Activation of Procollagenase IV by Cytochalasin D and Concanavalin A in Cultured Rat Mesangial Cells: Linkage to Cytoskeletal Reorganization¹

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ABSTRACT

The secretion and activation of procollagenase IV were studied in cultured rat mesangial cells. Under resting conditions, mesangial cells secrete predominantly a protein that, by gel zymography, exhibits gelatinase activity and also reacts with an anti-72-kd procollagenase IV antibody raised against a conserved region of the activation site of the enzyme. Cytochalasin D or concanavalin A treatment of mesangial cells causes disruption of actin stress fibers and results in the activation of procollagenase IV, yielding two lower molecular mass forms with gelatinase activity. Concanavalin A-induced actin filament disruption and procollagenase IV activation can be blocked by α-methyl-D-mannoside but not by D(+)-galactose. Procollagen IV as well as the activated forms all exhibit Ca²⁺ and Zn²⁺ dependency, characteristic of metalloproteinases. Mesangial cells in culture also secrete a specific tissue inhibitor of metalloproteinase, TIMP-2. Cytochalasin D treatment of mesangial cells reduces TIMP-2 expression. Cytochalasin D and concanavalin A both inhibited the serum-induced contraction of collagen gels embedded with mesangial cells. It was concluded that cytochalasin D-induced cytoskeletal disruption in mesangial cells may activate pro-collagenase IV by inhibiting TIMP-2 expression and that there is a concanavalin A-binding site on mesangial cells that is part of a transmembrane signaling system altering mesangial cell cytoskeletal organization and metalloproteinase secretion and activation.

Key Words: Glomerular mesangial cells, procollagenase IV, tissue inhibitor of metalloproteinase, actin filaments, cytochalasin D

Glomerulosclerosis, often accompanying progressive glomerular disease, is characterized by expansion of the mesangial extracellular matrix (ECM) and thickened glomerular basement membrane (1). Because the accumulation of ECM components could be a result of altered proteinase activity, attention has been focused on the role of proteinases in glomerular pathophysiology and kidney injury (2). A variety of proteinases are secreted by glomerular cells (3,4). Other proteinases may gain access to the glomerulus from the plasma or through release by leukocytes (2). The glomerular basement membrane comprises one of the major barriers restricting the ultrafiltration of plasma proteins. In addition, the mesangial ECM is recognized for its sieving and processing of circulating macromolecules (5). Even subtle changes in the composition of this highly structured fiber matrix may lead to distortions of function, manifested as altered permeability. In this way, the mechanisms and processes regulating the synthesis and breakdown of the glomerular basement membrane or mesangial ECM are critical to our understanding of the molecular basis for proteinuria. Among the family of metalloproteinases secreted by cells, collagenase IV secreted by mesangial cells (MC) (3) is of particular interest. For example, collagenase IV is implicated in basement membrane degradation during tumor metastasis (6). Thus, collagenase IV might play a critical and direct role in kidney injury through its degradative action on basement membrane and adjacent mesangial ECM.

Proteinases are known to be secreted in latent form and must be activated in order to exert their catalytic action. Latent collagenases can be activated chemically by chaotropic agents and organomercurial compounds by the "cysteine switch" mechanism (7–9).
The cellular mechanism(s) of activation in vivo, however, still remain incompletely understood. Yet, some information is available. For example, transforming growth factor-β and 12-O-tetradecanoylphorbol-13-acetate are both implicated in the cellular activation of a 72-kd collagenase IV in human tumorigenic cell lines (10). Cytochalasins, which have significant effects on cytoskeletal actin, lead to the cellular activation of an interstitial collagenase of synovial fibroblasts (11) and procollagenase IV in rat testicular peritubular myoid cells (12). Treatment with concanavalin A (ConA) of a variety of cells results in shape changes and activation of a 72-kd procollagenase IV (13,14). Finally, the specific inhibitor of procollagenase IV, TIMP-2 (tissue inhibitor of metalloproteinase) is involved in the activation of the latent enzyme (15). If glomerular pathology is linked to proteolysis, it is involved in the activation of the latent enzyme (15). If glomerular pathology is linked to proteolysis, it would be important to establish the cellular mechanisms leading to procollagenase IV activation in MC under normal culture conditions.

In this study, we investigated the expression of collagenase IV and TIMP-2 in cultured rat MC and describe a mechanism of cellular activation of the latent enzyme mediated by cytochalasin D–induced changes in the MC cytoskeleton. We also demonstrate that ConA not only induces similar shape change and activation of latent procollagenase in MC but also that the ConA effect is blocked specifically by α-methyl-D-mannopyranoside. These results implicate a transmembrane signaling mechanism linking cytoskeletal changes and procollagenase secretion.

MATERIALS AND METHODS

All reagents used in this study were purchased from Sigma (St. Louis, MO) unless otherwise indicated.

MC Culture

Cells used in this study were between passages 2 and 11. Glomeruli from 150 to 175-g Sprague-Dawley male rats were isolated by sieving as previously described (16). MC were trypsinized and plated in 24-well plates or 100-mm petri dishes in minimal essential medium (MEM) containing 20% fetal bovine serum, 1 mL and 10 mL, respectively, at a density of 5 x 10⁴ to 7.5 x 10⁴ cells/cm². For rhodamine-phalloidin staining, cells were plated on 12-mm glass slides positioned in the wells. After 24 h, cells were rinsed twice and incubated for 30 min in MEM to remove serum proteins. Cells were then treated with cytochalasins dissolved in dimethyl sulfoxide or ConA dissolved in MEM. After 24 h, medium was collected with 0.02% azide–0.02% Brij 35 (final concentration), spun to remove cell debris, and either used directly for analysis or further concentrated by dialysis and lyophilization. Viability and cell number after cytochalasin treatment were measured by trypan blue dye exclusion (17), by DNA measurement (see below), or by the ability of round, treated cells to reflatten after the change of serum-free medium back to serum-containing medium and incubation for 24 h (17).

Mesangial Cell Staining for Light Microscopy

Cells were stained for light microscopy as previously described (17). Briefly, fixed cells in wells were stained with 1% aceto-orcein for 5 min and with 1% Azur C for 7 min and were then rinsed three times with distilled water. Representative fields were photographed.

Actin Filament Staining

F-actin was localized by the use of rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR) as previously described (17). Briefly, cells plated on 12-mm glass coverslips in 24-well plates were rinsed three times in Hanks’ balanced salt solution (Gibco, Grand Island, NY), fixed 10 min with 4% paraformaldehyde, and washed again with Hanks’ balanced salt solution. Coverslips were transferred to a glass dish and permeabilized 10 min in cold (-20°C) acetone. Cells were air dried and rehydrated in phosphate-buffered saline (PBS), followed by incubation with 20 μL (0.4 U) of rhodamine-phalloidin for 30 min. Coverslips were washed three times with PBS, mounted in 0.1 M N-propyl gallate in 50% glycerol (18), and examined under a fluorescent microscope.

Contraction Assay

The contraction of MC was studied by the use of cells embedded in collagen gels, as described previously (17,19) with modifications. Briefly, the following stock solutions were mixed on ice: (1) one part 10× MEM, (2) one part 0.26 M NaHCO₃, (3) 3.2 parts 0.5% type I collagen (dissolved by mixing end over end in 0.1% acetic acid). To this mixture, a total of 4 x 10⁶ MC were added, after the total volume was adjusted to 10 parts with distilled water. Half-milliliter aliquots (2 x 10⁴ cells) were added to 24-well plates previously coated with 0.67% agarose and were incubated for 3 h at 37°C in 45% air–5% CO₂ in humidified atmosphere. The formed gels were flooded with 0.5 mL of MEM containing fetal bovine serum and two times the concentration of the required treatments indicated in the Results section. The longest and shortest axes of the collagen gels were measured 24 h after treatment, and the area was calculated. The mean of three measurements was recorded.
Measurement of DNA Concentration in Cells

Cells were washed with PBS, and 500 μL of TNE (10 mM Tris [pH 7.4], 1.5 M NaCl, 1 mM EDTA) was added. Cells were disrupted by sonication with 10 pulses of 0.5 s each. One hundred microliters of sonicated cells was transferred to 1.9 mL of TNE and vortexed. Ten microliters of Hoechst 33258 dye stock solution (20 μg/mL in TNE) was added to each sample, vortexed, and read promptly with a Hoefer TKO 100 mini-fluorometer (Hoefer, San Francisco, CA).

Detection of Activity of Metalloproteinases Secreted into the Medium

Samples were diluted with nonreducing buffer and applied to 10% (wt/vol) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels co-polymerized with 0.2% wt/vol gelatin (12,20). Proteins were resolved overnight with 7.5 mA/gel. Gels were washed with 2.5% Triton X-100 for 2 h, rinsed three times with distilled water, and incubated overnight in 50 mM Tris at pH 8.0 containing 5 mM CaCl₂. In some experiments, Ca²⁺ or Ba²⁺ ions, or gels were incubated in the presence of 10 mM EGTA or EDTA or 50 μM phenanthroline. Gels were then stained with 0.1% Coomassie blue R-250 in water/methanol/acetic acid (6:3:1 by vol). For activation of the latent enzyme, 90 μL of condition medium was incubated with 10 μL of 10 mM P-aminophenylmercuric acetate (APMA; 1 mM, final concentration) at 37°C overnight.

Detection of Latent Type IV Collagenase and TIMP-2 by Western Immunoblot Procedures

Conditioned medium was concentrated as described above and subjected to SDS/PAGE electrophoresis (24). Proteins were transferred to nitrocellulose paper (Schleicher and Schuell, Keene, NH) electrophoretically and processed as described earlier (25). Anti-TIMP-2 (a generous gift of Dr. W.G. Stetler-Stevenson, NIH) and anticollegenase IV antibodies were used at a dilution of 1:200. Anti-72-kd collagenase IV antibody against the activation site of the enzyme was raised in rabbits as described above. Anti-TIMP-2 was raised in rabbits against human TIMP-2 (26). Also used for comparison was anti-72-kd procollagenase IV antibody (a generous gift of Dr. W.G. Stetler-Stevenson) raised against amino acids 1 to 17 of the N terminus of the human latent enzyme (9).

RESULTS

MC Secretion of Procollagenase

Rat MC in culture secrete predominantly a latent procollagenase IV with an apparent molecular mass of 68 kd (Figure 1); we raised an antibody against a conserved region of procollagenase IV flanking the published (9) APMA activation site. With anticollegenase IV antibody in Western immunoblotting of MC conditioned medium, a single band was evident (Figure 1, Lane 2). This band was abolished when the antibody was preincubated with 20 μg/mL peptide (Figure 2, Lane 3). The band comigrated with the enzymatic activity detected by SDS/PAGE zymography (Figure 1, Lane 1). An antibody raised against peptides 1 to 17 of human procollagenase IV (9) on a Western immunoblot revealed a band migrating at a molecular mass of ~68 kd (data not shown). Two features of Figure 1 are noteworthy. First, although procollagenase IV is inactive, activation occurs under the denaturing conditions of zymography, presumably through the cysteine switch (7,8; see Discussion). Second, the apparent molecular mass of pro-
Figure 1. Gel zymography (Lane 1) and Western immunoblot (Lanes 2 and 3) of procollagenase IV present in MC conditioned medium. Conditioned medium was obtained from cells after 24-h culture in serum-free MEM. It was then applied directly (Lane 1) or concentrated 20x (Lanes 2 and 3) before being applied to gel electrophoresis. Samples were subjected to 10% SDS/PAGE with (Lane 1) or without (Lanes 2 and 3) copolymerization with gelatin. Proteins were transferred to nitrocellulose sheets (Lanes 2 and 3) and incubated with procollagenase IV antibody (1:200). Nitrrocellulose sheets were incubated with antibody without (Lane 2) or with (Lane 3) preincubation with 20 μg/mL peptide. For a description of the specific epitope recognized by this antibody, see the Materials and Methods section. Positions of molecular mass standards are indicated on the left and are expressed in kilodaltons.

Figure 2. ELISA of collagenase IV. Ninety-six-well plates were coated with 20 μg/mL peptide and processed as described in Materials and Methods. (A) No first antibody. (B) Preimmune serum. (C) Antibacterial IV antiserum. (D) Antibacterial IV antiserum preincubated with 40x concentrated MC conditioned medium. (E) Antibacterial IV antiserum preincubated with 20 μg/mL peptide. The treatment of MC with cytochalasin analogues E and B caused the cells to round up to varying degrees, but cytochalasin D was the most potent and cytochalasin E was the weakest compared with the nontreated control (data not shown). Similarly, collagenase IV activation potency by the different cytochalsins appeared to correlate with their ability to influence cytoskeletal actin (data not shown).

Both procollagenase IV and the activated lower molecular mass ~57-kd form were inhibited by the chelation of Ca²⁺ with EDTA or EGTA or by the Zn²⁺ chelator phenanthroline (Figure 6). Ca²⁺ was essential for activity and could not be replaced by other divalent cations like Mg²⁺ or Ba²⁺ (data not shown). These biochemical criteria indicate that the proteinase secreted by MC is a metalloproteinase.

The treatment of MC with cytochalasin D at 0.5 and 1 μg/mL concentration, and the conditioned medium from these treated cells was subjected to gel zymography. An inspection of Figure 4, Lanes 1 and 2, reveals the presence of a major band with an apparent molecular mass of 68 kd, identical to the latent form of collagenase IV shown in Figure 1B. Figure 4, Lanes 3 and 4, demonstrates dose-dependent activation yielding one major low-molecular-mass band of ~57 kd and a minor band migrating somewhat slower at ~59 kd. Also seen is a minor band at ~90 kd that appears to exhibit collagenase activity—presumably 92-kd collagenase IV. No further attempt was made to characterize this 90-kd entity in this study. The signal of the 68-kd band was decreased after treatment with cytochalasin D, suggesting activation of the latent procollagenase IV (Figure 5; see Discussion).

Collagenase IV under nonreducing conditions is ~68 kd. By the use of ELISA, the signal of the antibody (Figure 2C) could be abolished by preincubation of the antibody with 40x MC conditioned medium as a source of procollagenase IV or 20 μg/mL peptide (Figure 2D and E). No signal could be detected when first antibody was omitted (Figure 2A) or when preimmune serum was used (Figure 2B).

MC Treatment with Cytochalasin D

The effect of cytochalasin D (1 μg/mL) on the MC cytoskeleton is shown in Figure 3. Cytochalasin D added to MC plated on coverslips caused the cells to round up (compare Figure 3A versus B) and was associated with the disruption of actin stress fibers, as demonstrated by rhodamine-phalloidin actin staining (Figure 3, compare Panels C and D). Cytochalasin D–treated cells remained viable on the basis of trypan blue dye exclusion, DNA content, and re-flattening assay (data not shown).

MC were then exposed to cytochalasin D at 0.5 and 1 μg/mL concentration, and the conditioned medium from these treated cells was subjected to gel zymography. An inspection of Figure 4, Lanes 1 and 2, reveals the presence of a major band with an apparent molecular mass of 68 kd, identical to the latent form of collagenase IV shown in Figure 1B. Figure 4, Lanes 3 and 4, demonstrates dose-dependent activation yielding one major lower-molecular mass band of ~57 kd and a minor band migrating somewhat slower at ~59 kd. Also seen is a minor band at ~90 kd that appears to exhibit collagenase activity—presumably 92-kd collagenase IV. No further attempt was made to characterize this 90-kd entity in this study. The signal of the 68-kd band was decreased after treatment with cytochalasin D, suggesting activation of the latent procollagenase IV (Figure 5; see Discussion).

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The treatment of MC with cytochalasin analogues E and B caused the cells to round up to varying degrees, but cytochalasin D was the most potent and cytochalasin E was the weakest compared with the nontreated control (data not shown). Similarly, collagenase IV activation potency by the different cytochalasins appeared to correlate with their ability to influence cytoskeletal actin (data not shown).

Latent metalloproteinases can be activated chemically (i.e., without prior cellular treatment) by organomercural compounds such as APMA. Treatment of MC conditioned medium with APMA resulted in procollagenase IV activation. The active enzyme released by APMA under these conditions comigrated with the ~59-kd band of the cytochalasin D–activated en-

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Figure 3. Phase contrast light micrographs (A and B) and fluorescence micrographs (C and D) of rhodamine-phalloidin staining of MC cultured for 24 h in serum-free MEM in the absence (A and C) or presence (B and D) of 1 μg/mL cytochalasin D. MC were plated on coverslips on 24-Linbro plates in 20% serum MEM and cultured for 24 h. Medium was changed to serum-free MEM, and cells were treated with cytochalasin D. Cells treated with cytochalasin D round up (B), and actin filaments appear disrupted (D). Bar, 10 μm.

Figure 4. Effects of various concentrations of cytochalasin D on collagenase IV activation by rat MC in culture. Cells were plated on 24-Linbro plate wells in 20% fetal bovine serum–MEM, cultured as indicated in Materials and Methods. Nontreated cells (Lane 1) and cells treated with dimethylsulfoxide (Lane 2) secreted predominantly procollagenase IV with an apparent molecular mass of 68 kd. Treatment with cytochalasin D at concentrations of 0.5 and 1 μg/mL (Lanes 3 and 4, respectively) results in a dose-dependent activation of collagenase IV to a lower major 57-kd activity (Lanes 3 and 4). Also evident are a fainter activation band migrating at 59 kd and a stimulated band at the 90-kd region (Lane 4). Positions of molecular mass standards are indicated on the left and are expressed in kilodaltons.

zymes (compare Figure 4, Lane 4, and Figure 7A, Lane 2). Furthermore, the addition of APMA to the conditioned medium of cells that had previously been exposed to cytochalasin D did not lead to a further increase in the activation of the major 57-kd active form (Figure 7B, Lane 1). Because all zinc metalloproteinases are activated by APMA (8), this latter finding further suggests that the 57-kd band of collagenase IV induced by cytochalasin D is indeed activated.

Finally, TIMP-2 is involved in the inhibition of human collagenase IV activity as well as its activation (15,27). We therefore studied TIMP-2 secretion into MC conditioned medium, using a specific anti-TIMP-2 antibody (26). As shown in Figure 8, a unique protein of about 21-kd molecular mass was recognized by the anti-TIMP-2 antibody. Moreover, as also shown in Figure 8, the treatment of the cells with cytochalasin D inhibited the secretion of TIMP-2 into the medium.

MC Treatment With ConA

ConA has been reported to activate procollagenase IV in human fibroblasts and fibrosarcoma cells (13,14). We treated MC with increasing concentrations of ConA. As shown in Figure 9 (Lanes 3 to 10), a dose-related activation of the 68-kd procollagenase IV to a predominant 57-kd activity band was observed. This activity comigrated with the 57-kd activated band produced by cells treated with cytochalasin D (Figure 9, Lane 2). Interestingly, the activation of MC procollagenase by ConA (Figure 10, Lane 2) was inhibited by α-methyl-D-mannopyranoside (Figure 10, Lane 4) but not by D(+)galactose (Figure 10,
Figure 5. Western immunoblot of procollagenase IV present in MC conditioned medium without (right lane; control) or with (left lane; cyto. D) treatment with 1 μg/mL cytochalasin D. Note the reduction in the 68-kd signal of procollagenase IV after treatment with cytochalasin D. Additional details are described in the legend to Figure 1 and the Materials and Methods section. Positions of molecular mass standards are indicated on the left and are expressed in kilodaltons.

Figure 6. Dependence of latent and active collagenase IV activity on Ca²⁺ and Zn²⁺ ions. Conditioned media of MC incubated in the absence (−) or presence (+) of 1 μg/mL cytochalasin D (cyto. D) were subjected to SDS/PAGE zymography, and activity was developed in the presence of 10 mM EDTA or 50 μM phenanthroline. Both latent and active collagenase IV activity were abolished in the absence of Ca²⁺ or Zn²⁺. Similarly, collagenase IV activity was abolished in the presence of 10 mM EGTA, and Mg²⁺ or Ba²⁺ could not replace Ca²⁺ (data not shown). Positions of molecular mass standards are indicated on the left and are expressed in kilodaltons.

Lane 6). No apparent change in procollagenase levels was observed with the treatments of sugars alone (compare Figure 10, Lanes 3 and 5 with Lane 1).

Treatment of MC with ConA caused disruption of actin filaments (Figure 11D) that could be reversed by α-methyl-D-mannopyranoside (Figure 11E) but not by D(+)-galactose (Figure 11F). No apparent change in actin filament organization could be observed with the treatments of the sugars alone (compare Figure 11A with 11B and 11C). Because MC treatment with both cytochalasin D and ConA activated collagenase IV and also caused disruption of actin filaments, we attempted to establish a correlation between these cytoskeletal effects (which caused obvious rounding up of the cells) and MC "contractility." For this purpose, we used a so-called contraction assay. This system has been exploited for other cell types to explore the nature of cell-matrix interactions. Typically, when test cells are embedded under tissue culture conditions in collagen gel, exposure to serum results in the contraction of the collagen gel. Both cytochalasin D and ConA inhibited the serum-induced contraction of collagen gels embedded with MC. Moreover, this inhibition occurred in a dose-dependent fashion (Figure 12).

Data shown are representative of results obtained with at least three different cell preparations.

DISCUSSION

Proteolysis is implicated in complex processes of tissue restructuring including tissue maintenance, growth, development, and repair (28). Recently, there has been increasing attention paid to the role of proteolytic enzymes as causative agents in glomerular injury. These proteolytic agents might originate from plasma, from recruited leukocytes, or from resident glomerular cells (2). Whatever their origin, it will not be easy to differentiate between the disease-
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Figure 7. Activation pattern of latent procollagenase IV with APMA. Panel A shows SDS/PAGE zymography of conditioned medium from rat MC treated without (Lane 1) and with (Lane 2) 1 mM APMA. MC conditioned media from control cells (Lane 3) and from cytochalasin D-treated cells (Lane 4) are shown for comparison. Note that the predominant (57-kd) band of cytochalasin D-activated collagenase IV migrates faster than the APMA-activated enzyme (59 kd). Panel B shows SDS/PAGE zymography of conditioned medium from cells treated first with cytochalasin D and then incubated at 37°C for 24 h with APMA (Lane 1). For comparison, the same conditioned medium is shown without (Lane 2) and with (Lane 3) 1 μg/mL cytochalasin D activation. As can be seen in Lane 1, the latent procollagenase IV (68 kd, upper arrow) is activated to a lower band (59 kd, middle arrow) whereas the cytochalasin D-induced active collagenase IV (57 kd, lower arrow) is not influenced by APMA. Positions of molecular mass standards are indicated on the left and are expressed in kilodaltons.

Figure 8. Western immunoblot of TIMP-2 secreted into MC conditioned medium in the absence (control) or presence (cyto. D) of 1 μg/mL cytochalasin D. Samples were subjected to 12% SDS/PAGE and transferred to nitrocellulose paper. Sheets were incubated with anti-TIMP-2 antibody at 1:200 dilution. Note the reduction of TIMP-2 in the conditioned medium of cells treated with cytochalasin D. Cells were treated and media were processed as described in Material and Methods and in the legend to Figure 1. Positions of molecular mass standards are indicated on the left and are expressed in kilodaltons.

Figure 9. Effect of various concentrations of ConA on the activation of procollagenase IV in MC conditioned medium. MC were treated with 20, 10, 5, 2.5, 1.25, 0.265, 0.313, and 0.156 μg/mL of ConA (Lanes 3 to 10, respectively). Conditioned medium of untreated MC is shown in Lane 1. For comparison, conditioned medium of cells treated with 1 μg/mL of cytochalasin D is shown in Lane 2. Lanes 3 to 10 indicate a dose-dependent ConA activation of 68-kd procollagenase IV to a predominant 57-kd activity that comigrates with the activated 57-kd band of cytochalasin D–treated cells shown in Lane 2. Under control conditions and in the presence of both ConA and cytochalasin D, there is a band migrating somewhat slower than the 68-kd procollagenase, which may represent another higher molecular weight form of collagenase. Positions of molecular mass standards are indicated on the left and are expressed in kilodaltons.

It is well recognized, however, that the release of specific local proteolytic enzymes by both glomerular epithelial cells and MC might serve a regulatory role in controlling the balance between the synthesis and degradation of the basement membrane and the ECM and possibly even in controlling proliferative responses through the freeing of ECM-bound growth factors.

Specific neutral proteinases from MC and glomerular epithelial cells have been identified and to some extent biochemically characterized. The findings of this study confirm the original observations of Davies and coworkers (29) that rat MC in culture secrete a latent metalloproteinase. However, relatively little effort has so far been directed at uncovering the normal cellular mechanisms regulating its secretion and activation.
Cytochalasin D is a fungal product that prevents actin polymerization (30). Previous reports have correlated cytoskeleton disruption with signal transduction (31-33). Our results indicate a direct proportionality between the dose of cytochalasin D and cytoskeletal disruption in MC. Further, we have found that this effect on the cytoskeleton is correlated with increased expression of procollagenase IV in MC (34) and activation of the latent form of this enzyme. Unemori and Werb (35) have shown that disruption of synovial fibroblast actin filaments with various agents precedes an increase in interstitial collagenase gene expression. These findings demonstrating the influence of cytochalasin D on the expression of collagenase IV in rat MC suggests that these two distinct collagen genes may be influenced by cytochalsins via similar mechanisms. It should be pointed out, however, that the structure and control of human interstitial collagenase and human 72-kd collagenase IV are quite different (36). Clearly, there are both similarities and distinct differences in the structure and, hence, control of the different metalloproteinase genes.

The effect of cytochalasin D on the cytoskeleton was more rapid than that of ConA. This could be a consequence of the hydrophobicity of cytochalasin D, permitting its rapid intracellular partitioning. On the other hand, our evidence suggests that ConA binds to the extracellular domain of an MC plasma membrane and presumably causes disruption of the cytoskeleton via some transmembrane signaling pathway.

Both cytochalasin D and ConA inhibit the contraction of MC embedded in collagen gels. Motile cells generate three types of forces within the cortical cytogel underlying the leading lamela: (1) hydrostatic and osmotic pressure, (2) active forces (actomyosin sliding), (3) elastic forces from actin filaments (37). Because, in our system, MC migrate in a matrix of dense collagen I fibers and contact each other only over a small portion of their surface (unlike muscle cells in a muscle tissue for example), it is likely that the contraction forces generated by the MC are of the third type (i.e., actin filaments) and that these forces are transmitted primarily to the collagen I fibers via cell-extracellular attachments mediated by VLA integrins (38). It follows, then, that any disruption of actin filaments or perturbation of cell-ECM attachment will inhibit the MC-induced collagen gel contraction. The mechanism of cytochalasin D inhibition of MC-induced collagen contraction probably occurs by the direct disruption of actin filaments. The mechanism by which ConA causes inhibition of contraction remains to be clarified. The fact that the ConA effect could be reversed by the potent ConA-blocking sugar α-methyl-D-mannopyranoside but not by D (+)-galactose suggests that ConA action is extracellular and perhaps perturbs cell-ECM interaction, leading to actin filament disruption in the same manner as that reported for the fibronectin receptor perturbation (39).

Precisely how cytochalasin D or ConA causes procollagenase IV activation in MC or how these agents induce similar effects on other cell types (11-14) remains unknown. Moreover, cytochalasin D and ConA are not unique in this regard. Transforming growth factor-β and 12-O-tetradecanoylphorbol-13-acetate also selectively cause cellular activation of 72-kd human procollagenase IV in certain tumorigenic cells (10). In addition, as already demonstrated in this study and elsewhere (9, 12), the chemical activation of procollagenase IV can be produced by organomercurials such as APMA, presumably via the cysteine switch (7, 8), in which APMA disrupts zinc-cys 73 interaction and the resulting conformational change is followed by autocatalytic cleavage of an 80-amino-acid segment. The pattern of procollagenase IV activation by APMA and cytochalasin D, however, is quite different—as shown in Figure 7. These discrepancies suggest that the mechanism of cellular activation of latent collagenase IV is not the same as the autocatalytic process produced by APMA treatment. It has been shown that, by the addition of plasminogen to MC, the activation of 72-kd procol-
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Figure 11. Fluorescent micrographs of rhodamine-phalloidin-stained MC cultured for 24 h in serum-free MEM in the presence of 10 μg/mL of ConA (D through F) with no sugar (A and D) or with 500 μg/mL of α-methyl-D-mannopyranoside (B and E) or α(+)-galactose (C and F). Note disruptions of actin filaments with ConA (D) that are completely reversed by α-methyl-D-mannopyranoside (E) but not by α(+)-galactose (F). For further details, see legend to Figure 3 and Materials and Methods.

Bar, 10 μm.

Lagena IV occurs, presumably by plasmin generated by secreted plasminogen activator (40). In our study, however, cytochalasin D activation is probably not mediated by the plasminogen activator system because (1) plasmin activity is not documented in cultured MC and (2) cytochalasin D activation could not be inhibited by aprotinin and ε-amino caproic acid (M. Ailenberg and M. Silverman, unpublished data). These results concur with those from other studies that showed that activation of the 72-kd latent human procollagenase IV is not susceptible to inhibition by aprotinin and ε-amino caproic acid (10) or by trypsin treatment (21).

Perhaps the most appealing explanation is that cytochalasin D activation of procollagenase IV in MC is mediated through TIMP-2. If TIMP-2 acts by preventing the autocatalytic cleavage of procollagenase IV, then any reduction in TIMP-2 mRNA (34) leading to an inhibition of TIMP-2 protein, such as we have observed in this study after treatment with cytochalasin D, would correlate with enhanced collagenase IV activation. Indeed, it has been shown that the activation of 72-kd human procollagenase IV by APMA could be inhibited by TIMP-2 (15) and that TIMP-2 levels are reduced by the treatment of testicular cells with cytochalasin D (41). The possibility that cytochalasin D triggers a specific yet unknown activator of collagenase IV cannot be excluded. Whatever turns out to be the precise molecular basis for cytochalasin D–induced MC procollagenase IV activation, these results link MC cytoskeletal disruption to activation of the enzyme.

As a working hypothesis, therefore, we propose the following sequence of cell-mediated events. The inhibition of MC actin polymerization leads to increased gene expression of collagenase IV and reduced gene expression of TIMP-2, followed by activation of procollagenase IV. Conversely, agonist stimulation of the MC surface, causing actin polymerization, should bring about an increase in TIMP-2 with reduced activation of collagenase IV, culminating in a net increase in ECM deposition. More work is required to test this hypothesis with model culture systems as well as intact animals.
Figure 12. Inhibition of contraction of collagen gels embedded with rat MC. MC were embedded in collagen I solution and allowed to gel for 3 h, followed by treatment with various concentrations of cytochalasin D (upper panel) or ConA (lower panel) added in MEM containing 10% or 2.5% fetal bovine serum, respectively. Inspection reveals a dose-related inhibition of collagen contraction, expressed as a percentage of control gel area, for both cytochalasin D and ConA.

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