ADAM12 induces actin cytoskeleton and extracellular matrix reorganization during early adipocyte differentiation by regulating β1 integrin function

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Summary
Changes in cell shape are a morphological hallmark of differentiation. In this study we report that the expression of ADAM12, a disintegrin and metalloprotease, dramatically affects cell morphology in preadipocytes, changing them from a flattened, fibroblastic appearance to a more rounded shape. We showed that the highest levels of ADAM12 mRNA were detected in preadipocytes at the critical stage when preadipocytes become permissive for adipogenic differentiation. Furthermore, as assessed by immunostaining, ADAM12 was transiently expressed at the cell surface concomitant with the reduced activity of β1 integrin. Co-immunoprecipitation studies indicated the formation of ADAM12/β1 integrin complexes in these preadipocytes. Overexpression of ADAM12 at the cell surface of 3T3-L1 preadipocytes achieved by transient transfection or retroviral transduction led to the disappearance of the extensive network of actin stress fibers that are characteristic of these cells, and its reorganization into a cortical network located beneath the cell membrane. The cells became more rounded, exhibited fewer vinculin-positive focal adhesions, and adhered less efficiently to fibronectin in attachment assays. Moreover, ADAM12-expressing cells were more prone to apoptosis, which could be prevented by treating the cells with β1-activating antibodies. A reduced and re-organized fibronectin-rich extracellular matrix accompanied these changes. In addition, β1 integrin was more readily extracted with Triton X-100 from cells overexpressing ADAM12 than from control cells. Collectively, these results show that surface expression of ADAM12 impairs the function of β1 integrins and, consequently, alters the organization of the actin cytoskeleton and extracellular matrix. These events may be necessary for early adipocyte differentiation.

Key words: ADAM12, β1 integrin, Actin cytoskeleton, Extracellular matrix, Adipogenesis

Introduction
Adult stem cells and progenitor cells can integrate and respond to appropriate extracellular stimuli in the form of hormones and growth factors, or contact with the extracellular matrix (ECM) and neighboring cells. The delicate balance between the dormancy of progenitor cells and their timely proliferation and differentiation is a crucial parameter in tissue homeostasis that is often perturbed in disease. In vitro studies and animal implant experiments have revealed the multipotential nature of mesenchymal stem cells, which contribute to the regeneration of mesenchymal tissues such as bone, cartilage, muscle, tendon, stroma and adipose tissues (Pittenger et al., 1999; Prockop, 1997).

Adipogenesis is a complex process characterized by the strict temporal regulation of multiple and interacting signaling events that ultimately lead to the expression of adipocyte-specific genes (Gregoire et al., 1998; Rosen and Spiegelman, 2000). A cascade of transcription factors is induced that involves the sequential activation of the CCAAT/enhancer-binding proteins (C/EBPs) and the peroxisome proliferator-activated receptor γ (PPARγ), which leads to the activation of several genes, such as those responsible for lipid transport and metabolism. Initially, fibroblastic preadipocytes stop dividing and acquire a rounded morphology. The change of shape from fibroblastic preadipocytes to rounded, mature adipocytes is accompanied by changes in cytoskeletal organization and contacts with the ECM. The expression of fibronectin, integrins, actin and several cytoskeletal proteins is downregulated during adipogenesis (Rodriguez Fernandez and Ben-Ze’ev, 1989; Spiegelman and Farmer, 1982). In fact, the disruption of contacts with the ECM is a requirement for adipocyte differentiation (Spiegelman and Ginty, 1983).

Our understanding of the molecular events involved in adipogenesis is based mainly on in vitro, cell culture models,
especially 3T3-L1 preadipocytes (Gregoire et al., 1998; Rosen and Spiegelman, 2000). Several genetically modified mouse models have also contributed to the current knowledge of adipocyte differentiation (Moitra et al., 1998; Rosen et al., 1999; Wang et al., 1995). We have recently reported increased adipogenesis in transgenic mice overexpressing the disintegrin and metalloprotease, ADAM12, driven by the muscle creatine kinase promoter (Kawaguchi et al., 2002). Cells expressing early markers of adipogenesis were apparent in the perivascular space in the muscle tissue of 1- to 2-week-old transgenic mice, whereas mature, lipid-laden adipocytes were seen at 3 to 4 weeks of age, suggesting a crucial role for ADAM12 in adipogenesis in vivo.

ADAM12 belongs to the ADAMs, a family of adhesion proteins and metalloproteases comprising more than 30 members. The prototype ADAM molecule is a transmembrane protein composed of several distinct domains including a prodomain, metalloprotease, disintegrin, cysteine-rich, EGF-like and transmembrane domains, as well as a short cytoplasmic tail (Black and White, 1998; Schlondorff and Blobel, 1999). Human ADAM12 exists in two alternatively spliced forms – a long transmembrane form, ADAM12-L, and a shorter secreted form, ADAM12-S, which lacks the transmembrane and cytoplasmic domains (Gilpin et al., 1998). Only the long, membrane-anchored form of ADAM12 has been reported in mice (Yagami-Hiromasa et al., 1995). In our transgenic mouse models, increased adipogenesis occurred in the skeletal muscle of mice expressing human ADAM12-S, ADAM12-L, or ADAM12-L lacking the cytoplasmic domain (ADAM12-Dcyt) (Kawaguchi et al., 2002) (U.M.W., unpublished), indicating that it is primarily the extracellular domain of ADAM12 that elicits the robust adipogenic response observed in these mice. The phenotype of ADAM12-deficient mice was recently characterized and it was noted that the interscapular brown adipose tissue was reduced in some pups (Kurisaki et al., 2003).

The present study was undertaken to elucidate the molecular mechanisms underlying the stimulatory effects of ADAM12 on adipocyte differentiation. We present evidence that ADAM12 is transiently expressed at the cell surface just before the onset of adipogenesis, concomitant with the reduced activity of β1 integrin, when preadipocytes begin to change their morphology from a fibroblastic to a rounded shape. Overexpression and RNAi knockdown experiments show that ADAM12 induces several changes in β1 integrin-dependent functions in these mesenchymal cells. These changes in integrin function appear to involve lateral associations between ADAM12 and β1 integrin within the plasma membrane that result in increased detergent solubility of β1 integrin.

Materials and Methods

Cell lines

The following cell lines were used: mouse 3T3-L1 preadipocytes (ATCC CL173, Rockville, MD), C3H10T1/2 (ATCC CCL-226), CHO-K1 (ATCC CCL-61) and human liposarcoma LiSa-2 cells (Wabitsch et al., 2000). All cell lines, except for CHO-K1 and LiSa-2 cells, were maintained in growth medium consisting of Dulbecco’s Modified Eagle’s Medium (DMEM) with Glutamax I and 4500 mg/l glucose, 50 U/ml penicillin, 50 μg/ml streptomycin and 10% fetal bovine serum (FBS) (Invitrogen, Tåstrup, Denmark). Adipocyte differentiation of 3T3-L1 cells was induced by treating confluent cells with DMI, (0.25 μM dexamethasone, 0.5 mM methylisobutylxanthine, 10 μg/ml insulin) in growth medium for 2 days (Cook et al., 1985; Ntambi and Young-Cheul, 2000). Adipocyte differentiation of C3H10T1/2 cells was induced by treating confluent cells with DMI and 1 μM BRL49653 (kindly provided by Novo Nordisk A/S, Bagsvaerd, Denmark) in growth medium for 2 days (Lehmann et al., 1995). CHO-K1 and LiSa-2 cells were maintained in DMEM/F12 (1:1) (Invitrogen) containing 10% FBS. For differentiation of LiSa-2 cells, culture medium was replaced with serum-free DMEM/F12 (1:1) supplemented with 10 μg/ml transferrin, 15 mM HEPES, 1 nM insulin, 20 pM triiodothyronine and 1 μM cortisol (Wabitsch et al., 2000).

Antibodies

Antibodies to ADAM12 included mouse monoclonal antibodies (mAbs) 6E6, 6C10 and 8F8; and polyclonal antibodies rb122 and rb109 (Kronqvist et al., 2002). Mouse anti-human β1 integrin (clone 12G10) was obtained from Serotec (Oxford, UK). Rabbit-antiserum to β1 integrin was kindly provided by S. Johansson (Bottger et al., 1989) and mouse monoclonal antibody to vinculin was kindly provided by M. Glukhova. Mouse anti-human β1 integrin (clone 18) and phycocerythrin-conjugated CD29 (clone MAR4), anti-mouse β1 integrin (9EG7), anti-human fibronectin Ab (A245) and phycocerythrin-conjugated goat anti-mouse immunoglobulin G (IgG) were all obtained from BD Biosciences (Brondby, Denmark). Mouse anti-human β1 integrin (JB1A) was obtained from Chemicon Ltd (Hampshire, UK). Mouse anti-human β1 integrin (K20), isotype-controls, mouse IgG, goat anti-mouse–horse radish peroxidase (HRP) Ab, and goat anti-rabbit–HRP Ab were obtained from Dako A/S (Glostrup, Denmark).

Transient transfections

Cells were transfected using FuGene 6 transfection reagent (Roche Diagnostic, Mannheim, Germany) or Lipofectamine (Invitrogen) in serum-containing medium or Opti-MEM I according to the manufacturer’s instructions, and analyzed two days later. To create fusion proteins with enhanced green fluorescent protein (EGFP), human ADAM12 cDNAs were cloned into the pEGFP-N1 vector (Clontech Laboratories, Heidelberg, Germany). The following constructs were used (Hougaard et al., 2000): human ADAM12-L truncated at nt 2498, resulting in a membrane-inserted protein lacking the cytoplasmic tail, called ADAM12-Dcyt (#1442); and ADAM12-Dcyt with a E351-Q catalytic site mutation (#1455). As a control, the empty vector, pEGFP-N1 (#1422), was used. Mouse full-length ADAM12 (#1629) (kindly provided by J. Frey) or empty-vector control pcDNA3 (#1695) (Invitrogen) was transfected into CHO-K1 cells that were used as positive or negative controls, respectively, in western blots.

Retroviral transduction

ADAM12-Dcyt cDNA was subcloned into the SaI site of pBABEpuro (Morgenstern and Land, 1990) for retroviral transduction, as described previously (Porse et al., 2001). Briefly, retroviral stocks were obtained by transiently transfecting Phoenix ecotropic retroviral packaging cells with pBABE-based proviral constructs. Cell culture supernatants were harvested and used to infect 3T3-L1 and C3H10T1/2 cells. After infection, cells were cultured in the presence of 1 μg/ml puromycin (Sigma-Aldrich, Vallensbæk, Denmark) to select stable clones.

Northern blot analysis

TriZol reagent (Invitrogen) was used to isolate total RNA from embryonic muscle, white and brown adipose tissue, or cultured cells for RNA isolation. RNAs were analyzed by Northern blot analysis as described previously.
at various stages of differentiation. DNA (15 μg total DNA per lane) was separated on 1% formaldehyde gels and blotted onto Hybond N nylon membranes (Amersham Pharmacia Biotech, Uppsala, Sweden). Hybridization was carried out at 68°C for 1 hour using [32P]dCTP-labeled mouse ADAM12, PPARY (kindly provided by B. M. Spiegelman), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from Clontech. cDNAs in QuickHyb hybridization solution (Stratagene, Amsterdam, The Netherlands). After washing with 2x saline sodium citrate (SSC)/1% sodium dodecyl sulphate (SDS), 2x SSC/0.1% SDS and 0.2x SSC/0.1% SDS twice each at 65°C, the blots were exposed to Kodak X-OMAT AR film at ~80°C with intensifying screens, or analyzed by a PhosphorImager (Storm840, Molecular Dynamics, Amersham Biosciences, Hørsholm, Denmark).

Protein extraction, immunoprecipitation and western blot analysis

Proteins from growing 3T3-L1, C3H10T1/2, LiSa-2 cells and CHO-K1 cells transfected with control vector (#1629) were extracted at 4°C in 20 mM Tris, pH 8.0, 150 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 50 mM octylglucoside, and ADAM12 (#1629) were extracted at 4°C in 20 mM Tris, pH 8.0, 150 mM NaCl, 300 mM sucrose, 10 mM Pipes pH 6.8, 3 mM MgCl2, 0.5% Triton X-100 and 1.2 mM phenylmethylsulfonyl fluoride). Both extraction conditions yielded similar results. The cells were subsequently rinsed with PBS and incubated with polyclonal anti-β1 integrin antibodies for 30 minutes, rinsed again and fixed with 4% paraformaldehyde for 2 minutes. Secondary antibodies were applied as described above.

Cell attachment assays

Nunc-Immuno 96-well plates with a MaxiSorp surface (Nunc A/S, Roskilde, Denmark) were coated with 10 μg/ml 9EG7 rat anti-mouse β1 integrin antibody (BD Biosciences) or 10 μg/ml fibronectin in 0.1 M NaHCO3 buffer overnight at 4°C, rinsed with PBS and blocked with 1% bovine serum albumin in PBS for 1 hour at 37°C. The plate was washed once with PBS and 6x104 cells/well were added and allowed to attach for 1 hour at 37°C in 5% CO2 in a humidified atmosphere. For cell attachment assays, the plates were centrifuged in an inverted position at 25 g for 2 minutes. Cells were washed twice in serum-free DMEM, fixed for 20 minutes at room temperature in 2% glutaraldehyde in 0.1 M cacodylate buffer and stained with 0.1% crystal violet in 10% methanol. Absorbance was measured with a Multiscan ELISA reader (Lab-systems, Helsinki, Finland) at 590 nm. Each assay was carried out in eight separate wells in each of two repetitions.

Apoptosis assay

Cells were trypsinized and suspended in growth medium with or without 10 μg/ml of the β1 integrin-activating antibody, 9E8G7. After incubation for 20 minutes at 37°C in suspension, cells were plated and cultured for 20 hours. Cells were re-incubated with 9E8G7 (10 μg/ml) for 40 minutes immediately before treatment with tumour necrosis factor (TNF)-α (Strathmann Biotech AG, Hamburg, Germany) (100 ng/ml) and cycloheximide (Sigma-Aldrich) (5 μM). After 5 hours of TNF-α and cycloheximide treatment, cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and stained with 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI) (10 mM). The number of apoptotic cells were assessed by fluorescence microscopy (Higuchi et al., 2003).

Flow cytometry

Growing and confluent (day 0) LiSa-2 cells were detached with cell dissociation buffer (Invitrogen) and restored for 5 minutes at 37°C in growth medium supplemented with 10% FBS, then transferred to ice and washed twice in cold washing buffer (PBS supplemented with 1% BSA). Mouse anti-human 12G10 and MAR4 were diluted according to the description of the manufacturer and added to the cell suspension. After 20 minutes at 4°C, cells were washed twice with washing buffer, incubated with the secondary antibody using an inverted Zeiss Axiovert microscope equipped with phase contrast optics and connected to a PentaMAX chilled charge-coupled device camera. The images were processed using the Metamorph Software Program (Universal Imaging Corporation, Brock and Michelsen; Birkerød, Denmark). To estimate the degree of stress fiber and focal adhesion formation, more than 600 cells in 20 fields were analyzed and the percentage of cells with fewer than five stress fibers or focal adhesions was calculated. The experiments were repeated twice and with two to three dishes per experiment.

Triton X-100 extraction

Two experimental techniques were used to assess the extractability of β1 integrin. Adherent cells were treated for 5 minutes on ice with either 0.01% Triton X-100 in DMEM or, as previously described (Fey et al., 1984), with 0.5% Triton X-100 in a cytoskeletal (CSK) buffer (100 mM NaCl, 300 mM sucrose, 10 mM Pipes pH 6.8, 3 mM MgCl2, 0.5% Triton X-100 and 1.2 mM phenylmethylsulfonyl fluoride). Both extraction conditions yielded similar results. The cells were subsequently rinsed with PBS and incubated with polyclonal anti-β1 integrin antibodies for 30 minutes, rinsed again and fixed with 4% paraformaldehyde for 2 minutes. Secondary antibodies were applied as described above.

Immunostaining and imaging

Tissue specimens were fixed in formalin and embedded in paraffin. For immunoperoxidase staining, 4 μm tissue sections were deparaffinized, subjected to microwave treatments in citrate buffer at pH 6.0 and 80°C for 10 minutes, then incubated with hydrogen peroxide for 20 minutes. Sections were then incubated with a mixture of ADAM12 mAbs (6E6, 6C10 and 8F8; 7.5 μg total), or a control antibody (total IgG concentration per sample 7.5 μg) and protein-G Sepharose beads (Amersham Pharmacia Biotech), rinsed twice with extraction buffer and eluted by boiling in sample buffer. Samples were separated on 8% polyacrylamide gels. Western blots were performed, as described previously (Loechel et al., 1998), using a polyclonal anti-ADAM12 antibody (rb122), or – for co-immunoprecipitation studies – monoclonal antibodies against mouse (clone 18) or human (JB1A) β1 integrin.
(phycoerythrin-conjugated goat anti-mouse IgG) for 20 minutes at 4°C and rinsed twice in washing buffer. After the second wash, cell pellets were resuspended in 500 μl washing buffer. Flow cytometric analyses were performed according to standard settings on a FACStar PLUS flow cytometer with CELLQUEST software (both from BD Biosciences).

RNA interference and microinjection
Short interfering RNA (siRNA) oligos were purchased from Dharmacon (LaFayette, CO) in a 2′-deprotected, desalted, single-stranded form (option B), and annealed according to the manufacturer’s protocol. Oligo sequences were: AAUCCCACGAC- AAUGCUACGdTdT (mouse ADAM12 siRNA), and ACGACG-UCCACUCUACGAdTdT (scrambled siRNA). A mixture of siRNA (2 μM) and EGFP expression vector (0.1 μg/μl) was injected into 3T3-L1 cells using a FemtoJet microinjector and an Inject Man NI 2 manipulator (Eppendorf, Hamburg, Germany) at an injection pressure of 150 hPa and an injection time of 0.7 second. Confluent cells were injected, and differentiation was induced the following day as described above. At day 4 after differentiation, cells were processed for phalloidin staining or immunostaining with antibodies to ADAM12 and observed under a fluorescence microscope.

Statistical analysis
All data are expressed as means ± s.e. The unpaired Student’s t-test or the Mann Whitney test was used for comparisons. A value of P≤0.05 was considered statistically significant.

Results
Expression of ADAM12 in adipose tissue and during in vitro adipogenesis
We have previously shown that ADAM12 stimulates adipogenesis in a transgenic mouse model (Kawaguchi et al., 2002). Here, we investigated the potential mechanisms underlying this observation. First, the expression profile of ADAM12 was determined in normal adipose tissue and during adipogenesis in vitro. ADAM12 was detected by immunostaining in normal mouse white and brown adipose tissue (Fig. 1A,C). We were unable to detect ADAM12 in normal human adipose tissue (data not shown), but ADAM12 was expressed in lipomas, which are benign tumors of human white adipose tissue (Fig. 1B) and hibernomas, which are benign tumors of human brown adipose tissue (Fig. 1D). Expression of ADAM12 mRNA in abdominal and epididymal white adipose tissue, as well as in brown adipose tissue from normal mouse, was demonstrated by northern blotting (Fig. 1E). Similarly, two murine preadipogenic cell lines, 3T3-L1 and C3H10T1/2, and the human liposarcoma cell line, LiSa-2, all expressed ADAM12 mRNA (Fig. 2A,C and data not shown). The temporal expression of ADAM12 and PPARγ mRNA was determined in growing fibroblastic preadipocytes, in confluent preadipocytes at the time of induction of differentiation (day 0), and at days 4 and 9 following induction (the period of fat vacuole accumulation). The level of expression of the adipogenic marker PPARγ was low at day 0 and several fold higher at day 9, as expected (Fig. 2B). The level of ADAM12 mRNA expression was highest at day 0 and decreased during the process of adipogenic differentiation (Fig. 2B). Fig. 2B also shows that ADAM12 was transiently upregulated at day 0 and decreased thereafter. Thus, the expression of PPARγ and ADAM12 appears to be inversely correlated in the 3T3-L1 cells. Similarly, expression of ADAM12 mRNA in LiSa-2 cells appeared to decline during adipogenesis (Fig. 2C). ADAM12 protein expression in all three cell lines was confirmed by western blot (Fig. 2D).

Cell-surface expression of ADAM12 before the onset of adipogenesis
To study the subcellular distribution pattern of ADAM12 during different stages of 3T3-L1 adipogenesis, permeabilized adherent cells were immunostained (Fig. 3A-C). Growing cells displayed mostly intracellular staining (Fig. 3A), whereas day 0 confluent cells showed a distinct cell-surface staining (Fig. 3B). Most maturing adipocytes (day 4) exhibited intense cytoplasmic ADAM12 staining, which was confined to small separate vacuoles that probably represented late endosomes or lysosomes (Fig. 3C). The number of adipocytes with intracellular ADAM12

Fig. 1. ADAM12 expression in adipose tissues. (A-D) ADAM12 expression was demonstrated by immunostaining (arrows) in white adipose tissue from a normal mouse (A), in a human lipoma (B), in mouse brown and white adipose tissue (C), and in a human hibernoma (D). (E) The expression of ADAM12 mRNA in mouse abdominal (Ab)-, epididymal (Ep)- white adipose tissue (WAT) and brown adipose tissue (BAT), was shown by northern blot analysis. Normal adult muscle and embryonic muscle (E17) were used as negative and positive controls, respectively. The expression of GAPDH was analyzed as a loading control. The brownish color represents the positive immunostaining reaction product, and the blue color is hematoxylin counterstaining. Bars, 20 μm (A,B) and 30 μm (C,D).
Fig. 2. ADAM12 mRNA expression is transiently upregulated before adipocyte differentiation. (A) Total RNA was extracted from mouse 3T3-L1 cell cultures at various times during differentiation. The levels of ADAM12, PPARγ and GAPDH mRNA expression were analyzed by northern blot. GAPDH and rRNA served as loading controls. (B) Quantitative analysis of ADAM12 and PPARγ mRNA expression, normalized to GAPDH mRNA levels. (C) Northern blot analysis of ADAM12 RNA extracted from human liposarcoma-derived LiSa-2 cells at various stages of differentiation. (D) ADAM12 protein expression was analyzed by western blot in extracts from growing 3T3H10T1/2, 3T3-L1 and LiSa-2 cells. CHO-K1 cells transfected with control vector and full-length mouse ADAM12 cDNA were used as negative (NC) and positive (PC) controls, respectively. D0, day 0 confluent cells; D3, D4, D9 and D17, days 3, 4, 9 and 17 after the onset of differentiation, respectively; G, growing fibroblastic cells.

immunostaining decreased in later stages of differentiation (after 6-8 days, data not shown).

To more rigorously establish the cell-surface localization of ADAM12, suspended cells were immunostained for ADAM12 without previous permeabilization (Fig. 3D-F). Growing cells had little or no detectable cell-surface immunostaining (Fig. 3D). By contrast, day 0 cells had distinct, evenly distributed cell-surface staining (Fig. 3E). During the adipogenic stage (the 4 days following induction), maturing adipocytes exhibited cell-surface staining that was clearly fainter than the staining of day 0 cells (Fig. 3F). A similar temporal pattern of ADAM12 expression was observed in mouse C3H10T1/2 and in human LiSa-2 cells (data not shown).

The dynamic subcellular localization of ADAM12 during adipogenesis indicated that the protein is endocytosed after the onset of differentiation. To test this hypothesis, anti-ADAM12 IgG (a mixture of mAbs, 6E6, 6C10, 8F8; 7.5 µg total) or control IgG (7.5 µg) was incubated with growing 3T3-L1 cells. After 5 hours, 2 days or 6 days, the cultures were rinsed, fixed, permeabilized and stained with secondary antibody to detect the ADAM12:IgG complex (Fig. 3G-I). No ADAM12:IgG was detected in growing cell cultures (Fig. 3G); however, cell-surface ADAM12:IgG was detected in confluent preadipocytes (Fig. 3H). In day 4 cultures, ADAM12:IgG was located intracellularly (Fig. 3I). No intracellular staining was observed when control IgG was added (data not shown). These results suggest that ADAM12 is, in fact, endocytosed during adipocyte differentiation.

Together, these results indicate that the subcellular distribution of ADAM12 is highly regulated: ADAM12 is translocated to the cell surface of preadipocytes before the onset of differentiation, and internalized during adipogenesis.

Formation of ADAM12/β1 integrin complexes and reduced β1 integrin activity as assessed by 12G10 immunostaining upon ADAM12 cell-surface localization

Under nonadipogenic conditions, fibronectin in the ECM (Fig. 4A) and intracellular actin stress fibers in preadipocytic cells (Fig. 4B) appear to be arranged in parallel, most probably with β1 integrins as coordinating matrix points at the cell surface (Fig. 4C). Because ADAM12 can interact with β1 integrins in trans, i.e. ADAM12 present on one cell may bind to the integrin present on another cell (Eto et al., 2000), we hypothesized that ADAM12 itself could be part of a β1 integrin cell-adhesion complex and that this might influence the adipogenic differentiation pathway. To test this hypothesis, we performed co-immunoprecipitation experiments using antibodies against ADAM12 followed by western blotting with antibodies against β1 integrin. As shown in Fig. 4D, ADAM12 and β1 integrin formed complexes in LiSa-2, C3H10T1/2 and 3T3-L1 cells.

The localization pattern of β1 integrin at the cell surface was examined by immunostaining and fluorescence-activated cell sorter (FACS) analysis (Fig. 5). Growing LiSa-2 cells showed prominent β1 integrin cell-surface immunostaining using antibodies against the activated human β1 integrin (12G10) (Fig. 5C). Less intense β1 integrin immunostaining was detected on confluent day 0 cells stained in parallel (Fig. 5D), which was confirmed by FACS analysis (Fig. 5G). There was little or no detectable difference in the total amount of β1 integrin as determined by immunostaining using the K20 antibody (Fig. 5E,F), or by FACS analysis using the MAR4 antibody against β1 integrin (Fig. 5H).

These results indicate that upon ADAM12 cell-surface expression, β1 integrin/ADAM12 complexes are formed, which appears to result in reduced β1 integrin activity. To assess the consequences of reduced β1 integrin activity, potential changes in cell shape, actin cytoskeleton organization, cell adhesion, organization of ECM and cell survival were evaluated.

Cell-surface ADAM12 induces actin cytoskeleton re-organization

Concomitant with the onset of adipocyte differentiation, the fibronectin-rich ECM becomes degraded, the actin cytoskeleton undergoes a dramatic reorganization and the cell...
shape changes (Cornelius et al., 1994). Growing preadipocytes have a fibroblastic morphology and exhibit an elaborate network of stress fibers (Fig. 6A). At day 0 the preadipocytic cells are closely packed, and some begin to round up and lose their actin stress fibers (Fig. 6B). Within 4 days after induction of differentiation, maturing adipocytes are round, the actin stress fibers have disappeared and have reorganized into a cortical actin network located just beneath the cell membrane (Kanzaki and Pessin, 2001) (Fig. 6C). To determine whether ADAM12 influences the early changes in the organization of the actin cytoskeleton, growing 3T3-L1 preadipocytes were transfected with ADAM12-Dcyt. This construct allows the efficient translocation of ADAM12 to the cell surface in contrast to the full-length ADAM12-L construct, which results in a predominantly intracellular accumulation of protein (Hougaard et al., 2000). ADAM12-Dcyt, therefore, provides a good model to study the effect of ADAM12 at the cell surface. Two days after transfection with ADAM12-Dcyt, the cell morphology had changed dramatically. Transfected cells were smaller, and more rounded. They retracted their cytoplasm, and their actin stress fibers gradually disappeared and were replaced, first by dot-like actin elements, then by a distinct cortical localization of actin (Fig. 6D-F). Cells transfected with a protease-deficient ADAM12-Dcyt construct (ADAM12-Dcyt cat mut) had the same morphological appearance as cells transfected by ADAM12-Dcyt, i.e. the actin stress fibers in these cells disappeared and re-organized into a cortical localization (Fig. 6G-I). Cells transfected with a control EGFP vector maintained their stress fibers and did not exhibit morphological changes (Fig. 6J-L).

To test whether endogenous ADAM12 in preadipocytes influences reorganization of the actin cytoskeleton, we used an RNAi strategy to reduce ADAM12 expression. Confluent 3T3-L1 preadipocytes were microinjected with ADAM12 or scrambled siRNA oligos together with EGFP to identify injected cells, and differentiation was induced the following day. Distinct upregulation of ADAM12 at the cell surface before onset of 3T3-L1 adipocyte differentiation. Growing cells are shown in (A) and (D), confluent (day 0) cells in (B) and (E), and maturing cells after 4 days of differentiation in (C) and (F). (A-C) Adherent cells were immunostained after permeabilization. (D-F) Cells were immunostained in suspension without previous permeabilization, then subjected to cytospin centrifugation, as described in Materials and Methods. (G-I) ADAM12 mAbs were added to the cultures for 5 hours (G) 2 days (H) and 6 days (I), followed by detection of the ADAM12-IgG complex with a secondary Ab to visualize endocytosed ADAM12. The central red staining in the cells in panels A and B represent nonspecific nuclear immunoreactivity. Bars, 18 μm (A,B,D,E,G,H) and 30 μm (C,F,I).
Cells with increased cell-surface ADAM12 expression are less adhesive, are more prone to apoptosis and have a reorganized extracellular fibronectin matrix

We used retrovirial transduction to establish a 3T3-L1 cell line that constitutively expressed ADAM12-Δcyt at the surface (Fig. 8A,B), and confirmed protein expression by western blot (Fig. 8C). These cells, like preadipocytes transiently transfected with ADAM12-Δcyt (see Fig. 6D), showed fewer stress fibers compared with cells infected with a control vector (Fig. 8D,E). Compared with the control cells, a significantly higher percentage of the ADAM12-Δcyt-expressing cells had fewer than five stress fibers (Fig. 8F). As shown in Fig. 8G-I, the ADAM12-Δcyt-expressing cells also exhibited a marked reduction in vinculin-positive focal adhesions, suggesting reduced β1 integrin-mediated adhesion activity when ADAM12-Δcyt is expressed at the cell surface. We therefore tested the capacity of these cells to attach to 9EG7 mAb, to mouse β1 integrin (data not shown) and to fibronectin (Fig. 8I) in cell attachment assays. The 3T3-L1 cells expressing ADAM12-Δcyt were significantly less adherent than 3T3-L1 cells that did not express ADAM12-Δcyt (Fig. 8J). The same results were obtained with C3H10T1/2 cells infected with ADAM12-Δcyt (data not shown). These results led us to test whether ADAM12-Δcyt might also influence β1 integrin-mediated cell survival pathways. As shown in Fig. 9A,B, 3T3-L1 cells overexpressing ADAM12-Δcyt are more sensitive to TNF-α-induced apoptosis than are control cells. It should be noted that this effect of ADAM12-Δcyt was also observed in other cell types, including CHO-K1 cells (U.M.W., unpublished). The increased sensitivity to apoptosis was prevented by the β1 integrin-activating mAb 9EG7 (Fig. 9C).

Finally, the organization of the pericellular fibronectin-rich ECM was analyzed in 3T3-L1/ADAM12-Δcyt cells. We found that the fibronectin-matrix of these ADAM12-Δcyt-expressing cells no longer appeared as an extensive, dense network, as in the control fibroblastic preadipocytes (Fig. 9E), but instead was distributed as a dense basement membrane-like rim encircling the cell borders (Fig. 9D).

Together, these results indicate that ADAM12-Δcyt induces a negative regulation of typical β1 integrin functions.

**ADAM12 increases the Triton X-100 solubility of β1 integrin**

It is well known that integrins associate with the actin cytoskeleton and modulate cell shape – for example, during formation of filopodia and lamellipodia. The findings described above suggest that ADAM12 induces changes in the association between β1 integrin and the actin cytoskeleton. To test this hypothesis, the Triton X-100 extractability of β1 integrin was examined. We used two different experimental procedures: one was the standard 0.5% Triton X-100 in CSK buffer and the other was 0.01% Triton X-100 in DMEM. Under both experimental conditions ADAM12-Δcyt-expressing 3T3-L1 cells exhibited a dramatic reduction of β1 integrin immunostaining (Fig. 10A,E) compared with control 3T3-L1 cells, which exhibited intense β1 integrin immunostaining (Fig. 10B,F). The β1 integrin immunostaining in untreated ADAM12-Δcyt-expressing 3T3-L1 and control cells are shown in Fig. 10C,D for comparison. Triton X-100 solubility of β1 integrin is another demonstration of how overexpression of
ADAM12 mimics the dynamic changes that accompany early adipocyte differentiation (Fig. 10G,H).

**Discussion**

Changes in morphology are a hallmark of terminal differentiation. The present study provides novel insights into the role of ADAM12 at the critical stage of early adipogenesis in which fibroblastic preadipocytes change morphology and commence differentiation to mature adipocytes. We have provided direct evidence that ADAM12 induces alterations in the actin cytoskeleton, cell adhesion, cell survival and the fibronectin-rich ECM. Our data furthermore suggest that these effects of ADAM12 are mediated by inhibition of β1 integrin function.

Several studies have pointed to an important role of ADAMs during mesenchymal cell differentiation (Gilpin et al., 1998;
ADAM12 and the actin cytoskeleton

Yagami-Hiromasa et al., 1995). In the mouse embryo, ADAM12 is most prominently expressed in condensed mesenchymal cells in regions of muscle and bone formation, including cranial membranous bones, ribs and limbs, and in the bone marrow (Kurisaki et al., 1998). ADAM12 expression is not detectable in adult skeletal muscle; however, expression is upregulated during muscle regeneration (Galliano et al., 2000). Interestingly, ADAM12 has been shown to alleviate the pathology of the dystrophin-deficient mdx mice (Kronqvist et al., 2002). Furthermore, transgenic mice overexpressing ADAM12 show increased adipogenesis (Kawaguchi et al., 2002), whereas ADAM12-deficient mice showed decreased interscapular brown adipose tissue (Kurisaki et al., 2003). In the present study we examined the expression profile of ADAM12 during adipogenesis. We found that ADAM12 mRNA was detected throughout most stages of adipogenesis but was present at maximal levels in confluent preadipocytes just before the onset of differentiation. These committed preadipocytes were characterized by increased levels of ADAM12 immunostaining at the cell surface that was present in complexes with β1 integrin. ADAM12 mRNA expression decreased in later stages of adipogenesis, and ADAM12 located at the cell-surface disappeared, which was most probably due to endocytosis.

Adhering fibroblastic cells, including preadipocytes, are characterized by an elaborated network of stress fibers and focal adhesions and show an intense cell-surface β1 integrin immunostaining with 12G10 mAb. As preadipocytes become confluent and round up, their stress fibers become reorganized into a cortical network and their adhesion to the ECM is weakened. This is thought to be crucial for the subsequent induction of adipogenic transcription factors. Direct evidence
that ADAM12 is critically involved in the process of reorganization of stress fibers was obtained from experiments in which ADAM12-D cyt was overexpressed in 3T3-L1 preadipocytes by either transient transfection or by retroviral transduction. Thus, fibroblastic cells overexpressing ADAM12-D cyt attained a rounded morphology, and their actin stress fibers disappeared and reorganized into a distinct F-actin cortical localization, even before they reached confluence. Furthermore, we found that 3T3-L1 preadipocytes microinjected with ADAM12 siRNA oligos maintained their stress fibers even 4 days after the induction of adipogenic differentiation.

We hypothesized that the effect of ADAM12-D cyt on the actin cytoskeleton is mediated through β1 integrin, and therefore asked whether other typical integrin-mediated functions were also altered. We found that ADAM12-expressing cells had decreased 12G10 immunostaining at the cell surface, had fewer vinculin-positive focal adhesions and adhered less efficiently to β1 integrin antibodies and fibronectin. Even integrin-mediated cell-survival pathways appeared to be altered – that is, cells overexpressing ADAM12-D cyt were more prone to TNFα-induced apoptosis (Fig. 9) as well as apoptosis induced by ultraviolet light and thapsigargin (U.M.W., unpublished). The finding that 9EG7 mAb could prevent TNF-α-induced apoptosis in 3T3-L1 cells presents strong evidence that this effect of ADAM12-D cyt is, in fact, mediated through β1 integrin.

In other cell systems, it has been shown that the interaction between ADAMs and β1 integrin is mediated through the disintegrin or cysteine-rich domains. For example, the disintegrin domains of ADAM2 and 9 supports cell adhesion through α6β1 integrin (Chen et al., 1999; Nath et al., 2000), and that of ADAM12 through α9β1 integrin (Eto et al., 2000). The recombinant cysteine-rich domain of ADAM12, however, interacts with syndecans and mediates integrin-dependent cell spreading (Iba et al., 2000). The intriguing role of the cysteine-rich domain was elucidated further in a recent study on ADAM13 (Smith et al., 2002) in which the authors showed that this domain could cooperate intramolecularly with the metalloprotease domain to regulate its function in vivo. Further studies are needed to determine which of these adhesion domain(s) of ADAM12 are involved in mediating β1 integrin-mediated changes in cell behavior.

ADAM12 induced a reorganization of the fibronectin-rich ECM – that is, preadipocytes overexpressing ADAM12-D cyt showed reduced fibronectin-rich ECM that was organized into a basement membrane-like rim encircling the cells while control preadipocytes maintained an elaborate meshwork of fibronectin-rich ECM covering the cells. This is an important finding as reduction of the fibronectin-rich ECM is a crucial determinant for adipose differentiation. The role of the ECM in adipogenesis was first suggested in 1983 when it was reported that adipogenesis was inhibited in preadipocytes grown on fibronectin-coated dishes (Spiegelman and Ginty, 1983). Subsequent studies showed that transforming growth factor (TGF)-β inhibits adipogenesis by increasing the expression of fibronectin and collagen (Bortell et al., 1994). More recent studies have pointed to an important role for matrix metalloproteases (MMPs)-2 and -9 and plasmin in promoting adipogenesis by degrading the fibronectin-rich ECM (Alexander et al., 2001). Although, on the basis of the studies presented here, we suggest that the effect of ADAM12 on the reorganization of the fibronectin-rich ECM is mediated via the reduced activity of β1 integrin, we cannot exclude the contribution of the metalloprotease activity of ADAM12. Little is known about the physiological substrate for the ADAM12 protease, although it was recently proposed to cleave heparin-
binding epidermal growth factor-like growth factor (HB-EGF) in the mouse heart (Asakura et al., 2002). We have previously shown that human ADAM12-S can degrade insulin-like growth factor binding protein (IGFBP)-3 and IGFBP-5 (Loechel et al., 2000; Shi et al., 2000). Transgenic mice expressing a form of ADAM12-S lacking the prodomain and metalloprotease domains (called ADAM12-S minigene) did not exhibit increased adipogenesis (Kawaguchi et al., 2002), suggesting a role for the protease activity of ADAM12 in adipogenesis.

The data presented here strongly suggest that ADAM12 may directly or indirectly influence the function of β1 integrin. The exact molecular mechanisms involved, however, warrant further consideration. We found that β1 integrin co-immunoprecipitates with ADAM12, suggesting that these two proteins can be part of the same adhesion complex. This indicates that ADAM12 in-cis could modulate β1 integrin function through lateral associations, and this further raises the interesting question as to whether ADAM12 may induce a redistribution of β1 integrins in the cell membrane, i.e. out of the rafts or caveolae microdomains. In this context, the recent demonstration that an extracellular matrix component, laminin-2, could induce a colocalization of α6β1 integrins and platelet derived growth factor (PDGF)αR into the same lipid rafts during oligodendrocyte differentiation is of interest (Baron et al., 2003). The exact localization of ADAM12 in the cell membrane is not yet known, but ADAM10 (α-secretase) seems not to be present in lipid microdomains (Kojro et al., 2001).

Even if the exact localization of ADAM12 and β1 integrin in the membranes of these cells is not yet determined, it was observed that the effect of the association between the two proteins was to disengage β1 integrin from the actin cytoskeleton. This conclusion is based on the striking finding that 0.5% Triton X-100 extracted β1 integrin from ADAM12Δcyt-expressing 3T3-L1 cells but not from control cells.

The investigations described in this study focused on the role of ADAM12 in early adipogenesis before the onset of terminal differentiation. However, the ADAM family contains at least 33 members, and it is likely that other ADAMs function in parallel with ADAM12 in a synergistic or antagonistic fashion. Preliminary RT-PCR data showed that ADAM9, 10, 17 and 19 are also expressed in the 3T3-L1 cells during adipogenesis, and additional immunohistochemical data demonstrated the expression of ADAM15 (U.M.W., unpublished). The finding that ADAM12 null mice do not exhibit striking defects in the white adipose tissue (Kurisaki et al., 2003) further suggests functional overlap among the ADAMs. Additional studies, including those using double-knockout technology, are needed to definitively determine the contribution of other ADAMs during adipogenesis.

In conclusion, the findings reported here show that ADAM12 induces reorganization of the actin cytoskeleton, reduced cell adhesion, changes in cell survival pathways and reorganization of the fibronectin-rich ECM in fibroblastic preadipocytes. Furthermore, our observations strongly suggest that ADAM12 at the cell surface mediates its effects via a direct or indirect interaction with β1 integrin that results in an impairment of β1 integrin function, possibly by decreasing the association of β1 integrin with the actin cytoskeleton. These results may have implications, not only for adipocyte differentiation and maturation, but also for mesenchymal cell differentiation in general.

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