Paradigmatic Identification of MMP-2 and MT1-MMP Activation Systems in Cardiac Fibroblasts Cultured as a Monolayer

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Abstract   Activations of MMP-2 and membrane type 1-matrix metalloproteinase (MT1-MMP) have been correlated with cell migration, a key cellular event in the wound healing and tissue remodeling. We have previously demonstrated furin-dependent MMP-2 and MT1-MMP activations induced by type I collagen in cardiac fibroblasts. To understand mechanistic aspects of the regulation of MMP-2 and MT1-MMP activations by potential non-matrix factor(s) in cardiac fibroblasts, in the present study, we examined the effects of various agents including concanavalin A (ConA), a proteolytic phenotype-producing agent. We showed that treatment of cells with ConA activated pro-MMP-2, and that this activation concurred with elevated levels of cellular MT1-MMP and TIMP-2. The presence of active MT1-MMP and 43 and 36 kDa processed forms of MT1-MMP in a fraction of intracellular proteins prepared from ConA-treated cells suggests the possible internalization of differential forms of MT1-MMP. The appearance of 36 kDa processed form of MT1-MMP in conditioned media prepared from ConA-treated cells indicates the possible extracellular release of the further processed MT1-MMP fragment. Inhibition of furin in ConA-treated cells attenuated pro-MT1-MMP processing and the cellular TIMP-2 level, plus it reduced cell-released active MMP-2 in a time-dependent manner. These results suggest the involvement of furin in the ConA-induced activations of MT1-MMP and MMP-2. Furthermore, the existence of furin inhibitor-insensitive pro- and active MMP-2 species associated with ConA-treated cells implies that a mechanism independent of furin may perhaps account for the binding of the MMP-2 species to the cells. Supplementary material for this article can be found at http://www.mrw.interscience.wiley.com/suppmat/0730-2312/suppmat/94/suppmat_guo.tif. J. Cell. Biochem. 94: 446–459, 2005.   © 2004 Wiley-Liss, Inc.

Key words: MMP-2; MT1-MMP; furin; TIMP-2; cardiac fibroblasts; concanavalin A

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Matrix metalloproteinases (MMPs) are a family of zinc-containing enzymes that degrade extracellular matrix proteins. Most MMPs are secreted as a latent form (pro-MMPs) and proteolytic removal of the propeptide domain is necessary for MMP processing to the active form [Nagase and Woessner, 1999]. Activations of MMP-2 and membrane type 1-matrix metalloproteinase (MT1-MMP) have been correlated with cell migration [Deryugina et al., 1997; Hotary et al., 2000], a key cellular event in the wound healing and tissue remodeling.

Cell-mediated MMP-2 activation in vitro is generally believed to be a process that takes place on the cell surface and requires the participation of both MT1-MMP and the tissue inhibitor of matrix metalloproteinase-2 (TIMP-2). Generation of a sufficient amount of active MT1-MMP by intracellular processing of the latent form of MT1-MMP (pro-MT1-MMP), plus transference of this active enzyme to the cell

surface are generally accepted as being critical steps for the occurrence of MMP-2 activation [Seiki and Yana, 2003]. It has been found that intracellular pro-MT1-MMP processing can be achieved by the action of furin or furin-like proprotein convertase(s), via cleavage at the C-terminal side of [108]RRKR (furin recognition motif) in the propeptide domain of MT1-MMP, within the trans-Golgi network of various cell types [Maquoi et al., 1998; Yana and Weiss, 2000]. However, the existence of an alternative furin-independent pathway for pro-MT1-MMP processing has also been demonstrated using MT1-MMP/furin co-transfected COS-1 cells [Cao et al., 1996] and MT1-MMP-transfected MCF breast carcinoma cells [Rozanov et al., 2001]. Furthermore, inhibition of furin reduced the levels of active MMP-2 released by con-canavalin A (ConA)-treated human uterine cervical fibroblasts, but did not seem to affect the levels of active MMP-2 released by ConA-treated rabbit dermal fibroblasts [Sato et al., 1999], raising the possibility of a cell type-dependent role for furin in ConA-induced MMP-2 activation.

MMPs have been implicated in the pathogenesis of ventricular remodeling. Broad spectrum MMP inhibition resulted in delayed infarct healing in a mouse myocardial infarction model [Rohde et al., 1999]. The delayed infarct healing has been speculated to be due to the reduction of collagen deposition as a result of the impaired migration of fibroblasts-like cells to injury sites, caused by the MMP inhibition [Creemers et al., 2001]. The fibroblasts-like cells are thought to originate from cardiac fibroblasts [Weber, 1997], the cell type responsible for collagen synthesis in the myocardium and known to produce MMPs [Siwik et al., 2000; Bergman et al., 2003]. Recently, we have demonstrated furin-dependent MT1-MMP and MMP-2 activations induced by type I collagen, the principal component of the interstitial fibrillar collagen network, in cardiac fibroblasts, and have correlated these activations with the in vitro migration of cardiac fibroblasts [Guo and Piacentini, 2003].

To understand mechanistic aspects of the regulation of MMP-2 and MT1-MMP activations by potential non-matrix factor(s) in cardiac fibroblasts cultured as a monolayer, in the present study, we examined the effects of two cardiac pro-fibrotic peptides: endothelin-1 (ET-1) and angiotensin II (Ang II), and two

pharmacological agents: phorbol 12-myristate 13-acetate (PMA) and ConA, both of which have been recognized as producing a proteolytic phenotype in a cell type-dependent manner [Lohi et al., 1996; Foda et al., 1999].

MATERIALS AND METHODS

Cell Culture

Rat cardiac fibroblasts were obtained as described previously [Guo and Piacentini, 2003]. Identical numbers of first and second passage cardiac fibroblasts were grown in minimal essential medium containing 10% newborn bovine calf serum either in 100 mm culture dishes (seeding density of 4×10^5 cells/dish) or in 6-well culture plates (seeding density of 8×10^4 cells/well) until confluence was reached. To ensure cells quiescence and to avoid interference from exogenous MMPs, TIMPs, and other protease inhibitors in the serum, the medium was then changed to a serum-free medium supplemented with apo-transferrin (10 μg/ml), insulin (10 μg/ml), and selenium (5 ng/ml) for 48 h before further treatment. Upon treatment, an equivalent volume of fresh serum-free medium containing various agents was applied to each dish (10 ml) or well (3 ml). Human fibrosarcoma cells (HT1080; European Collection of Cell culture) were maintained in Dulbecco's modified minimal essential medium containing 10% (v/v) Fetal bovine serum (Gibco Life Technologies, Paisley, UK) and 1% (v/v) non-essential amino acids. Once confluent, the cells were cultured in serum-free media for 48 h after which some cells were treated with PMA (100 nM) for 24 h.

Preparation of Medium Samples

Conditioned medium samples were collected, centrifuged at 400g to remove cell debris, and then stored at −20°C before zymographic or Western blot analysis. In some experiments, medium samples were normalized for volume and then concentrated (approximately 10 folds) using concentrators (Micron YM-10, Millipore, MA) before Western blot analysis.

Preparation of Whole Cell Lysates

Whole cell lysates were prepared from mono-layer cardiac fibroblasts using a modified method [Sato et al., 1999]. Briefly, cells were thoroughly washed twice with PBS and then

scraped into a chilled buffer containing Tris-HCl (50 mM), NaCl (150 mM), CaCl$_2$ (1 mM), IGEPAL CA-630 (1%, v/v), Brij 35 (0.05 %, v/v), PMSF (1 mM), E-64 (10 µM), aprotinin (10 µg/ml), leupeptin (1 µg/ml), and pepstain A (10 µg/ml), pH 7.5. Cells were then solubilized by trituration with a 22-G needle for 30 passages, vigorously vortexed and lysed on ice for 60 min. Homogenates were centrifuged at 4,000g for 15 min at 4°C. Supernatants, which corresponded to the whole cell lysates, were collected. Protein concentrations in all cell lysates were determined using the Bio-Rad protein assay against a bovine serum albumin standard. All samples were kept at −20°C before zymographic or Western blot analysis.

Preparation of Intracellular Soluble Proteins

Intracellular soluble proteins were prepared as previously described [Pei et al., 2000] with some modifications. Following 24 h culture, cells were washed three times with PBS and incubated with PBS (pH 7.2) containing 0.1% (w/v) saponin, 75 mM potassium acetate, and 25 mM Hepes, for 30 min at room temperature. The supernatant was collected and a proteinase inhibitor mixture including PMSF 1 mM, 10 µM E-64, 10 µg/ml aprotinin, 1 µg/ml leupeptin, and 10 µg/ml pepstain A was immediately added. The post-saponin extraction residual components were then lysed with the chilled lysis buffer as stated above. Following protein concentration determination using the Bio-Rad protein assay, saponin-extracted supernatant was concentrated using Micron YM-10 concentrators.

Preparation of Cell Membrane Fraction

Cell membrane fraction was prepared from cardiac fibroblasts using a modified ultracentrifugation-based method [Itoh et al., 1998]. Cells were maintained in the absence or presence of ConA (10 µg/ml) under serum-free conditions in 100 mm dishes for 24 h. At the end of this time period, the cells were thoroughly washed twice with PBS, scraped into a chilled buffer containing Tris-HCl (100 mM), sucrose (250 mM), NaN$_3$ (0.02%, w/v), PMSF (2 mM), and E-64 (10 µM), pH 8.0, and homogenized by trituration with a 22-G needle for 30 passages on ice. Under these circumstances, phase-contrast microscopic examination showed that the extent of cell breakage exceeded 80%. The resultant homogenate was subjected to a centrifugation at 12,000g for 20 min at 4°C. The post-

nuclear supernatant was pelleted at 100,000g for 1 h at 4°C. Precipitates were washed with a chilled buffer containing Tris-HCl (50 mM), NaCl (150 mM), CaCl$_2$ (10 mM), and NaN$_3$ (0.02%, w/v), pH 7.5, and centrifuged again at 100,000g for 1 h at 4°C. Precipitates corresponding to cell membrane fractions were then resuspended in this chilled buffer. All samples were kept at −80°C until used for experiments. Protein concentrations in cell membrane fractions were determined via measuring samples dissolved in NaOH (50 mM), with the BCA protein assay using bovine serum albumin as a standard.

Zymography

Zymographic analysis was performed as described previously [Kleiner and Stetler-Stevenson, 1994]. A gelatin (type I, Sigma, Poole, UK) concentration of 2 mg/ml was employed in 10% polyacrylamide resolving gels. Following quantitation of gelatinolytic bands corresponding to MMP-2 using NIH Image 1.62 software program, the ratio of the active MMP-2 (A), which includes intermediate and full active forms of MMP-2, to the total MMP-2 (T), which combines pro- and active forms of MMP-2, was calculated. The A/T ratio (%) was adopted in this study as an index for the evaluation of the extent of MMP-2 activation as described previously [Guo and Piacentini, 2003]. Each zymogram presented is representative of at least three experiments carried out using different cell populations.

Reverse-Zymography

TIMP activity in a cell membrane fraction prepared from ConA-treated cardiac fibroblasts was detected using reverse-zymography as described previously [Oliver et al., 1997]. A gelatin (type I, Sigma) concentration of 1 mg/ml and recombinant human pro-MMP-2 (1 µg/10 ml) (Oncogene, Nottingham, UK) were employed in 12% polyacrylamide resolving gels. Coomassie brilliant blue positive stain bands corresponding to the inhibitory activity of TIMP were quantified using NIH 1.62 Image software program. Each reverse-zymogram presented is representative of four experiments carried out using different cell populations.

Western Blot Analysis

For immunodetection of MT1-MMP, TIMP-2, or furin in whole cell lysates, cell membrane fractions, or conditioned culture media, Western

blot analysis was performed. Briefly, medium and lysate samples were normalized for volume and protein, respectively, and separated on 10–12% SDS–polyacrylamide resolving gels under denatured and reducing conditions, and then transferred onto polyvinylidene difluoride (PVDF) membranes. To ensure equal loading of samples, proteins either in the resolving gels or on PVDF membranes were stained using coomassie brilliant blue (see Supplementary Material http://www.mrw.interscience.wiley.com/suppmat/0730-2312/suppmat/94/suppmat_guo.tif for details). Non-specific binding sites on PVDF membranes were blocked with 10% (w/v) skimmed non-fat milk in PBS supplemented with 0.1% (v/v) Tween-20 (PBS-T). The membranes were then incubated with appropriate primary antibodies (monoclonal antibodies (mAbs) against MT1-MMP (1C1, generously donated by Dr. Akiko Okada, Department of Cancer Cell Research, Institute of Medical Science, University of Tokyo, Japan, used in 1:1,000 dilution; 2D7, generously donated by Dr. Marie-Christine Rio, Institut de Genetique et de Biologie Moleculaire et Cellulaire, Strasbourg, France, used in 1:50 dilution), TIMP-2 (Ab-2, Oncogene, used in 1:1,000 dilution), or furin (Mon-139, Alexis Biochemicals, Nottingham, UK, used in 1:1,000 dilution), and a polyclonal antibody against TIMP-2 (C-20, Santa Cruz Biotechnology, Calne, UK, used in 1: 1,1000 dilution) diluted in PBS-T containing 4% (w/v) skimmed non-fat milk overnight at 4°C or 1 h at room temperature. Unbound primary antibody was removed by three washes with PBS-T. The membranes were sequentially incubated with secondary antibodies (goat anti-mouse HRP conjugated IgG (Santa Cruz Biotechnology, used in 1:5,000 dilution) for monoclonal antibody procedures or donkey anti-goat HRP conjugated IgG (Santa Cruz Biotechnology, used in 1:10,000 dilution) for polyclonal antibody procedures) diluted in PBS-T containing containing 4% (w/v) skimmed non-fat milk. Following three washes with PBS-T to remove unbound secondary antibodies the membranes were further cleaned by final washes with PBS and deionized water. Finally immunoreactive bands in the membranes were detected with a standard chemilumescence procedure (Amersham, Bucks, UK). Each immunoblot presented is representative of at least three experiments carried out using different cell populations.

Statistics

For comparisons between two time-matched groups, a paired student's t-test with two-tail probability (P) values was used. All values are presented as the mean ± standard error of the mean (SE) in n experiments as indicated. P values less than 0.05 ($P < 0.05$) were considered statistically significant.

RESULTS

ConA-Induced MMP-2 Activation in Cardiac Fibroblasts Concurs With the Up-Regulation of Cellular MT1-MMP and TIMP-2

After treatment of cardiac fibroblasts with ConA (10 µg/ml) for 24 h, but not ET-1, Ang II, or PMA, gelatinolytic species corresponding to the latent and active MMP-2 forms were detected in both conditioned media (Fig. 1A) and whole cell lysates (Fig. 1B) prepared from these cells. Additionally, zymographic analysis indicated the presence of a single low molecular mass gelatinolytic band (~45 kDa) that is likely to be the fully activated MMP-2 species minus the C-terminus in the lysates prepared from the ConA-treated cells [Ward et al., 1994] (Fig. 1B). It should be noted that a faint gelatinolytic band near 97 kDa, that is most likely to be pro-MMP-9, was apparent in the both conditioned media (Fig. 1A) and whole cell lysates (Fig. 1B) after the treatment of cells with PMA (100 nM).
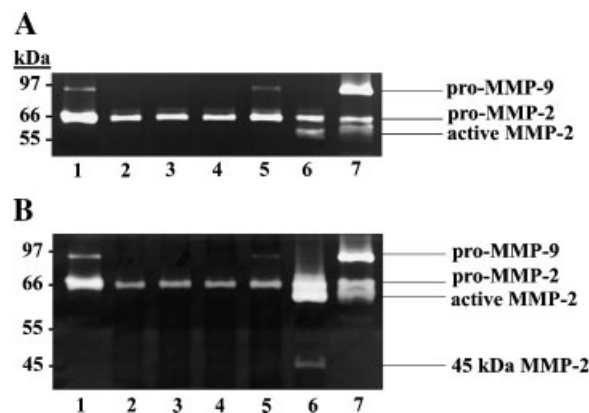

Fig. 1. MMP-2 activation induced by ConA in cardiac fibroblasts. Cells were treated for 24 h with ET-1 (10^{-7} M, **lane 3**), Ang II (10^{-6} M, **lane 4**), PMA (100 nM, **lane 5**), or ConA (10 µg/ml, **lane 6**) in 100 mm dishes. Gelatinolytic activities in conditioned media (A: 2.5 µl per lane) and whole cell lysates (B: 6 µg per lane) were assayed using zymography. A sample from time-matched untreated monolayer cells was included as a control (A and B, **lane 2**). Additionally medium samples from untreated and PMA (100 nM)-treated HT1080 cells (A and B, **lanes 1** and **7**, respectively) were included as positive controls.

Concurrently, treatment of cardiac fibroblasts with ConA (10 µg/ml) for 24 h, but not ET-1, Ang II, or PMA, induced the up-regulation of cellular MT1-MMP (Fig. 2A,B) and TIMP-2 (Fig. 2D). Here, two antibodies raised against the amino acid sequence of MT1-MMP, were employed to clarify which form(s) of MT1-MMP were being expressed by ConA-treated cardiac fibroblasts. Western blot analysis using the 1C1 mAb, which reacts with active MT1-MMP [Okada et al., 1997], detected a species with a molecular mass of ∼57 kDa (Fig. 2A). Further analysis was then performed using the 2D7 mAb, which recognizes the hemopexin-like domain of MT1-MMP. This antibody has previously been successfully used to detect different forms of MT1-MMP in HT1080 cells [Maquoi et al., 2000a]. With reference to molecular mass markers and whole cell lysates prepared from untreated and PMA-treated HT1080 cells (Fig. 2B), multiple bands that were immunoreactive to 2D7 in whole cell lysates prepared from ConA-treated cardiac fibroblasts were identified as the pro-, active, and processed forms of MT1-MMP (Fig. 2B). The pro-form migrated with a molecular mass (∼65 kDa; with occasionally two bands, ∼65 and 68 kDa, detectable) slightly higher than its counterpart (∼63 kDa) in HT1080 cells. The active form (∼57 kDa) had a molecular mass similar to the active MT1-MMP form found present in HT1080 cells. The processed form corresponding to 43 kDa MT1-MMP in HT1080 cells appeared to be a doublet (∼40−43 kDa). Additionally, a second doublet species with an estimated molecular mass of ∼33−36 kDa was identified in the lysates prepared from ConA-treated cardiac fibroblasts. This doublet band, and a single band with a similar molecular mass (∼36 kDa) observed in the lysates prepared from PMA-treated HT1080 cells, is possibly a further processed form of MT1-MMP, containing the hemopexin-like domain as described previously [Hernandez-Barrantes et al., 2000] and has therefore been designated as a "further processed MT1-MMP fragment/form." Furthermore, expression of MT1-MMP in the differential fractions of ConA-treated cardiac fibroblasts was detected. According to the Western blotting results (Fig. 2C), substantial levels of pro-, active, 43 kDa, and further processed MT1-MMP species were all present in cell lysates prepared from the membrane-containing fraction, the post-saponin-extraction residual
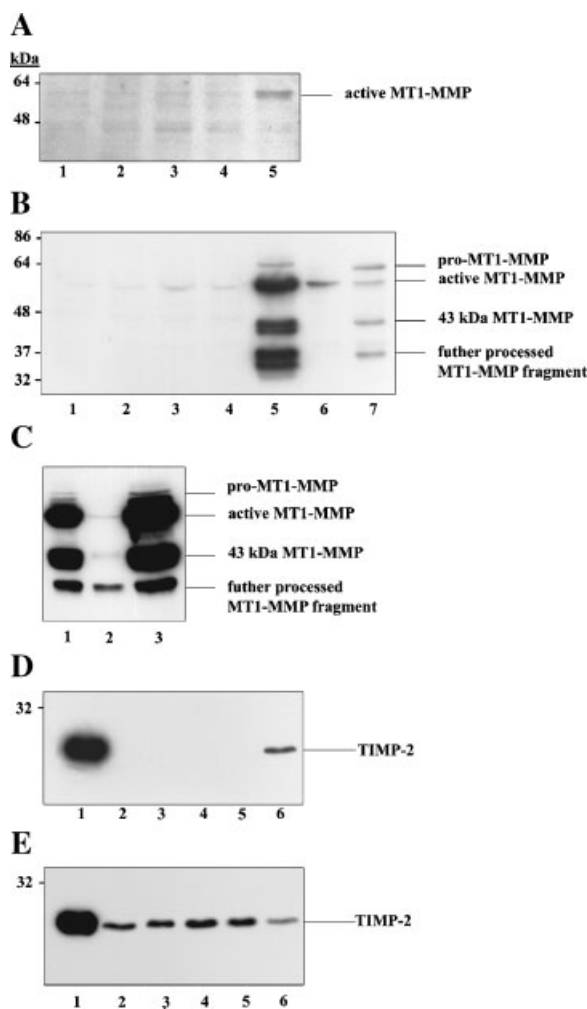

Fig. 2. ConA-induced MMP-2 activation concurs with elevated levels of cellular active MT1-MMP and TIMP-2 and decreased secreted levels of TIMP-2. Cells were treated for 24 h with ET-1 (10^{-7} M, **lane 2** in A and B or **lane 3** in D and E), Ang II (10^{-6} M, **lane 3** in A and B or **lane 4** in D and E), PMA (100 nM, **lane 4** in A and B or **lane 5** in D and E), or ConA (10 µg/ml, **lane 5** in A and B or **lane 6** in D and E) in 100 mm dishes. **A** and **B**: Expression of MT1-MMP in whole cell lysates was detected by Western blotting using 1C1 (A, 40 µg per lane) and 2D7 (B, 25 µg per lane) mAbs. A sample from time-matched untreated cells was included as a control (A and B: **lane 1**). Additionally, lysate samples from untreated and PMA (100 nM)-treated HT1080 cells (B: **lanes 6** and **7**, respectively; 40 µg per lane) were included as positive controls. **C:** Subcellular distribution of MT1-MMP species in ConA (10 µg/ml)-treated cells detected by Western blotting using 2D7 mAb (**lane 1**, 50 µg whole cell lysates; **lane 2**, concentrated sample corresponding to 50 µg saponin-extracted intracellular soluble proteins; **lane 3**, 50 µg cell lysates prepared from post-saponin-extraction residual cellular components). **D** and **E**: Expression of TIMP-2 in whole cell lysates (D: 40 µg per lane) or conditioned media (E: a concentrated sample corresponding to 300 µl conditioned media per lane) was detected by Western blotting using Ab-2 mAb. A sample from time-matched untreated cells was included as a control (D and E: **lane 2**). Additionally, recombinant human TIMP-2 (D and E: **lane 1**, 150 ng per lane) was included as a positive control.

cellular components, whilst the saponin-extracted intracellular protein fraction contained low levels of active, 43-kDa, and further processed MT1-MMP species.

Cellular TIMP-2 expression was detectable when the cells were treated with ConA (10 µg/ml), but not ET-1, Ang II, or PMA (Fig. 2D). Additionally, Western blot analysis indicated that the treatment of cardiac fibroblasts with ConA decreased the levels of TIMP-2 in conditioned media (Fig. 2E).

ConA-Induced MMP-2 Activation Is Temporal Process, Which Coincides With Elevated Cellular MT1-MMP and TIMP-2 Levels

ConA-induced MMP-2 activation was found to be time-dependent (Fig. 3A,B). The profile of MMP-2 activities in both conditioned media and whole cell lysates gradually changed over the first 8 h of ConA (10 µg/ml) incubation. Appearance of the active MMP-2 species in the lysates was earlier than that in the media: this species was detectable within 8 h in the lysates, but was not apparent in the media until 19 h of incubation with ConA. After 19 h of the incubation substantial levels of active MMP-2 species were observed in both the media and the lysates.

Following treatment with ConA (10 µg/ml) the pattern of cellular MT1-MMP expression gradually changed over the time period examined (Fig. 3C). Pro-MT1-MMP was detectable after an 8 h or longer period of ConA incubation. Immunoreactive bands attributable to active MT1-MMP did not appear to increase during the early period of ConA incubation, but became stronger with prolonged periods of the incubation. Forty-three kilodalton of MT1-MMP was detectable throughout the period considered. The further processed form of MT1-MMP was detected as early as 4 h after ConA incubation. Unexpectedly, an MT1-MMP monoclonal antibody (2D7) reactive band, with a low molecular mass, was detectable in concentrated conditioned media from cells incubated with ConA (10 µg/ml) for prolonged periods (Fig. 3D). This species seemed to correspond to the further processed MT1-MMP form observed in whole cell lysates prepared from ConA-treated cells, and it is likely to be an MT1-MMP fragment containing the hemopexin-like domain, which is recognized by this antibody. TIMP-2 was detectable in whole cell lysates prepared from ConA-treated cardiac fibroblasts but only after longer periods of incubation with ConA (Fig. 3E).
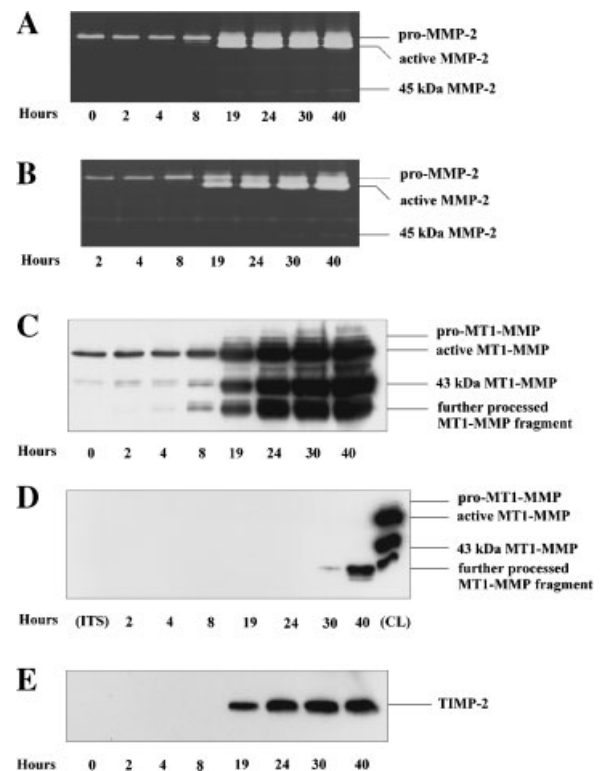

Fig. 3. ConA-induced MMP-2 activation is correlated with up-regulation of MT1-MMP and TIMP-2 in cardiac fibroblasts. Cells were treated with ConA (10 µg/ml) for variable durations in 100 mm dishes. Lysate and medium samples were collected at the indicated time points. **A** and **B**: MMP-2 activities in whole cell lysates (A: 6 µg per lane) and conditioned media (B: 10 µl per lane) were assayed using zymography. **C** and **D**: Expression of MT1-MMP in whole cell lysates (C: 50 µg per lane) and conditioned media (D: a concentrated sample corresponding to 200 µl conditioned media per lane) were detected by Western blotting using 2D7 mAb. Additionally serum-free medium containing insulin, apo-transferrin, and selenium (ITS in D) and whole cell lysates (CL in D) prepared from cardiac fibroblasts treated with ConA (10 µg/ml) for 24 h were included as controls. **E**: TIMP-2 expression in whole cell lysates (50 µg per lane) was detected by Western blotting using Ab-2 mAb.

Cell Membranes Prepared From ConA-Treated Cardiac Fibroblasts Activates Pro-MMP-2

As confirmed by zymography (Fig. 4), incubation of the cell membrane fraction prepared from ConA-treated cardiac fibroblasts with either conditioned media from untreated monolayer cells containing latent MMP-2 species (Fig. 4B) or recombinant human pro-MMP-2 (Fig. 4D), resulted in a concentration-dependent increase of gelatinolytic band intensity corresponding to the active MMP-2 species. This indicated that this membrane fraction was capable of activating both rat and human
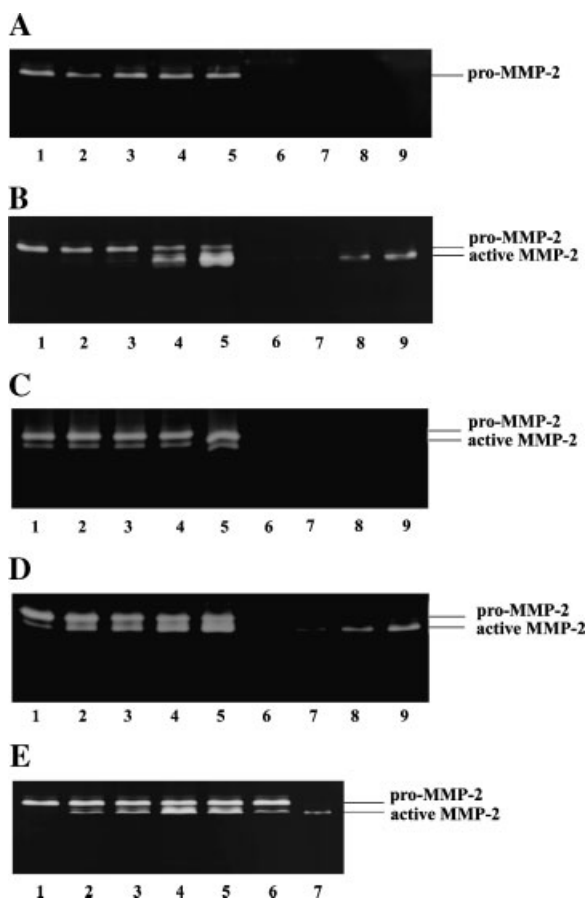
Fig. 4. Cell membranes prepared from ConA-treated cardiac fibroblasts activate pro-MMP-2. **A** and **C**: Effect of cell membrane fractions prepared from control cells on activation of either (A) pro-MMP-2 in conditioned media from untreated cells (**lane 1**: 5 μl media alone; **lanes 2–5**: 5 μl media incubated with 75, 150, 300, and 600 ng membrane fractions, respectively; **lanes 6–9**: 75, 150, 100, 300, and 600 ng membrane fractions, respectively) or (C) recombinant human pro-MMP-2 (**lane 1**: 5 ng human pro-MMP-2 alone; lanes 2–5: 5 ng human pro-MMP-2 incubated with 75, 150, 300, and 600 ng membrane fractions, respectively; lanes 6–9, 75, 150, 300, and 600 ng membrane fractions, respectively). **B** and **D**: Effect of cell membrane fractions prepared from ConA-treated cells on activation of either (B) pro-MMP-2 in the conditioned media from untreated cells (**lane 1**: 5 μl media alone; **lanes 2–5**: 5 μl media incubated with 75, 150, 300, and 600 ng membrane fractions, respectively; **lanes 6–9**, 75, 150, 100, 300, and 600 ng membrane fractions, respectively) or (D) recombinant human pro-MMP-2 (**lane 1**: 5 ng human pro-MMP-2 alone; **lanes 2–5**: 5 ng human pro-MMP-2 incubated with 75, 150, 300, and 600 ng membrane fractions, respectively; **lanes 6–9**, 75, 150, 300, and 600 ng membrane fractions, respectively). **E**: Biphasic effect of TIMP-2 on MMP-2 activation mediated by cell membrane fractions prepared from ConA-treated cardiac fibroblasts (**lanes 2–6**: 300 ng membrane fractions was first reacted with 0, 0.1, 0.5, 1, and 10 ng TIMP-2, respectively, and then incubated with 5 μl conditioned media from untreated cells; **lane 1**, conditioned media only; **lane 7**, cell membrane fraction only).

pro-MMP-2 species. In contrast, the cell membrane fraction prepared from untreated time-matched control cells did not have this property (Fig. 4A,C).

To further characterize this membrane-mediated MMP-2 activation, the effect of recombinant human TIMP-2 was tested. TIMP-2 had a pronounced effect on the MMP-2 activation mediated by the membrane fraction prepared from ConA-treated cardiac fibroblasts: small amounts of recombinant TIMP-2 enhanced MMP-2 activation whilst amounts greater than 1 ng attenuated the activation (Fig. 4E).

High Levels of MT1-MMP and TIMP-2 Are Associated With Cell Membranes Prepared From ConA-Treated Cardiac Fibroblasts

In contrast to the untreated control, the cell membrane fraction prepared from ConA-treated cardiac fibroblasts contained higher levels of the active MT1-MMP species (Fig. 5A). Coincidently, high levels of TIMP-2 were also present in the cell membrane fraction prepared from ConA-treated cardiac fibroblasts, but not in that prepared from time-matched untreated cells (Fig. 5B). TIMP inhibitory activities associated with the cell membrane fraction were also examined using reverse-zymography. An inhibitory species with a similar molecular mass to recombinant TIMP-2 ($\sim$21 kDa) was present in higher levels in the membrane fraction prepared from the ConA-treated cells, compared to that prepared from the time-matched control (Fig. 5C).

Inhibition of Furin Reduces Levels of Active MMP-2 in Conditioned Media but do not Modify Levels of Cell-Associated Active MMP-2

In this present study, the protocol used by Sato et al. [1999] was initially used to test the effect of furin inhibitor I (FI, Decanoyl-Arg-Val-Lys-Arg-chloromethylketone), on ConA-induced MMP-2 activation in cardiac fibroblasts cultured as a monolayer. Cells were pretreated with variable concentrations of FI (0.1–100 μM) for 6 h before the addition of ConA (10 μg/ml) for a further 18 h. As shown by zymography, FI did not alter the pattern of active MMP-2 gelatinolytic activity in either conditioned media (Fig. 6A) or whole cell lysates (Fig. 6B). In later experiments using a single FI concentration (100 μM) and extending the incubation time with ConA (10 μg/ml) to 24 h, it was
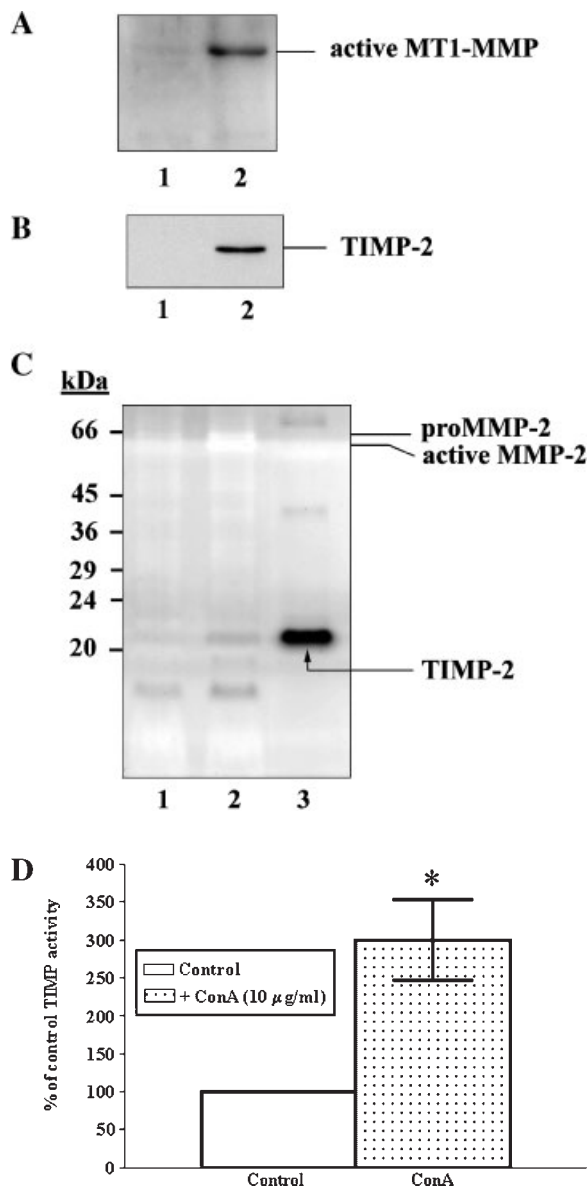
A

active MT1-MMP

1   2

B

TIMP-2

1   2

C

kDa

66 —

45 —

36 —

29 —

24 —

20 —

proMMP-2
active MMP-2

TIMP-2

1   2   3

D

% of control TIMP activity

□ Control
□ + ConA (10 μg/ml)

*

400
350
300
250
200
150
100
50
0

Control   ConA

Fig. 5. High levels of active MT1-MMP and TIMP-2 are associated with cell membranes prepared from ConA-treated cardiac fibroblasts. **A** and **B**: Expression of MT1-MMP (A) or TIMP-2 (B) in a cell membrane fraction prepared from ConA(10 μg/ml)-treated cells (**lane 2** in A and B: 5 μg per lane) was detected by Western blotting using 1C1 (A) or Ab-2 (B) mAb. A cell membrane fraction prepared from time-matched untreated cells was included as a control (**lane 1**: 5 μg per lane). **C**: Levels of TIMP activity in a cell membrane fraction prepared from ConA-treated cells (lanes 1 and 2: control and ConA-treated cells, respectively, 5 ng per lane; lane 3: 15 ng recombinant human TIMP-2). **D**: Histogram showing the levels of TIMP activity associated with cell membrane fractions prepared from ConA-treated cells Each value indicates the mean ± SE in four experiments and is expressed as the percentage of control value. *, $P < 0.05$ versus control.

A

pro-MMP-2
active MMP-2

+ FI (μM)   0   1   5   10   25   50   100

B

pro-MMP-2
active MMP-2

+ FI (μM)   0   1   5   10   25   50   100

C

pro-MMP-2
active MMP-2

1   2

D

pro-MMP-2
active MMP-2

1   2

E

A/T ratio (%)

□ Control
□ + FI (100 μM)

60
50
40
30
20
10
0

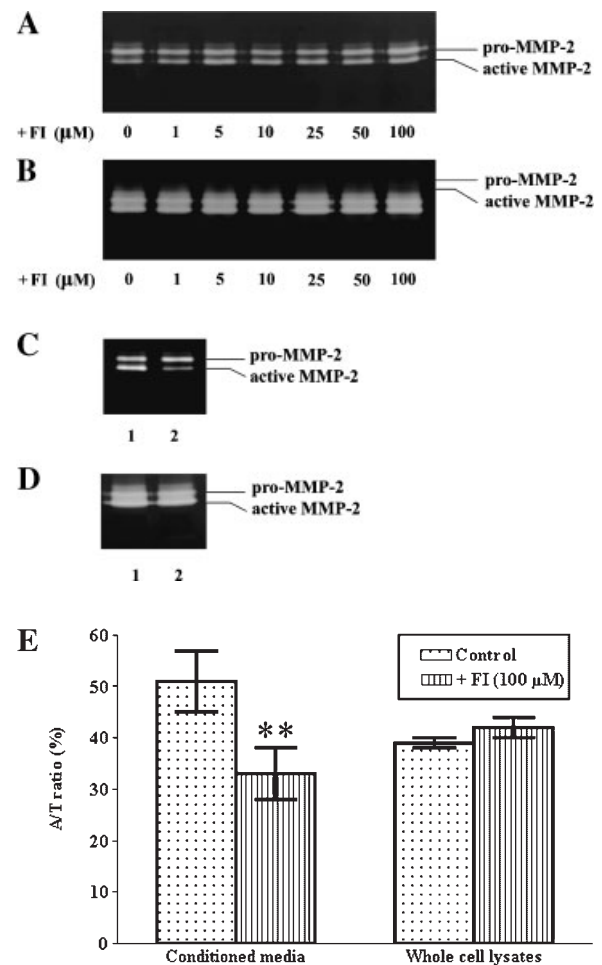**

Conditioned media   Whole cell lysates

Fig. 6. FI decreases release of active MMP-2 species from ConA-treated cardiac fibroblasts in a time-dependent manner. **A** and **B**: Cells were pretreated with FI at the indicated concentrations for 6 h before the addition of ConA (10 μg/ml) for a further 18 h in 6-well plates. MMP-2 activity in conditioned media (A: 10 μl per lane) and whole cell lysates (B: 6 μg per lane) were measured using zymography. **C** and **D**: Cells were incubated in the absence (**lane 1**) or presence (**lane 2**) of FI (100 μM) for 6 h before the addition of ConA (10 μg/ml) for a further 24 h in a 6-well plate. MMP-2 activity in conditioned media (C: 5 μl per lane) and whole cell lysates (D: 6 μg per lane) and were measured using zymography. **E**: Effect of FI (100 μM) on the A/T ratios in conditioned media and whole cell lysates prepared from cells co-incubated with FI and ConA for 24 h. Each value indicates the mean ± SE in three experiments. **, $P < 0.01$ versus control untreated with FI.

found that FI decreased the levels of active MMP-2 in conditioned media (Fig. 6C,E) but not in whole cell lysates (Fig. 6D,E).

Inhibition of Furin Prevents Processing of Pro-MT1-MMP to Active MT1-MMP

To examine the effect of FI on ConA-induced MT1-MMP expression by cardiac fibroblasts,

the cells were pretreated with FI (100 μM) for 6 h and then incubated with ConA (10 μg/ml) for a further 18 h. Two mAbs (2D7 and 1C1) for MT1-MMP were employed for subsequent Western blot analysis. The concurrent treatment of cells with FI and ConA resulted in lower levels of active MT1-MMP in whole cell lysates compared to cells treated solely with ConA (Fig. 7A,B). Moreover, immunoblot results using 2D7 showed that the concurrent treatment with FI and ConA increased cellular levels of pro-MT1-MMP (Fig. 7A). Furthermore, this treatment led to the appearance of another immunoreactive band (with an estimated molecular mass of ∼62 kDa) located above the active MT1-MMP (Fig. 7A). This band is likely to be an intermediate form between the pro- and active MT1-MMP.

Inhibition of Furin Reduces the Levels of Cell-Associated TIMP-2

To examine the effect of furin inhibition on ConA-induced TIMP-2 expression by cardiac fibroblasts, the cells were pretreated with FI (100 μM) for 6 h and subsequently incubated with ConA (10 μg/ml) for a further 18 h. As demonstrated by Western blotting (Fig. 7C), concurrent treatment of cells with FI and ConA reduced levels of TIMP-2 in whole cell lysates compared to those prepared from cells treated solely with ConA.

Cellular Expression of Furin in ConA-Treated Cardiac Fibroblasts

As demonstrated by Western blotting (Fig. 7D), a mAb (Mon-139), which recognizes epitope region 4 in the cytoplasmic tail of furin, reacted with a species with an estimated molecular mass of ∼87 kDa in whole cell lysates from both control and ConA-treated cardiac fibroblasts. Treatment of cells with ConA (10 μg/ml) for 24 h did not alter the expression of 87 kDa furin in whole cell lysates.

DISCUSSION

Profile of ConA-Induced MMP-2 Activation in Cardiac Fibroblasts

In the present study, it was found that, consistent with results from rat mesangial cells, human lung fibroblasts, and HT1080 cells [Allenberg et al., 1994; Gervasi et al., 1996], MMP-2 activation induced by ConA in rat cardiac
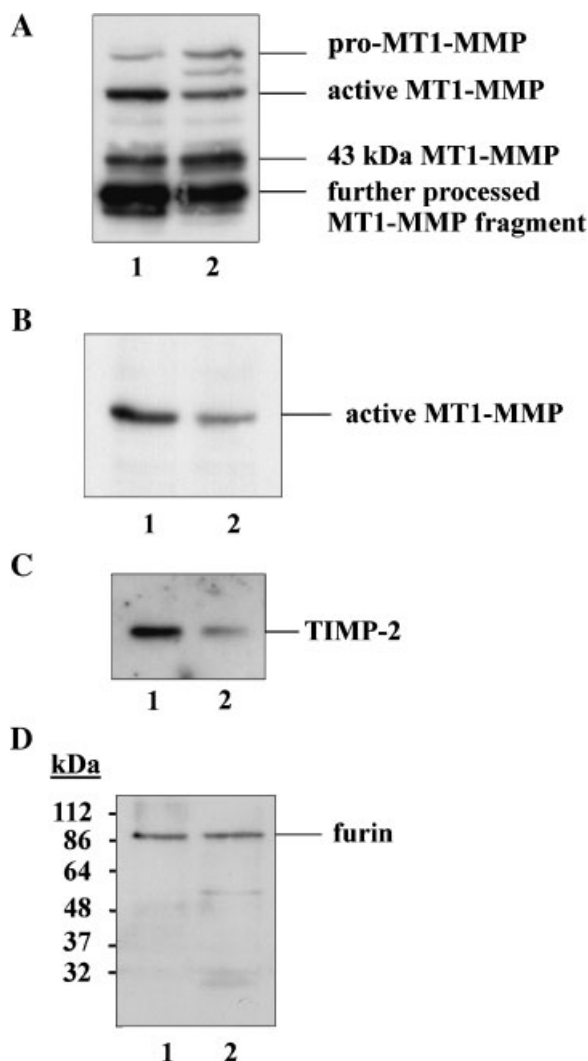

Fig. 7. Inhibition of furin prevents pro-MT1-MMP processing and reduces levels of cell-associated TIMP-2. **A, B** and **C**: Cells were incubated in the absence (**lane 1** in A, B, and C) or presence (**lane 2** in A, B, and C) of FI (100 μM) for 6 h before the addition of ConA (10 μg/ml) for a further 18 h in a 6-well plate. Expression of MT1-MMP in whole cell lysates was detected by Western blotting using 2D7 (A: 25 μg per lane) and 1C1 (B: 40 μg per lane) mAbs. Expression of TIMP-2 in whole cell lysates was detected by Western blotting using a polyclonal antibody C-20 (C: 25 μg per lane). **D**: Cells were incubated in the absence (**lane 1**, 80 μg per lane) or presence (**lane 2**, 80 μg per lane) of ConA (10 μg/ml) for 24 h. Expression of furin in whole cell lysates was detected by Western blotting using Mon-139 mAb.

fibroblasts is a concentration-dependent process: ConA at a concentration of 5 μg/ml or greater induced the appearance of active MMP-2 species both in conditioned media and whole cell lysates prepared from cardiac fibroblasts cultured as a monolayer (data not shown). Similar to MMP-2 activation by cardiac fibro-

blasts embedded in three-dimensional type I collagen lattice [Guo and Piacentini, 2003], ConA-induced MMP-2 activation by cardiac fibroblasts grown as a monolayer coincided with enhanced cellular levels of MT1-MMP and TIMP-2. This similarity indicates that MT1-MMP and TIMP-2 may be important factors for both MMP-2 activation systems in this cell type.

Subcellular Distributions of MT1-MMP in ConA-Treated Cardiac Fibroblasts

Interestingly, active, 43 kDa, and further processed forms of MT1-MMP were all found in the intracellular protein fraction prepared from ConA-treated cardiac fibroblasts, whilst in the intracellular protein fraction prepared from untreated control cells grown as a monolayer, in agreement with our previous observation [Guo and Piacentini, 2003], only 43 kDa MT1-MMP was detected (data not shown). These results suggest that ConA treatment modulates the subcellular distributions of MT1-MMP in this cell type.

Internalization of active MT1-MMP via endocytosis has been demonstrated in HT1080 and MDCK cells [Jiang et al., 2001; Remacle et al., 2003]. Moreover, internalization of the 43 kDa MT1-MMP can be detected in HT1080 cells either stably transfected with full-length human MT1-MMP cDNA or treated with PMA [Remacle et al., 2003]. Considering these findings, it is tempting to speculate that the presence of differential forms of MT1-MMP in the intracellular protein fraction prepared from ConA-treated cardiac fibroblasts could be due to internalization since ConA can trigger endocytosis in fibroblasts [Rosenblith et al., 1973].

Extracellular Release of MT1-MMP Fragment by ConA-Treated Cardiac Fibroblasts

MT1-MMP antibody-reactive polypeptides have been observed in conditioned media from ConA-treated cells before where a 56 kDa MT1-MMP species, recognized using an antibody against the catalytic domain of MT1-MMP, was detected in conditioned media from human breast carcinoma cells treated with ConA in a concentration and time-dependent manner [Harayama et al., 1999]. In contrast, in the present study, a smaller polypeptides was detected using an antibody (2D7) against the hemopexin-like domain of MT1-MMP in conditioned media after 30 h of ConA treatment. This dissimilarity

between the two studies is likely to be attributable to the utilization of different antibodies, the difference in ConA concentrations and ConA exposure time, or cell type used. The smaller polypeptides detected in this study is likely to be an MT1-MMP fragment released by ConA-treated cardiac fibroblasts. This raised the possibility that cell may clear the proteolytically inactivated form of MT1-MMP either from the cell surface or from intracellular compartments to the extracellular milieu.

Contribution of TIMP-2 to ConA-Induced MMP-2 Activation

Similar to results obtained from two previous studies using tongue squamous carcinomas cells (HSC-4) [Shofuda et al., 1998] and human uterine cervical fibroblasts [Itoh et al., 1998], in this study a high level TIMP-2 was found in both whole cell lysates and cell membranes prepared from ConA-treated cardiac fibroblasts: this coincided with elevated active MT1-MMP levels and MMP-2 activation. In contrast, TIMP-2 was undetectable in either whole cell lysates or cell membranes prepared from untreated control cells. This suggests that, similar to our findings in type I collagen-induced MMP-2 activation [Guo and Piacentini, 2003], the presence of TIMP-2 may also be a prerequisite for the occurrence of MMP-2 activation following ConA treatment in this cell type. The gradual appearance of TIMP-2 in whole cell lysates prepared from the ConA-treated cells during the examined time periods suggests that this protein may be steadily integrated into the MMP-2 activation "machinery." Additionally, reverse-zymography of of cell membrane samples revealed that treatment of cells with ConA resulted in a decrease in gelatinase activity corresponding to a species with a similar molecular mass as the TIMP-2 recombinant standard. The identity of this species remains unresolved because the molecular mass of TIMP-2 has been demonstrated to be identical to that of TIMP-4 (another TIMP member highly expressed in the myocardium [Greene et al., 1996; Leco et al., 1997] and previously identified utilizing reverse-zymography [Bigg et al., 2001]). In this present study, the profile of TIMP-4 expression by cardiac fibroblasts was not defined. Therefore, this inhibitory species could be TIMP-2, TIMP-4, or a combination of the two inhibitors.

As previously shown in human uterine cervical fibroblasts [Itoh et al., 1998], in the present

study, the presence of high levels of TIMP-2 in whole cell lysates was concomitant to reduced levels of this protein in conditioned media following ConA treatment. The reduced secreted levels of TIMP-2 could potentially have been attributed to either the recruitment of this protein to the cell surface or to the decreased synthesis of this protein via down-regulation of Timp-2 gene expression. However, at least in HT1080 cells, the latter possibility has been convincingly disproved [Maquoi et al., 2000a,b].

Profile of MMP-2 Activation Mediated by Cell Membranes Prepared From ConA-Treated Cardiac Fibroblasts

In this study, cell membranes were prepared from ConA-treated cardiac fibroblasts, and proved to have potential to process both rat latent MMP-2 (in conditioned media) and human pro-MMP-2 to their active enzymes. Similar to observations made in a previous study using human uterine cervical fibroblasts [Itoh et al., 1998], high levels of MT1-MMP and TIMP-2 were associated with cell membranes prepared from ConA-treated rat cardiac fibroblasts. It can therefore be deduced that the two proteins are key components of cell membranes-based MMP-2 activation "machinery." Furthermore, in reference to the tri-molecular complex model established previously [Strongin et al., 1995; Butler et al., 1998], the potentiation of MMP-2 activation observed with small amounts of exogenous TIMP-2 (0.1−1 ng), may be due to formation of rat MT1-MMP/human TIMP-2 complexes (the putative "receptor" for pro-MMP-2). The attenuation of MMP-2 activation with a larger amount of exogenous TIMP-2 ($\geq$10 ng) may result from the saturation of previously free MT1-MMP molecules. This result strongly suggests that the ratio of TIMP-2/MT1-MMP serves as an important factor to regulate MMP-2 activation mediated by cell membranes prepared from ConA-treated cardiac fibroblasts.

Role of Furin in ConA-Induced MMP-2 Activation in Adult Rat Cardiac Fibroblasts

With reference to a previous study carried out using rabbit dermal fibroblasts, in which ConA-induced MMP-2 activation was not modulated either by FI or furin antisence oligonucleotide [Sato et al., 1999], a similar experimental protocol was adopted in this study to test the effect of FI on ConA-induced MMP-2 activation in cardiac fibroblasts. The A/T ratios in both conditioned media and whole cell lysates were not altered after treatment with FI. However, this treatment did cause a substantial decrease in active MT1-MMP levels and an increase in pro-MT1-MMP levels in whole cell lysates, indicating that furin is essential for the pro-MT1-MMP processing induced by ConA in this cell type. In view of the possibility that MMP-2 activation is a temporal event secondary to the extent of pro-MT1-MMP processing, later experiments were carried out to test the effect of a high concentration (100 μM) of FI on MMP-2 activation by cardiac fibroblasts treated with ConA (10 μg/ml) for an extended incubation time (24 h). Under these conditions, inhibition of furin attenuated the release of active MMP-2 species by the ConA-treated cardiac fibroblasts. The conclusion of the previous study remains questionable since direct information about the effect of either FI or the furin antisense oligonucleotide on pro-MT1-MMP protein processing was not included. The discrepancy between the two studies could also be attributed to differences in species, tissues, or cell types used.

It is currently difficult to explain why the A/T ratio in whole cell lysates from ConA-treated cardiac fibroblasts remained unchanged following FI treatment, since the levels of both components (TIMP-2 and active MT1-MMP) of the putative pro-MMP-2 "receptor" (active MT1-MMP/TIMP-2 complex) and "activator" (active MT1-MMP) were down-regulated. One possibility is the presence of some unknown mechanisms that are responsible for the binding of pro-MMP-2 to the cells or the release of active MMP-2 from the cells. Several molecules could be considered. It has been demonstrated in human uterine cervical fibroblasts that TIMP-2 can binds to the cell membrane by two different mechanisms upon ConA treatment: one is sensitive to a peptidyl hydroxamic acid MMP inhibitor and is presumably dependent on active MT1-MMP, and the other is insensitive to the inhibitor and is likely to be dependent on an as yet unidentified binding protein [Itoh et al., 1998]. Provided that these two mechanisms are applicable to ConA-treated cardiac fibroblasts, furin inhibition could only down-regulate levels of those TIMP-2 molecules dependent on active MT1-MMP on the cell surface. The TIMP-2 molecules that are bound to the cell surface in

an MT1-MMP independent manner could have a role for the binding of both pro- and active forms of MMP-2. Another molecule that can bind to pro-MMP-2 via its C-terminal domain is TIMP-4 [Bigg et al., 1997]. However, there is no current evidence to show that TIMP-4 can form a complex with active MT1-MMP as an alternative "receptor" on the cell surface for pro-MMP-2 when cell-associated TIMP-2 is down-regulated. Additionally, it has been demonstrated that $\alpha_v\beta_3$ integrin can bind to the hemopexin-like domains of both pro- and active forms of MMP-2 in hamster melanoma cells (CS-1) [Brooks et al., 1996]. However, possibility of the involvement of the $\alpha_v\beta_3$ integrin in the binding of MMP-2 to cardiac fibroblasts can be discounted from the following findings: (i) both furin and active MT1-MMP are functional convertases for the generation of mature α_v via processing of pro-α_v [Deryugina et al., 2002; Ratnikov et al., 2002], and (ii) FI treatment can prevent the processing of the pro-α_v subunit [Strongin et al., 1995]. FI would therefore reduce the effect of any $\alpha_v\beta_3$ integrin-dependent protein rather than potentiate it.

Perspective

Although it is still difficult to ascertain the direct physiological context of MMP-2 and MT1-MMP activations, induced by ConA in cardiac fibroblasts cultured as a monolayer, data obtained in this study may potentially be useful for identifying pathophysiologically relevant factors contributing to changes in the activation state of MMP-2 and/or MT1-MMP in this cell type. For example, cardiac fibroblasts are likely to be the target for a wide range of cytokines such as IL-1 β, TNF-α, and IL-6, released by infiltrated inflammatory cells following myocardial infarction [Ono et al., 1998; Yue et al., 1998]. These cytokines in vitro have previously been shown to be capable of inducing active MMP-2 secretion by cardiac fibroblasts [Siwik et al., 2000]. The mechanism underlying this secretion, however, remains to be investigated in depth.

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