Matrix metalloproteinase synthesis and expression in isolated LV myocyte preparations

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Coker, Mytsi L., Melissa A. Doscher, Chadwick V. Thomas, Zorina S. Galis, and Francis G. Spinale. Matrix metalloproteinase synthesis and expression in isolated LV myocyte preparations. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H777–H787, 1999.—In several cardiac disease states, alterations in myocyte and extracellular matrix (ECM) structure occur with left ventricular (LV) remodeling and are associated with changes in matrix metalloproteinase (MMP) activity. Although nonmyocyte cell types have been implicated as sites for synthesis and expression of MMPs within the ECM, whether the LV myocyte itself expresses specific types and active forms of MMPs remains unknown. Accordingly, isolated Ca2+-tolerant LV porcine myocytes (106 cells/ml) in which selective disaggregation and resuspension was performed (13 independent experiments) were plated on basement membrane substrates including Matrigel, collagen IV, laminin, and fibronectin as well as poly-L-lysine. After 24-h incubation, LV myocyte conditioned media were subjected to zymography, a specific MMP-2 proteolytic capture assay, immunoblotting, and ELISA for detection of MMP activity and relative content of the 72-kDa gelatinase MMP-2. Although robust zymographic activity (pixels·mm2/cell) was observed in conditioned media from LV myocytes plated on collagen IV (1,673 ± 297), fibronectin (1,530 ± 281), and poly-L-lysine (2,545 ± 560), proteolytic activity appeared to be lower in conditioned media from LV myocytes plated on Matrigel (842 ± 83) and laminin (1,329 ± 238). MMP-2 proteolytic activity was increased by approximately eightfold in conditioned media taken from LV myocytes plated on poly-L-lysine compared with that of Matrigel. With respect to each of the adhesion substrates, MMP-2 content was at least 50% lower in LV myocyte conditioned media taken from Matrigel and laminin. Immunofluorescent labeling of LV myocytes yielded a strong signal for MMP-2 within the myocyte and along the sarcolemmal surface. In conclusion, this study demonstrated for the first time that adult LV myocytes synthesize and express members of the MMP family and thus may potentially participate in the LV remodeling process through synthesis and secretion of MMPs.

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artery (5 x 5 cm) was dissected free and cannulated. The LV section was then perfused with a modified Krebs solution containing bacterial collagenase (0.5 mg/ml, type II; 273 U/mg; Worthington Biochemical, Freehold, NJ) for 20 min. After perfusion, the LV myocardial tissue was then minced and added to an oxygenated solution containing 2% BSA (Sigma Chemical, St. Louis, MO), 51 Kunitz units/ml deoxyribonuclease (type IV; Sigma), 400 µM CaCl$_2$, and 0.5 mg/ml bacterial collagenase (Worthington). The LV myocardial tissue and myocyte trituration solution were transferred to a centrifuge tube and gently agitated. After 15 min, the trituration solution was drawn off, and the myocyte pellet was vigorously washed three times. First, the myocyte pellet was resuspended in Krebs solution and allowed to settle for 10 min. After the myocyte pellet had reformed, the Krebs solution was discarded, and the myocytes were resuspended in fresh serum-free culture medium (2 mM Ca$^{2+}$; medium 199; Gibco Life Technologies, Grand Island, NY). This washing step in medium was then repeated again. These vigorous wash/resuspension steps were performed to remove any residual collagenase. After the final wash step, the myocytes were resuspended in fresh medium at ~1 x 10^6 cells/ml.

Myocyte primary culture. The myocyte suspensions were plated on 35-mm petri dishes that had been previously coated with the following four different basement membrane substrates: Matrigel (2.26 mg/ml; Collaborative Research, Bedford, MA), laminin (50 µg/ml), fibronectin (50 µg/ml), and collagen IV (500 ng/ml) or poly-L-lysine (0.30 µg/ml; Sigma). The compound Matrigel was chosen for myocyte attachment since it is an artificial basement membrane preparation that contains a number of basement membrane components. The concentrations for the basement membrane components laminin, fibronectin, and collagen IV were selected on the basis of a previous study performed by this laboratory (45). Poly-L-lysine was used to provide a positively charged surface that would allow for nonspecific myocyte attachment. The myocytes were incubated in the presence of these different substrates for 4 h (37°C, 95% O$_2$ and 5% CO$_2$), and then the medium was replaced with fresh serum-free medium. At this point, the number of myocytes adherent to the different substrates was determined using computer-assisted image analysis. Briefly, three independent areas of 0.49 mm of each petri dish were imaged using a x10 objective on an inverted microscope (IM35; Zeiss, Oberkochen, Germany). Field areas were digitized (DT3851; Data Translation, Marlboro, MA), the images were input to a computer (80486 computer; Zeos International), and the total number of myocytes in each petri dish was computed. The myocyte suspensions were then incubated for 24 h (37°C, 95% O$_2$ and 5% CO$_2$), after which the medium was drawn off, separated into aliquots, and frozen in liquid nitrogen. From these media samples, MMP zymographic activity, content, and abundance were determined.

MMP zymography. Before electrophoresis, the LV myocyte media were concentrated using centrifugation (CentraPlus; Amicon, Beverly, MA). Briefly, the media were centrifuged (6,000 rpm for 20 min), and the protein concentration of the LV myocyte media was then determined using a standardized colorimetric assay (Bio-Rad Protein Assay; Bio-Rad, Richmond, CA). The LV myocyte media (0.50 µg total protein) were then directly loaded on electrophoretic gels (SDS-PAGE) containing 1 mg/ml of gelatin (Sigma) under nonreducing conditions as described previously (32). After SDS-PAGE, the gels were washed and incubated for 18 h in a substrate buffer (32). After incubation, the gels were stained using 0.1% amido black, destained, and analyzed as described below.

MMP-2 activity by antibody capture assay. To more carefully examine the specificity of MMP activity in the LV myocyte conditioned media, an additional series of studies was performed in which specific MMP-2 activity was measured by an antibody capture method. For this assay, purified MPP-2 or LV myocyte conditioned media (50 µg total protein) were incubated on a 96-well microtiter plate in which a monodonal MMP-2 antibody was immobilized (RPN2631; Amersham Pharmacia). We have confirmed previously the specificity of this MMP-2 antibody using immunoblotting techniques (10, 32). The specificity and concentration dependency of this MMP-2 activity assay system was first examined using full-length MMP-2 standards (1–12 ng/ml) purified from a transfected HT-1080 cell line as described previously (22, 32). The standards or LV myocardial extracts were incubated overnight at 4°C and then washed vigorously with phosphate buffer solution containing 0.05% Tween 20. Next, p-aminophenylmercuric acetate (1 mM), an organomercurial, was added to activate captured latent MMP-2 (22, 25, 41), after which an enzyme substrate solution containing 50 mM Tris-HCl, 1.5 mM NaCl, 0.5 mM CaCl$_2$, 1 µM ZnCl$_2$, 0.01% BRIJ 35, and chromogenic peptide substrate S-2444 (Amerham Pharmacia Biotech) was added. The reaction was allowed to proceed at 37°C for 2 h, and the absorbance at 405 nm was recorded. Preliminary studies demonstrated that longer incubation times did not result in a further increase in absorbance values. The absorbance from the cleaved chromogenic substrate was linear with increasing concentrations of the purified MMP-2 standards [y = 3.76x; r = 0.99, P < 0.006]. LV myocyte MMP-2 activity was expressed as units of MMP-2 activity based on the linearized results of the purified MMP-2 standards and was expressed as picograms per microgram per square millimeter per cell.

MMP immunoblotting and ELISA. Before immunoblotting, the LV myocyte media were concentrated as stated above. LV myocyte media (4.0 µg total protein) were loaded on an 8% SDS-PAGE and subjected to electrophoretic separation (32). The separated proteins were then transferred to a nitrocellulose membrane. Membranes were blocked with 0.2 M Tris base and 1.4 M NaCl, pH 7.6, containing 5% powdered goat milk, 5% BSA, 0.1% Tween 20, and 0.02% NaN$_3$. After being washed with 0.2 M Tris base and 1.4 M NaCl, pH 7.6, containing 0.1% Tween 20, membranes were incubated overnight at 4°C in a specific monoclonal antibody corresponding to MMP-2 or MMP-9 (1.0 µg/ml; Oncogene Research Products, Cambridge, MA; see Ref. 26). Membranes were then washed, incubated in horseradish peroxidase-conjugated goat anti-mouse antibody (1:5,000 dilution; Bio-Rad), washed again, and conjugated with an activated secondary antibody (enhanced chemiluminescence Western blotting reagents; Amersham Life Science, Arlington Heights, IL). The luminescent signal was detected by exposure to X-ray film (Eastman Kodak, Rochester, NY) for exactly 5 min. Prestained molecular weight markers (Bio-Rad) were used to assess molecular weight and to ensure adequate protein separation and transfer.

A two-site ELISA system was used to quantitate MMP-2 content in the concentrated LV myocyte media (Amersham Life Sciences, Amersham, UK). MMP standards and LV myocyte media were incubated at 25°C for 2 h and washed. The wells were then filled with a horseradish peroxidase-conjugated anti-rabbit sera and incubated for an additional hour. After a final series of washes, the reaction was initiated by the addition of 3,3′,5,5′-tetramethylbenzidine/hydrogen peroxide in dimethylformamide (30% vol/vol) and allowed to proceed for 30 min. The colorimetric reaction was then terminated and stabilized by the addition of 100 µl of M
sulfuric acid, and the plate was read at 450 nm ($V_{\text{max}}$ Kinetic Microplate Reader; Molecular Devices). The MMP content in the LV myocyte media was determined by linear regression analysis obtained from MMP standards. Samples were performed in duplicate, and the optical density (OD) values obtained from the LV myocyte media were in the linear range of the MMP standards.

Immunofluorescent labeling. The LV myocyte primary cultures were plated on microscope slides that were previously coated with 1.0 µg/ml poly-L-lysine. The myocytes were fixed using 3.7% formaldehyde for 10 min and were stored in a buffer (65 mM PIPES, 25 mM HEPES, 10 mM EGTA, and 3 mM MgCl$_2$) at 4°C. The primary antisera used in the present study were smooth muscle actin (1:400 dilution; Sigma), α-actinin (1:700 dilution; Sigma), and MMP-2 (1:200 dilution; Oncogene). Immediately before immunostaining, the myocytes were permeabilized with 1% Triton X-100 for 10 min at room temperature and then washed with PBS. Myocyte preparations were incubated with 10% goat serum or mouse serum for 1.5 h at 4°C and washed with PBS. The myocyte preparations were then flooded with primary antisera and incubated overnight at 4°C. After the overnight incubation, myocytes were washed with PBS and then incubated for 30 min at room temperature with a 1:60 dilution of either goat anti-mouse fluorescein-labeled secondary antibody or goat anti-mouse Texas red-labeled secondary antibody (Sigma). This secondary antibody was diluted using a 1:40 goat serum-PBS mixture. The concentrations for the primary and secondary antibodies employed were chosen on the basis of preliminary dilution studies. The myocytes were examined using fluorescence microscopy (Zeiss Axioplan) at a magnification range of ×20–100 and were imaged using a high-sensitivity video camera (CCD 100; Dage-MTI, Michigan City, IN). The images were acquired using a Flashpoint 128 image capture card (Integral Technologies, Indianapolis, IN) and were processed using Image Pro Plus (Media Cybernetics, Silver Spring, MD). For the images obtained from LV myocytes in which double immunofluorescent staining was performed, single-level digital deconvolution was performed using Micro Tome Software (Vay Tek, Fairfield, IA). In addition, after immunostaining for MMP-2, LV myocytes were analyzed using a volume method of deconvolution (27, 39). With the use of this method, multilevel images at 5-µm intervals were acquired on LV myocytes that provided different optical planes through the cell. These optical planes were integrated, and deconvolution was performed. For assessment of antibody specificity, LV myocytes were imaged using this approach under phase contrast as well as fluorescence, and images were obtained from LV myocytes in which the primary antibody was substituted with nonimmune mouse serum.

Positive cell culture control lines. To provide an internal reference for the experiments performed in the present study, the appropriate positive controls were included. For the zymograms, the conditioned medium from HT-1080 cells (2 µg total protein; ATCC, Rockville, MD) was used. The HT-1080 cells were grown to confluence in DMEM (GIBCO) supplemented with 10% fetal calf serum. Confluent cells were washed with PBS and transferred to serum-free DMEM-F-12 (1:1) for 24 h. The conditioned medium from the HT-1080 cells was then concentrated and handled in the same manner as the LV myocyte media. Confluent HT-1080 cells were also used as a positive control for MMP-2 in the experiments involving immunofluorescent labeling. These cells were handled in the same manner as the LV myocytes.

With respect to immunoblotting, the positive controls for MMP-2 and MMP-9 were obtained from a human epithelial cell line (AG771; Chemicon). This MMP-2/MMP-9 positive control (1:3 dilution with sample buffer) was included in all immunoblots.

Immunofluorescent labeling of smooth muscle actin was employed to confirm the absence of other cell types in the myocyte primary cultures. Smooth muscle cells were used as positive controls for this set of experiments. Smooth muscle cells were isolated and grown to confluence as previously described (24). Briefly, a 10-cm segment of porcine carotid artery was harvested and subjected to sequential incubations in trypsin (0.1%; GIBCO, Detroit, MI) and collagenase (CLS type I; 630 U/ml). Pellets containing 8 × 10$^5$ cells (range 4–15 × 10$^5$), obtained by centrifugation at 150 g, were resuspended in two 0.5-ml aliquots of culture medium (medium 199; GIBCO) and plated on 30-mm plates. Upon reaching confluence, the smooth muscle cells were fixed and stained in the same manner as the LV myocytes.

MMP data analysis. The zymograms were digitized (CCD 72; Dage-MTI) using a constant light intensity. The size-fractionated banding pattern of the zymograms, which indicated MMP activity, was determined by quantitated image analysis (Gel Pro; Media Cybernetics). A densitometric profile was generated by placing a fixed area of interest (0.5 × 0.5 mm) over each of the lysis areas. Next, a two-dimensional integrated OD was computed as $\text{SOD}(x,y) = \Sigma i/\text{-log(intensity}(x,y) - \text{background intensity} - \text{incident light} - \text{background reference})$. The area under the densitometric curve spanning from 100 to 70 kDa was integrated, and this value reflects total zymographic activity.

MMP zymographic activity, specific MMP-2 activity, and MMP content were compared using ANOVA. With respect to the zymograms, values of total proteolytic activity were reported as absolute values expressed in pixels per square millimeter per cell for each of the substrates. Specific MMP-2 activity was reported as picograms per microgram per square millimeter per cell. Total MMP content was reported as nanograms per microgram per square millimeter per cell. These values of MMP activity and MMP content were then analyzed using a one-way ANOVA to examine the effect of substrate type. If the one-way ANOVAs performed on the MMP activity and MMP content data revealed significant differences, pairwise tests of individual group means were compared using Bonferroni probabilities. All statistical procedures were performed using the BMDP statistical software package (BMDP Statistical Software). Results are presented as means ± SE. Values of $P < 0.05$ were considered to be statistically significant.

RESULTS

LV myocyte zymography. Total zymographic activity for the LV myocyte preparations after a 24-h incubation was computed using densitometric methods. A representative densitometric profile obtained from a gelatin zymogram lane is shown in Fig. 1. Myocyte cell density (cell/mm$^2$) was highest with Matrigel (21 ± 2) but was similar in the other substrates: collagen IV (13 ± 2), laminin (15 ± 2), fibronectin (14 ± 2), and poly-L-lysine (15 ± 3). Total LV myocyte zymographic activity was normalized for cell density. A representative gelatin zymogram containing conditioned media from LV myocyte preparations plated on Matrigel, collagen IV, laminin, fibronectin, or poly-L-lysine is shown in Fig. 2. In LV myocyte media conditioned for 24 h, significant zymographic activity was observed between the 100- and 70-kDa region. Zymographic activity in the LV myocyte preparations plated on Matrigel was de-
creased compared with that of the remaining substrates (collagen IV, laminin, fibronectin, and poly-L-lysine). In a parallel set of experiments, LV myocyte suspensions were subjected to centrifugation in pre-

Fig. 1. Isolated left ventricular (LV) myocyte zymographic activity was quantitated by densitometry. After background subtraction, a 5-pixel-wide scan line was placed over each lane, and an intensity profile was generated with respect to molecular weight. A: representative gelatin zymogram lane containing LV myocyte conditioned media. B: integrated optical density for specific proteolytic bands was quantitated from the densitometric profile.

formed gradients of 40% Percoll (Sigma; see Ref. 17) to separate myocytes from nonmyocyte cell populations on the basis of density. These isolated LV myocytes were then resuspended in serum-free medium and handled in the same manner as the previous LV myocyte preparations. Quantitative analysis of the media taken from the Percoll-isolated LV myocytes revealed no difference in zymographic activity when compared with that of the previous LV myocyte preparations, which were not subjected to density centrifugation.

LV myocyte MMP-2 activity. To more carefully characterize which MMP species may be contributing to the total zymographic activity measured in the LV myocyte preparations, a specific MMP-2 antibody capture assay was performed (n = 6). Specific MMP-2 activity for the LV myocyte preparations plated on each of the basement membrane substrates is summarized in Fig. 3. MMP-2 activity was increased in conditioned media taken from LV myocytes plated on poly-L-lysine compared with that of Matrigel. Although MMP-2 activity was higher in conditioned media from LV myocytes plated on collagen IV (P = 0.14), laminin (P = 0.17), and fibronectin (P = 0.16), these values did not reach statistical significance.

LV myocyte MMP abundance/content. In light of the fact that LV myocyte zymographic activity was observed at the 100- to 70-kDa region, immunoblotting for MMP-2, which has a molecular weight of ~72 kDa (41, 44), was performed. A representative immunoblot for MMP-2 using the media of LV myocytes plated on basement membrane components is shown in Fig. 4. A strong signal for MMP-2 was observed at 72 and 32 kDa, which likely represent the latent and processed forms of this MMP species, respectively (41, 44). In the conditioned media taken from LV myocytes plated on Matrigel, a positive signal was observed at ~60 kDa. To ensure that the relative abundance of MMP-2 detected in the isolated LV myocyte media was not due to the residual presence of reagents used during isolation and resuspension as well as in basement membrane sub-

Fig. 2. Total isolated LV myocyte zymographic activity was examined in myocytes plated on Matrigel (MTG), collagen IV (CIV), laminin (LAM), fibronectin (FIB), and poly-L-lysine (PL) using gelatin as a proteolytic substrate. Conditioned media from an HT-1080 cell line were included in all zymograms. LV myocyte zymographic activity was observed at the 100- to 70-kDa region. Isolated LV myocyte zymographic activity was increased in myocytes that were plated on collagen IV, fibronectin, and poly-L-lysine compared with that of Matrigel. Although LV myocyte zymographic activity was higher in myocytes plated on laminin, this was not significantly different (n = 14 preparations; *P < 0.05 vs. Matrigel).
strates, immunoblotting for MMP-2 was performed using bacterial collagenase and serum-free media that had been incubated in Matrigel-coated dishes for 24 h (not shown). There were no immunoreactive bands for MMP-2 observed for bacterial collagenase. With respect to the serum-free media incubated on Matrigel, only one immunoreactive band was observed at 60 kDa; there were no immunoreactive signals observed at 72 and 32 kDa. Thus the MMP-2 signals observed at 72 and 32 kDa in the immunoblots were specific for the conditioned LV myocyte media. Because of the strong signal observed for MMP-2 in the immunoblots performed using LV myocyte conditioned media, a quantitative MMP ELISA system was used to measure MMP-2. MMP-2 content for the LV myocytes plated on different basement membrane substrates is summarized in Fig. 5. MMP-2 content was increased in conditioned media taken from LV myocytes plated on collagen IV, fibronectin, and poly-L-lysine compared with that of Matrigel. There was no change in MMP-2 content in conditioned media from LV myocytes plated on laminin when compared with that of Matrigel.

In a separate set of preliminary studies, immunoblotting for MMP-9 was also performed using the remaining LV myocyte conditioned media (n = 4). A representative immunoblot for MMP-9 using the media of LV myocytes plated on basement membrane components is shown in Fig. 6. An immunoreactive signal was observed for MMP-9 at ~92 kDa, which is consistent with that reported for this MMP species (41, 44). As with the MMP-2 immunoblots, in the conditioned media taken from LV myocytes plated on Matrigel, an immunoreactive signal for MMP-9 was observed at ~80 kDa and may be due to the presence of MMPs in the Matrigel.

LV myocyte immunofluorescent labeling. The isolated LV myocyte preparations used in the previous experiments were stained with α-actinin and/or smooth muscle cell actin to demonstrate that the LV myocyte preparations consisted primarily of cardiac myocytes. Isolated LV myocytes exhibited abundant positive immunostaining for α-actinin, which illustrated a sarcomeric registration (Fig. 7). For smooth muscle cell actin, no immunofluorescence was observed in the isolated myocytes.

Fig. 3. Specific matrix metalloproteinase (MMP)-2 activity in LV myocyte conditioned media was examined using an antibody capture assay. With the use of this approach, MMP-2 activity was measured in the conditioned media from LV myocytes plated on Matrigel, collagen IV, laminin, fibronectin, and poly-L-lysine. MMP-2 activity was increased in conditioned media taken from LV myocytes plated on poly-L-lysine compared with that of Matrigel (n = 6; *P < 0.05 vs. Matrigel). Although MMP-2 activity was higher in conditioned media from LV myocytes plated on collagen IV (P = 0.14), laminin (P = 0.17), and fibronectin (P = 0.16), this did not reach statistical significance compared with that of Matrigel.

Fig. 4. Immunoblotting for MMP-2 was performed on the media of isolated LV myocytes that were plated on the following substrates: Matrigel (MTG), collagen IV (C4), laminin (LAM), and fibronectin (FIB) (n = 14). In addition to the substitution of nonimmune antisera, which abolished all staining, a positive control for MMP-2 (+) obtained from a human epithelial cell line (AG771; Chemicon) was used. A positive immunoreactive band for MMP-2 was observed at 72 and 32 kDa, which is consistent with this MMP species.

Fig. 5. Because MMP-2 immunoblotting of the isolated LV myocyte conditioned media yielded such a robust immunoreactive signal at 72 kDa, a quantitative MMP-2 ELISA was performed. MMP-2 content in the LV myocyte conditioned media was measured in myocytes plated on Matrigel, collagen IV, laminin, fibronectin, and poly-L-lysine. Values obtained from the MMP-2 ELISA were the result of linear regression analysis using purified MMP-2 standards. MMP-2 content was increased in LV myocytes plated on collagen IV, fibronectin, and poly-L-lysine compared with that of Matrigel; however, no change was observed with those plated on laminin (n = 14; *P < 0.05 vs. Matrigel).
cytes, indicating that the myocyte preparations were not associated with smooth muscle cell contamination (Fig. 7).

To examine the distribution of MMP-2 in the isolated LV myocytes, immunostaining for MMP-2 was also performed (Figs. 8 and 9). The isolated LV myocytes exhibited punctate immunostaining for MMP-2 and strong perinuclear staining. A well-distributed and focal pattern of MMP-2 immunostaining was also observed along the intracellular interface of the sarcolemmal border. Colocalization of α-actinin and MMP-2 was also performed (Fig. 10), which illustrated a strong immunofluorescent signal for MMP-2 within the LV myocytes.

DISCUSSION

A family of metal-dependent enzymes, the MMPs, has been implicated in the tissue remodeling process in a number of normal developmental and disease states (6, 21, 28, 38, 41, 44). Changes in MMP activity have been reported to occur with cardiac morphogenesis, acute ischemia, and cardiomyopathies (6, 10, 21, 30, 32). Specifically, previous clinical and experimental forms of dilated cardiomyopathy have provided evidence to suggest that LV dilation and remodeling were associated with changes in MMP expression (10, 32). Past studies indicated that endothelial cells, fibroblasts, and vascular smooth muscle cells express a variety of MMP species (13, 15, 38, 43). Although the LV myocardium contains these nonmyocyte cell types, 90% of myocardial mass is composed of myocytes (15). However, whether and to what degree adult ventricular myocytes synthesize and secrete MMPs remains explored. Accordingly, the present study examined MMP activity and expression in isolated mammalian LV myocyte preparations. The significant and unique findings of the present study were twofold. First, LV myocytes plated on a variety of basement membrane substrates synthesized and released abundant MMPs, as evidenced by robust zymographic activity. Second, LV myocytes expressed MMP species, such as the 72-kDa gelatinase MMP-2. Thus the results of the present study suggest that LV myocytes may actively

Fig. 6. In an additional set of preliminary experiments, immunoblotting for MMP-9 was performed on the LV myocyte preparations that had been plated on Matrigel, laminin, and collagen IV (n = 4). A positive signal was observed in LV myocyte conditioned media at the 92-kDa region. This immunoreactive band was also observed in a positive control (+) obtained from a human epithelial cell line (AG771; Chemicon) and likely reflects MMP-9. Because of limited sample volumes, the conditioned media from LV myocytes plated on fibronectin and poly-L-lysine could not be used in these experiments.

Fig. 7. To ensure that the isolated LV myocyte primary cultures were not associated with smooth muscle cell contamination, immunofluorescent labeling was performed using antisera for α-actinin and smooth muscle actin. A: isolated LV myocytes exhibited abundant positive immunostaining for the cytoskeletal protein α-actinin, which illustrated the myofibrillar structure of the myocytes (magnification ×40). B: as a positive control, a smooth muscle cell culture was stained with smooth muscle actin (magnification ×20). C: isolated LV myocytes were stained with smooth muscle actin (magnification ×40). No immunofluorescence was observed in the isolated LV myocyte primary cultures, indicating the absence of smooth muscle cells.
participate in the LV remodeling process through elaboration of MMPs. LV myocytes originate from mesenchymal cells found in the lateral plate mesoderm of embryos. Past in vitro studies have indicated that differentiated cells of mesenchymal origin, such as fibroblasts, synthesize and express MMPs (42, 43). For example, Werb et al. (42) demonstrated that synovial fibroblasts synthesized and released several MMP species that were influenced by a number of physical and chemical stimuli. In the present study using zymography and immunoblotting techniques, abundant zymographic activity and MMP expression were observed in LV myocyte preparations, which suggests that adult LV myocytes retained the capacity to synthesize MMPs. Nonmyocyte cell types that exist in the LV myocardium include endothelial cells, smooth muscle cells, mast cells, and pericytes. Several past studies have demonstrated that these nonmyocyte cell types produce MMPs (13, 15, 38, 43). Therefore, the results of the present study indicate that all myocardial cell types possess the capacity to synthesize and release MMPs.

In the present study, differences in zymographic activity and MMP-2 expression were observed in the conditioned media of LV myocytes plated on the basement membrane substrates Matrigel, collagen IV, laminin, and fibronectin, as well as poly-L-lysine. A fundamental mechanism for myocyte interaction with the extracellular matrix is through the family of transmembrane receptors, termed integrins. Furthermore, LV myocyte-integrin engagement may be an important transducing mechanism for influencing protein expression and synthesis (9, 11, 16). In LV myocytes, there was a robust increase in zymographic activity and MMP-2 content in the conditioned media from myocytes plated on fibronectin and collagen IV when compared with the relative levels observed for Matrigel or laminin. It has been demonstrated previously that LV myocytes express integrin receptors that are selective for laminin, fibronectin, and collagen IV (4, 31). Past in vitro studies reported evidence to suggest that laminin and fibronectin exposure may influence MMP activity in certain cell types (26, 36, 42). Thus the relative changes in LV myocyte zymographic activity and MMP-2 levels observed in the present study may reflect selective integrin engagement. To determine whether integrin-independent adhesion would affect MMP synthesis, LV myocyte preparations were plated on a nonspecific substrate, poly-L-lysine, which mediates attachment via electrostatic interaction. Both gelatinolytic activity and MMP-2 content were increased in the conditioned media of LV myocytes plated on poly-L-lysine when compared with that of Matrigel. Taken together, the results of the present study suggest that selective integrin engagement in LV myocytes may influence MMP activity and expression.

Interestingly, LV myocyte preparations plated on the artificial basement membrane substrate Matrigel contained zymographic activity that was lower than that found with the purified basement membrane substrates and with poly-L-lysine. This decrease in MMP zymographic activity could be due to several factors, such as multiple integrin engagement or the presence of inhibitory components in the Matrigel. For example, it has been reported previously that Matrigel contains transforming growth factor-β (per Product Specification Sheet; Collaborative Research), which represses MMP production (28, 41, 44). Therefore, the decreased MMP zymographic activity observed in the LV myocyte preparations plated on Matrigel was likely due to both physical and chemical determinants. However, the...
extent to which MMP expression is influenced by integrin-mediated adhesion warrants further study. In the present study, LV myocyte proteolytic activity was observed between 100 and 70 kDa, which is consistent with gelatinase activity or that of MMP-2 and MMP-9 (28, 41, 44). Because previous clinical and experimental studies have demonstrated the existence of MMP-2 in the myocardium (10, 32, 37), the first step of the present study was to identify MMP-2 in the LV myocyte media using an ELISA system. Distinct and quantifiable MMP-2 exists in the LV myocyte conditioned media and was increased with nonspecific attachment to poly-L-lysine. To more carefully characterize the degree of MMP-2 activity in the LV myocyte conditioned media, an additional set of experiments was performed using an MMP-2 antibody capture assay. Again, heightened MMP-2 activity as elicited from the antibody capture assay was highest in the LV myocytes plated on poly-L-lysine when compared with Matrigel. These results suggest that changes in LV myocyte release of MMP-2 occur when exposed to different binding substrates. Although the results of this MMP-2 capture assay indicate that this MMP species may have contributed to some of the zymographic activity observed, the molecular weight location of the lytic zones in the zymograms suggests that the zymographic activity observed may be due to other MMPs.

A secondary objective was to perform immunoblotting for MMP-9. An immunoreactive signal was observed for MMP-9 between 127 and 72 kDa. This likely represents the 92-kDa proenzyme form of MMP-9 (28, 41, 44). These preliminary studies confirmed the existence of MMP-9 in the isolated LV myocyte preparations. However, because of limited sample volumes, quantitative immunoblotting for MMP-9 and other MMP species could not be performed in the present study. There are at least 20 MMP species known to date, and these are classified based on substrate specificity (28, 41, 44). Moreover, there appear to be differences in MMP expression and affinity across animal species (19, 40). In the present study, MMP gelatinolytic activity and MMP-2 expression were examined in adult porcine LV myocytes. The gelatinolytic activity observed in the present study, with respect to molecular weight localization, was similar to that observed previously in human samples (32). Furthermore, the primary sequence of porcine MMP-1 and that of human MMP-1 are 80% identical, supporting the suggestion that substrate specificity is similar between these MMP species (8, 33). However, future studies are necessary to characterize the species identity and substrate specificity of MMPs synthesized by adult LV myocytes.

The fact that LV myocytes synthesize and release MMPs may have particular relevance in the LV remodeling processes, such as those of cardiac development, hypertrophy, and heart failure. Changes in the expression of basement membrane components and integrin attachment have been reported to occur between neonatal and adult myocytes (4). Also, increased expression of basement membrane components has been described with the application of a pressure overload stimulus (3). Finally, alterations in the basement membrane...
adhesion capacity of LV myocytes have been described in a model of heart failure (45). Thus changes in the expression of basement membrane components or myocyte adhesion would, in turn, influence LV myocyte-integrin engagement and potentially alter MMP expression and activity. Future studies are necessary to more carefully define the relationship between integrin engagement, focal adhesion complex formation, and MMP expression. The present study also sets the stage for future experiments in which cytokines and neurohormonal factors, which are operative in LV remodeling and may influence MMP expression and activity, can be examined. For example, the cytokine tumor necrosis factor-α (TNF-α) has been reported to contribute to the LV remodeling process in LV failure (5), and abundant TNF-α receptors exist on LV myocytes (35). Therefore, determining whether and to what degree TNF-α activation influences MMP expression and activity in the isolated myocyte system described herein would be a fruitful avenue of investigation.

LV myocytes were isolated and resuspended in a stepwise manner to eliminate nonmyocyte cell contamination. However, the possibility that a small number of nonmyocyte cells persisted in the LV myocyte preparations cannot conclusively be ruled out. In an additional series of studies, LV myocyte preparations were subjected to density centrifugation using a Percoll gradient (not shown). The MMP zymographic activity for these preparations was similar to that of the LV myocyte preparations not subjected to density centrifugation, suggesting minimal nonmyocyte cell contamination. Furthermore, immunofluorescent labeling studies revealed that the LV myocyte preparations were not significantly contaminated by smooth muscle cells. Finally, double immunofluorescent labeling of the LV myocyte preparations clearly demonstrated the presence of MMP-2 within the myocyte. Thus these findings suggest that the MMP zymographic activity observed in the present study was primarily due to LV myocytes.

Because the present study examined LV myocyte MMP expression in vitro in the absence of hormonal and physical stimuli, future studies concerning the regulation of MMP expression in LV myocytes are needed. Nevertheless, the results of the present study indicate that LV myocytes may actively participate in the LV remodeling process through the synthesis and secretion of MMPs.

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