Mesangial cells (MC) occupy the core of the renal glomerulus and are surrounded by a mesangial matrix. In certain diseases, MC migrate through this matrix into the pericapillary space. The mechanisms involved, however, are poorly understood. Members of the ADAM (A Disintegrin And Metalloproteinasenase) family of membrane proteins have the potential to be key modulators of cell-matrix interactions through the activities of their constituent domains. We have studied the possible role of ADAM 15 in human (H) MC migration in vitro. HMC ADAM 15 was expressed at low levels in serum-free medium but was increased during migration. Antibodies to the individual domains of ADAM 15 and the incorporation of antisense ADAM 15, (but not control oligonucleotide) inhibited this migration. Furthermore, inhibition of migration by the broad spectrum metalloproteinase inhibitor BB3103, demonstrated that metalloproteinase activity was essential for migration. ADAM 15, extracted from HMC membranes, was an active metalloproteinase, which degraded both type IV collagen and gelatin prepared from fibrillar collagen. Activity was inhibited by EDTA but not by phenylmethylsulfonyl fluoride. This is the first report of the potential of ADAM 15 for involvement in the restructuring of the mesangial matrix and in the migration of MC in disease.

Mesangial cell migration through the mesangial matrix and into the pericapillary space is a feature of a number of renal diseases, including mesangiocapillary glomerulonephritis (1). In addition, it appears that extracellular matrix components such as fibronectin (2), thrombospondin (3), and heparin-like glycosaminoglycans (4) can modulate this migration. This movement of cells must involve disengagement from and remodeling of the surrounding extracellular matrix. Possible mediators for this remodeling include a variety of serine proteinases and matrix metalloproteinases (MMPs)1 but also the newly described ADAM family of molecules.

The ADAMs are a family of cell surface molecules that possess both disintegrin and MMP domains. The disintegrin domain of these molecules resembles the sequence of the snake venom disintegrins and binds to integrins on the cell surface.

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1 The abbreviations used are: MMP(s), matrix metalloproteinase(s); ADAM, a disintegrin and metalloproteinase; HMC, human mesangial cell; FCS, fetal calf serum; GST, glutathione S-transferase; PMSF, phenylmethylsulfonyl fluoride; RT, reverse transcription; APMA, p-aminoethylmercuric acetate; RANTES, regulated on activation normal T cell expressed and secreted.

The Role of ADAM 15 in Glomerular Mesangial Cell Migration*

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present report examines the expression of ADAM 15 by human mesangial cells in vitro and its involvement in the migration of these cells following injury.

EXPERIMENTAL PROCEDURES

**Human Mesangial Cell (HMC) Culture and Identification**—Human glomeruli were obtained by the serial sieving of normal human kidney cortex recovered at nephrectomy. HMC were maintained in RPMI 1640 containing 20% v/v FCS. The cells were confirmed as mesangial cells by morphology and by the use of immunohistochemistry as previously described (19). Mesangial cells showed positive staining for intracellular myosin filaments and were negative for factor VIII and cytokeratin. All experiments were repeated using cells from at least three different sources. Before experimental procedures HMC were growth-arrested for 48h by culture in medium containing 0.2% w/v lactalbumin hydrolysate in the absence of serum (20).

**Fusion Protein Source and Synthesis**—ADAM 15 cytoplasmic/GST fusion protein plasmid was kindly provided by Dr. Carl Blobel, Sloan-Kettering Institute, Memorial Sloan-Kettering Cancer Centre, New York. This protein was purified by the use of glutathione beads, characterized, and used to raise antisera by standard techniques.

**Preparation of anti-ADAM 15 Antibodies**—Antibodies were raised in rabbits to each of the individual domains (cytoplasmic, metalloproteinase, and disintegrin) of the ADAM 15 molecule. Synthetic peptides corresponding to these domains (6) were synthesized (Enzyme Research Laboratories Ltd., Swansea, UK) and used to immunize rabbits by intraperitoneal injection. cDNA was prepared by the reverse transcription of 1 μg of total RNA using random primers, and the equivalent of 0.05 μg was amplified by PCR using primers specific for ADAM 15 or α actin (15). The following primer sequences were used: ADAM 15, 5′-CCGACGGGCCCCTGGGAAAGAGG-3′ and 5′-GCTGGGCATAGGAGGCACAAC-3′; α actin: 5′-GGAGCAATGATCTTGATCTT-3′ and 5′-GCCCTCCGAAAATGCATGGG-3′.

**Northern Blot Analysis**—Total RNA (up to 40 μg) was run on a denaturing gel and transferred by vacuum blotting onto a nylon membrane (Amersham Biosciences, Hybond N+, Amersham Biosciences, Buckingham, UK) and used to detect the presence of ADAM 15 mRNA by hybridization with a 32P-labeled probe prepared from PCR products and detected as previously described (15).

**Cell Migration Assay**—HMC were sub-cultured into gridded (2-mm square) 35-mm Petri dishes (Nunc International) and grown to confluence. Cells were growth-arrested and then carefully removed from more than half of the dish, up to the edge of a line of grids, using a cell scraper. The culture was then continued for a further 96 h in the absence of FCS or for 48 h in the presence of FCS at which time the cells were fixed with paraformaldehyde (3% v/v) and stained with a solution of crystal violet (0.5% w/v) for 3 mins. The cells were washed thoroughly with phosphate-buffered saline, and the number of cells that had migrated into the squares adjacent to the line of origin was determined.

To examine the effect of MMP inhibition on mesangial cell migration, the hydroxamine inhibitor BB-3103 (British Biotech Pharmaceuticals Ltd, Oxford, UK), prepared in Me2SO, was initially added to the cells at a range of concentrations from 100 nM to 10 μM. For the subsequent inhibition experiments, the inhibitor was used at a concentration of 1 μM for 48 h at which time there were no toxic effects on the cells. To determine the effect of the anti-ADAM antibodies on HMC migration, the affinity-purified antibodies were added at a concentration of 2 μg/ml to the dishes following the removal of cells, and the migration was determined as before. To collect RNA and cellular protein from the maximum number of migrating cells, this cell migration protocol was subsequently modified to allow the infliction of multiple scratches using a 10-mm false floor on confluent monolayer cultures of HMC in 35-mm Petri dishes as described by Kinsella and Wight (22).

In a second migration system, HMC were seeded into 8-well chamber slides (Permanox Slides, Lab-Tek) and grown to confluence. Cells were growth-arrested as before and an area of each well was then carefully denuded of cells using a plastic pipette tip. The migration of the cells into the denuded area was then followed by time-lapse photography, and the area covered by the migrating cells was measured using an “Openlab” software package. (Improvement, UK).

A third method of studying cell migration was used in experiments to analyze the effect of antisense ADAM 15 oligonucleotides. This migration assay was performed as described by Belien et al. (23) using a “cell sedimentation manifold” (CSM Inc., Phoenix, AZ). Ten-well Teflon printed microscope slides were precoated with 1% bovine serum albumin in phosphate-buffered saline for 30 min at room temperature before use and stored at 4 °C. Medium (50 μl) was added to each well, and the cell sedimentation manifold was placed on the slide. Two thousand cells were added in a volume of 1 μl to each chamber and allowed to sediment and adhere to the slide for 1 h on ice then overnight at 37 °C. The manifold was then removed, and the medium was aspirated and replaced with 40 μl of fresh medium with the presence of the test reagents. The area covered by cells was recorded at regular intervals, and the ratio of each area to that at the time of addition of the test materials was quantified by image analysis as above.

**Effect of Antisense ADAM 15 Oligonucleotides on Migration**—Antisense ADAM 15 oligonucleotides together with matched controls were purchased from Biognostic (TCS Biologicals, Buckingham, UK) and treated with 12% SDS-PAGE and transferred onto nitrocellulose membrane. ADAM 15 was then detected with purified antibody and visualized by ECL (Amersham Biosciences).

Cell Proliferation—Cell proliferation was assessed in two ways. (i) Cytoskeletons were grown either in the presence of serum or in serum-free conditions for 48 h, and BrdUrd (Sigma) was added to the cells at a concentration of 0.1 mM in medium, and the incubation continued at 37 °C. The amount of BrdUrd incorporated into cell DNA was determined by the addition of mouse anti-BrdUrd antibodies (Amersham Biosciences), followed by two cycles of anti-mouse immunoglobulin secondary antibodies and the application of APAAP complex (Dako Ltd., Cambridgeshire, UK), and finally visualized by staining with Fast Red. The culture was then continued for a further 96 h in the absence of FCS or for 48 h in the presence of FCS at which time the cells were fixed with paraformaldehyde (3% v/v) and stained with a solution of crystal violet (0.5% w/v) for 3 mins. The cells were washed thoroughly with phosphate-buffered saline, and the number of cells that had migrated into the squares adjacent to the line of origin was determined.

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added at a concentration of 2 μM to confluent HMC in 8-well chamber slides. The efficiency of transfection was determined using a fluorescein isothiocyanate-labeled oligonucleotide. This demonstrated >90% uptake of the oligonucleotide within 8 h. For experimental procedures the medium containing the oligonucleotide was therefore aspirated after 8 h and an area of cells was removed as before. The remaining cells were then washed, and the oligonucleotide-containing medium was replaced in the well. The effect of the antisense oligonucleotide compared with a matched control oligonucleotide was determined by time-lapse photography as described. In additional experiments, the effect of the antisense ADAM 15 oligonucleotide on migration was studied in the cell sedimentation manifold system as described, using the same protocol.

Collagen Degradation Assay—Affinity-purified ADAM 15 (at concentrations up to 1.111 μg) was incubated with Collagen IV (10 μg) (Sigma) in vitro at 37 °C for up to 96 h. The reaction was carried out in a final volume of 25 μl, containing 5 μl of assay buffer (400 mM Tris, 10 mM Ca²⁺, pH 8.0) in the presence or absence of the inhibitors EDTA (10 mM), PMSF (10 μg/ml), or leupeptin (2 μg/ml). At the end of the incubation period the reaction was terminated by addition of reducing PAGE buffer and boiled and separated on a 7.5% polyacrylamide gel. The degradation of collagen was assessed by Western blotting of the gel using rabbit anti-human Type IV collagen antibody (ICN, Basingstoke, UK) and visualized by ECL (Amersham Biosciences, Bucks, UK).

Zymography—Zymography of ADAM 15 was carried out as previously described (15) using 7.5% polyacrylamide gels incorporating gelatin at a concentration of 1 mg/ml. In some experiments APMA (p-aminophenylmercuric acetate) (Sigma) was preincubated with the ADAM 15 at a concentration of 1 mM to investigate whether ADAM 15 was present as a latent MMP. Proteolytic activity was demonstrated by zones of lysis in the Coomassie Blue-stained gel.

Metalloproteinase Activity Assay—Affinity-purified ADAM 15 (at concentrations up to 1.111 μg) was incubated with the fluorogenic substrate Dnp-Pro-β-cyclohexyl-Ala-Gly-Cys(Me)-His-Ala-Lys(N-Me-Abz)-NH₂ (product M-2055 (Bachem, Essex, UK) (10 μM) in a final volume of 200 μl in 200 mM Tris, pH 8.0, containing 5 mM CaCl₂ overnight at 37 °C. The proteolytic activity of the ADAM 15 was determined using a Denley Wellfluor fluorescence reader with an excitation wavelength of 360 nm and emission at 460 nm. To assess the inhibitory potential of the antibodies to the individual ADAM 15 domains, purified antibodies or control IgG were included in separate incubations, and the resulting proteolytic activity were compared with incubations in the absence of antibody.

FIG. 2. HMC express ADAM 15 mRNA and protein. HMC cultured either in serum-free medium (a) or 20% FCS (b) were lysed and extracted for mRNA analysis by RT-PCR using primers specific for ADAM 15 (A) or protein expression using SDS-PAGE and Western blotting with the anti-cytoplasmic domain antibody (B). Actin was used as the housekeeping gene for RT-PCR, and the PCR products were separated on 2% agarose gels. Results shown are representative of three separate experiments.

FIG. 3. Induction of ADAM 15 mRNA following multiscratch wounding. Multi scratch wounds were inflicted on HMC and RNA extracted at the indicated times. The RNA was then either amplified by RT-PCR and products separated in 2% agarose gels with actin as housekeeping gene (A) or analyzed by Northern blotting, with ethidium bromide staining of the gels to confirm equivalent loading as described under “Experimental Procedures” (B). Results shown are representative of five separate experiments.

FIG. 4. Time-dependent increase in ADAM 15 protein expression following multiscratch wounding of HMC. Cells were lysed at the indicated times, and the lysates were separated by SDS-PAGE as described. Western blotting was carried out using the anti-cytoplasmic domain antibody. Results shown are representative of five separate experiments.
RESULTS

Western Blot and PCR Analysis of ADAM 15 in HMC—Following extraction, ADAM 15 mRNA was shown by RT-PCR to be present in growth-arrested HMC and was up-regulated in the presence of serum (Fig. 2A). ADAM 15 protein was barely detectable by Western blotting in growth-arrested HMC lysates. Its expression was increased, however, by incubating the cells with FCS (Fig. 2B).

ADAM 15 Expression in a Multiscratch Model of Cell Injury—Following the induction of multiscratch wounds in confluent HMC monolayers, ADAM 15 mRNA levels increased rapidly to a maximum at around 4–6 h and remained elevated for up to 24 h (Fig. 3A). The RT-PCR data was confirmed by Northern blotting of total cellular RNA collected 3 h following the infliction of multiscratches (Fig. 3B). In addition, ADAM 15 protein expression was shown by Western blotting to be increased at 24 h (Fig. 4).

ADAM 15 Mediates HMC Migration—The potential role of ADAM 15 in cell migration was investigated using the domain-specific antibodies. In the presence of serum, the antibodies to the disintegrin and metalloproteinase domains significantly decreased HMC migration (Fig. 5).

To establish that the observed changes were not due to an effect on cell proliferation, HMC were stained with BrdUrd to identify the number of proliferating cells. The anti ADAM 15 antibodies had no effect on HMC proliferation. This finding was confirmed by assaying cell number using the MTT assay. These results suggested that ADAM 15 expression was essential for HMC migration. To confirm this, the effect of transfecting the cells with the antisense ADAM 15 oligonucleotide was determined. Initially migration was monitored in 8-well chamber slides by time-lapse photography. The antisense ADAM 15 oligonucleotide decreased the rate of migration relative to controls and to the matched control oligonucleotide, (which itself had no significant effect on migration) (Fig. 6). Migration was also inhibited after cell sedimentation, where the area of the expanding circle of cells was measured over time. There was a significant effect of the antisense ADAM 15 oligonucleotide relative to controls after 72 h of incubation, whereas the matched control oligonucleotide had no significant effect (Fig. 7).

RT-PCR for ADAM 15 in cells exposed to the antisense ADAM 15 oligonucleotide demonstrated a reduction in the amount of ADAM 15 mRNA relative to control cells (Fig. 8A). In addition, antisense ADAM 15 decreased the induction of ADAM 15 following multiscratching (Fig. 8B).

Metalloproteinase Activity Is Integral to HMC Migration—MMP activity is involved in migration in many cell systems. The effect on HMC migration, of inhibiting MMP activity, was examined using the hydroxamine inhibitor BB-3103. Preliminary experiments determined that the optimal effective non-toxic concentration of BB-3103 was 1 μM. At this concentration BB-3103 inhibited by 36% the number of cells migrating into 2-mm squares (Fig. 9). This supported previous reports of MMP involvement in migration (24), and the degree of inhibition observed was similar to the maximum achieved by incubation with the domain-specific antibodies to ADAM 15.

ADAM 15 Is a Metalloproteinase with Gelatinolytic Activity—The data presented above demonstrate a role for ADAM 15
in HMC migration and together with the BB3103 inhibition data suggest that the MMP domain of the molecule may be actively involved. The possibility that this domain is proteolytic, however, has not previously been addressed in any system. The first possibility examined was that ADAM 15 might act in a similar manner to the membrane type MMPs (MMP14–17) and convert other latent MMPs to an active form. Affinity-purified ADAM 15 was, therefore, incubated for up to 72 h with HMC-conditioned medium containing latent MMP2 (gelatinase A). The incubation mixture was then analyzed by gelatin zymography. No shift in the zone of lysis corresponding to the activation of latent MMP2 was observed. There was, however, an obvious zone of lysis in the non-reduced gel at a higher molecular size, which was present only in those lanes that contained the purified ADAM 15 (Fig. 10). This activity was not increased by prior incubation with APMA.

To confirm this proteolytic activity, affinity-purified ADAM 15 was incubated with Collagen IV in vitro at 37 °C for up to 96 h. There was a dose-dependent degradation of this basement membrane protein (Fig. 11), which was inhibited by the addition of EDTA (10 mM) but was not affected by the serine proteinase inhibitors, PMSF and leupeptin (Fig. 12).

Inhibition of ADAM 15 Proteolytic Activity by Anti-ADAM 15 Antibodies—Incubation of purified ADAM 15 with the fluorogenic substrate Dnp-Pro-β-cyclohexyl-Ala-Gly-Cys(Me)-His-Ala-Lys(N-Me-Abz)-NH₂ resulted in substrate cleavage, measured by strength of fluorescent emission at 460 nm. This cleavage was inhibited both by the antibody to the metalloproteinase domain of the ADAM 15 molecule and by the antibody to the disintegrin domain (Fig 13). No inhibition of proteolytic activity was seen when non-immune IgG was substituted for the antibodies in the incubations (not shown).

**Fig. 8. Effect of ADAM 15 antisense oligonucleotides on ADAM 15 mRNA expression.** A, HMC in serum-free medium were incubated alone (a), with the oligonucleotide control (b), or with ADAM 15 antisense oligonucleotides (c). After 8 h the RNA was extracted and RT-PCR was carried out using primers specific for ADAM 15. B, HMC in serum-free medium were left uninjured (a) or multiscratched (b–d), in the absence of oligonucleotides (b), in the presence of ADAM 15 antisense oligonucleotides (c), or in the presence of control oligonucleotides (d). The RNA was extracted 3 h following multiscratching, and RT-PCR was carried out using primers specific for ADAM 15. Actin was used as the housekeeping gene in all experiments, and the PCR products were separated on 2% agarose gels. Results are representative of three separate experiments.

**Fig. 9. The effect of the metalloproteinase inhibitor BB-3103 on HMC migration.** The number of HMC migrating into 2-mm grid squares following removal of half the monolayer was measured after 48 h in medium containing 20% FCS either in the absence (a) or presence (b) of 1 μM BB3103. The results are the mean of four separate experiments.

**DISCUSSION**

The role of the ADAM molecules in tissue homeostasis is poorly understood. These molecules have separate domains that would enable the cell to both interfere with integrin binding to the extracellular matrix and to degrade that matrix, suggesting a role in cell migration. However, their ability to influence cell migration has not previously been demonstrated. We have shown here for the first time that the ADAM 15 molecule may be involved in the migration of human mesangial cells following the initiation of migration in vitro.
purified from HMC lysates were incubated with 10 mM EDTA (\(\text{H9262}/\text{H9262}\)) for 18 h before electrophoresis. ADAM 15 in the presence of 1 mM APMA, MMP-2 were incubated together for 18 h before electrophoresis. c, ADAM 15 in the presence of 1 mM APMA. Results are representative of three separate experiments.

**Fig. 10.** The gelatinolytic activity of ADAM 15. ADAM 15, purified from lysed HMC, was analyzed by zymography in 7.5% acrylamide gels containing gelatin at 1 mg/ml as described. Following staining with Coomasie Blue clear bands on the gel demonstrated zones of proteolytic activity. a, MMP-2 derived from HMC-conditioned medium as positive control. b, ADAM 15 and MMP-2 were incubated together for 18 h before electrophoresis. c, ADAM 15 in the presence of 1 mM APMA. d, zymogram control mixture of active and latent MMP-2. Results are representative of three separate experiments.

**Fig. 11.** ADAM 15 degrades type IV collagen. Increasing amounts of ADAM 15, 0.055 \(\mu g\) (a), 0.277 \(\mu g\) (b), 0.555 \(\mu g\) (c), and 1.111 \(\mu g\) (d), purified from HMC lysates were incubated with 10 \(\mu g\) of collagen IV for 96 h. The mixture was then separated on a 7.5% polyacrylamide gel and transferred to nitrocellulose, and the collagen was visualized by immunoblotting with anti-collagen IV antibody as described.

**Fig. 12.** ADAM 15 is a metalloproteinase. Collagen IV (10 \(\mu g\)) was incubated at 37 °C for 72 h alone (a) or with ADAM 15 (1 \(\mu g\)) purified from HMC as above in the absence of proteinase inhibitors (b), in the presence of 10 mM EDTA (c), in the presence of 1 mM PMSF (d), and in the presence of 1 mM PMSF (e). The mixture was then separated on a 7.5% polyacrylamide gel, and the collagen was visualized by immunoblotting with anti-collagen IV antibody as described.

Low levels of ADAM 15 mRNA were detected by RT-PCR in HMC and were increased following exposure to serum. Subsequently, the levels of ADAM 15 mRNA and protein were both shown to increase following the initiation of migration, suggesting a degree of transcriptional up-regulation. The fact that the antisense oligonucleotide and antibodies raised against the ADAM 15 molecule nor the metalloproteinase inhibitor BB-3103 affected HMC proliferation as measured by labeling with BrdUrd. This was somewhat unexpected, as it has been previously reported that the metalloproteinase activity of MMP-2 is involved in the proliferation of mesangial cells (25). Our find-
ings were confirmed by MTT assay where there were no changes in cell number or viability following incubation with either the antibodies or the metalloproteinase inhibitor. These results confirm a separate series of studies suggesting that factors affecting proliferation in MC are separate from those that effect migration (26, 27).

The RGD integrin-binding sequence of ADAM 15 (18) is reported to facilitate the interaction of this molecule with both the αvβ3 and αvβ5 integrin (32, 33). Considered together with its proteolytic activity, this gives ADAM 15 the potential for involvement in cell migration at several levels. The migration of the mesangial cell from the glomerulus into the pericapillary space in diseases such as mesangiocapillary glomerulonephritis is a major feature of this disease. Little is known about the mechanism controlling the migration of the mesangial cells, although it has been reported that MC stimulated with a com-

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