Journal of Cellular Biochemistry 63:185–198 (1996)

Differential Gene Expression of Extracellular Matrix Components in Dilated Cardiomyopathy

Suresh C. Tyagi, Suresh Kumar, Donald J. Voelker, Hanumanth K. Reddy, Joseph S. Janicki, and Jack J. Curtis

Departments of Medicine, Biochemistry (S.C.T., S.K., D.J.V., H.K.R.), and Surgery (J.J.C.), The Dalton Cardiovascular Research Center, University of Missouri–Health Sciences Center, Columbia, Missouri 65212; Departments of Physiology and Pharmacology Auburn University, Auburn, Alabama 36849 (J.S.J.)

Abstract Extracellular matrix metalloproteinases (MMPs) are activated in dilated cardiomyopathic (DCM) hearts [Tyagi et al. (1996): Mol Cell Biochem 155:13–21]. To examine whether the MMP activation is occurring at the gene expression level, we performed differential display mRNA analysis on tissue from six dilated cardiomyopathy (DCM) explanted and five normal human hearts. Specifically, we identified three genes to be induced and several other genes to be repressed following DCM. Southern blot analysis of isolated cDNA using a collagenase cDNA probe indicated that one of the genes induced during DCM was interstitial collagenase (MMP-1). Northern blot analysis using MMP-1 cDNA probe indicated that MMP-1 was induced three- to fourfold in the DCM heart as compared to normal tissue. To analyze posttranslational expression of MMP and tissue inhibitor of matrix metalloproteinase (TIMP) we performed immunoblot, immunoassay, and substrate zymographic assays. TIMP-1 and MMP-1 levels were 37 ± 8 ng/mg and 9 ± 2 ng/mg in normal tissue specimens ($P < 0.01$) and 2 ± 1 ng/mg and 45 ± 11 ng/mg in DCM tissue ($P < 0.01$), respectively. Zymographic analysis demonstrated lytic bands at 66 kDa and 54 kDa in DCM tissue as compared to one band at 66 kDa in normal tissue. Incubation of zymographic gel with metal chelator (phenanthroline) abolished both bands suggesting activation of neutral MMP in DCM heart tissue. TIMP-1 was repressed approximately twentyfold in DCM hearts when compared with normal heart tissue. In situ immunolabeling of MMP-1 indicated phenotypic differences in the fibroblast cells isolated from the DCM heart as compared to normal heart. These results suggest disruption in the balance of myopathic-fibroblast cell ECM-proteinase and antiproteinase in ECM remodeling which is followed by dilated cardiomyopathy.

Key words: extracellular matrix, remodeling, collagenase, collagen, dilated cardiomyopathy, congestive heart disease, end-stage heart failure, matrix metalloproteinase, tissue inhibitor of metalloproteinase, differential display mRNA analysis, gene expression

Structural anatomic abnormalities are the primary cause of congestive heart disease and not the effect [Richardson et al., 1996]. A great many studies have been focused on cardiac dysrhythmias or conduction abnormalities. Structural changes may also lead to cardiac dysfunction. Little is known about the structural alteration leading to dilated cardiomyopathy. The extracellular matrix (ECM), including type I fibrillar collagen and its receptor integrins which surround the cardiac cell, forms a three-dimensional network and bridges the ECM and the cellular cytoskeletal myofibril [Borg and Burgess, 1993]. This skeletal framework provides tensile strength to the tissue, governs tissue stiffness, and preserves the alignment of cardiac myocytes [Borg and Burgess, 1993; Weber et al., 1994b]. It, therefore, indirectly governs the structural architecture and geometry of the myocardium and its ventricular chambers [Gerdes et al., 1992]. Morphologic evidence of fibrillar collagen disruption and myocyte slippage has been observed in the dilated failing heart [Olivetti et al., 1990]. During the development of

Abbreviations used: MMPs, matrix metalloproteinases; ECM, extracellular matrix; TIMPs, tissue inhibitor of metalloproteinases; PMSF, phenyl methyl sulfonyl fluoride; DCM, dilated cardiomyopathy; LA, left atrium; LV, left ventricle; RV, right ventricle; phen, phenanthroline; H & E, hematoxylin and eosin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate buffer saline; DTT, dithiothreitol; RT, reverse transcriptase; PCR, polymerase chain reaction.

Received March 1, 1996; accepted April 26, 1996.

Address reprint requests to Suresh C. Tyagi, University of Mississippi Medical Center, Department of Physiology and Biophysics, 2500 North State Street, Jackson, MS 39216.

dilated cardiomyopathy (DCM) in patients and animals disruption and discontinuity in collagen fibers has been observed leading to cardiac remodeling [Cannon et al., 1983; Zhao et al., 1987]. Remodeling of ECM implies an alteration in the extracellular matrix and in the spatial orientation of cells and intracellular components. Specialized proteinases that are capable of breaking down the extracellular matrix (i.e., matrix metal*loproteinases* [MMPs]) and the inhibitors of proteinases (i.e., tissue inhibitor of metalloproteinases [TIMPs]) appear to be balanced in the normal myocardium, thereby maintaining the integrity of the myocardium [Tyagi et al., 1993a, 1995a, Tyagi and Simon, 1994]. It is possible that the balance equilibrium between proteinase and antiproteinase is altered following DCM.

The genetic basis of ECM disruption following DCM is not well addressed [Kelly and Strauss, 1994]. The inability of ECM to maintain cardiovascular integrity is the primary result of weakened connective tissue seen in rare single-gene disorders of structural artitechure, such as Ehlers-Danlos IV, which is a defect in type III collagen gene [O'Connor et al., 1985]. Fibrillin is required for ECM assembly at the cell surface, and Marfan's syndrome is due to a defect in the fibrillin-1 gene [Boileau et al., 1993]. Elastin deficiency causes Williams' syndrome and vasculopathy [Ewart et al., 1994]. The mutations in the TIMP-3 gene [Weber et al., 1994] in patients with Sorsby's fundus dystrophy have been identified. Disruption of connective tissue turnover and the consequent effect on matrix stability has been implicated in a number of diseases [Tyagi and Simon, 1990; Tyagi et al., 1995b, 1996a], and the role of MMPs has been demonstrated in the ECM/MMP/TIMP family [Tyagi et al., 1995b, 1996a]. Collectively, these studies suggested that disruption of MMP-mediated matrix remodeling does affect the composition and function of tissues. It also suggests that more common genetic variation in these genes may contribute to the pathogenesis of common multifactorial disorders in cardiovasculature. It seems likely that mild mutations in some of the components of ECM may cause severe cardiomyopathic abnormalities. For the first time we indicated a MMP gene malupregulation during DCM that regulates ECM remodeling. This will provide target for therapeutic intervention.

Previously, in a hamster model of DCM, we demonstrated temporal expression of MMP activity [Janicki et al., 1993, 1994]. MMP activity was increased at day 180 and remained elevated up to 310 days when the heart failed [Tyagi et al., 1993b]. This MMP activity was associated with a reduction in TIMP expression [Tyagi et al., 1993b]. In human heart end-stage failure secondary to ischemic cardiomyopathy, we demonstrated activation of collagenase and disruption of ECM components [Tyagi et al., 1996a]. Here we report that MMP-1 was specifically and differentially induced and the level of TIMP was repressed following dilated cardiomyopathy.

MATERIALS AND METHODS

Tissue Sources

Human heart tissue from different regions of explanted idiopathic dilated cardiomyopathic (DCM) hearts were obtained during transplant surgery at the University of Missouri–Columbia. Institutional Review Board (IRB) approval was obtained for the study. Normal left atrial (LA) tissue was obtained from donor hearts prior to implantation. Previously we have demonstrated similar MMP activity in LA and LV [Tyagi et al., 1996a]. Tissue blocks, from the epimyocardium, endomyocardium, and transmural, were collected from the left and right ventricles from all explanted hearts ($n = 6$ for DCM and $n = 5$ for normal). Normal left atrial (LA) tissue was obtained from donor hearts prior to implantation. Similar MMP expression was observed in normal LA and LV [Tyagi et al., 1996a]. The tissue was directly frozen in liquid nitrogen and stored at $-80°C$ until used. Tissue for histological analysis was preserved in 10% zinc formalin. DCM was diagnosed by identifying thin wall and an increase in ventricular size by echocardiography. The coronary in these hearts were apparently normal.

Histology

Tissue sections from DCM and normal hearts were prepared using standard histological techniques. Briefly, tissue was fixed with 10% buffered formalin and embedded in paraffin and the sections were stained with hematoxylin and eosin (H & E) for histological examination [Bradbury and Gordon, 1982; Tyagi et al., 1995b].

Preparation of Cardiac Tissue Extract

Cardiac tissue extracts were prepared as described previously and used within 1–2 h [Tyagi et al., 1993c]. Bradford [1976] dye binding assay

was applied to estimate the total protein concentration in the tissue extract.

Electrophoresis and Zymography

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with or without reduction according to the method of Laemmli [1970]. Proteins were stained with Coomassie Brilliant Blue R 250. Intrinsic myocardial matrix metalloproteinase activity in the gel was measured as described previously using type I collagen, gelatin, or casein (Sigma, St. Louis, MO) as substrates [Tyagi et al., 1993c].

Western Blot

Following electrophoresis and under reducing conditions, gels were equilibrated in transfer buffer. Proteins in the gel were transferred to nitrocellulose paper using a Bio-Rad (Richmond, CA) Trans Blot apparatus as described [Tyagi et al., 1995a]. Before probing, the nitrocellulose paper was blocked in PBS containing 5% fat-free milk for 2 h and then probed in fresh blocking buffer containing either anti-proMMP-1 or anti-TIMP-1 (Binding Site Corp., CA) antibody (1/200 dilution) and 0.5% fat-free milk. After extensive washing with PBS containing 0.05% Tween-20, alkaline phosphatase–conjugated secondary antibody (Sigma) was added and incubated for 1 h. Following a final wash, nitrocellulose paper was stained with buffer containing 5-bromo-4-chloro-3-indolyl phosphate (p-toluidine salt) and nitro blue tetrazolium chloride. MMP-1 and TIMP-1 were purified as described [Tyagi et al., 1995a]. A similar protocol was employed for type I collagen-derived fragments with antibody to rabbit anti-collagen type I (Bio-Design, Medford, MA).

Immunoassay

Microtitration plates were coated with 100 μl/well of proMMP-1 or TIMP-1 antibody diluted 1/200 in 0.1 M carbonate buffer, pH 9.6, for 8 h. Plates were washed three times with TBS-T (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.05% Tween-20). Wells were filled with either 100 μl of tissue sample or a MMP-1 or TIMP-1 standard diluted in TBS-T, followed by a 16 h incubation. The concentrations of MMP and TIMP were estimated using $A^{1\%}_{280nm}$ of 12 and 10, respectively [Suzuki et al., 1990; Ward et al., 1991]. After blocking empty sites with TBS-T containing rabbit antisheep IgG (Sigma) (1/200), 100 μl primary antibody diluted 1:200 with TBS-T was incubated for 8 h. Antibody dilution (1/200) was identified by serial dilution and color formation at a fixed total protein concentration from the extract. After washing with TBS-T, 100 μl of secondary antibody (alkaline phosphatase conjugate) diluted 1:200 with TBS-T containing 5% nonfat dry milk was added to each well. Microwell plates were incubated for 2–3 h at room temperature and then washed three times with TBS-T. Enzyme substrate (p-nitrophenylphosphate in diethanolamine buffer; Bio-Rad) was added until color developed (10–20 min), and the reaction was stopped by NaOH. Plates were read at 405 nm. A standard plot was developed between purified MMP-1 or TIMP-1 concentration and OD_{405}. Amounts of MMP-1 and TIMP-1 were calculated from the standard plot.

Northern Blot Analysis

Total tissue RNA was isolated from 200 mg of tissue using 4 M guanidine thiocyanate buffer [Churgwin et al., 1979]. The mRNA blot were prepared as described [Tyagi et al., 1995a]. In Northern blot analysis MMP-1 was probed using MMP-1 cDNA. The plasmid containing TIMP cDNA probe was obtained from Synergen Corp. (Boulder, CO). The TIMP-1 probe was a 0.7 kb EcoRI fragment of a human TIMP-1 cDNA. A 4.5 kb EcoRI fragment from 18 S RNA (18SR) gene was used as an internal control (a gift from Dr. R. Guntaka). The membrane was washed in 0.1 standard saline citrate plus 0.1% SDS at 42°C for 1 h and then exposed to X-ray films at −70°C for 24 h. Scan values for TIMP-1 and MMP-1 mRNA signals were normalized for the scan reading of the internal standard 18SR. Ratios were expressed in arbitrary units.

Isolation of Nuclei

The procedure described by Franke [1966] has been modified to minimize contamination with cytoplasmic membranes. Human heart tissue (1 g) was frozen in liquid nitrogen and crushed and powdered under liquid nitrogen in a porous clay tray. The powder was homogenized with a glass homogenizer in STM buffer (250 mM sucrose, 50 mM Tris-Cl (pH 7.4 at 4°C), and 5 mM $MgSO_4$). The homogenate was filtered through cheese cloth and centrifuged at 800*g* for 10 min. The pellet was washed with STM buffer. The pellet was suspended in STM buffer and layered onto DS buffer (2.1 M su-

crose, 50 mM Tris-Cl (pH 7.4 at 4°C), and 5 mM $MgSO_4$). The suspension was centrifuged in a SW 27 rotor at 4°C for 60 min at 70,000*g*. The pellet was suspended in STM buffer, layered on DS buffer, and recentrifuged at 70,000*g* for 30 min. The nuclei were extracted from the pellet in nuclei extraction buffer (20 mM Hepes, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF, 10% v/v glycerol) by incubating for 30 min at 4°C and centrifuging at 14,000 rpm for 20 min. Morphologically, the nuclei appeared as intact bilayer spheres as judged by phase contrast light transmission microscopy.

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay (EMSA)

Nuclear proteins were purified by a modification of the Dignam et al. [1983] protocol. Briefly, after washing with PBS, isolated nuclei were centrifuged, and the nuclei pellet was suspended in 500 μl buffer A (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, and 1 mM DTT). After recentrifugation, the nuclei were resuspended in 80 μl buffer A containing 0.1% Triton X-100 by gentle pipeting. After incubating for 10 min at 4°C, the homogenate was centrifuged, and the nuclear pellet was washed once with buffer A and resuspended in 70 μl of buffer C (10 mM Hepes, pH 7.9, 25% (v/v) glycerol, 0.4 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 1 mM DTT). The suspension was incubated for 30 min at 4°C followed by centrifugation at 20,000*g* for 10 min, and the resulting supernatant was stored at −70°C. Protein concentrations was determined by the Bradford method [1976]. To minimize proteolytic degradation of nuclear proteins, the buffer contained 1 mM PMSF. The oligonucleotide containing part of AP1 of human collagenase promoter, −77 to −54 nucleotides [Angel et al., 1987] sequence (5′-AAGCATGAGTCAGACACCTCTGGC-3′), was synthesized. A double-stranded oligonucleotide containing the collagenase promoter was prepared by annealing complementary synthetic oligonucleotide. All nucleotides were prepared by the University of Missouri–Columbia DNA core facility. Radiolabeled double-stranded DNA was made by annealing an oligonucleotide complementary to the 3′ end of the sequence and the end labeled with [^{32}P]ATP and polynucleotide kinase. Unincorporated nucleotides were removed by column chromatography over a Sephadex G-50 column. Cold double-stranded DNA was similarly constituted. The DNA binding reaction was performed at 30°C for 15 min in a volume of 20 μl, which contained 2–5 μg of nuclear extract, 225 μg/ml BSA, 1×10^5 cpm ^{32}P-labeled probe (~1 ng), 0.1 mg/ml poly(dI-dC), and 15 μl binding buffer (12 mM Hepes, pH 7.9, 4 mM Tris, 60 mM KCl, 1 mM EDTA, 12% glycerol, 1 mM DTT, and 1 mM PMSF). Samples were subjected to 6% Tris-glycine-PAGE.

Differential Display Analysis of mRNA in the DCM and Normal Hearts

To identify alterations in the gene expression following dilated cardiomyopathy, mRNA was analyzed by differential display methodology [Liang and Pardee, 1992; Zimmermann and Schultz, 1994]. This methodology visualizes mRNA compositions by displaying subsets of mRNAs as short cDNA bands (100–600 bp). The tissue (50 mg) was processed directly for RNA isolation as described [Tyagi et al., 1995a]. Polyadenylated RNAs was isolated using the Quickprep mRNA purification kit from Pharmacia-LKB Biochemicals (Piscataway, NJ). The purified mRNA (0.5 mg) was reverse-transcribed to cDNA by AMV reverse transcriptase (RT) with dNTP and oligo(dT)CA/GC primers. The cDNA was amplified by polymerase chain reaction (PCR) with either T_{11}CA (5′-TTTTTTTTTTTCA-3′) or T_{12}GC (5′-TTTTTTTTTTTTGC-3′) as anchor and 10-mers (5′-CTGATCCATG-3′ or 5′-CTTGATTGCC-3′) as random sets of primers using dNTP plus [^{32}P]dCTP and *Taq* DNA polymerase. Identical amounts of total RNA were used in each experiment. The PCR products were analyzed by resolving in 6% acrylamide urea gels and by autoradiography. The PCR products were observed only in samples with RNA during RT. Negative control reactions containing water instead of mRNA showed no RT activity. From a band(s) induced in the DCM tissue, DNAs were isolated by cutting out the bands and eluting the samples by boiling the gel piece.

Polymerase Chain Reaction

The PCR reaction was carried out in buffer containing 10 mM Tris-Cl (pH 8.3), 3 mM $MgCl_2$, 2 μM dNTP, and 1 mCi [^{32}P]dCTP plus 1 unit of Taq polymerase. Reaction was carried out in a Perkin-Elmer (Oak Brook, IL) 9600, using thermal cycling at 94°C for 30 s, 40°C for 2 min, and 72°C for 30 s for a total of 40 cycles with a final 10 min extension at 72°C. The reaction product was dried and dissolved in sample buffer prior to loading onto the sequencing gel.

Southern Blot Analysis

Bands separated by differentially expressed cDNAs were excised from the gel, soaked in 100 μl of distilled water for 10 min, boiled for 15 min, and centrifuged to clarify the supernatant. The gel-free supernatant material was precipitated with 10 μl of 3 M sodium acetate, 5 μl of glycogen (10 mg/ml) (Boehringer-Manneheim, Indianapolis, IN), and 450 μl of 100% ethanol overnight at −20°C. After centrifugation the pellet was rinsed with ice-cold 100% ethanol. These cDNAs were dissolved in 10 μl water. For each band the total RNA was amplified in a total volume of 50 μl using the same primers sets and PCR condition as described in section of polymerase chain reaction. After PCR reaction the products were analyzed by 1% agarose gel and visualized by ethidium bromide. The cDNAs were blotted onto nitrocellulose membrane. The transferred cDNAs were immobilized by UV illumination. The membrane was prehybridized and then hybridized with collagenase probe-labeled with ^{32}P by the random primer labeling method (Pharmacia) at 42°C for 36 h. The plasmid containing fibroblast collagenase and collagen cDNA were obtained from American Type Culture Collection (ATCC, Rockville, MD). The MMP-1 probe was a 2.05 kb Hind III and Sma I fragment from human MMP-1 cDNA. The membrane was first washed with 1× SSC containing 0.1% SDS and then with 0.1 M SSC and kept for autoradiography.

Isolation of Myopathic Fibroblast Cells and In Situ Immunolabeling

DCM interstitial fibroblast cells (p-2) were isolated as described [Tyagi et al., 1995c]. The cells were grown on coverslips in 5% serum plus 10 ng/ml monensin (Sigma) to block protein secretion. The MMP-1 immunostaining was performed as described [Tyagi et al., 1995c].

Statistical Analysis

Data are expressed as mean ± SD. Differences between experimental condition of DCM vs. normal tissue were assessed by paired t-test. A value of $P < 0.05$ was considered significant.

RESULTS

Gene Expression in Dilated Cardiomyopathy

To identify induction of the gene responsible for interstitial structural alteration in the DCM heart, we analyzed tissue at the cellular and nuclear levels. The morphometric analysis revealed an increase in cardiac cell size and fibrous tissue in between the myocytes in the DCM tissue as compared to normal tissue (Fig. 1A,B). The size of nuclei was increased twofold in the DCM heart when compared with normal heart (Fig. 1C,D).

To identify whether the morphological changes in the cardiac cell are associated with induction of ECM gene expression and to identify transcriptionally regulated genes potentially involved in the progression of DCM, we compared differential mRNA display patterns for DCM and normal hearts. We performed PCR amplifications with eight primer combinations on all six DCM samples and five normal samples and identified three PCR products greater than 300 bp. The differential display mRNA approach allows for the identification of candidate factors that may be induced in the DCM hearts. The limitation of differential display analysis is that some of the lower molecular size gene fragments may be a premature tunacated product(s) of the high molecular size differentiated and/or com-

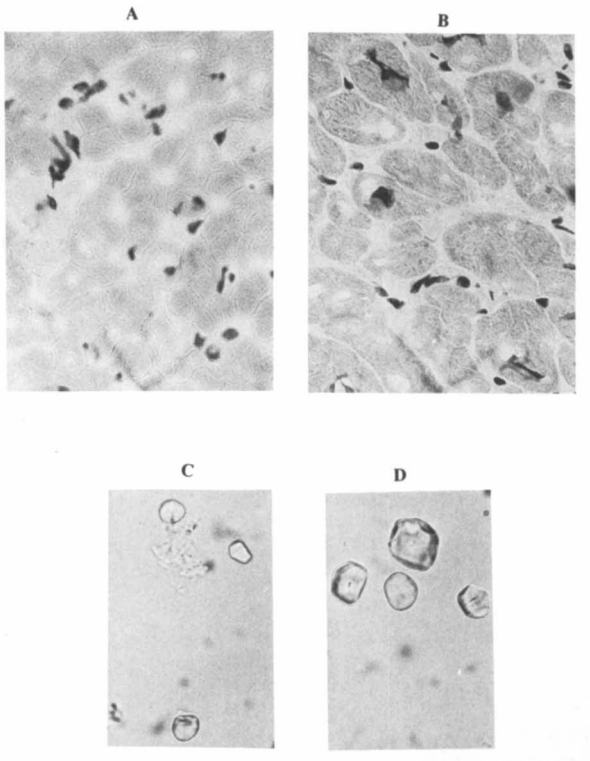

Fig. 1. Morphologic analysis of nuclei in the DCM heart. Nuclei from normal and DCM heart tissue were isolated (Materials and Methods). **A,B:** H & E staining of normal and DCM tissue, respectively. ×40. **C,D:** Normal and DCM nuclei, respectively. ×400. Note the presence of a bilayer nuclear membrane and that the size of DCM nuclei is larger than normal nuclei.

mon gene(s). Therefore, we analyzed only the longer size (~ 400 bp) fragments in the differential display mRNA analysis. Our analysis compared a series of six explanted hearts simultaneously; therefore, we avoided isolating factors that might have been related to a single heart or the procedure rather than to the disease itself. Most importantly, we performed each differential display analysis at least three times to reduce nonspecific (background) PCR signal interference, and we restricted the selection of cDNA bands for further study to those that reproduced the regulation pattern for the first differential display analysis in at least three subsequent analyses. Figure 2A shows representative PCR amplification obtained with two sets of primers. Normal and DCM samples that ran in parallel revealed differences in their mRNA patterns. The distinct three bands marked + (Fig. 2A) were differentially expressed in DCM hearts when compared with the normal samples. There were some genes which were specifically repressed in the DCM hearts and marked with − sign (Fig. 2A). These results suggested differential (up and down) regulation of genes following dilated cardiomyopathy.

Southern Blot Analysis With PCR-Amplified Fragments

To confirm the gene regulation patterns observed in the differential display experiments, we recovered the three selected bands which were upregulated in the DCM heart, reamplified them, and used them to probe with cDNA for MMP-1 (Fig. 2B). The MMP-1 cDNA probe cross-reacted with band 1. The other bands did not react with the MMP-1 cDNA probe. This indicated that band 1 obtained from differential mRNA analysis is most likely to be the MMP-1 gene induced following DCM.

Northern Blot Analysis

To analyze whether the differential induced bands in DCM heart are initiated at the gene transcription level following DCM, we performed Northern blot analysis using cDNA obtained from differentially expressed mRNA as the probe for MMP-1. TIMP-1 was analyzed using cDNA clone (Fig. 3). The level of MMP-1 mRNA was increased three- to fourfold in DCM ventricle when compared with normal tissue ($P < 0.01$). The level of TIMP-1 mRNA was repressed in DCM (Fig. 3). These results suggest

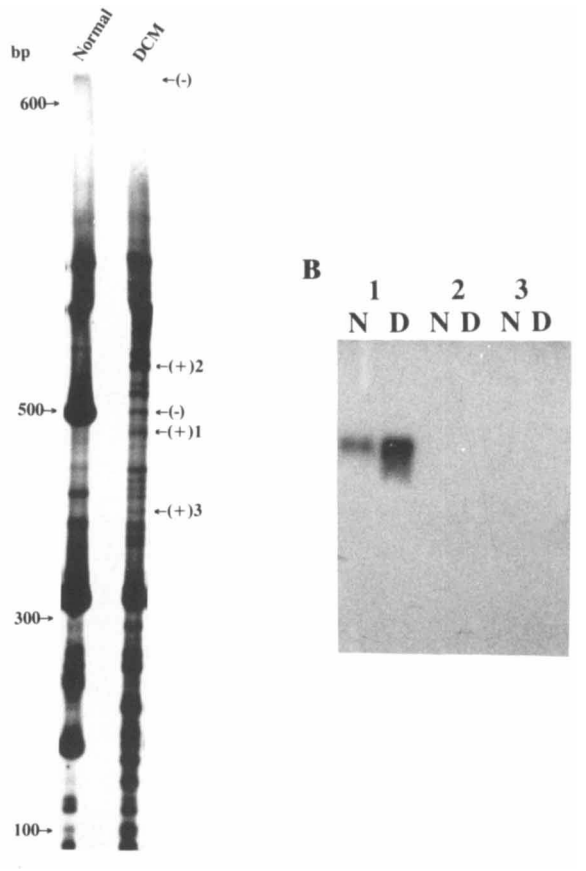

Fig. 2. A typical differential display of mRNA analysis from human normal and DCM hearts. **A:** [α-^{32}P]dCTP was incubated in the PCR reaction (Materials and Methods). The samples were loaded onto 6% urea gels. The gel was exposed to X-ray film for 4 h. The marker at the left represents the base pair (bp) size of the PCR product. The (+) and (−) signs represent bands present and absent in the DCM tissue relative to normal tissue. The bands noted by *arrows* were excised, reamplified, and selected for Southern blot (B) analysis using a collagenase [^{32}P]cDNA probe. Molecular weight markers (bp) are shown on the left. **B:** Southern analysis of bands 1, 2, and 3 isolated from A and reamplified in the PCR reaction as described. The reamplified product was run on agarose gel and transferred to nitrocellulose membrane and blotted using [^{32}P]cDNA probe of human collagenase. Results indicated cross-reaction between band 1 in A and collagenase probe, suggesting that band 1 is due to collagenase expression. Note the induction of collagenase expression in the DCM heart as compared to normal tissue.

that MMP-1 is induced and TIMP-1 is repressed at the gene transcription level during DCM.

Induction of Collagenase Promoter Binding Protein Activity in DCM Hearts

To determine whether MMP-1 induction is related to the increase in collagenase promoter activity through transcriptional regulatory fac-

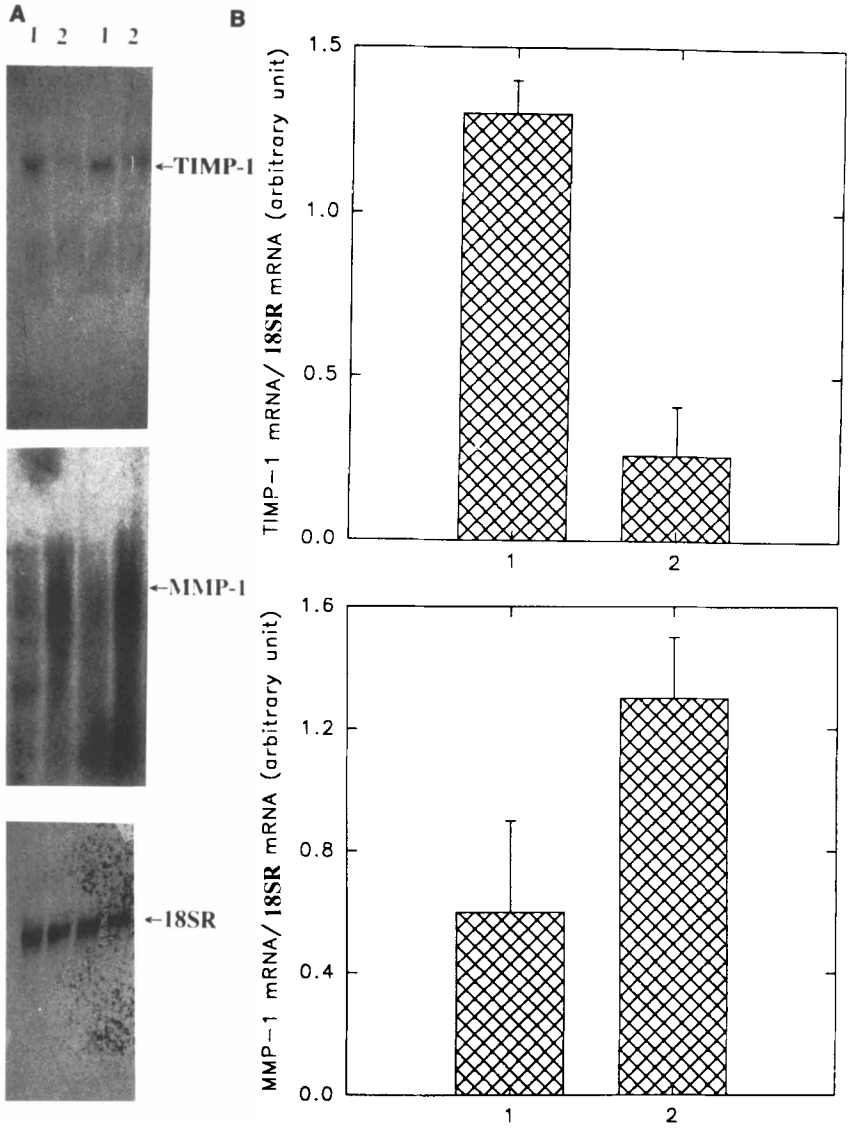

Fig. 3. **A:** Steady-state mRNA (Northern blot) analysis of TIMP-1 and MMP-1 and their comparison with the 18S RNA gene (18SR) in end-stage DCM human heart tissue. Total RNA from frozen tissue samples (220 mg) was separated by agarose gel, transferred to nitrocellulose membrane, and hybridized with cDNA probes for MMP-1, TIMP-1 and 18SR. LA, left atrium (*lanes 1*); DCM, left ventricle (*lanes 2*). The size of transcript is 2.4 kb for MMP-1, 1.5 kb for TIMP-1, and 4.5 kb for 18SR. **B:** Histographic representation of the scanned data normalized to 18SR mRNA (arbitrary unit). Results suggest induction of MMP-1 and repression of TIMP-1 expression at the gene transcription level in DCM tissue. Mean values ± SD of triplicates are reported.

tor, nuclear extracts from DCM and normal hearts tissue were assayed for DNA binding activity to a double-stranded oligonucleotide which contained part of AP1 collagenase promoter elements located at positions −77 to −54 [Angel et al., 1987]. As shown in Figure 4, the nuclear extract from DCM tissue contained a factor which forms a complex with a specific promoter sequence. The specificity of this protein-DNA complex for the MMP promoter sequence was determined by competition binding assays. Competition with excess cold (nonradioactive) DNA probe resulted in 95% inhibition of binding, which suggests specificity of binding between MMP promoter and the nuclear transcription factor. The nuclear extracts from normal tissue demonstrates no DNA binding activity and no complex formation, which indicates that in the DCM heart MMP promoter activity is induced.

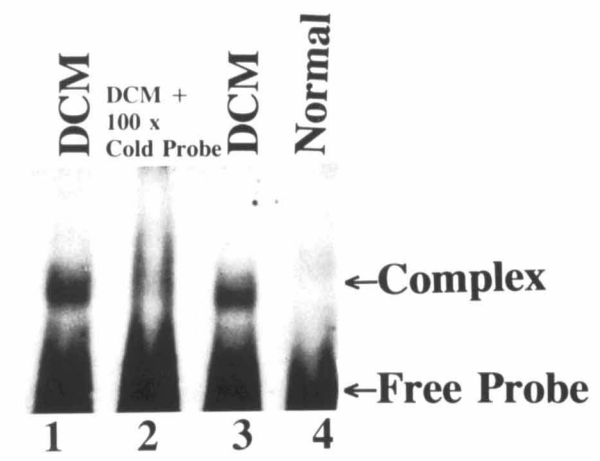

Fig. 4. Electrophoretic mobility shift assays (EMSA) of collagenase promoter binding protein in DCM heart tissue. Double-stranded oligonucleotide of promoter sequence was labeled with [^{32}P] at the 3'-end, and 10 μM was incubated with in vitro nuclear extract isolated from normal and DCM heart tissue. Normal (*lane 4*) and DCM (*lane 3*) represent nuclear extract from normal and DCM heart tissue, respectively. DCM (*lane 1*) represents from dilated heart tissue. DCM + 100 × Cold Probe (*lane 2*) indicates displacement of nuclear DNA binding protein from [^{32}P]-DNA in competition with cold probe. Results indicate induction of specific transcription factor activity following DCM on collagenase promoter.

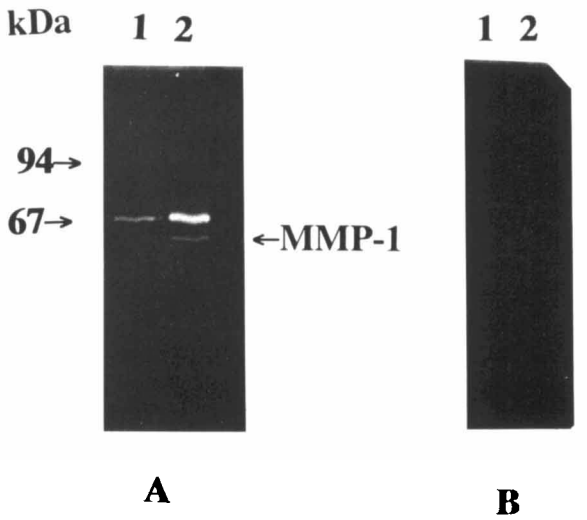

Fig. 5. **A:** Zymographic analysis (10% SDS-PAGE) of MMP activity in normal (LA) (*lane 1*) and in end-stage dilated cardiomyopathic (DCM) (*lane 2*) human heart tissue. Numbers on the side refer to the size of molecular markers. The band below gelatinase A (66 kDa) is 54 kDa MMP-1. **B:** Gel impregnated with phenanthroline indicates the elimination of MMP bands in both the normal (*lane 1*) and DCM (*lane 2*) tissues. Identical amounts of total protein (40 μg) were loaded onto each lane in the gel. Gelatinase A (66 kDa) and MMP-1 are induced following DCM.

Collagenolytic/Gelatinolytic Activity in DCM Hearts

To measure MMP activation in DCM hearts, we analyzed MMP activity expression by zymography. MMP-2 (66 kDa) activity in normal human heart was constitutively expressed (Fig. 5). However, in the DCM heart MMP-2 activity was induced. Interstitial collagenase (MMP-1) is a poor gelatinase. However, the interstitial collagenase band, at 54 kDa, below MMP-2 was specifically induced in the DCM tissue when compared with normal heart tissue (Fig. 5). The activity of both MMP-1 and MMP-2 was completely abolished by treatment with phenanthroline, suggesting that the lytic activity in the zymographic gels was due to metalloproteinases (Fig. 5B).

Intrinsic Activity of Interstitial Collagenase

In the myocardium 90% of collagen is type I fibrillar collagen, and interstitial collagenase is the enzyme responsible for initial breakdown and solubilization of collagen. Functional activity of MMP-1 was further confirmed by the analysis of soluble collagen-derived peptide (CDP) of type I fibrillar collagen in the cardiac extracts which was obtained from DCM and normal hearts. To determine whether the activity of MMP-1 related specifically to the collagen degradation, we performed immunoblot analysis on the protein in cardiac extracts, including soluble collagen-derived fragments using a specific antibody to type I collagen. The antibody recognizes soluble CDP (i.e., ¾ and ¼ fragments of α-chain). There was more CDP (i.e., collagen degradation) in the DCM endocardium than in normal endocardium (Fig. 6), suggesting active MMP-1 expression and collagen degradation in the dilated cardiomyopathic heart.

Interstitial Collagenase Protein Expression

To further determine that the collagenolytic band at 54 kDa in Figure 5 is due to interstitial collagenase, we measured the protein level of MMP-1 in normal and the DCM heart using Western blot and ELISA analysis (Fig. 7). The antibody to MMP-1 recognized a band at 54 kDa in immunoblot analysis as compared to standard MMP-1 when used in the same gel (Fig. 7). The results suggest the presence of MMP-1 in the normal and DCM extract. The antibody labeling was increased by increasing the amount of tissue extract, suggesting specificity of the antibody. MMP-1 protein expression was remarkably higher in DCM tissue. Based on immunoassay we observed a four- to fivefold increase in

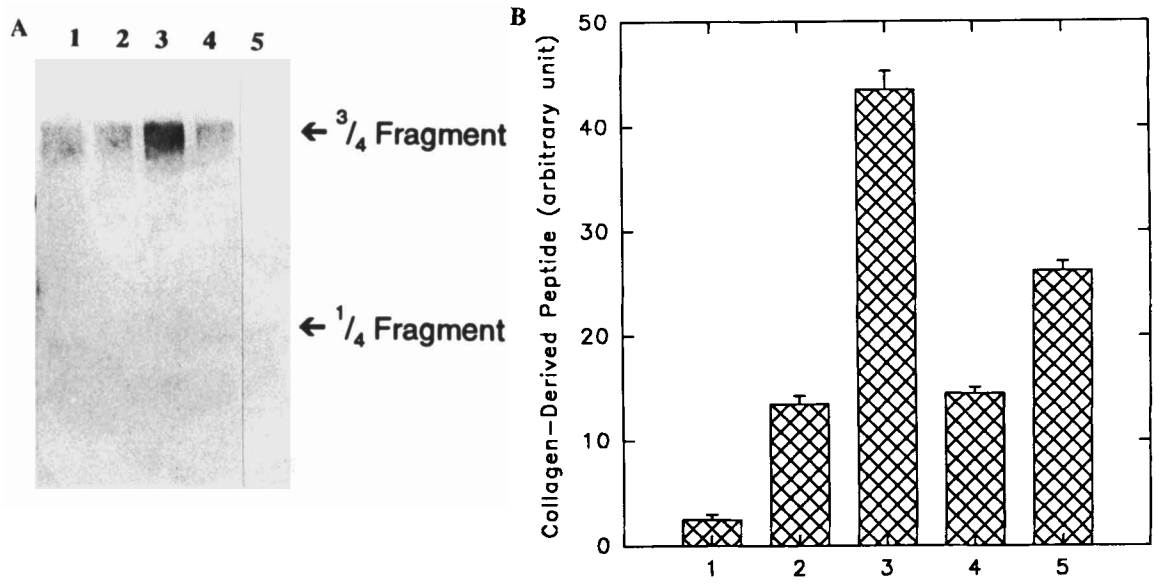

Fig. 6. **A:** Analysis of collagen degradation in DCM tissue of end-stage heart failure. Soluble collagen-derived peptide (¾ and ¼ fragments of α-chain) were identified by immunoblot analysis. Total protein (100 μg) from extracts of DCM heart was loaded onto each lane in SDS-PAGE under reducing conditions. The gel was transferred to nitrocellulose membrane and blotted using type I collagen antibody (Bio-Design). *Lanes 1, 2:* Endo- and epimyocardium of RV. *Lanes 3, 4:* Endo- and epimyocardium from LV. *Lane 5:* Normal LA tissue. **B:** Histographic representation of densitometric scanned intensity values (arbitrary unit) of collagen-derived peptide. *Bar 1:* Normal tissue. *Bars 2, 3:* LV epi- and endomyocardium. *Bars 4, 5:* RV epi- and endomyocardium. Mean average ± SD is presented. Enhanced collagen degradation was observed in the endomyocardium of DCM heart. Collagen degradation by intrinsic activation of interstitial collagenase (MMP-1) produces ¾ and ¼ fragments of type I fibrillar collagen. Sometimes the ¼ fragment is easily diffused during gel transfer to the nitrocellulose paper as compared to the ¾ fragment. Therefore, in quantitation we scanned mostly the ¾ fragment.

MMP-1 protein expression in the DCM heart compared to normal tissue (Fig. 7B). The level of MMP-1 was 45 ± 11 ng/mg in the DCM heart and 9 ± 2 ng/mg in normal tissue.

Expression of Tissue Inhibitor of Metalloproteinase

Activity of MMP-1 is regulated by TIMP-1 posttranslationally. To determine whether the elevated MMP-1 level is due to reduction of the TIMP-1 protein expression level, we measured the TIMP-1 level in DCM and compared it with normal tissue by using immunoblot and ELISA analyses (Fig. 8). To validate the antibody for TIMP-1, we have analyzed tissue extracts from normal and DCM hearts using anti-TIMP-1 antibody on Western blot analysis. Extract from both normal and DCM tissue showed different amounts of immunoreactivity to the TIMP-1 band at ~28 kDa (Fig. 8), suggesting greater reduction in the TIMP-1 protein level in the DCM tissue than in normal tissue. Furthermore, the TIMP-1 antibody reacted only to the band corresponding to TIMP-1 at 28 kDa when compared with standard TIMP-1 protein, suggesting no evidence of cross-reactivity with other proteins in the extracts.

To quantitate TIMP-1 levels in normal and DCM tissue, we employed a sandwich immunoassay for TIMP-1 (Fig. 8B) and compared tissue extracts from six different DCM and five different normal tissue lesions. Based on ELISA the TIMP-1 level was 2 ± 1 ng/mg in DCM tissue and 37 ± 8 ng/mg ($P < 0.01$) in normal tissue. These results suggested repression in the TIMP level following dilated cardiomyopathy.

To identify the cellular origin of MMP expression in the DCM heart, we isolated interstitial fibroblast cells from the DCM tissue. The DCM fibroblast cells were roundish in shape and bigger in size as compared to elongated cells in the normal tissue. The immunostaining of MMP-1 indicated apparent increased expression of MMP-1 in the myopathic fibroblast cells compared to the normal fibroblast cells (Fig. 9).

DISCUSSION

Dilated cardiomyopathy includes a disruption of the myocardial collagen matrix. Differential display mRNA analysis reveals an induction of a

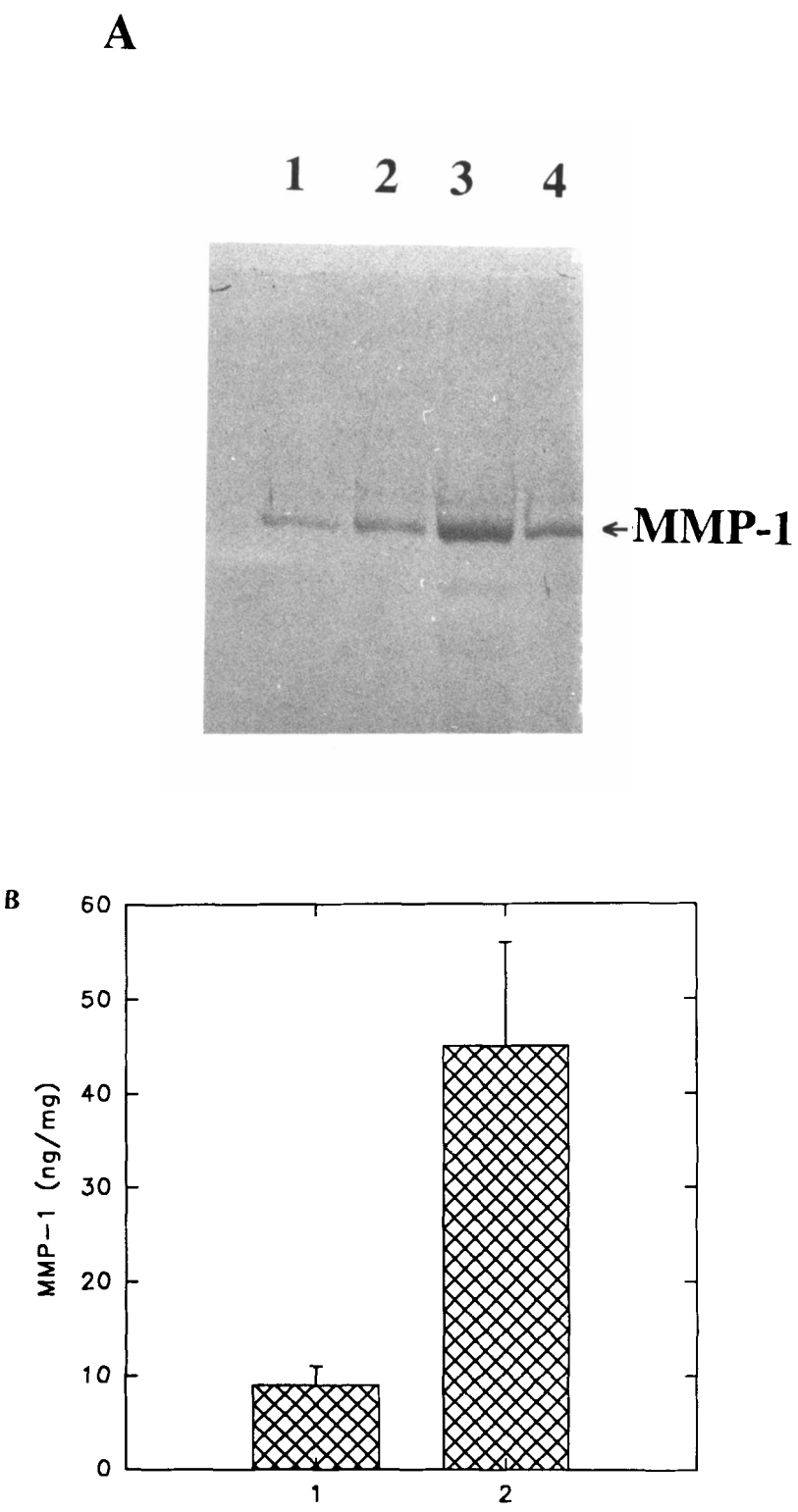

Fig. 7. **A:** Immunoblot analysis of human myocardial MMP-1 in normal and DCM tissue extract. *Lanes 1, 2:* Normal extract (20 and 40 μg, respectively). *Lane 3:* DCM cardiac tissue extract (20 μg). *Lane 4:* Standard MMP-1 (1 μg). Proteins were electrophoresed by SDS-PAGE (8%) gels and transferred to nitrocellulose membrane and blotted using MMP-1 antibody. **B:** Immunoassay data for MMP-1 obtained from normal (*bar 1*) and DCM (*bar 2*) tissue extracts. Errors bars are based on the deviations from the mean value. MMP-1 was induced in the DCM tissue.

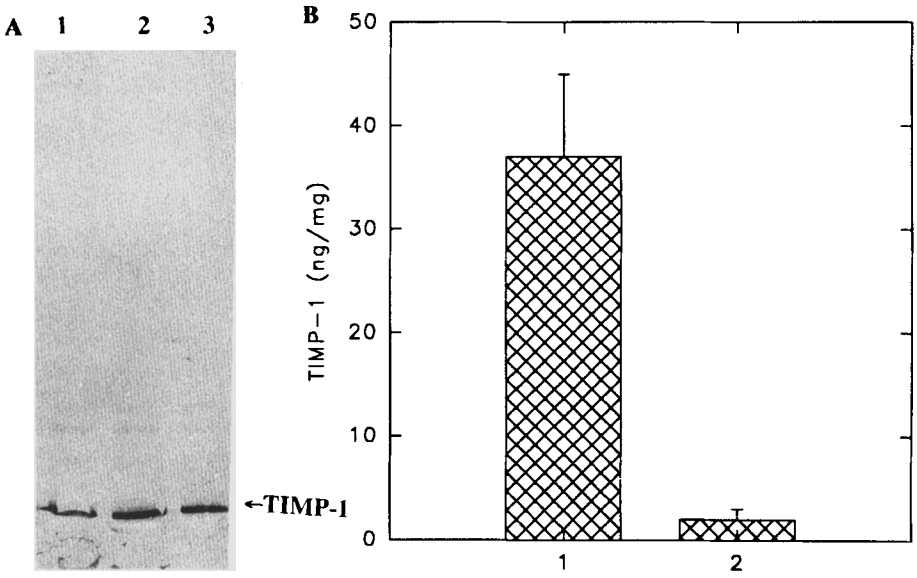

Fig. 8. **A:** Immunoblot analysis of human myocardial TIMP-1 in normal and DCM tissue extract. *Lane 1:* DCM extract. *Lane 2:* Normal extract. *Lane 3:* Standard TIMP-1. Extract (40 μg) was electrophoresesed by SDS-PAGE (8%) gels and transferred to nitrocellulose membrane and blotted using TIMP-1 antibody. **B:** Sandwich ELISA data for TIMP-1 obtained from normal (*bar 1*) and DCM (*bar 2*) tissue extracts. Errors bars are based on the deviations from the mean value. TIMP-1 was repressed in the DCM heart. When comparison was made between the levels of MMP and TIMP, all experiments were carried out under identical conditions.

number of genes in DCM. The gene for MMP-1 and its promoter activity was found to be elevated in the DCM heart. Protein analysis at the activity level indicated that indeed the gene of MMP-1 which was elevated during DCM was translated. Collagen degradation was observed in the DCM heart. The elevation of MMP-1 activity coincided with the repression of TIMP-1 levels leading to an induction of MMP-1 activity and ECM disruption following dilated cardiomyopathy.

Human cardiac nuclei display polyploidy in congenital heart disease [Vander Laarse et al., 1989; Gerdes et al., 1991]. We observed that in DCM the cardiac nuclei are doubled in size compared to nuclei from normal hearts (Fig. 1), and this increase in size is associated with an increase in nuclear transcription activity of activator protein (AP-1) on collagenase promoter (Fig. 4). The AP-1 is a strong promoter in the −77 to −55 region of the collagenase gene [Angel et al., 1987]. Transcription factors associated with cardiomyocyte differentiation and enhancement are expressed early in cardiac embryogenesis and are critical in activation of cardiac specific genes [Komuro and Izumo, 1993; Lints et al., 1993]. Lints et al. [1993] created mice homozygous for ablation of the transcription factor responsible for the development of the heart. In these mice they observed early embryonic lethality secondary to arrested cardiac development, with a thin-walled, poorly functioning (similar to DCM hearts), incompletely septated heart. These studies indicated a critical role of gene promoter binding proteins in proper adaptive function in maintaining cardiac-specific gene structure and expression at different stages of cardiac development and function. The up- and/or downregulation of these genes may cause cardiac maladaption and the progression of certain disease states. Ye et al. [1995] described a common stromelysin promoter variant that was associated with the progression of atherosclerosis. For the first time we demonstrated that collagenase promoter (AP-1) function is altered in DCM. Explanations for this abnormality include mutations in transcription factor or the overproduction of the transcription factor causing uncontrolled gene expression. However, these possibilities remain to be examined.

Our study demonstrated that differential mRNA display techniques can be adapted to identify mediators associated with a complex multicellular disease such as dilated cardiomyopathy. Differential display has been used to study breast cancer [Liang et al., 1992]. We demonstrated for the first time that extracellular matrix gene(s) are induced during the chronic

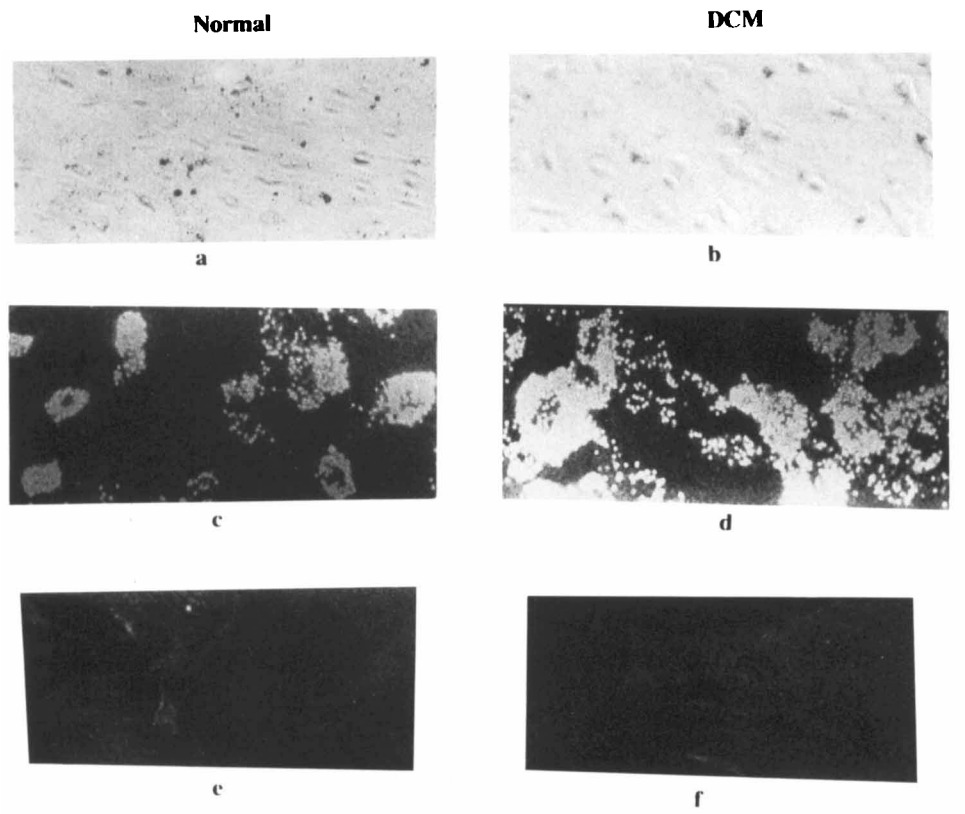

Fig. 9. Light transmission micrograph of fibroblast cells (p-2) isolated from normal (**a**) and DCM (**b**) hearts. ×20. In situ immunolabeling of MMP-1 in fibroblast cells isolated from normal (c) and DCM (**d**). ×200. **e,f:** Immunostaining with the secondary antibody in absence of primary antibody of normal and myopathic fibroblast cells, respectively. The DCM fibroblast cells are phenotypically different from the normal and MMP-1 staining in the myopathic fibroblast cells is greater than normal fibroblast cells.

cardiovascular disease process. We identified primarily three differential displayed cDNA bands that were reproducible and upregulated in tissue from DCM hearts. From these three bands, we identified that one cross-reacted with MMP-1 cDNA probe on Southern blot analysis (Fig. 2). Furthermore, we demonstrated by Northern blot analysis that the increase in MMP-1 cDNA was at the gene transcription level (Fig. 3). There are two possible consequences of this specific MMP-1 gene induction in DCM. One is associated with the mutation at the propeptide site of MMP-1 which will produce constitutively active MMP-1. This possibility will avoid the posttranslational requirement of MMP activation. The isolation of a full-length cDNA clone of MMP-1 from DCM tissue and fibroblast cells is in progress. However, the temporal expression of this gene may explain partly the presence of active MMP-1 in the DCM tissue. Further animal studies are needed to support this conclusion. The other possible consequence is that the level of TIMP is dramatically repressed. The reduced level of TIMP in the DCM heart may be due to the mutation in the TIMP gene. The mutations in the TIMP-3 gene have been identified in noncardiac tissue [Weber et al., 1994a]. However, this remains to be elucidated in the heart.

It is important to correlate the differentially displayed mRNAs to the protein level to indicate the mechanistic role of gene induction. We performed protein activity assays and indicated that MMP-1 is indeed induced at the functional level (Figs. 5, 6). Previously, we demonstrated that tissue plasminogen activator (tPA) converts plasminogen to plasmin which, in turn, activates MMPs and inactivates TIMP-1 posttranslationally following ischemic cardiomyopathy [Tyagi et al., 1996a]. Furthermore, in ischemic DCM we also observed the induction of gelatinase B (92 kDa), gelatinase A (66 kDa), and interstitial collagenase (MMP-1). Contrary to this, in DCM heart we found induction of only gelatinase A (66 kDa) and interstitial collagenase (MMP-1) and no expression of gelatinase B. In DCM patients with cardiac amyloidosis, elevated myocar-

dial collagenase activity was associated with fibrillar collagen degradation [Tjahja et al., 1993]. The collagenase activity in hypertrophic cardiomyopathy was also increased. However, in hypertrophic cardiomyopathy TIMP level was ~100-fold higher than the normal control, suggesting a role of TIMP in hypertrophic cardiomyopathy [Tyagi et al., 1993d]. Collectively, our data suggested generalized induction of MMP during DCM and indicated differential gene expression of proteinase antiproteinase under various myopathic conditions.

The cells responsible for the abnormality of ECM metabolism following DCM is possibly the phenotypically transformed myopathic fibroblast (Fig. 9). Previously, we have demonstrated that the myocardial fibroblast and endothelial and myocytic cells are the primary source of the synthesis of ECM components, and these cells produced MMP and actin in response to serum-containing growth factors, suggesting transformation of these cells to myofibroblastic and actin-producing cells [Tyagi et al., 1995c]. It is known that type I and type II collagen-derived peptides induce collagenase expression in rheumatoid synovial tissue [Fisher et al., 1982]. We observed an increased level of collagen peptide in the DCM heart (Fig. 6) and demonstrated that collagen peptide induces phenotypic shift in the fibroblast cells [Tyagi et al., 1996b].

In conclusion, we have demonstrated induction of MMP-1 by differential display mRNA analysis. This induction in collagenase expression was associated with an increased level of collagenase promoter activity. The increase in collagenase gene expression coincided with the expression at the protein level in DCM hearts. The level of TIMP-1 was reduced significantly in the DCM heart as compared to normal heart tissue. However, expression of other ECM components (i.e., collagen, elastin, and proteoglycans) in the DCM hearts remains to be elucidated. These studies are in progress.

ACKNOWLEDGMENTS

We acknowledge Dr. A. Martin Gerdes for discussion. We thank Susan Borders and John Burton for their expert technical assistance in this work. This work was supported in part by NIH grant GM-48595 and by a grant in aid from the American Heart Association, Missouri Affiliate (92-10517).

REFERENCES

Angel P, Baumann I, Stein B, Delius H, Rahmsdorf HJ, Herrlich P (1987): 12-O-tetradecanoyl-phorbol-13-acetate induction of the human collagenase gene is mediated by an inducible enhancer element located in the 5'-flanking region. Mol Cell Biol 7:2256–2266.

Boileau C, Jondeau G, Barbron (1993): Autosomal dominant marfan-like connective-tissue disorder with aortic dilation and skeletal anomalies not linked to the fibrillin genes. Am J Hum Genet 53:46–54.

Borg TK, Burgess ML (1993): Holding it all together: Organization and function(s) of the extracellular matrix of the heart. Heart Failure, 8:230–238.

Bradbury P, Gordon KC (1982): Connective tissue and stains. In Bancroft JD, Stevens A, (eds): *Theory and Practice of Histological Techniques* Edinburgh, London, Melborne, New York. Churchill Livingstone, p. 122.

Bradford MM (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Cannon RO, Butany JW, McManus BM, Speir E, Kravits AB, Billi R, Ferrans VJ (1983): Early degradation of collagen after acute myocardial infarction in the rat. Am J Cardiol 52:390–395.

Churgwin JM, Przybyla AZ, MacDonal RJ, Rutter WJ (1979): Isolation of biological active ribonucleic acid from sources enriched in ribonucleases. Biochemistry 132:6–13.

Dignam J, Lebovitz R, Roeder R (1983): Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res 11: 1476–1489.

Ewart AK, Jin W, Atkinson D, Morris CA, Keating MT (1994): SVAS associated with a deletion disrupting the elastin gene. J Clin Invest 93:1071–1077.

Fisher WD, Golds EE, van der Rest M, Cooke TD, Lyons HE, Poole AR (1982): Stimulation of collagenase secretion from rheumatoid synovial tissue by human collagen peptides. J Bone Joint Surg [Am] 64:546–557.

Franke WW (1996): Isolated nuclear membranes. J Cell Biol 31:619–623.

Gerdes AM, Morales MC, Handa V, Moore JA, Alvarez MR (1991): Nuclear size and DNA content in rat cardiac myocytes during growth, maturation and aging. J Mol Cell Cardiol 23:833–839.

Gerdes AM, Kellerman SE, Moore JA, Muffy KE, Clark LC (1992): Structural remodeling of cardiac myocytes in patients with ischemic cardiomyopathy. Circulation 86:426–430.

Janicki JS, Tyagi SC, Henegar JR, Campbell SE (1993): Myocardial collagenase activity and ventricular dilatation in cardiomyopathic hamsters. Circulation 88:I-381.

Janicki JS, Tyagi SC, Campbell SE, Reddy HK, Henegar JR (1994): Progressive dilatation in heart failure: The role of myocardial collagenase. Can J Cardiol 10(Suppl A):45A.

Kelly DP, Strauss AW (1994): Inherited cardiomyopathy: The spectrum of genetic etiologies. N Engl J Med 330:913–919.

Komuro I, Izumo S (1993): Csx: A murine homeobox-containing gene specifically expressed in the developing heart. Proc Natl Acad Sci USA 90:8145–8149.

Laemmli UK (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227:680–685.

Liang P, Pardee AB (1992): Differential display of eukaryotic mRNA by means of the polymerase chain reaction. Science 257:967–971.

Liang P, Averboukh L, Keyomarsi K, Sager R, Pardee AB (1992): Differential display and cloning of mRNAs from human breast cancer versus mammary epithelial cells. Cancer Res 52:6966–6968.

Lints TJ, Parsons LM, Hartley L (1993): Nkx-2.5: A novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendants. Development 119:419–431.

O'Connor WN, Davis JB, Geissler R, Cottrill CM, Noonan JA, Todd EP (1985): Supravalvular aortic stenosis. Arch Pathol Lab Med 109:179–185.

Olivetti G, Capasso JM, Sonnenblick EH, Anversa P (1990): Side-to-side slippage of myocytes participates in ventricular wall remodeling acurely after myocardial infarction in rats. Circ Res 67:23–34.

Richardson CP, McKenna RW, Bristow M, Maisch B (1996): Report of the 1995 WHO/ISFCTF on the definition and classification of cardiomyopathies. Circulation 93:841–842.

Suzuki K, Enghild JJ, Morodomi T, Salvesen G, Nagase H (1990): Mechanisms of activation of tissue procollagenase by matrix metalloproteinase 3 (stromelysin). Biochemistry 29:10261–10270.

Tjahja IE, Reddy HK, Tyagi SC, Campbell SE, Heggins JP, Diaz-Arias A, Weber KT (1993): Elevated myocardial collagenase activity associated with fibrillar collagen degradation in patients with cardiac amyloidosis. Clin Res 41: 630A.

Tyagi SC, Simon SR (1990): Inhibitors directed to binding domains in neutrophil elastase. Biochemistry 29:9970–9977.

Tyagi SC, Simon SR (1994): Hydrophobic binding sites of elastin-derived peptide on neutrophil elastase. Biochem Cell Biol 72:419–427.

Tyagi SC, Ratajska A, Weber KT (1993a): Myocardial matrix metalloproteinase(s): Activation and localization. Mol Cell Biochem 126:49–59.

Tyagi SC, Janicki JS, Henegar JR, Campbell SE (1993b): Post-transcriptional and translational modification of myocardial collagenase leads to ventricular dilatation in cardiomyopathic hamsters. Clin Res 41:630A.

Tyagi SC, Matsubara L, Weber KT (1993c): Direct extraction and estimation of collagenase(s) activity by zymography in microquantities of rat myocardium and uterus. Clin Biochem 26:191–198.

Tyagi SC, Zhou G, Ratajska A, Weber KT (1993d): Quinapril-induced myocardial collagenase in spontaneously hypertensive rats (SHR). Am J Hypertens 6:67A.

Tyagi SC, Kumar SG, Banks J, Fortson W (1995a): Co-expression of tissue inhibitor and matrix metalloproteinase in myocardium. J Mol Cell Cardiol 27:2177–2189.

Tyagi SC, Meyer L, Schmaltz RA, Reddy HK, Voelker DJ (1995b): Proteinases and restenosis in human coronary artery: Extracellular matrix production exceeds the expression of proteolytic activity. Atherosclerosis, 116:43–57.

Tyagi SC, Kumar SG, Glover G (1995c): Induction of tissue inhibitor and matrix metalloproteinase by serum in human heart-derived fibroblast and endomyocardial endothelial cells. J Cell Biochem 58:360–371.

Tyagi SC, Campbell SE, Reddy HK, Tjahja E, Voelker DJ (1996a): Matrix metalloproteinase activity expression in infarcted, noninfarcted and dilated cardiomyopathic human hearts. Mol Cell Biochem 155:13–21.

Tyagi SC, Kumar SG, Alla SR, Reddy HK, Voelker DJ, Janicki JS (1996b): Extracellular matrix regulation of metalloproteinase and antiproteinase in human heart fibroblast cells. J Cell Physiol 167:137–147.

Vander Laarse A, Hoollaar L, Vliegen HW, Dijkshoorn NJ, Cornelisse CJ, Bogers AJJC, Quaegebeur JM (1989): Myocardial (iso)enzyme activity, DNA concentration and nuclear polyploidy in hearts of patients operated upon for congenital heart disease, and in normal and hypertrophic human heart. Eur J Clin Invest 19:192–200.

Ward RV, Hembry RM, Reynolds JJ, Murphy G (1991): The purification of tissue inhibitor of metalloproteinase-2 (TIMP-2) from its 72 kDa progelatinase complex; demonstration of the biochemical similarities of TIMP-2 and TIMP-1. Biochem J 278:179–187.

Weber BHF, Vogt G, Pruett RC, Stohr H, Felbor U (1994a): Mutations in the TIMP-3 in patients with Sorsby'dundus dystrophy. Nat Genet 8:352–355.

Weber KT, Sun Y, Tyagi SC, Cleutjens J (1994b): Collagen network of the myocardium: Function, structural remodeling and regulatory mechanisms. J Mol Cell Cardiol 26: 279–292.

Ye S, Watts GF, Mandalia S (1995): Preliminary report: Genetic variation in the human stromelysin promoter is associated with the progression of coronary atherosclerosis. Br Heart J 73:209–215.

Zhao M, Zhang H, Robinson TF, Factor SM, Sonneblick EH, Eng C (1987): Profound structural alterations of the extracellular collagen matrix in postischemic dysfunctional ("stunned") but viable myocardium. J Am Coll Cardiol 10:1322–1334.

Zimmermann JW, Schultz RM (1994): Analysis of gene expression in the preimplantation mouse embryo: Use of mRNA differential display. Proc Natl Acad Sci USA 91: 5456–5460.