Type I Collagen-induced MMP-2 Activation Coincides with Up-regulation of Membrane Type 1-Matrix Metalloproteinase and TIMP-2 in Cardiac Fibroblasts*

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Received for publication, July 7, 2003, and in revised form, September 4, 2003
Published, JBC Papers in Press, September 11, 2003, DOI 10.1074/jbc.M307238200

Migration of cardiac fibroblasts is implicated in infarct healing and ventricular remodeling. Activation of matrix metalloproteinases induced by three-dimensional type I collagen, the principal component of the myocardial interstitium, is hypothesized to be essential for this migration. By utilizing primary cultures of cardiac fibroblasts and collagen lattice models, we demonstrated that type I collagen induced MMP-2 activation, and cells undergoing a change from isometric tension to mechanical unloading were associated with increased levels of total and active MMP-2 species. The collagen-induced MMP-2 activation coincided with up-regulated cellular levels of both membrane type 1-matrix metalloproteinase (MT1-MMP) and TIMP-2. A fraction of cellular membrane prepared from cells embedded in the collagen lattice containing active MT1-MMP and TIMP-2 was capable of activating pro-MMP-2, and exogenous TIMP-2 had a biphasic effect on this membrane-mediated MMP-2 activation. Interestingly, the presence of 43-kDa MT1-MMP species in a fraction of intracellular soluble proteins prepared from monolayer cells but not cells embedded in the lattices indicates that MT1-MMP metabolizes differently under the two different culture conditions. Treatment of cells embedded in the lattice with furin inhibitor attenuated pro-MT1-MMP processing and MMP-2 activation and impeded cell migration and invasion. These results suggest that the migration and invasion of cardiac fibroblasts is furin-dependent and that the active species of MT1-MMP and MMP-2 may be involved in both events.

Cardiac fibrosis, a consequence of infarct healing and ventricular remodeling, is characterized by hyperplasia of α-smooth muscle actin expressing fibroblast-like cells and deposition of excessive extracellular matrix (ECM)1 proteins by these cells in the myocardium. These cells are thought to originate from cardiac fibroblasts, which are recruited from the interstitium of the heart following myocardial injury (1). In vitro cardiac fibroblasts grown as a monolayer have been proved to be migratory cells (2, 3). However, cardiac fibroblasts in vivo reside in the interstitial fibrillar collagen network; the migratory/invasive potential of cardiac fibroblasts, by which the cells detach from the collagen network where they normally reside in vivo and then penetrate basement membrane surrounding necrotic myocytes, has not been investigated previously.

Type I collagen, the principal component of the interstitial fibrillar collagen network, is a known matrix effector for MMP-2 activation in various cell types including neonatal rat cardiac fibroblasts (4). However, the mode of MMP-2 activation induced by three-dimensional type I collagen remains largely undefined (5–11). Furthermore, reorganization of the cytoskeleton subsequent to changes in mechanical tension in the collagen lattice has been reported to underlie three-dimensional type I collagen-induced MMP-2 activation in human skin fibroblasts (12) but not rat capillary endothelial cells (8), raising the possibility of a differential role for mechanical tension dependent on cell type.

Considerable evidence supports a role for MMP-2 and MT1-MMP in cell migration. First of all, MMP-2 activation and high levels of MT1-MMP are well correlated with tumor spread (13–15). Second, MMP-2 (16, 17) and MT1-MMP (18, 19) have been localized to the migration front of invasive cells, and addition of exogenous active MMP-2 facilitates invasion of tumor cells (20, 21). Finally, transfection of cells with MMP-2 (17) and MT1-MMP (22–26) promotes their migratory and invasive potentials. Moreover, generation of active MT1-MMP and mature MMP-2, which is necessary for proteolytic degradation of ECM during cell migration and invasion, requires the action of furin in many cell types studied (29, 30). Inhibition of furin prevents collagen-induced pro-MT1-MMP processing and MMP-2 activation in HT1080 cells (31) and melanoma MV3 cell line (32), and this inhibition impairs invasive potential of these cells measured by their ability to penetrate a barrier composed of type IV collagen or reconstituted basement membrane. Alternatively, transfection with α1-PDX (a protein-based specific furin inhibitor) cDNA prevents pro-MT1-MMP processing and MMP-2 activation in head and neck squamous cell carcinoma cells and attenuates the invasive capacity of these cells (33). These findings suggest that furin modulates cell migration and invasion.

We hypothesized that the activation of matrix metalloproteinases induced by three-dimensional type I collagen would be essential for the migration and invasion of cardiac fibroblasts residing in the collagen matrix in vitro. By utilizing primary cultures of cardiac fibroblasts and collagen lattice models, we

* This work was supported by grants (to L. P.) from De Montfort University and the UK National Heart Research Fund. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: ECM, extracellular matrix; MMP, matrix metalloproteinase; pro-MMP, matrix metalloproteinase with propeptide domain; TIMP, tissue inhibitor of matrix metalloproteinases; MT1-MMP, membrane type 1-matrix metalloproteinase; FI, furin inhibitor I, decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone; PET, polyethylene terephthalate; ConA, concanavalin A; MEM, minimal essential medium; DMEM, Dulbecco’s modified minimal essential medium; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride.

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demonstrated that three-dimensional type I collagen-induced MMP-2 activation coincided with up-regulated cellular levels of MT1-MMP and TIMP-2. High levels of active MT1-MMP and TIMP-2 were associated with a fraction of cellular membrane prepared from cells embedded in the collagen lattice, and 43-kDa MT1-MMP species was found in a fraction of intracellular soluble proteins prepared from monolayer cells but not cells embedded in a polymerized collagen lattice. Inhibition of furin-like protease activity in a stabilized MT1-MMP and MMP-2 activation induced by three-dimensional type I collagen and impedes the migration and invasion of cells embedded in the collagen lattice. Furthermore, the expression profile of cellular furin protein by cells embedded in the collagen lattice was different from that expressed by their counterparts cultured as a monolayer. These results suggest that the migration and invasion of cardiac fibroblasts is furin-dependent and that the active species of MMP-2 and MT1-MMP may be involved in both events.

EXPERIMENTAL PROCEDURES

Cell Culture—Cardiac fibroblasts were prepared by enzymatic dissection of left ventricular tissue from adult male Sprague-Dawley rats (250–450 g), as described previously (34), and maintained in minimal essential medium (MEM) (Invitrogen) containing 10% (v/v) newborn bovine calf serum (Invitrogen) at 37 °C, in humidified air supplemented with 5% CO2 until the cells were almost confluent. Two to three rats were used for each preparation. First and second passage cardiac fibroblasts were used for experiments. Human fibrosarcoma cell lines (HT1080 cells, European Collection of Cell Culture, EC number 8511505) were maintained in Dulbecco’s modified essential medium (DMEM), 10% (v/v) fetal bovine serum (Invitrogen), and 1% (v/v) non-essential amino acids at 37 °C in humidified air supplemented with 5% CO2.

Preparation of Collagen Lattices—Three-dimensional type I collagen lattices were prepared as described previously in other cells type (5, 12) with some modifications. Type I collagen (BD Biosciences), 10% concentrated MEM, NaOH (1 N), and deionized H2O were used to make up the solution that was added to three parts of collagen lattice solution. The final collagen concentration was 1% (v/v) and the pH was adjusted to 2.6 using NaOH. The collagen lattice solution was constituted in the pH-adjusted buffer. The collagen lattices were washed three times with PBS and incubated with PBS (pH 7.2) containing 0.1% (v/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 1 mM PMSF, 10 μM E-64, 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 10 μg/ml pepstatin A was immediately added. The post-saponin extraction residual components were then lysed with the chilled lysis buffer as stated above. Following protein precipitation, supernatants were washed with cold ethanol. Anti-MMP-2 antibodies (Abcam), 250 μg/ml pestatin A, 10 μM E-64, 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 25 mM PMSF were added. The precipitates were then resuspended in this buffer. Cell membrane fractions were prepared from time-matched untreated cells grown as a monolayer using an identical protocol.

Preparation of Whole Cell Lysates—Polymerized collagen lattices containing embedded cells were centrifuged at 400 × g to remove as much water as possible, then suspended in a large volume of ice-cold PBS, mixed well, and centrifuged again. This wash procedure was performed 5 times. The entire lattice was then mixed with a chilled lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM CaCl2, 1% (v/v) IGEFA, 0.05% (v/v) Brij 35, 1 mM PMSF, 10 μM E-64, 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 10 μg/ml pepstatin A, homogenized by trituration with a 22-gauge needle for 30 passages, vigorously vortexed, and lysed on ice for 60 min. After a centrifugation at 4,000 × g for 15 min, supernatants corresponding to the whole cell lysates were collected. Meanwhile, lysate samples from time-matched cells grown as a monolayer were prepared using an identical protocol. The cells were thoroughly washed twice with PBS and scraped into the chilled lysis buffer.

Preparation of Intracellular Soluble Proteins—Intracellular soluble proteins were prepared as described previously (36) with some modifications. Following 24 h of culture, monolayer cells or cell-populated collagen lattices were washed three times with PBS and incubated with PBS (pH 7.2) containing 0.1% (v/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 1 mM PMSF, 10 μM E-64, 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 10 μg/ml pepstatin A was immediately added. The post-saponin extraction residual components were then lysed with the chilled lysis buffer as stated above. Following protein precipitation, supernatants were washed with cold ethanol. Anti-MMP-2 antibodies (Abcam), 250 μg/ml pestatin A, 10 μM E-64, 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 25 mM PMSF were added. The precipitates were then resuspended in this buffer. Cell membrane fractions were prepared from time-matched untreated cells grown as a monolayer using an identical protocol. Western blot analysis was performed. Sample proteins were prepared under reducing conditions, size-fractionated in a 10% SDS-polyacrylamide resolving gel, and transferred onto Hybond-P hydrophobic polyvinylidene difluoride membrane. Monoclonal antibody against MMP-2 (AB-3, used in 1:100 dilution) and anti-TIMP-2 (2D7, generously gifted by Dr. Marie-christine Rio, Institut de Genetique et de Biologie Moleculaire et Cellulaire, Strasbourg, France, used in 1:50 dilution), furin (Mon-139, Alexius Biochemicals, used in 1:1,000 dilution), and a polyclonal antibody against TIMP-2
Matrigel matrix for 2 hi nahumidified tissue culture incubator at 37 °C was added to the interior of the inserts and wells to rehydrate the storage and allowed to come to room temperature. Warm MEM (37 °C) was used in invasion experiments, and the chambers were termed "migration chambers." Cell culture inserts coated with a thin layer of the Matrigel basement membrane matrix were employed in cell invasion experiments, and the chambers were termed "invasion chambers." Before each experiment, invasion chambers were removed from −20 °C storage and allowed to come to room temperature. Warm MEM (37 °C) was added to the interior of the inserts and wells to rehydrate the Matrigel matrix for 2 h in a humidified tissue culture incubator at 37 °C, 5% CO2 atmosphere. After rehydration, the medium was carefully removed without disturbing the layer of Matrigel matrix on the PET membrane. When migration or invasion experiments were performed, trypsinized cardiac fibroblasts were preincubated with 50 μM furin inhibitor 1 (FI, decanoyl-RVVR-chloromethyl ketone, Calbiochem) for 20 min and then seeded in type I collagen solution at a density of 3.0 × 104 cells/ml. The final concentration of the collagen was 1 mg/ml. 800 μl of collagen lattice solution was layered over the upper surface of each cell culture insert to make a stabilized lattice. After 60 min of incubation at 37 °C to allow for polymerization of the collagen, 2.5 ml of serum-free MEM containing 10−6 M angiotensin II (a known chemoattractant) (2) was added to each well of the accompanying 6-well plate. The serum-free MEM containing 50 μM FI was also layered over the collagen lattice immediately. The assembled invasion and migration chambers were incubated for 24 h in a humidified tissue culture incubator at 37 °C, 5% CO2 atmosphere. At the end of the incubation period, collagen lattices were removed from the upper surface of the inserts, and the surface of the insert was then gently but firmly rubbed using a cotton-tipped swab to remove all remaining cells and/or the Matrigel matrix on the PET membrane. The bottom side of the insert was then sequentially passed through PBS (pH 7.4) containing 10 mm sodium periodate, 75 mm l-lysine, and 2% (w/v) paraformaldehyde for 2 min, 0.1% crystal violet for 2 min, and two beakers of deionized water to remove excess stain. The PET membrane was air-dried and then cut from the insert housing using the tip of a sharp scalpel blade and mounted, bottom side down, on a microscope slide. Cells on the membrane were observed under a microscope. For migration experiments, each membrane, cells in four random microscopic fields were counted at 400× magnification (39). The mean of total cell numbers in duplicate membranes from three independent experiments using different cell populations was used for statistical comparisons.

Cell Counting—To assess the effect of FI on cell numbers, confluent cardiac fibroblasts grown as a monolayer were serum-starved and then counted at 400× magnification (39). Additionally, a lysate sample from ConA (10 μg/ml)-treated monolayer cells was included as a positive control (C, lane 5–40 μg per lane). A lysate sample from time-matched untreated monolayer cells (C, lane 1–40 μg per lane) was included as a control. The differences between the two studies indicate that age may be a factor to influence the pattern of MMP-2 secretion. In contrast to findings from human skin fibroblasts (12) and in agreement with findings using rat capillary endothelial cells (8), there were no significant differences between A/T ratios or levels of total MMP-2 activity in the conditioned media from the three lattice types (Table I). However, values of A/T ratio and levels of total MMP-2 activity in whole cell lysates prepared from cardiac fibroblast-populated released lattices were higher than those in the lysates prepared from cardiac fibroblast-populated stabilized lattices (Table I). These findings suggested that alterations in mechanical tension might regulate the development of a cellular "proteolytic" phenotype or the extent of MMP-2 activation in the three-dimensional lattice in a cell-dependent manner. In addition, a gelatinolytic species corresponding to a band near 97 kDa, and likely to be pro-MMP-2, was also found in conditioned media and whole cell lysates prepared from cardiac fibroblasts embedded in all three lattice types (Fig. 1, A and B).

RESULTS

Cellular MT1-MMP Expression—As demonstrated by zymog-
The estimated molecular mass of MT1-MMP was measured using zymography (Fig. 2), which is a method to detect MMP activity. The processed 43-kDa MT1-MMP form was longer (Fig. 2C), corresponding to pro-MT1-MMP species, was detectable in the lysates after the cells were seeded into the lattices for 19 h or longer. Substantial levels of this species were detected as early as 6 h after the cells were seeded into the lattice, and substantial levels of this species were observed in both the lattices and media after 19 h or longer. Coincidentally, as shown by Western blotting (Fig. 2C), cellular expression of MT1-MMP in cardiac fibroblasts embedded in the lattice under conditions of contact with lattices indicated that MT1-MMP expression goes through a dynamic metabolism.

Levels of active MT1-MMP in whole cell lysates prepared from cardiac fibroblasts embedded in three-dimensional type I collagen lattice gradually changed over the period examined. The active MMP-2 species appeared in both the lysates and media as early as 2 h after the cells were seeded into the lattice, and substantial levels of this species were detected in both the lysates and media after 19 h or longer. A species with an estimated molecular mass of 65 kDa, corresponding to pro-MT1-MMP species, was detectable only after the cells were seeded into the lattice for 19 h or longer (Fig. 2C). The processed 43-kDa MT1-MMP form was evident throughout the period considered. The species with an estimated molecular mass of 34 kDa detected at some time points is likely to be a further processed fragment containing the hemopexin-like sequences of MT1-MMP. The gradual changing profile of cellular MT1-MMP during variable durations of contact with lattices indicated that MT1-MMP expressed by cardiac fibroblasts embedded in the lattice undergoes a dynamic metabolism.

**Cellular Levels of MT1-MMP and TIMP-2 Are Up-regulated in Cardiac Fibroblasts Embedded in Three-dimensional Type I Collagen Lattices**—Compared with time-matched monolayer control cells, cardiac fibroblasts embedded in three-dimensional type I collagen lattice expressed high levels of active MT1-MMP and pro-MT1-MMP 24 h after seeding (Fig. 3A). TIMP-2 was detectable only after the cells were seeded into the lattice for 19 h or longer (Fig. 3B). Similarly, cellular expression of TIMP-2 in HT1080 cells embedded in three-dimensional type I collagen lattice was higher than that in HT1080 cells grown as a monolayer (Fig. 3B). However, cellular levels of MT1-MMP and TIMP-2 in the three type lattices were comparable (Fig. 3, C and D).

**Cell Membrane Fraction Prepared from Cardiac Fibroblasts Embedded in Three-dimensional Type I Collagen Lattices Activates Pro-MMP-2**—As demonstrated by zymography (Fig. 4), cell membrane fraction prepared from the stabilized lattices was capable of activating the latent form of MMP-2 secreted by untreated monolayer cells (Fig. 4B) and recombinant human pro-MMP-2 (Fig. 4D). In both cases this activation was concentration-dependent. In contrast, neither cell-secreted pro-MMP-2 (Fig. 4A) nor the recombinant proenzyme (Fig. 4C) was activated by cell membrane fractions prepared from the monolayer control cells. Furthermore, small amounts of recombinant human TIMP-2 promoted this activation of the secreted pro-MMP-2, whereas larger amounts of the TIMP-2 inhibited the activation (Fig. 4, E and F).

**Cell Membrane Fraction Prepared from Cardiac Fibroblasts Embedded in Three-dimensional Type I Collagen Lattices Contains Higher Levels of Active MT1-MMP Than Their Counterparts Grown as a Monolayer and Detectable Levels of TIMP-2**—As shown by Western blotting (Fig. 4G), levels of active MT1-MMP present in the cell membrane fraction prepared from cardiac fibroblasts embedded in three-dimensional type I collagen lattices were higher than those in the cell membrane fraction prepared from cardiac fibroblasts grown as a monolayer. TIMP-2 was detectable only in the cell membrane fraction prepared from cells embedded in the lattice (Fig. 4H).

**Fraction of Intracellular Soluble Proteins Prepared from Cardiac Fibroblasts Embedded in Three-dimensional Type I Collagen Lattices Contains Pro- and Active MMP-2 but Not MT1-MMP**—Lee et al. (5) suggested that three-dimensional type I collagen-induced MMP-2 activation by human skin fibroblasts occurs intracellularly. In order to identify if this was also the case for cardiac fibroblasts, gelatinolytic MMP activities in differential fractions of cells embedded in three-dimensional type I collagen lattice was examined. Zymographic analysis showed that although there were gelatinolytic activities in the saponin-extracted intracellular protein fraction of cardiac fibroblasts embedded in the lattice corresponding to pro- and active MMP-2 species, higher levels of gelatinolytic activity attributable to both species were observed in cell lysates prepared from the membrane-containing fraction, the post-saponin-extraction residual cellular components (Fig. 5A). This indicates that the MMP-2 activation induced by type I collagen occurs in the intracellular compartment and is associated with
Inhibition of Furin Decreases Cellular Levels of Active MT1-MMP and Increases Cellular Levels of Pro-MT1-MMP—Following treatment with FI (100 μM), the levels of pro-MT1-MMP in whole cell lysates prepared from cardiac fibroblasts embedded in three-dimensional type I collagen lattices increased, and the levels of active MT1-MMP in the lysates decreased (Fig. 6D). It has been noticed that, for these cells treated with FI, the extent of decrease in the levels of active MT1-MMP is larger than the extent of increase in the levels of pro-MT1-MMP. It is unlikely that the larger decreased extent of active MT1-MMP was because of further down-regulation of the active enzyme via an autocatalytic mechanism because inhibition of furin did not lead to increased cellular levels of the 43-kDa form and a species lower than 43 kDa. Rather, it is possible that accumulated pro-MT1-MMP due to inhibition of furin served as a negative feedback signal for pro-MT1-MMP synthesis to result in a moderate increase in the levels of pro-MT1-MMP. In contrast to the changes in MT1-MMP expression profile, cellular levels of TIMP-2 did not change following this treatment (Fig. 6E).

Cardiac Fibroblasts Embedded in Three-dimensional Type I Collagen Lattice Express Furin Protein Differently Than Their Counterparts Grown as a Monolayer—Whole cell lysates prepared from cardiac fibroblasts, grown either as a monolayer or embedded in a three-dimensional type I collagen lattice, contained a protein of ~87 kDa that was recognized by a monoclonal antibody (Mon-139) against the furin cytoplasmic tail. Another band with a higher molecular mass (~97 kDa) was found only in whole cell lysates prepared from cells embedded in the lattice (Fig. 6F).

Inhibition of Furin Impedes Cell Migration and Invasion—After 24 h of incubation, many cardiac fibroblasts were observed on the bottom side of the PET membranes in both migration (Fig. 7A) and invasion (Fig. 7C) chambers. Treatment with FI (50 μM) decreased the number of cells on the bottom side of the membrane in migration chambers (Fig. 7, B and E), indicating the impaired capability of cardiac fibroblasts to detach from surrounding type I collagen matrix, whereas following this treatment only few cells were seen on the bottom side of the membrane in invasion chambers (Fig. 7D), suggesting the markedly reduced ability of cardiac fibroblasts with the impaired migratory capability to penetrate the layer of the Matrigel matrix. In addition, incubation of FI (25–100 μM) with cardiac fibroblasts grown as a monolayer under serum-free conditions for 48 h did not modify cell number (Fig. 7F).

**DISCUSSION**

Cardiac fibroblasts embedded in three-dimensional type I collagen lattices secrete, and are associated with, high levels of active MMP-2. This raises the possibility that an *in vivo* interaction between the fibrillar collagen network and cardiac fibroblasts works as a “motor” to generate active MMP-2 species which may, in turn, be involved in myocardial ECM turnover. Previous studies (40–42) carried out in fibroblast-populated collagen lattices suggest that changes in mechanical tension play an important role in synthesis and remodeling of new connective matrix proteins deposited at the sites of injury. In these studies, the floating lattice resembles a mechanically relaxed tissue at healthy state or very early stage of wound healing; the stabilized lattice is comparable with stressed granulation tissue, and the released lattice represents granulation tissue in which mechanical tension is gradually unloaded. In light of this, and based on the data obtained from the present study, it is likely that moderate MMP-2 activation by cardiac fibroblasts occurs at an early stage of infarct healing and during the formation of granulation tissue, which would favor synthesis of ECM. Once the area of myocyte loss is replaced...
Type I Collagen-induced MMP-2 Activation in Cardiac Fibroblasts

Evidence has accumulated that shows that expression of MT1-MMP is essential for MMP-2 activation by several cell types embedded in three-dimensional type I collagen lattice (8–11). We provided further data to support a role of MT1-MMP in the collagen-induced MMP-2 activation, for the first time demonstrating that elevated cellular levels of active MT1-MMP protein in cardiac fibroblast-populated type I collagen lattice coincides with MMP-2 activation. The capability of cell membranes containing high levels of active MT1-MMP, prepared from cardiac fibroblasts embedded in three-dimensional type I collagen lattice to activate pro-MMP-2, suggests that the presence of a sufficient amount of active MT1-MMP protein on the cell surface may be essential for an occurrence of the MMP-2 activation. However, the presence of active MMP-2 species, which was not associated with active MT1-MMP, in the intracellular protein fraction prepared from cardiac fibroblasts embedded in the lattice raises two possibilities for type I collagen-induced MMP-2 activation: (i) an intracellular MMP-2 activation pathway not involving active MT1-MMP may run in parallel with a MMP-2 activation pathway involving active MT1-MMP on the cell surface, or (ii) intracellular activation of pro-MMP-2 may be subsequent to intracellular activation of pro-MT1-MMP prior to or during transferring of active MT1-MMP to the cell surface, where it is known to act as the activator of pro-MMP-2. In the present study, the elevated extent of MMP-2 activation, as indicated by an increase of about 10% in A/T ratio value (Table I), was observed in whole cell lysates prepared from cardiac fibroblasts embedded in the released lattice. The putative intracellular pathway not involving active MT1-MMP might be responsible for this elevation when cells underwent a change from isometric tension to mechanical unloading, because cellular levels of active MT1-MMP were comparable in three types of lattices. However, it should be noted that in this study it remains undetermined whether the levels of Mmp-2 gene expression by cardiac fibroblasts are equal in three type lattices. Therefore, another possibility cannot be excluded that if releasing lattice from isometric tension triggered an up-regulated Mmp-2 gene expression and this led to an increased pro-MMP-2 protein production, then the increased amount of pro-MMP-2 protein might be processed and thus might result in increased levels of total and active MMP-2 species in the presence of free active MT1-MMP molecule. In contrast to the findings in whole cell lysates, MMP-2 expression profile in conditioned media prepared from cardiac fibroblasts embedded in released lattices was similar to those displayed in conditioned media prepared from cells embedded in other two type lattices. Although there is no clear answer for this discrepancy, some clues may shed light on it. As shown in Fig. 5A, compared with the intracellular protein fraction, the membrane-containing fraction contained much higher levels of active MMP-2 species, suggesting that the majority of the active species might be generated on the cell surface. Given that the presence of active MMP-2 species in conditioned media was a heterogeneous composition resulting from cell release of the active enzyme from the cell surface and intracellular compartments, the putative cell surface-based pathway would be a main source of the active species. Comparable levels of active MT1-MMP and TIMP-2 in three type lattices could result in a

dimensional type I collagen lattice (lane 2, −5 (G) or 10 (H) μg per lane). Cell membrane fraction prepared from time-matched monolayer cardiac fibroblasts was included as controls (lane 1, −5 (G) or 10 (H) μg per lane). The immunoblot presented is representative of three experiments carried out using different cell populations.

Fig. 4. Cell membranes prepared from cardiac fibroblasts embedded in three-dimensional type I collagen lattices activates pro-MMP-2. A and C, effect of cell membrane fractions prepared from monolayer control cells on activation of either pro-MMP-2 released by untreated monolayer cardiac fibroblasts (A, lane 1, 5 μl of media alone; lanes 2–4, 5 μl of media incubated with ~100, 200, and 400 ng of membrane fractions, respectively; lanes 5–7, ~100, 200, and 400 ng of membrane fractions, respectively) or recombinant human pro-MMP-2 (C, lane 1, ~5 ng of human pro-MMP-2 alone; lanes 2–4, ~5 ng of human pro-MMP-2 incubated with ~100, 200, and 400 ng of membrane fractions, respectively; lanes 5–7, ~100, 200, and 400 ng of membrane fractions, respectively). B and D, effect of cell membrane fractions prepared from cardiac fibroblasts embedded in three-dimensional type I collagen lattices on activation of either pro-MMP-2 released by untreated monolayer cardiac fibroblasts (B, lane 1, 5 μl of media alone; lanes 2–4, 5 μl of media incubated with ~100, 200, and 400 ng of membrane fractions, respectively; lanes 5–7, ~100, 200, and 400 ng of membrane fractions, respectively) or recombinant human pro-MMP-2 (D, lane 1, ~5 ng of human pro-MMP-2 alone; lanes 2–4, ~5 ng of human pro-MMP-2 incubated with ~100, 200, and 400 ng of membrane fractions, respectively; lanes 5–7, ~100, 200, and 400 ng of membrane fractions, respectively). E, biphasic effect of TIMP-2 on MMP-2 activation mediated by cell membrane fraction prepared from cardiac fibroblasts embedded in three-dimensional type I collagen lattices under cell-free conditions. Lanes 2–6, ~200 ng of the membrane fractions was first reacted with 0, 0.1, 0.5, 1, and 10 ng of TIMP-2, respectively, and then incubated with 5 μl of conditioned media from untreated monolayer cardiac fibroblasts; lane 1, conditioned media only; lane 7, cell membrane fraction only. F, inhibitory effect of TIMP-2 on the MMP-2 activation. Lanes 2–6, ~400 ng of the membrane fractions was first reacted with different amounts of 0, 1, 10, 50, and 100 ng of TIMP-2, respectively, and then incubated with 5 μl of culture media from untreated monolayer cardiac fibroblasts; lane 1, conditioned media only; lane 7, cell membrane fraction only. Each zymogram presented is representative of three experiments carried out using different cell populations. G and H, expressions of MT1-MMP (G) and TIMP-2 (H) in cell membrane fraction prepared from cardiac fibroblasts embedded in three-dimensional type I collagen lattice (lane 2, −5 (G) or 10 (H) μg per lane). Cell membrane fraction prepared from time-matched monolayer cardiac fibroblasts was included as controls (lane 1, −5 (G) or 10 (H) μg per lane). The immunoblot presented is representative of three experiments carried out using different cell populations.
similar extent of MMP-2 activation occurring on the cell surface, and this could lead to similar levels of active MMP-2 species released from the cell surface. As discussed above, the putative intracellular pathway might be attributable to the small scale increase in the extent of cellular MMP-2 activation by cells embedded in the released lattice. It is possible that this small scale increase was not enough to make a significant increase of active MMP-2 levels in conditioned media prepared from cells embedded in the released lattice under the culture conditions used in the present study. However, it is actually unknown what the function of intracellular active MMP-2 species is and how cells regulate this species. In this regard, the differential MMP-2 expression profiles displayed in conditioned media and whole cell lysates prepared from cardiac fibroblasts embedded in three type lattices also could be caused by an unknown mechanism underlying release or binding of cellular MMP-2 species.

Metabolism of MT1-MMP in monolayer culture appears to be different from that in a three-dimensional lattice system. In comparison to cells embedded in three-dimensional type I collagen lattice, cardiac fibroblasts grown as a monolayer expressed a lower level of active MT1-MMP. 43-kDa MT1-MMP was found in the whole cell lysates, and this species was mainly present in the intracellular soluble protein fraction. This finding supports a novel notion that in untreated monolayer cells, active MT1-MMP may be intracellularly processed into the 43-kDa form which may in turn be routed to lysosomes for destruction because no released species around or lower than 43 kDa were found in time-matched culture media (data not shown), and this internalization could be due to endocytosis for down-regulation of the levels of MT1-MMP expressed on the cell surface as observed previously (43) in HT1080 cells.

The exact role of TIMP-2 in type I collagen-induced MMP-2 activation remains undetermined. Cellular levels of TIMP-2 did not change when human skin fibroblasts (44) and rat capillary endothelial cells (8) were cultured in type I collagen lattices. In contrast to those findings, our results demonstrated that the levels of cardiac fibroblast-associated TIMP-2 increased when these cells were embedded in three-dimensional type I collagen lattices. Similar changes in cellular TIMP-2 levels were observed when HT1080 cells were embedded in the lattice. Moreover, a cell membrane fraction containing TIMP-2 in addition to active MT1-MMP, prepared from cardiac fibroblasts embedded in the lattice, was capable of activating pro-MMP-2. Furthermore, a biphasic effect of TIMP-2 was observed in MMP-2 activation mediated by the cell membrane fraction prepared from cardiac fibroblasts embedded in the lattice, suggesting that the ratio of TIMP-2/MT1-MMP is a factor that regulates this cell-free membrane-mediated activation. All these observations support a role for TIMP-2 in type I collagen-induced MMP-2 activation in this cell type. Additionally, in the absence of detectable levels of cell-associated TIMP-2, MMP-2 activation did not occur in cardiac fibroblasts grown as a monolayer, albeit these cells did express detectable levels of active MT1-MMP, implying that the presence of cell-associated TIMP-2 is likely to be a prerequisite for an occurrence of MMP-2 activation in the lattice.

It has been established that pro-MT1-MMP is intracellularly processed to the active MT1-MMP species by furin or a furin-like proprotein convertase due to the existence of a furin recognition motif, 10RRKR, between the propeptide and catalytic domains (29). Similarly to findings using HT1080 cells cultured on type IV collagen (31) and in a high invasive melanoma cell line, MV3, cultured in type I collagen lattice (32), in this study inhibition of furin decreased pro-MT1-MMP processing in whole cell lysates prepared from cardiac fibroblasts embedded in three-dimensional type I collagen lattice, and this coincided with attenuation of active MMP-2 secretion and decreased levels of cell-associated active MMP-2. Treatment with F1 did not alter levels of cell-associated TIMP-2 in cardiac fibroblasts embedded in the lattice. This finding was unexpected because furin inhibition of ConA-treated cells down-regulated cellular levels of TIMP-2. 2 Although it is possible that the detection techniques used in this study were not sensitive enough to detect subtle alterations in TIMP-2 levels, this finding suggests

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2 C. Guo and L. Piacentini, unpublished results.
that the mechanism for the collagen-induced MMP-2 activation differs from that for ConA-induced MMP-2 activation in that the association of TIMP-2 with cells embedded in the lattice may be in a manner independent of active MT1-MMP. Altogether, these findings indicate that three-dimensional type I collagen-induced MMP-2 activation in cardiac fibroblasts is furin-dependent, and it is likely that this activation requires active MT1-MMP.

Little is known about the expression profile of furin by cells embedded in the lattice. In this study, a furin-like protein, possibly pro-furin, is detectable in whole cell lysates prepared from cardiac fibroblasts embedded in the lattice but not cells grown as a monolayer. This novel finding suggests that cardiac fibroblasts might increase synthesis of pro-furin upon its interaction with three-dimensional type I collagen. Furthermore, this raises the possibility that increased pro-furin levels may be essential for pro-MT1-MMP processing and MMP-2 activation in cells embedded in three-dimensional type I collagen lattice.

Results from the migration and invasion experiments performed in this study indicate, for the first time, that primary cardiac fibroblasts are capable of detaching from three-dimensional type I collagen matrix, penetrating the Matrigel basement membrane, and passing through microscopic pores in the PET membrane. Considering the nature of the three-dimensional collagen lattice culture system, a close equivalent of the in vivo myocardial collagen network, these results further suggest that, under certain circumstances, cardiac fibroblasts residing in the interstitium of the heart could migrate and invade cellular compartments. Vracko et al. (45) described an interesting phenomenon, observed in a myocardial infarction model: at the stage of tissue repair following necrosis of myocytes, fibroblast-like cells were found in the empty compartment surrounded by basement membranes, and numerous holes were observed in the membranes. Results in the present study could provide an explanation: to enter the areas of the spaces once occupied by healthy myocytes, cardiac fibroblasts "drill" holes in the surrounding basement membranes by degrading specific ECM component.

The capability of cardiac fibroblasts to detach from three-dimensional type I collagen and penetrate the reconstituted basement membrane could be attributable to active MT1-MMP generated from pro-MT1-MMP processing and mature MMP-2 arising from MMP-2 activation. Active MT1-MMP could be involved in degradation of type I collagen (46) and processing of CD44 (a major hyaluronic acid receptor expressed in migratory cells) (27). This would allow cardiac fibroblasts to detach from the collagen matrix, migrate, and participate in the digestion of laminins, entactin, and heparan sulfate proteoglycans (46, 47) present in the Matrigel basement membrane matrix. Active MMP-2 could be responsible for the degradation of type I collagen (48) when cardiac fibroblasts migrate from the lattice, and digestion of type IV collagen (49), laminins (50), and entactin (51) when the cells traverse the Matrigel basement membrane matrix. Accordingly, future work is necessary to clarify the exact roles of these MMP species in migration and invasion of cardiac fibroblasts from the three-dimensional lattice. Impairment of both the migration and invasion of cardiac fibroblasts prepared from cardiac fibroblasts embedded in three-dimensional type I collagen lattices and incubated in the absence (lane 1 in D and E) or presence (lane 2 in D and E) of FI (100 µM) were detected using Western blotting (~50 D or 80 E µg per lane). F, cells were maintained in the stabilized lattice for 24 h. Expression of furin in lysate samples was detected using Western blotting (lanes 1 and 2, monolayer control and lattice samples, respectively; ~80 µg per lane).
blasts from three-dimensional type I collagen lattice by furin inhibition is likely to be subsequent to reduced levels of active MT1-MMP and MMP-2 species, as postulated by previous studies (31–33). However, there are other possibilities to account for this impairment. It has been demonstrated that both furin and MT1-MMP are functional convertases for pro-\(\alpha_\text{i}\) integrin receptor, and this, in turn, might affect cell locomotion. Another possibility is related to a possible cytotoxic effect of FI due to its irreversible inhibition of furin (53). However, if this were the case for cardiac fibroblasts, FI is likely to have had a profound effect on cell behavior rather than on cell numbers, as the case for cardiac fibroblasts, FI is likely to have had a profound effect on cell behavior rather than on cell numbers.

Acknowledgments—We thank Dr. Marie-christine Rio, Institut de Genetique et de Biologie Moleculaire et Cellulaire, Strasbourg, France, for generous donations of monoclonal antibodies for MT1-MMP and Dr. Gayle Hosford for critical reading of the manuscript.

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**Fig. 7.** Inhibition of furin impedes migration or invasion of cardiac fibroblasts. A–D, representative photomicrographs (magnification ×400) demonstrating the migration (A and B) or invasion (C and D) of cardiac fibroblasts from three-dimensional type I collagen lattice in the absence (A and C) or presence (B and D) of FI (50 μM). Examples of a pore (arrow) and a cell (arrowhead) on the PET membrane are indicated. E, inhibitory effect of FI on migration of cardiac fibroblasts from the lattice. Each value indicates the mean ± S.E. mean of three experiments and is expressed as percentage of control (number of migratory cell in the absence of FI) value. **, \( p < 0.01 \) versus control. F, effect of FI on cell number. Each value indicates the mean ± S.E. mean of three experiments and is expressed as percentage of control value (number of cells incubated in the absence of FI).**
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J. Biol. Chem. 2003, 278:46699-46708.
doi: 10.1074/jbc.M307238200 originally published online September 11, 2003

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