The ADAMs have been referred to by other names, including cellular disintegrins and MDCs (for metalloprotease/disintegrin/cysteine).

The ADAMs have a unique domain organization, including metalloprotease, disintegrin-like, cysteine-rich, transmembrane, and cytoplasmic domains. The closest homologues of ADAMs are the highly toxic snake venom metalloproteases (SVMPs). Both SVMPs and ADAMs are members of the repolysin/adamalysin subfamily of zinc-dependent metalloproteases.^{9–11} The SVMPs are known to degrade basement membrane components, including type-IV collagen, laminin, and fibronectin, thereby leading to hemorrhage in the tissue.^{12–14} Some SVMP disintegrin domains contain an arginine-glycine-aspartic acid (RGD) integrin ligand sequence in a β -loop structure that binds with high affinity to $\alpha v \beta 3$ and $\alpha IIb \beta 3$ integrins and can inhibit the function of platelet integrin $\alpha IIb \beta 3$.^{15–17} Interestingly, such disintegrins are 3000 to 30,000 times more active than small RGD-containing peptides in inhibiting adenosine diphosphate (ADP)-induced platelet aggregation.¹⁸ It has been suggested that SVMP disintegrin domains can disrupt other types of integrin-mediated cell-cell and cell-matrix interactions; in fact there is evidence that eristostatin can reduce the number of lung metastases following tail vein injection of B16F10 melanoma cells and in the liver following injection via mesenteric veins.^{19,20}

Their intriguing composition and homology to the SVMPs suggest that the ADAMs could be active in cell adhesion and proteolysis in a wide variety of biological processes. Although at least 23 ADAMs have been cloned so far, the tissue distribution pattern and function of some of these proteins are only beginning to be unraveled.^{7,8} Several ADAMs such as fertilin α/β and cyritestin have been implicated in fertilization and/or spermatogenesis, in fact targeted disruption of the fertilin β in the mouse results in impaired fertilization.^{7,21,22} There is ample evidence that the ADAMs on the sperm bind to an integrin on the egg plasma membrane, leading to cell fusion.²³ Interest in the ADAM proteins mounted when it was shown that ADAM 10 and 17 (TACE) can process

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tumor necrosis factor- α ²⁴⁻²⁷ and that the *Drosophila* ADAM KUZ is involved in the processing of Notch.²⁸ Of additional interest is the proposed role for ADAMs in proteolyzing insulin-like growth factor binding proteins.²⁹ These results point to an important role of the ADAM proteins in ectodomain shedding.⁸

We have recently cloned and begun to characterize human ADAM 12.^{30,31} We found that in addition to the expected membrane-anchored form, designated ADAM 12-L, an alternatively spliced, secreted form exists, designated ADAM 12-S. Furthermore, we demonstrated that a minigene of *adam 12-S*, encoding the disintegrin-like, the cysteine-rich, and the unique carboxy-terminus, provoked myogenesis in a nude mouse-model system. Our results and those obtained by Yagami-Hiromasa et al³² using the mouse C2C12 myoblast model system point to a role of ADAM 12 in cell-cell interactions and differentiation.

In the present study we show by immunostaining and reverse transcriptase-polymerase chain reaction (RT-PCR) that ADAM 12 is up-regulated in human carcinoma specimens and that ADAM 12 appears to be located at the tumor cell surfaces. This led us to hypothesize that ADAM 12 is involved in cellular interactions in cancer, and we explored the interaction between ADAM 12 and the cell surface of several cultured tumor cell lines. We found that a recombinant polypeptide from the cysteine-rich domain of human ADAM 12 expressed in *Escherichia coli* supports cell adhesion by engaging a cell surface heparan sulfate proteoglycan receptor.

Materials and Methods

Tissue Samples and Cell Lines

Tissue specimens from 37 histologically confirmed cases of human carcinomas comprised 15 infiltrating ductal breast carcinoma, 14 adenocarcinoma of the colon and rectum, four squamous cell carcinoma of the lung, and four adenocarcinoma of the stomach. Adjacent nontumorous tissue, including 10 samples of normal breast tissue of which nine corresponded to samples from patients with carcinoma, were also investigated. Tissue samples were either snap-frozen in liquid nitrogen and stored at -80°C or were fixed in 96% ethanol/glacial acetic acid (99:1 v/v) overnight, embedded in paraffin, and stored at 4°C .³³ Tissue samples were obtained from the Department of Surgical Pathology, University of Copenhagen and Nykøbing Falster Hospital, Denmark.

The following 11 human tumor cell lines were used: MDA-MB-231 breast carcinoma (HTB 26), MDA-MB-435 breast carcinoma,³⁴ MDA-MB-468 breast carcinoma (HTB 132), MCF-7 breast carcinoma (HTB 22), RKO colon carcinoma,³⁵ Clone A colon carcinoma,³⁶ A431 squamous cell carcinoma (CRL 1555), A375 melanoma (CRL 1619), SK-MEL-28 melanoma (ATCC 72), HT1080 fibrosarcoma (CCL 121), and A204 rhabdomyosarcoma (HTB 82). The MDA-MB-435, RKO, and Clone A cells were obtained from Dr. A. M. Mercurio, Harvard Medical School (Boston, MA) and the remainder from the American Type Culture Collection (Rockville, MD). The cells

were grown in Dulbecco's modified Eagle's medium (DMEM) with Glutamax I and 4500 mg/ml glucose, 50 U/ml penicillin, 50 $\mu\text{g/ml}$ streptomycin, and 10% fetal bovine serum (Gibco-BRL, Grand Island, NY) at 37°C in 5% CO_2 in air and serially passaged using trypsin/EDTA.

Antibodies

A number of monoclonal and polyclonal antibodies to human ADAM 12 were used.³⁰ Rabbit polyclonal antisera included rb104, rb950, R20, R21, R23, M11. Rat monoclonal antibodies included the 14E3 hybridoma and a newly developed hybridoma, 16E8. The antibodies were raised against recombinant cysteine-rich domain of human ADAM 12 (aa 564-708; p1053, see below) and characterized as described.³⁰ The antibodies react in immunostaining and on Western blots with COS-7 cells transiently transfected with an ADAM 12 expression construct (p1095), but not with COS-7 cells transfected with a control vector³⁰ (not shown).

The integrin function-blocking monoclonal antibody A1B2 developed by C. H. Damsky was obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa, Department of Biological Sciences (Iowa City, IA). The integrin $\alpha 6$ function-blocking 2B7 monoclonal antibody³⁶ was a kind gift from Dr. A. M. Mercurio, Harvard Medical School. The IgGs were purified using Protein G-Sepharose as described by the manufacturer (Amersham-Pharmacia, St. Louis, MO). Mouse monoclonal antibodies against β -actin (A-5441, Sigma-Biotech, Horsholm, Denmark) were also used. Fluorescein- and mouse rhodamine-conjugated antibodies against rabbit, rat, and mouse immunoglobulins were purchased from DAKO (Glostrup, Denmark).

Immunohistochemistry on Tissue Sections

For immunostaining on fixed, paraffin-embedded sections, the indirect immunoperoxidase staining technique was used as described.³³ Briefly, sections were deparaffinized, and endogenous peroxidase activity was blocked with 10% hydrogen peroxide in methanol for 10 minutes at room temperature. Some sections were subsequently pretreated with pronase (10 $\mu\text{g/ml}$ in buffer for 5 minutes) and rinsed. The primary antisera were applied and incubated with the sections overnight at 4°C in a humidified chamber. Following a thorough rinse, the sections were incubated with peroxidase-coupled swine anti-rabbit, rabbit anti-mouse, or rabbit anti-rat immunoglobulins. Incubations with both primary and secondary antibodies were performed in 0.05 mol/L Tris-HCl (pH 7.2) and rinses in 0.05 mol/L Tris-HCl (pH 7.2) containing 0.15 mol/L NaCl. The primary antibodies were used in the following dilutions: rb 104 and rb 950 1:200; R20, R21, and R23 1:100. On control sections, the specific antibodies were omitted or replaced with irrelevant mouse or rat monoclonal antibodies of the same isotype or with non-immune mouse, rat, or rabbit serum. As a further control, the inhibitory effect of simultaneously incubating the antisera with purified recombinant ADAM 12 polypeptide

was examined. To this end, sections were incubated with serial dilutions of the antisera together with 25 $\mu\text{g/ml}$ of recombinant ADAM 12 or another irrelevant recombinant protein (a protein of same M_r and purified in the same way). This experiment was repeated three times on different tissue samples with the same results. The slides were mounted in buffered glycerol and examined under a Zeiss LSM-10 laser scan confocal microscope.

Immunostaining on frozen sections were performed essentially as above with the following modifications. Cryostat sections were air-dried and fixed in precooled acetone at 4°C for 15 minutes. Each of the respective antibodies (diluted 1:100) were applied to the sections and incubated for 1 hour. Following a thorough rinse, the sections were incubated with fluorescein isothiocyanate-coupled secondary antibodies 1:50 for 30 minutes.

Recombinant Proteins

A series of plasmids for the expression of His-tagged ADAM 12 polypeptides in *E. coli* were constructed. Plasmid p1053, which codes for subdomain b' and c of the cysteine-rich domain of human ADAM 12 and the first four amino acids of carboxy terminus of ADAM 12-S (aa 564–708 of ADAM 12-S) has been described previously.³⁰ Inserts for the other ADAM 12 plasmids were amplified from an ADAM 12 cDNA template with Pfu DNA polymerase (Stratagene), using the following primers:

Plasmid	Primers	ADAM 12	Domain
p1048	5'-dCAGGATCCAGAGAGA- CCCTCAAG 5'-dCAACTCGAGCGGCA- GGTTAAACAG	aa 207–416	metallo- protease
p1219	5'-dCAGGATCCTCATGTC- AGGATGTG 5'-dCAACTCGAGTGCTTG- CCGGATGGG	aa 513–704	cys a+b'+c
p1222	5'-dCAGGATCCGGCAAA- GTCTCGAAG 5'-dCAACTCGAGTGCTTG- CCGGATGGG	aa 563–704	cys b'+c
p1053	<i>Bam</i> HI/ <i>Xho</i> I fragment of ADAM 12-S	aa 564–708	cys b'+c
p1347	5'-dCAGGATCCGAAGTCA- GGGAGTCT 5'-dCAACTCGAGGTGCCC- ATCGTGCAG	aa 417–512	disintegrin

Inserts for the ADAM 15 plasmids were amplified from a human fetal skeletal muscle RT-reaction with Pfu DNA polymerase (Stratagene, La Jolla, CA), using the following primers:

Plasmid	Primers	ADAM 15	Domain
p1345	5'-dCAGGATCCATGGCT- GCTTTCTGCGG 5'-dCAACTCGAGCTCGC- CATCCCCTAGGCTG	aa 420–510	disintegrin
p1346	5'-dCAGGATCCGGGCGC- AACCCAGTGG 5'-dCAACTCGAGGGAGC- TGTTGCTTTGA	aa 561–693	cys b'+c

All plasmids except p1053 were constructed by digesting the appropriate PCR product with *Bam*HI and *Xho*I and then cloning it into the *Bam*HI/*Sal*I sites of pQE30 (Qiagen GmbH, Hilden, Germany). Plasmid p1048 contains a 604-bp PCR fragment that codes for the metalloprotease domain of human ADAM 12 (aa 207–416). Plasmid p1219 contains the full-length cysteine-rich domain of ADAM 12 (aa 513–704), which can be subdivided into regions a, b', and c;³⁷ p1222 contains the b'+c regions (aa 563–704) and is almost identical to plasmid p1053 (aa 564–708). The b'+c region of human ADAM 15 cysteine-rich domain is contained in p1346 (aa 561–693). Plasmid 1347 contains the disintegrin-like domain of ADAM 12 (aa 417–512), whereas p1345 contains the disintegrin-like domain of ADAM 15 (aa 420–510).

The cysteine-rich domain polypeptides (p1053, p1219, p1222, and p1346) and the disintegrin-like domain polypeptides (p1345 and p1347) were expressed in *E. coli* and purified by metal affinity resin chromatography as previously described.³⁰ The metalloprotease domain polypeptide (p1048) precipitated when purified by this protocol, so an alternative method of purification was used. *E. coli* cells expressing the p1048 polypeptide were lysed in 0.02 mol/L Tris-HCl, pH 7.9, 0.5 mol/L NaCl, 6 mol/L guanidine HCl, and bound to the Talon metal affinity resin (Clontech, Palo Alto, CA), which was washed using the same buffer. Bound material was eluted with the same buffer supplemented with 0.05 mol/L EDTA. The His-tag purified protein was refolded by dialyzing for 24 hours at 4°C against 6 mol/L urea, 0.05 mol/L Tris-HCl (pH 8.0), 2 mmol/L reduced glutathione, 0.02 mmol/L oxidized glutathione, 0.005% Tween 80, and 3 $\mu\text{mol/L}$ ZnCl₂. Dialysis was continued for another 24 hours in the same buffer with 1 mol/L urea and finally for a third 24-hour period in 0.02 mol/L Tris-HCl (pH 8.0), 0.15 mol/L NaCl, 0.005% Tween 80, 1 $\mu\text{mol/L}$ ZnCl₂. The purified recombinant proteins were quantitated using the Bio-Rad (Hercules, CA) #500–0006 protein assay system using IgG as a standard.

RT-PCR

Total RNA was extracted from tissue specimens and cultured cells using the TRIzol reagent (Gibco-BRL). For RT-PCR, 5 μg of total RNA was used to synthesize cDNA using murine Moloney leukemia virus (MMLV) reverse transcriptase as recommended by the manufacturer (Stratagene). Aliquots of cDNA equivalent to 125 ng of total RNA were amplified using primers that flanked a known intron at the point of divergence between ADAM 12-L and ADAM 12-S (GenBank numbers AF023476 and AF023477) and also with primers specific for laminin α 2 chain (GenBank number Z26653).

Specific for	Primers	Nucleotides amplified
ADAM 12-L	5'-dTGCAGGCAGAGGCTTCTGAGG 5'-dGTTTGGCTTTGGAGGAAGCACAG	2276–2792
ADAM 12-S	5'-dCCATCTAGATCAGATGAGTGTCA- GTGA	2131–2523

Specific for	Primers	Nucleotides amplified
	5'-dCAGAATTCAAGGAGGCCGGATT-CTGTG	
Laminin $\alpha 2$	5'-dGAAGCTACAAACTGGCAACA-GGT	5900-6418
	5'-dTAGGTTATCCTCAAGTTCCTTGAT	

After an initial denaturation at 95°C for 60 seconds, 35 cycles of denaturation at 94°C for 15 seconds, annealing at 60°C (for ADAM 12-L) or 55°C (for ADAM 12-S and laminin $\alpha 2$) for 20 seconds, and extension at 72°C for 40 seconds were carried out. The ADAM 12 reaction products were separated on agarose gels, blotted onto Hybond N+ nylon membranes, and hybridized with [³²P]dCTP random primed labeled probes specific for ADAM 12-S or ADAM 12-L according to standard procedures.³⁸ Blots were exposed to Kodak X-Omat AR film for up to 2 hours. To further confirm the identity of the PCR products, the amplified DNA fragments were cloned into the vector pCR 2.1 using the TA cloning kit (Invitrogen, Groningen, The Netherlands), and sequenced with vector primers using the Vistra DNA sequencer 725 (Amersham Pharmacia Biotech).

Cell Attachment Assays

Cells were released from the tissue culture flasks with 10 mmol/L EDTA or with trypsin/EDTA. When trypsin/EDTA was used, cells were incubated at 37°C for 5 minutes in DMEM with 10% fetal bovine serum to allow regeneration of cell surface proteins before rinsing. Both EDTA- and trypsin-released cells were rinsed once in serum-free DMEM and resuspended in the same medium at the concentrations indicated. Cell viability was checked by nigrosin dye exclusion test. The recombinant polypeptides of human ADAM 12 (see above) and for comparison, laminin-1 purified from the Engelbreth-Holm-Swarm (EHS) tumor and human laminin (Gibco-BRL) were used as substrates. An irrelevant recombinant polypeptide of the same M_r and purified in the same way and bovine serum albumin (10 mg/ml) served as negative controls. Nunc-Immuno™ 96-well plates with MaxiSorp™ surface (Nunc) were coated with purified recombinant ADAM 12 polypeptides in 0.1 mol/L NaHCO₃ buffer overnight at 4°C, rinsed with PBS, and incubated with 10 mg/ml bovine serum albumin in PBS for 1 hour at 37°C. Following a rinse with PBS, 100 μ l of cell suspension (0.6×10^6 /ml) was added to the wells, which were then incubated at 37°C in 5% CO₂ in a humidified atmosphere. After allowing the cells to attach for 1 hour, the wells were rinsed twice in serum-free DMEM, fixed for 20 minutes in 2% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.2, rinsed in PBS, and stained with 0.1% crystalviolet in 10% methanol (v/v). Absorbance was measured with a Multiscan enzyme-linked immunosorbent assay reader (Lab-systems, Helsinki, Finland) at 590 nm. A blank value corresponding to an empty well was automatically subtracted. Each assay point was derived from 3 to 6 separate wells and repeated at least two times. To obtain a

100% maximum attachment control, cells were plated in parallel on culture quality 96 well-plates (Costar, Cambridge, MA) in DMEM with 10% fetal bovine serum for 1 hour. To test for adhesion strength the plates were centrifuged in an inverted position at 500 rpm ($60 \times g$) for 2 minutes (PR-6000 centrifuge, Damon/IEC), and the attachment of cells was compared with that of cells in plates that were not subjected to this centrifugal force. To examine the morphology of tumor cells attached, an inverted microscope (Zeiss axiovert) equipped with phase contrast optics was used.

The following experimental conditions were tested: 1) 10 mmol/L EDTA was added to the incubation medium; 2) the incubation temperature was lowered to 4°C; 3) the cells were pretreated for 1 hour with the combination of 50 mmol/L 2-deoxy-D-glucose plus 10 mmol/L sodium azide to inhibit energy production;³⁹ 4) the cells were pretreated with cytochalasin B dissolved in dimethyl sulfoxide to inhibit actin polymerization and as a control with dimethyl sulfoxide at the same final concentration; 5) the cells were preincubated for 15 minutes at room temperature with function-blocking monoclonal antibodies to integrin $\alpha 6$ (2B7) or integrin $\beta 1$ (A1B2); 6) the cells were grown in sulfate-free Fischers Medium + 10% dialyzed fetal bovine serum in the presence of 20 mmol/L sodium chlorate (Sigma C 3171), an inhibitor of sulfation in living cells,^{40,41} or as a control with both sodium chlorate and 10 mmol/L sodium sulfate, for 24 hours before the assay; 7) the cells or the plates were preincubated with heparin (Sigma H 2149), heparan sulfate (Sigma D-9808), chondroitin A, B, and C (Sigma C 9819, C 0320, C 4384, respectively), and hyaluronic acid (H1751); and 8) the cells were pretreated for 30 minutes with 1 mU/ml heparitinase or 50 mU/ml protease-free chondroitinase ABC (# 100703 and 100332, respectively, Seikagaku Corporation, Japan). Unless otherwise indicated, the various reagents were present throughout the 1-hour attachment assay period.

Indirect Immunofluorescence Microscopy and Time Lapse Microscopy of Attaching Cells

For immunofluorescence staining, cells were rinsed and fixed in cold methanol for 3 minutes, rinsed and incubated with anti- β actin 1:100 for 1 hour at room temperature, and rinsed and incubated with secondary antibodies as described above.

For time-lapse microscopy, cells were plated at low density in a dish, sealed with parafilm, and placed on the microscope stage heated to 37°C. The Axiovert inverted microscope was connected to a PentaMAX-chilled charge-coupled device camera (Princeton Instruments, Inc. Trenton, NJ) and a Dell computer. A frame-by-frame analysis of the cell surface projections at intervals of 2 minutes for 1 hour was used to discriminate between filopodia and retraction fibers. Images were analyzed with the MetaMorph Imaging System (Universal Imaging Corporation, West Chester, PA). For migration studies, the cells were allowed to attach for 30 minutes before placing them on the heated stage. The cell displacement

as a function of time was monitored at 15 minutes intervals for 1 hour and analyzed as described.³⁶

Binding of Human ADAM 12 to Heparin Sepharose

Full-length ADAM 12-S protein encoded by the plasmid p1151 was produced by transfection of COS-7 cells as previously described.³¹ One ml of conditioned serum-free UltraDOMA medium was incubated for 3 hours at room temperature with 30 μ l of heparin Sepharose CI-6B beads (Pharmacia). The beads were washed with PBS, and bound protein was then eluted by boiling in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. ADAM 12 protein was detected by SDS-PAGE and Western blotting using the 14E3 mAb as previously described.³¹ Binding to heparin Sepharose was performed either in the presence of 0.1 mol/L NaCl (as present in UltraDOMA medium) or after the addition of 0.5 mol/L or 1.0 mol/L NaCl.

Results

ADAM 12 Is Present at Tumor Cell Surfaces

The distribution of ADAM 12 in a series of 37 human carcinomas compared with the normal counterpart tissue was investigated by immunohistochemistry. Representative samples are shown in Figure 1. All 15 cases of breast carcinomas exhibited intense ADAM 12 immunoreactivity (Figure 1A) using several different antibodies, whereas in normal breast tissue, only a few scattered luminal cells of the ducts exhibited ADAM 12 immunoreactivity (Figure 1E). Half of the 14 cases of colon carcinomas, 2 of the 4 cases of gastric, and all 4 cases of lung carcinomas examined exhibited ADAM 12 immunoreactivity (Figure 1, B-D). Little or no immunostaining was observed in the corresponding normal colon epithelium (Figure 1F). In the ADAM 12 positive carcinomas, intense cytoplasmic immunostaining was observed in most of the carcinoma cells. Strikingly, in several areas (Figure 1D) the immunoreactivity was located between tumor cells and at the tumor-stroma interface, an indication that ADAM 12 is found on the tumor cell plasma membrane. The immunostaining was completely abolished when purified recombinant ADAM 12 was added together with the primary antibodies (not shown). In both normal and tumor specimens a strong immunoreactivity for ADAM 12 was present in the smooth muscle of the blood vessel wall. The endothelial cells of the blood vessels and the stromal cells including myofibroblasts did not show any detectable immunoreactivity. The available antibodies did not allow us to discriminate between the presence of ADAM 12-L and ADAM 12-S in the tissue sections. To investigate this question and to confirm the existence of ADAM 12 transcripts in the tissue specimens, we performed RT-PCR.

A series of normal and cancerous human breast tissue specimens were analyzed by RT-PCR using primers that were specific for ADAM 12-L and ADAM 12-S. Breast

carcinoma tissue appeared to contain more ADAM 12-L transcript than normal breast tissue (Figure 1G). ADAM 12-S transcript was present in both normal and tumor tissue and appeared to vary among the tissue samples (not shown). Both forms of ADAM 12 could be detected in a range of cultured tumor cell lines (not shown). The identity of the RT-PCR products was confirmed by nucleotide sequencing.

The expression of ADAM 12 in normal tissue, in particular in muscle, has previously been documented.^{30,31,42} The finding in the present study of an apparent up-regulation of ADAM 12 in human carcinoma, and in particular the intriguing immunostaining pattern, raised the question whether ADAM 12 might play a role in cell-cell and/or cell-matrix interactions in cancer.

rADAM 12-cys Promotes Tumor Cell Adhesion

We decided to use an *in vitro* system to study tumor cell adhesion to human ADAM 12. Our strategy was to express individual domains of human ADAM 12 as recombinant polypeptides in *E. coli* and assay for their ability to promote tumor cell attachment, similar to the approach that was used for defining the specific interaction between the disintegrin-like domain of human ADAM 15 with integrin α v β 3.⁴³ The metalloprotease, the disintegrin-like, and cysteine-rich domains were individually expressed in *E. coli* as His-tagged polypeptides. These domains make up the extracellular moiety of ADAM 12 after removal of the prodomain by a furin-type protease,³¹ and any specific cell-binding activity would be expected to reside in one or more of these domains. As a control, recombinant polypeptides of the disintegrin-like and cysteine-rich domains of human ADAM 15 were generated. A standard cell-attachment assay was used in which tumor cells were allowed to attach for 1 hour in wells coated with the respective substrates. As expected based on the work by Zhang et al,⁴³ we found that α v β 3 integrin-expressing A375 melanoma cells⁴⁴ attached well to recombinant ADAM 15 disintegrin-like domain, whereas MDA-MB-231 cells that express only low levels of α v β 3 integrin⁴⁵ did not attach (Figure 2A, 2B). Neither of these two cell lines attached to the recombinant cysteine-rich domain of ADAM 15 (Figure 2B). Interestingly, however, we found that for ADAM 12 the recombinant cysteine-rich domain served as a specific adhesion substrate for the MDA-MB-231 breast carcinoma cells in a concentration-dependent manner, whereas neither the recombinant metalloprotease nor disintegrin-like domains were biologically active in this attachment assay (Figure 2B, 2C). The cysteine-rich domain is composed of three subdomains a, b', and c.³⁷ To further localize the binding site, we expressed various subdomains of the cysteine-rich domain. Recombinant polypeptides of subdomain b'+c (p1053, p1222) were the most active (Figure 2D). The recombinant b'+c subdomain of the ADAM 12 cysteine-rich domain, hereafter referred to as rADAM 12-cys, was used in further experiments. The attachment to rADAM-cys was dose-dependent, and maximum attachment was obtained at concentrations of 10 to 20 μ g/ml. Attachment to

Figure 1. ADAM 12 is up-regulated in carcinoma tissue compared with normal tissue by immunohistochemistry and RT-PCR. Intense ADAM 12 immunostaining is shown in the tumor cells of a breast carcinoma (A), a gastric carcinoma (B), and a colon carcinoma (C and D). The insert in B is a higher magnification of the area indicated by an arrow. A section of normal breast tissue with a few scattered luminal cells of the ducts with ADAM 12 immunoreactivity is demonstrated in E and normal colon epithelium with essentially no ADAM 12 immunoreactivity in F. The sections are counterstained with hematoxylin. Normal human breast and carcinoma tissue was examined by RT-PCR and subsequent Southern hybridization (G) to detect the 516-bp human ADAM 12-L PCR product. Human term placenta served as a positive control. Lanes 1–7 are a series of different breast carcinomas. Lanes 8 and 9 are pairs of normal and tumor tissue from the same patient. NC is a negative control in which template is omitted in the PCR reaction. The bottom panel is a control RT-PCR of the same samples after amplification with primers specific for human laminin $\alpha 2$ chain, yielding a 518-bp product that was detected on an ethidium-bromide stained agarose gel. Scale bars, 35 μ m (A and C); 78 μ m (B); 18 μ m (insert); 17.5 μ m (D); 41 μ m (E); 44 μ m (F).

Figure 2. Cell attachment of human tumor cell lines to recombinant ADAM 12 fragments demonstrates the specificity and dose-dependency of the cysteine-rich domain as a substrate. **A** shows cell attachment of the A375 melanoma cell line to multiwell plates coated with different concentrations of EHS laminin (∇); rADAM 15-disintegrin-like domain (∇); and the MDA-MB-231 breast carcinoma cell line on EHS laminin (∇) and on rADAM 15-disintegrin-like domain ($\bullet$). **B** shows cell attachment of the A375 cell line (hatched bars) and the MDA-MB-231 breast carcinoma cell line (black bars) to rADAM 15-cysteine-rich domain, rADAM 15-disintegrin-like domain, rADAM 12-cysteine-rich domain, rADAM 12-disintegrin-like domain, (20 $\mu\text{g/ml}$). **C** shows cell attachment of the MDA-MB-231 breast carcinoma cell line to multiwell plates coated with different concentrations of human laminin (∇); rADAM 12-disintegrin-like domain (∇); rADAM 12-metalloprotease domain ($\circ$); and rADAM 12-cys (b'+c) ($\bullet$). **D** demonstrates cell attachment of MDA-MB-231 to various subdomains of the cysteine-rich domain: rADAM 12 cys-(a+b'+c) p1219 ($\circ$); rADAM 12-cys (b'+c) p1053 ($\bullet$) and p1222 (∇). Each point represents the mean $\pm$ SEM of triplicate wells. In all experiments the absorbance value of cell attachment to bovine serum albumin was below 0.05 at 590 nm (not shown). Data present the mean $\pm$ SEM. Abcissa (**A**, **C**, and **D**) are the concentration of substrate in $\mu\text{g/ml}$ and ordinate (**A**, **C**, and **D**) is O.D. value at 590 nm. **E** demonstrates ADAM 12-cys mediated cell attachment of a series of human tumor cell lines. Cell attachment of several cultured cell lines to wells coated with 20 $\mu\text{g/ml}$ of rADAM 12-cys was compared with wells coated with 20 $\mu\text{g/ml}$ of EHS-laminin. Data present the mean $\pm$ SEM. The cell lines are: 1) MDA-MB-231; 2) MDA-MB-435; 3) MDA-MB-468; 4) MCF-7; 5) RKO; 6) Clone A; 7) A431; 8) A375; 9) SK-MEL-28; 10) HT1080; and 11) A204. **F** demonstrates a Coomassie-blue-stained SDS-PAGE with some purified recombinant polypeptides used. **Lane 1**, the disintegrin-like domain of human ADAM 15 (p1345); **lane 2**, the cysteine-rich domain of human ADAM 12 (p1053); **lane 3**, the cysteine-rich domain of human ADAM 12 (p1222). Molecular mass markers (Mark 12, Novex) are indicated.

rADAM12-cys was approximately 60% of maximum attachment obtained on tissue culture plates (not shown). The tumor cells attached firmly to both rADAM-12 cys and laminin substrates as evidenced by their unaltered attachment when the plates were inverted and centrifuged at $60 \times g$ for 2 minutes (not shown). We tested a large series of tumor cells lines (Figure 2E) and found that MDA-MB-231, MDA-MB-435, MDA-MB-468, RKO, Clone A, A431, SK-MEL-28, A375, HT1080, and A204 cells adhered as well to rADAM 12-cys as they did to laminin, whereas MCF-7 cells adhered significantly better to laminin (Figure 2C, column 4).

Adhesion mediated via rADAM 12-cys has distinct physical requirements (Figure 3). The addition of 10 mmol/L EDTA to the incubation medium inhibited the cell attachment, indicating that the cell adhesion to the rADAM 12-cys was dependent on divalent cations (Figure 3A). The rADAM 12-cys mediated adhesion process was temperature independent and, in contrast to the adhesion to laminin, was efficient at 4°C (Figure 3B). The binding of tumor cells to rADAM 12-cys required metabolic energy, because pretreatment and incubation with a mixture of 50 mmol/L 2-deoxy-D-glucose plus 10 mmol/L sodium azide³⁹ resulted in an almost complete inhibition of attachment (Figure 3C). Cytochalasin B, an inhibitor of actin polymerization, had only a minor effect on attachment but inhibited the formation of cell surface projections (not shown).

Tumor Cells Attach to rADAM 12-cys with a Rounded Morphology Accompanied by Numerous Actin-Containing Filopodia

Morphological analysis of tumor cell adhesion to rADAM 12-cys after 1 hour revealed a distinct pattern of cell shape. The tumor cell lines, such as MDA-MB-231 breast carcinoma cells, remained rounded and had a number of cell surface projections radiating from the central soma without any apparent polarization (Figure 4A). In contrast nearly all cells plated on laminin were spread (Figure 4B). The numerous cell surface projections contained actin as evidenced by immunostaining with monoclonal antibodies to β -actin (Figure 4C, 4D). A critical role for actin was also indicated by the observation that these projections did not form on adhering cells in the presence of cytochalasin B (not shown). To examine whether the actin-containing projections were caused by membrane protrusions (filopodia) or by membrane retractions (retraction fibers), we used time-lapse microscopic analysis.^{2,36} We found that the thin cell surface projections actively protruded from the cells (not shown) and began to appear a few minutes after plating the cells. In some cells, lamellae developed around the cells secondary to the formation of cell surface projections. These cell surface projections were therefore considered to be filopodia.

A combination of a rounded morphology, filopodia, and lamella formation is characteristic of migrating cells and led us to test the ability of rADAM 12 to stimulate tumor cell migration. Because Clone A cells have been demonstrated to migrate spontaneously and can be fur-

Figure 3. Cell attachment to rADAM 12-cys has specific physical requirements. **A** demonstrates cell attachment of MDA-MB-231 cells to rADAM 12-cys (●); rADAM 12-cys + 10 mmol/L EDTA (○); EHS-laminin (▼); and EHS-laminin + 10 mmol/L EDTA (▽). **B** demonstrates cell attachment of MDA-MB-231 cells to rADAM 12-cys (●) and to EHS-laminin (▼) at 4°C. **C** demonstrates cell attachment of MDA-MB-231 cells to rADAM 12-cys (●); rADAM 12-cys + a mixture of 50 mmol/L 2-deoxy-D-glucose plus 10 mmol/L NaNa₃ (○); EHS-laminin (▼); and EHS-laminin + a mixture of 50 mmol/L 2-deoxy-D-glucose plus 10 mmol/L NaNa₃ (▽). In all experiments the absorbance value of cell attachment to bovine serum albumin was below 0.05 at 590 nm (not shown). Data present the mean $\pm$ SEM. Abscissa is substrate in μ g/ml and the ordinate is absorbance value at 590 nm.

ther stimulated by laminin,³⁶ we compared the migration rate of these cells on rADAM 12-cys substrate with migration on uncoated tissue culture plastic or on EHS-laminin. Clone A cells exhibited a random migration at a rate of $19.9 \pm 5.4 \mu\text{m/h}$ on plastic, $36.7 \pm 5.1 \mu\text{m/h}$ on EHS-laminin, and $9.0 \pm 1.1 \mu\text{m/h}$ on rADAM 12. We conclude that rADAM 12-cys does not appear to stimu-

Figure 4. Morphology of tumor cells attaching to rADAM 12-cys. MDA-MB-231 breast carcinoma cells were seeded on rADAM 12-cys (A) or EHS-laminin (B) for 1 hour, fixed, stained with crystal violet, and photographed using phase contrast optics, as described in Materials and Methods. The MDA-MB-231 cells attained a rounded morphology with many cell surface projections on the rADAM 12-cys substrate (A) compared with a flattened and spread morphology on EHS-laminin (B). MDA-MB-231 (C) and RKO carcinoma cells (D and E) were seeded on rADAM 12-cys for 1 hour, were fixed, and reacted with antibodies to β -actin. Numerous cell surface projections irradiating from the cells stained positively (C and D). Note in (D) that the projections of adjacent cells appeared to contact each other. E is a control staining where the primary antibody was omitted. Scale bar, 14.5 μ m (A and B); 7.5 μ m (C-E).

late cell migration but appears to retard spontaneous migration ($P < 0.0001$; Student's *t*-test).

rADAM 12-cys Mediates Tumor Cell Adhesion Through Cell a Surface Heparan Sulfate Proteoglycan

The results obtained so far indicated that rADAM 12-cys represents a tumor cell attachment stimulus, but what are the receptor(s) and the molecular mechanisms involved? The integrin family of cell surface proteins are primary participants in cell adhesion to extracellular matrix molecules and to other cells.⁴⁶ We found that function-blocking monoclonal antibodies to integrin $\alpha 6$ (2B7) and $\beta 1$ (A11B2) did not inhibit significantly attachment to rADAM 12-cys (Figure 5A and not shown) of the MDA-MB-231 cells. As expected, attachment to laminin was perturbed by both antibodies (Figure 5A). Another candidate cell receptor family is the sulfated cell membrane proteoglycans.⁴⁷ Sulfation of glycosaminoglycans was inhibited by growing cells in culture media containing sodium chlorate.^{40,41} At a concentration of 20 mmol/L chlorate, more than 95% decrease in cell attachment to rADAM 12-cys was seen (Figure 5B). The effect of chlorate on cell attachment could be almost completely reversed by inclusion of 10 mmol/L sodium sulfate in the cell cultures

(Figure 5B). No effect of sodium chlorate was observed in MDA-MB-231 cell attachment to laminin. These results suggest that sulfated glycosaminoglycans are of critical importance for cell attachment to rADAM 12-cys.

To determine which class of sulfated cell membrane proteins are involved, we examined the effect on cell attachment to rADAM 12-cys and laminin of a series of inhibitors of glycosaminoglycan function, including heparin, heparan sulfate, chondroitin sulfate A, B, and C, and as a control nonsulfated hyaluronic acid. Cell attachment to rADAM 12-cys was completely inhibited by heparin and heparan (Figure 5C, 5D). Notably, cell attachment was inhibited when heparin was preincubated for 30 minutes with the rADAM 12-cys coated wells and the wells rinsed before addition of the cells. Thus, heparin was not binding to the tumor cell surfaces but rather was binding to the rADAM 12-cys substrate and thereby blocking cell adhesion to rADAM 12-cys by competitive inhibition. We found no dose-dependent effect of chondroitin sulfate A, B, and C or hyaluronic acid (Figure 5C and not shown). Furthermore, enzymatic removal of glycosaminoglycans by treatment of the cells with 1 mU/ml heparatinase significantly reduced the cell attachment, whereas 50 mU/ml chondroitinase ABC had no effect (Figure 5C). No significant effect of any of these compounds was observed in MDA-MB-231 cell attachment to laminin (Figure 5D and

Figure 5. MDA-MB-231 cell attachment to rADAM 12-cys is mediated through a cell surface heparan sulfate proteoglycan. **A** shows the effect of function-blocking $\beta 1$ integrin antibodies on attachment to rADAM 12-cys and to EHS-laminin of MDA-MB-231 cells. MDA-MB-231 cells on rADAM 12-cys (●) and on EHS-laminin (○). Note that the antibodies completely inhibited attachment to laminin as expected, but that no dose response was obtained on attaching to rADAM 12-cys. **B** shows that MDA-MB-231 cells grown in the presence of 20 mmol/L sodium chlorate (hatched bars) are inhibited in their cell attachment on rADAM 12-cys compared with cultures without the addition of sodium chlorate (open bars) or with the addition of 10 mmol/L sodium sulfate (cross-hatched bars). No effect of sodium chlorate (hatched bars) on cell attachment to laminin compared with untreated cells (open bars) was observed. Data present the mean $\pm$ SEM. **C** demonstrates the effect of 10 $\mu\text{g/ml}$ each of heparin, heparan, chondroitin sulfate A, B, C, and hyaluronic acid as well as 1 mU/ml heparitinase or 50 mU/ml chondroitinase ABC. Note that MDA-MB-231 cell attachment is sensitive to heparin, heparan, and heparitinase. **D** demonstrates a dose response of heparin on cell attachment to rADAM 12-cys (●) and to laminin (○). Note that low doses of heparin inhibited the attachment of MDA-MB-231 cells. No effect of heparin was observed on cell attachment to laminin. Data present the mean $\pm$ SEM.

not shown). These results suggest that for MDA-MB-231 cells, heparan sulfate chains are critically involved in rADAM 12-cys-mediated cell attachment. Several other cell lines (RKO, Clone A, and A375 cells) were tested, and the same inhibitory effect of heparin was observed.

The data shown above indicate that ADAM 12 interacts with tumor cell surface heparan-sulfate proteoglycans. It should be stressed that the data were obtained using recombinant fragments of ADAM 12 expressed in *E. coli*, which may be not folded in the same configuration as in full-length native ADAM 12. As a way of testing whether authentic and full-length human ADAM 12 can interact with heparan-sulfate proteoglycan(s), we tested the abil-

ity of such protein to bind to heparin Sepharose. ADAM 12-S protein was produced by transfecting COS cells with an expression plasmid for full-length human ADAM 12-S.³¹ Conditioned medium containing the 92-kD pro-form and the 68-kD active protease was incubated with heparin Sepharose in the presence of increasing amounts of NaCl. Bound material was eluted and analyzed by SDS-PAGE. Figure 6 shows that both forms of ADAM 12-S bind to heparin Sepharose and that this binding is eliminated at increased ionic strength as is seen for known heparan-sulfate binding proteins. Both the 68- and 92-kD forms of ADAM 12 contain the cysteine-rich domain; therefore this result is consistent with

Figure 6. Authentic full-length human ADAM 12-S binds to heparin Sepharose. ADAM 12-containing conditioned culture medium was incubated under various conditions with heparin Sepharose, washed and eluted, and bound ADAM 12 protein detected by Western blot as described in Materials and Methods. **Lane 1** represents the starting material (the conditioned culture medium); **lane 2** represents bound material under physiological ionic strength; **lane 3** represents bound material on the addition of 0.5 mol/L NaCl, and **lane 4** in the addition of 1.0 mol/L NaCl. Molecular mass are indicated.

the results obtained in this study using individual domains expressed in *E. coli*.

Discussion

The concept that ADAM proteins serve as cell adhesion proteins through binding to an integrin has emerged from a series of studies of the role of fertilins and cyritestin in fertilization.^{7,21–23} Moreover, a recombinant disintegrin-like domain of human MDC 15 (metargidin, ADAM 15) expressed in *E. coli* was shown to function as an RGD-dependent adhesion molecule.⁴³ In the present study we obtained results that provide further support for a role for ADAM proteins in cellular interactions and more specifically with a relevance to cancer cell biology. We used an *in vitro* attachment assay to test the ability of various recombinant fragments of human ADAM 12 to support cell adhesion and found that a series of tumor cell lines were capable of attaching to rADAM12-cys in a specific and dose-dependent manner. The finding that the cys-

teine-rich domain of human ADAM 12 supports cell adhesion was somewhat unexpected because the disintegrin domain of SVMPs and ADAMs, but not the cysteine-rich domain, has been previously associated with a role in cell adhesion.^{7,15,43} In agreement with the work of Zang et al,⁴³ we showed that the recombinant disintegrin-like domain of human ADAM 15 supported cell adhesion of cells that express $\alpha\beta 3$ integrin but not of cells lacking this integrin. The recombinant disintegrin-like domain of human ADAM 12 did not support cell adhesion in our assay. This may be due to the fact that ADAM 12 does not contain an RGD tripeptide as does ADAM 15, although we cannot exclude the possibility that it results from a difference in the ability of the *E. coli*-produced polypeptides to fold properly. Because most ADAMs in fact do not have an RGD peptide in the disintegrin-like domain, it will be interesting to explore whether the cysteine-rich domain of other ADAMs may also be assigned a role in cell adhesion.

We explored the molecular mechanism of rADAM 12-cys mediated cell attachment, aiming first at defining the receptor complex(es) involved. Cell attachment to rADAM 12-cys required metabolic energy and involved reorganization of the actin microfilaments. These observations suggest that ADAM 12-cys mediated cell attachment results in intracellular signaling events. For MDA-MB-231 cells that attached but did not spread, we found that the cells attached to rADAM 12-cys by engaging cell surface heparan sulfate chains but apparently not chondroitin sulfate chains. This conclusion is based on attachment assays in which heparin, heparan, and pretreatment of the cells with heparitinase almost completely inhibited attachment. The cysteine-rich domain of human ADAM 12 has no distinct heparin-binding site based on the amino acid sequence, although it is rich in basic amino acids. Candidate cell surface receptors containing heparan sulfate chains include the syndecan family of cell surface proteoglycans, the phosphatidylinositol-linked glypican, and part-time proteoglycans betaglycan and CD44E.^{47–50} Several studies have demonstrated a role of syndecans in regulating cell adhesion and morphology.^{51–57} There are four members of the syndecan family (for review see Refs. 47–50). Their heparan sulfate chains bind to a large number of molecules, including extracellular matrix components such as fibronectin, collagen, thrombospondin, and heparin-binding growth factors such as the FGF's. Syndecans have a distinct, sometimes overlapping tissue distribution pattern.⁵⁸ There is evidence that syndecan-1 is required for maintenance of normal epithelial cells,^{59,60} that it can suppress tumor growth,⁶¹ and that the expression is down-regulated in cancer.^{62–64} Little is known about the expression pattern and function of syndecan-4 in cancer; however, in keeping with previous results,⁶⁴ we found that syndecan-4 transcripts are readily detectable in several of the tumor cell lines, including MDA-MB-231, Clone A, and A375 cells (Gilpin and Wewer, unpublished observations). Interestingly, syndecan-4 localizes to focal adhesions and regulates the distribution and activity of protein kinase C, thus providing direct transmembrane signaling.^{54,65,66} Although it is tempting to speculate, more rigorous bio-

chemical studies are obviously needed to define if and by which mechanism syndecan-4 or another of the heparan sulfate bearing cell membrane proteins transduce the rADAM 12-cys-mediated cell adhesion response.

We have found that ADAM 12 expression is up-regulated in human carcinoma tissue, that it appears to be located at the tumor cell surfaces, and furthermore that the cysteine-rich domain of ADAM 12 supports cell adhesion in an *in vitro* assay. This new class of interactions between an ADAM protein and tumor cell surfaces appears to involve heparan sulfate proteoglycan as a receptor. These results lead us to ask how ADAM 12 may operate in authentic tumor tissue. Tumor cells displaying ADAM 12-L on their cell surface may use heparan sulfate proteoglycan(s) as receptors or as co-receptors in cell-cell interactions. Alternatively, the secreted form of ADAM 12, ADAM 12-S, may be incorporated in the extracellular matrix and serve as a ligand for the same cell surface receptors. Our results indicate that human ADAM 12 mediates cell-cell or cell-matrix adhesion, but we do not yet know whether ADAM 12-L itself functions as a receptor and initiates a distinct downstream signaling on its own, nor how this may influence tumor cell behavior. In any event, our results may initiate a new avenue of studies aiming at defining the role of the cysteine-rich domain of other ADAM proteins in cell adhesion.

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