Identification, Cellular Distribution and Potential Function of the Metalloprotease-Disintegrin MDC9 in the Kidney

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Abstract. The complex interactions of glomerular and tubular epithelial cells with the basal laminae play a critical role in renal function. Disruption of these interactions has been widely implicated in glomerular diseases and acute renal failure. MDC are a large family of membrane-bound proteins containing metalloprotease, disintegrin (integrin interaction sites), and cysteine-rich domains. Little information is available concerning the presence of MDC in the kidney or their role in renal pathophysiology. Using degenerate PCR primers for the conserved metalloprotease and disintegrin domains of this protein family, cDNA templates from tubules, whole glomeruli, and glomerular epithelial cells (GEC) yielded a single, 195-bp product, which on sequence analysis corresponded to a region in the disintegrin domain of MDC9. Northern analysis of poly(A)⁺ RNA from tubules, whole glomeruli, and GEC revealed a 3.9-kb transcript, identical to that of mouse MDC9. Using antibodies generated against a 21-amino acid peptide present in the metalloprotease domain of MDC9, Western analysis of concanavalinA-enriched glomerular microsomal extracts demonstrated both processed (76 kD) and unprocessed (116 kD) forms of MDC9, which upon reduction changed to the corresponding 84- and 124-kD forms. Histochemical studies revealed a basolateral localization of intrinsic MDC9 protein in renal cortical tubule cells and glomerular visceral epithelial cells, which colocalized with the β1 integrin chain. Expression of green fluorescence protein MDC9 chimeric constructs in GEC or polarized Madin-Darby canine kidney epithelial cells revealed a similar punctate basolateral surface localization. Transient overexpression of the soluble disintegrin-domain-green fluorescence protein chimera in GEC led to dramatic changes in cellular morphology with rounding and detachment from cell monolayers. These studies document the presence of MDC9 in renal epithelial cells and suggest an important role for MDC9 in renal epithelial cellular interactions with the basal lamina and adjoining cells.

The interactions of epithelial cells with the basal laminae of the renal tubules and glomerulus have been the object of intense scrutiny over the past two decades. These investigations have underscored the complexity of cell-cell and cell-matrix interactions and the relationship of these interactions to renal epithelial cell structure and function. Many of these studies have focused on renal epithelial cell expression and distribution of several distinct classes of adhesion molecules, including the cadherins (1–3) and the integrin family (4,5). Adler and Chen have elegantly described the role of α3β1 integrin, which is recognized by the anti-Fx1A antibody in glomerular epithelial cell (GEC) attachment to various extracellular matrix components (6).

Recent reports have documented the existence of a large family of membrane-bound proteins containing metalloprotease, disintegrin (integrin ligand), and cysteine-rich domains (7,8), referred to as MDC proteins (for metalloprotease-like, disintegrin, cysteine-rich). The MDC proteins have a broad tissue distribution and play important roles in several critical cellular processes, including sperm-egg interaction (9), myocyte binding and fusion (10), and neurogenesis (11). Only a few members of this family of proteins possess an active catalytic domain, and the role of this metalloprotease domain has mainly been elucidated as an “ectodomain sheddase” rather than degradation of the extracellular matrix components (12–14). The one exception is ADAM10, which cleaves type IV collagen in vitro (15). The disintegrin domains of molecules of this family have been implicated in cellular adhesion (9,10) and shown in the case of metargidin to interact with the integrin αvβ3 (16).

The combination of metalloprotease and disintegrin domains was first observed in soluble snake venom proteins, where autodigestion leads to the production of soluble disintegrins that can disrupt cell-cell and cell-basal laminae interactions by binding to integrins (17–19). These observations suggested that MDC may play a similar role in mammalian tissues.

Thus, the focus of the current study was to identify the MDC family proteins present in rat kidney and to investigate their...
potential role in modulation of interactions between glomerular and tubular epithelial cells with the basal lamina. We present evidence for the presence of MDC9 in rat kidney using a combination of biochemical and cellular approaches. We also present preliminary evidence for the role of the disintegrin domain in epithelial cell attachment to the basal laminae.

Materials and Methods

**Tissues and Cells**

Sprague Dawley rats (150 g body wt) were used as the source of renal cortical glomerular and tubular fractions using standard sieving techniques. The purity of each fraction was greater than 95% as judged by phase-contrast microscopy. GEC were prepared and extensively characterized as reported previously (20,21). Morphologic characterization included a polygonal shape with a cobble-stone growth pattern, while by scanning electron microscopy the cells were round-to-polygonal with large nuclei and numerous short, finger-like projections. Exposure of the cells to puromycin was cytotoxic. Immunofluorescence staining was positive for the Fx1A antigen and podocalyxin, while negative staining was observed for factor VIII and Thy1.1 antigens. GEC were grown in a defined, serum-free medium as reported (22). The polarized renal epithelial Madin-Darby canine kidney cell line (MDCK) was obtained from American Type Culture Collection (Manassas, VA) and maintained in minimal essential medium (Life Technologies) supplemented with 5% fetal bovine serum, 2m M -glutamine, and 10 mM Hepes, pH 7.4.

Cell and tissue (glomeruli, tubules) microsomal extracts were prepared by suspension in a lysis buffer consisting of 20 mM sodium borate, pH 8.0, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, 1 mg/ml aprotinin, 1 mM pepstatin, and 1 mM ethylenediaminetetra-acetic acid for 20 min at 4°C. Thereafter, the debris were removed by centrifugation at 5000 rpm, 1 mM leupeptin, 1 mg/ml aprotinin, 1 mM pepstatin, and 1 mM borate, pH 8.0, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and stored at 80°C until use. Protein determination was performed using the BCA assay (Pierce, Rockford, IL).

**Preparation of cDNA and PCR**

cDNA was reverse-transcribed from RNA isolated from renal tubules, glomeruli, and cultured GEC (Superscript kit, BRL). The nested PCR amplification strategy was as defined by Weskamp and Blobel (8) (Figure 1). PCR was performed with the following primer sets, using first-strand cDNA and Taq polymerase (Boehringer Mannheim). A sense primer (primer A) for the metalloprotease domain (5'-GAACNAYGARAGGNNCAAYA-3') and an antisense primer (primer C1) for the disintegrin domain (5'-CARTAYTCNG-GNARRTCRCA-3') were used in the first amplification reaction, followed by nested PCR on a 1:200 dilution of the primary reaction using the primer sets B2 (5'-GGNGARGAYTYGGAYTG-3') and C2 (5'-TAYTCNGGNNARRTCRCAYTC-3'). Primers B2 and C2 included EcoRI cloning sites to permit subcloning of the PCR products (195 bp) into plasmid pGEM-7zf (Promega, Madison, WI). Thirty individual plasmid clones from each cellular source were sequenced using the Taq-Tracks sequencing kit (Promega).

**Northern Blot Analysis**

Poly(A)+ RNA (10 µg) from isolated renal cortical tubules, glomeruli, and cultured GEC was electrophoresed on 1.25% denaturing agarose gels and transferred to nylon membranes. The blots were hybridized with the 195-bp nested PCR product labeled by incorporation of α-32P-dCTP (New England Nuclear, Boston, MA). After hybridization, the blots were washed with 0.1× SSC at 60°C for 1 h, followed by autoradiography.

**Preparation of Anti-MDC-9 Antibodies**

An anti-MDC rabbit antibody was prepared by immunization with a murine MDC9 metalloprotease domain synthetic peptide sequence, REKFLITRRHDSAQLVLKK coupled to keyhole limpet hemocyanin. IgG-enriched fractions from immunized and nonimmunized animals were prepared by standard methodology on diethylaminoethoxy-dextran columns. Immune IgG was subjected to peptide-affinity chromatography using the metalloprotease domain peptide coupled to SulfoLink gel (Pierce) according to the manufacturer’s instructions.

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**Figure 1.** Nested PCR strategy for recovery of MDC (metalloprotease-like, disintegrin, cysteine-rich) family members in the kidney. Total RNA from cortical tubules, glomeruli, and cultured glomerular epithelial cells (GEC) was reverse-transcribed and used as the template for nested PCR designed to amplify multiple members of the ADAM (a disintegrin and metalloprotease) family. Primer pair A/C1 was used in the first round of amplification, followed by a second round of amplification using primer pair B2/C2. The primer pair B2/C2 is designed to amplify within the disintegrin domain of the MDC molecules.
Western Blots
Concanavalin A (ConA)-enriched glomerular extracts were prepared by incubation of 200 μg of microsomal protein in 100 μl of a binding buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40) along with an equal volume of ConA-coupled agarose beads for 18 h at 4°C. The beads were thereafter washed in a large volume of binding buffer, followed by elution with 500 mM α-methyl mannoside. The eluates were electrophoresed in either reducing or nonreducing sample buffer and transferred to nitrocellulose membranes. The membranes were blocked in phosphate-buffered saline (PBS)/0.2% casein, followed by incubation overnight at 4°C in blocking buffer with 5 μg/ml affinity-purified anti-MDC9 metalloprotease IgG or an equivalent concentration of preimmune IgG. Thereafter, the blots were washed in blocking buffer and incubated for 2 h at 4°C with 2 μg/ml biotinylated F(ab’)2 goat anti-rabbit IgG. Finally, washed blots were incubated sequentially with avidin/biotin-conjugated alkaline phosphatase (Vector Laboratories, Burlingame, CA) and CDP-Star (Tropix, Bedford, MA) substrate according to the manufacturer’s specifications and exposed to x-ray film for 3 min.

Immunohistochemistry
Kidneys from euthanized Sprague Dawley rats were perfusion-fixed with 4% ice-cold buffered paraformaldehyde, cut into 5-mm blocks, and snap-frozen in liquid nitrogen-cooled isopentane. Frozen sections (5 to 6 μm) were fixed for 20 min with ice-cold 4% buffered paraformaldehyde and blocked with sequential applications of 5% normal goat serum in PBS, and avidin/biotin blocking solutions (Vector). Rinsed sections were then incubated overnight at 4°C with 5 μg/ml biotinylated F(ab’)2 goat anti-rabbit IgG. Finally, washed sections were incubated sequentially with avidin/biotin-conjugated alkaline phosphatase (Vector Laboratories, Burlingame, CA) and CDP-Star (Tropix, Bedford, MA) substrate according to the manufacturer’s specifications and exposed to x-ray film for 3 min.

Chimeric Green Fluorescence Protein/MDC-9 Constructs
A 2548-bp cDNA fragment consisting of the entire open reading frame of murine MDC9 was amplified by PCR from the full-length cDNA (23), using a 5’ primer (5’-ACTGGTCGACACCAT-GGGCCCGCGCGCTCTCG-3’) and a 3’ primer (5’-CAGTG-
GATCCGGGTAGGGGAGCTATATGAAGG-3') to permit subcloning of the PCR product into the SalI and BamHI cloning sites of the EGF-N1 (Clontech, Palo Alto, CA) expression vector, which contains an optimized variant of *Aequorea victoria* green fluorescence protein (GFP). The GFP component of this chimeric protein is located at the carboxy terminus of the MDC9 molecule.

A secreted MDC9 disintegrin domain/GFP fusion protein was prepared by PCR using the murine MDC-9 cDNA as a template, a 5' primer (5'-GCGTCTGACCCCAATCAGGCGGCTCCTG-3'), and a 3' primer (5'-ACGTGGATCCAAATGATTGGGCTG-3'). The PCR reaction yielded a 270-bp product encoding the MDC-9 disintegrin domain extending from amino acids 413 to 503. The disintegrin cDNA fragment was subsequently subcloned into a modified EGFP-N1 vector that included a 5' oncostatin M leader sequence cDNA to permit cellular secretion of the expressed fusion protein. Thus, this chimeric construct consists of the N-terminal oncostatin leader sequence, the disintegrin domain, and a carboxy-terminal GFP sequence. A secreted GFP control chimeric construct, i.e., lacking the disintegrin domain, was prepared in a similar manner.

Transient transfections of GEC with the respective GFP fusion constructs were performed using the DNA/diethylaminoethyl dextran method as modified by McMillan et al. (24), and the cells were examined by fluorescence microscopy at 48 h. Transient transfections of MDCK cells were performed with lipofectin (Life Technologies, Gaithersburg, MD), and the cells were examined by scanning confocal microscopy at 48 h.

**Results**

Identification of MDC9 in the Kidney

To identify the transcripts of the MDC family members in the kidney, we used a nested PCR approach with degenerate primers encoding highly conserved sequences in the disintegrin and metalloprotease domains present in previously defined members of the MDC protein family (8) (Figure 1). PCR was performed on cDNA templates prepared from cortical tubules, glomeruli, and cultured GEC and yielded in each case a 195-bp product (Figure 2). Thirty individual colonies of the subcloned PCR products from the glomerular, tubular, and GEC templates were sequenced. All sequences from the glomerular, tubular, and GEC templates were identical to the corresponding domain of the murine cDNA sequence for MDC9 (23).

Northern blot analysis, using the 195-bp rat nested PCR product as a probe, of RNA isolated from the respective tubular, glomerular, and GEC fractions (Figure 3) demonstrated a single transcript of 3.9 kb, consistent with the transcript size of MDC9 in human and murine tissues (23). Western blot analyses of ConA-enriched glomerular microsomal membrane extracts were carried out with the affinity-purified anti-MDC9 metalloprotease domain antibody. In nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis, two proteins with relative mobilities of 116 and 76 kD were observed (Figure 4). Inclusion of β-mercaptoethanol in the loading buffer changed the mobilities of the bands to 120 to 124 kD and 84 kD, respectively. No bands were observed with the preimmune control IgG. Similar results were reported by Weskamp et al. (23) and are in keeping with the 45 cysteine residues predicted from the cDNA sequence of murine MDC9.

**Immunohistochemical Localization of MDC9 in the Kidney**

Frozen sections of rat renal cortex were evaluated for the distribution of MDC9 protein, using both immunofluorescence and immunoperoxidase techniques. While preimmune IgG did not yield significant fluorescence signal (Figure 5A), incubation with the affinity-purified IgG raised against the anti-MDC9 metalloprotease domain yielded very bright glomerular and cortical tubular staining (Figure 5, B and C). The glomerular staining for MDC9 protein occurred within approximately 20 to 25 cells per 5-μm section, while cortical tubular staining was evident within all tubular segments. Staining was particularly intense within proximal tubular cells (which are the dominant segment shown in Figure 5C), and was distributed in a punctate, apparent basolateral pattern. To more precisely localize the cellular sites of MDC9 expression within the kidney, immunoperoxidase histochemistry was used in conjunction with Nomarski optics, which facilitates delineation of glomerular structure (25). Immunoperoxidase reaction product in the glomerulus (Figure 6A) was limited to the basal surfaces of visceral epithelial cells at the contact regions with the
glomerular basement membrane. No staining was detected within glomerular endothelial or mesangial cells. Immunoperoxidase staining of cortical tubular sections demonstrated reaction product concentrated along the basilar aspects of the cells (Figure 6B).

Given the punctate pattern of basolateral MDC9 staining and the association of MDC proteins with certain integrin types, we performed confocal laser microscopy on sections stained for MDC9 and the common renal epithelial integrin chain β1. As detailed in Figure 7, there is a very high correlation between MDC9 and β1 integrin staining in a punctate basolateral pattern, consistent with a heteromeric association of these molecules.

**Figure 5.** Immunofluorescence localization of MDC9 in the kidney. Frozen sections (5 μm) of rat renal cortex were incubated with preimmune IgG (A) or affinity-purified anti-MDC9 IgG (B and C) followed by sequential incubations with biotinylated secondary antibody and rhodamine-coupled streptavidin. No signal is detected with the preimmune IgG. Incubation with anti-MDC-9 IgG (A) yielded bright immunofluorescent staining of both glomerular and tubular structures (B). Within a section composed primarily of proximal tubules (C), anti-MDC9 staining is present in a punctate pattern that is particularly prominent within the basolateral infoldings of these cells. Magnification, ×300.

**Figure 6.** Immunoperoxidase staining for MDC9 in the kidney. Frozen sections of rat renal cortex were processed as described in Figure 5, with the exception that development used horseradish peroxidase histochemistry. The glomerular section (A) demonstrates a GEC (note large nucleus, extended cytoplasm) abutting the basal lamina of a glomerular capillary loop (CL). Dense peroxidase reaction product is limited to the basal surface of the GEC at points of contact with the underlying glomerular basement membrane (arrows). Immunoperoxidase reaction product is also concentrated on the basal surfaces of tubular cells from a cortical section (B). Magnification: × 900 in A; ×300 in B.

**Distribution of MDC9-GFP Chimeric Proteins in GEC and MDCK Cells**

To confirm the localization of MDC9 described using immunohistochemical methods, we transfected cultured GEC with an MDC9-GFP chimeric protein construct and compared the cellular distribution of this intrinsically fluorescence protein with cells transfected with GFP alone. Expression of GFP alone in GEC resulted in a bright cellular signal without concentration or localization within specific cellular compartments (Figure 8A). In contrast, the chimeric MDC9-GFP was concentrated on the periphery of the GEC and was clustered at sites of cell-cell contact (Figure 8B).

Cultured GEC are considerably dedifferentiated from their
in vivo counterparts and do not exhibit fully polarized features (26). Therefore, we repeated these transfections using the polarized renal tubular epithelial cell line MDCK and examined the transfectants by scanning confocal microscopy. Figure 9 shows a series of cellular sections extending from the apical to the basal aspect of MDC9-GFP transfected MDCK cells. Bright, punctate GFP signal is evident on the both the lateral and basal aspects of these cells. No GFP signal is present on the apical surfaces, confirming the observations obtained by the immunohistochemical analyses of whole cortical sections.

**Cellular Effects of MDC9-Disintegrin Domain Expression**

The disintegrin domain of the MDC family of proteins has been postulated to act as a potential integrin ligand to facilitate cell-cell or cell-matrix interactions. The cellular distribution of the intact MDC9 protein described above also supports a role for this protein in cellular interactions. To specifically evaluate the potential role of the MDC9 disintegrin domain in this activity, we constructed a secreted GFP chimera that included the 90-amino acid disintegrin domain and the oncostatin M
secretory sequence. Transfection of the oncostatin M-disintegrin-GFP construct into GEC had profound effects upon both cellular morphology and attachment to fibronectin matrices. Compared with oncostatin M-GFP controls (Figure 10A), oncostatin M-disintegrin-GFP–transfected cells demonstrated rounding up (Figure 10B), loss of cell-cell and cell-matrix contacts, and frequent detachment from the substratum (Figure 10C). These experiments suggest an important role for the disintegrin domain of MDC9 in mediating cell-cell and cell-matrix interactions of GEC.

Discussion

The primary goal of this study was to examine the cellular localization and potential function of MDC in the kidney. Our PCR-based strategy for the amplification of transcripts containing highly conserved components of the MDC family revealed a single gene product whose sequence corresponded to MDC9 from all studied templates. The presence of MDC9 was confirmed by Northern analysis and Western blotting. Other members of the MDC protein family reported in the kidney, including ADAM10 and metargidin (15,27), were not detected. This may be related to significant differences in sequence at the priming sites or the relative abundance of the MDC9 transcript.

A significant proportion of MDC9 protein detected in freshly prepared glomerular or tubular extracts was 124 kD in size, indicating, within the context of the kidney, that MDC9 is present in a precursor form. Overexpression of MDC family proteins MDC9 (28), metargidin (29), and ADAM12 (30) in COS cells yields primarily the processed forms. This processing has been ascribed to intracellular conversion by furin or related endopeptidases. We observed, based on the Western analysis, that MDC9 isolated from renal tissues exists in both the precursor and processed forms. The precursor material detected by this method presumably represents newly synthesized protein on its way through the secretory apparatus.

The immunohistochemical studies reported here demonstrate a specific cellular distribution of MDC9 protein. The protein is present in all segments of the renal tubule and is
concentrated in a punctate manner on the basolateral surfaces of these cells. Furthermore, MDC9 expressed by glomerular visceral epithelial cells in vivo is limited to the areas of contact between the cell and the underlying glomerular basement membrane. This pattern of distribution is most consistent with a role of MDC9 in epithelial cell-cell or cell-matrix interaction. The disintegrin components of snake venoms are defined by their interactions with integrin proteins (18,19). It has been suggested that the disintegrin-like domains of the MDC proteins serve a similar function (9,10). This recently was confirmed in the case of metargidin, in which the disintegrin domain specifically binds to the \( \alpha v \beta 3 \) integrin (16). The dual labeling studies of renal cortex for MDC9 and the \( \beta 1 \) integrin proteins revealed a very high degree of colocalization, consistent with a specific MDC9-\( \beta 1 \) integrin interaction on the surface of renal epithelial cells.

Cultured GEC express only the \( \alpha 3 \beta 1 \) integrin, which has been shown to mediate attachment of these cells to laminin, fibronectin, and type IV collagen (31,32). Transfection of GEC cultured on fibronectin matrices with the MDC9 disintegrin domain resulted in cellular retraction and detachment from the underlying matrix. Antibodies to the \( \beta 1 \) integrin chain inhibit adhesion of GEC to these substrates (6,33), strongly suggesting that the disruption of cellular attachment following transient transfection of the MDC9 disintegrin domain is the result of specific interaction with the \( \alpha 3 \beta 1 \) integrin. The integrin profile of the specific renal tubular segments is more complex than GEC (34,35). While the \( \beta 1 \) chain is a universal feature, the \( \alpha 2 \) chain is limited to the distal tubule (35). The \( \alpha 3 \) chain is found on GEC and in only limited amounts on the distal tubular cells. The \( \alpha 6 \) chain is not found in GEC, but is present on both proximal and distal tubule cells. Given the presence of the MDC9 within all tubular segments and the GEC, it is likely that this protein is capable of interactions with several distinct integrin species of the \( \beta 1 \) type.

The catalytic role of MDC9 as a stimulus-coupled heparin-binding epidermal growth factor-processing enzyme was recently reported (14). Immunohistochemistry has localized heparin-binding epidermal growth factor to the tubular epithelial cells of the S3 segment and to epithelial cells of the proximal tubules (36). This suggests that in the glomerulus, MDC9 may act as a “sheddase” for other membrane molecules. Retraction and rounding of GEC, with associated development of proteinuria, is a common feature of many forms of glomerular disease, and could conceivably be the result of MDC9 disintegrin domain interference with \( \alpha 3 \beta 1 \)-mediated adhesion to the basement membrane. A similar process may also explain the frequent occurrence of tubule cell exfoliation and basal lamina exposure observed in postischemic injury to the kidney. Future analysis of the functional role of MDC9 in the kidney may provide important new insights into the pathophysiology and treatment of these critical clinical problems.

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