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Degradation of Type IV Collagen by Matrix Metalloproteinases Is an Important Step in the Epithelial-Mesenchymal Transformation of the Endocardial Cushions

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Morphogenesis of some tissues and organs in the developing embryo requires the transformation of epithelial cells into mesenchyme followed by cell motility and invasion of surrounding connective tissues. Details of the mechanisms involved in this important process are beginning to be elucidated. The epithelial-mesenchymal transformation (EMT) process involves many steps, one of which is the upregulation and activation of specific extracellular proteinases including members of the matrix metalloproteinase (MMP) family. Here we analyze the role of MMPs in the initiation of the mesenchymal cell phenotype in the developing heart, and find that they are necessary for the invasion of mesenchymal cells into the extracellular matrix of the endocardial cushion tissues. An important requirement in the formation of this mesenchyme is the turnover of type IV collagen along the basal surface of endocardial cells. *In vitro* experiments suggest that type IV collagen does not provide a suitable migratory substrate for endocardial cushion cells unless MMP-2 and MT-MMP are active. Relevant MMPs were found to be upregulated by factors known to be involved in the induction of the EMT such as TGFβ3. These results provide evidence of an important role for MMPs during a specific stage of the epithelial mesenchymal transformation in the embryonic heart, and suggest that specific cell-matrix interactions which facilitate cell migration only occur when the composition of the surrounding extracellular matrix is proteolytically altered.

Key Words: **epithelial-mesenchymal transformation; endocardial cushions; matrix metalloproteinases; extracellular matrix; embryonic heart development; type IV collagen.**

INTRODUCTION

The transformation of epithelial cells into mesenchyme is a unique and basic process which occurs during embryonic development and is important in the morphogenesis of organs and tissues in the embryo. The epithelial-mesenchymal transformation (EMT) has been shown to occur at discrete sites and at specific times in the embryo, and gives rise to a population of elongated, highly motile cells which invade the surrounding extracellular matrix (Hay, 1995; Hay and Zuck, 1995). In some cases, these cells can migrate to sites in the embryo which are considerable distances from the originating epithelium. Examples of the EMT process include the transformation of the ventral portion of the somite into sclerotome cells which contribute to the formation of the vertebral column (Balling *et al.*, 1996), the fusion of the palatal structures through the transformation of the midline epithelial seam (Shuler *et al.*, 1992; Shuler, 1995), and the formation of the neural crest which differentiate into a wide variety of cell types including melanocytes of the skin, various components of the peripheral nervous system, and a variety of skeletal and connective tissue structures (Duband *et al.*, 1995).

This study focuses on the EMT which occurs in the endocardial cushions during the early stages of heart development (Markwald *et al.*, 1996). At a specific stage of development endocardial cells of the cushions form mesenchyme which invades the underlying cardiac jelly matrix. These cells eventually differentiate into the connective tissue cells of the valves and contribute to portions of the atrial and ventricular septae. This process appears to be

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driven by signals derived from the underlying myocardium in the form of extracellular matrix (ECM) components and/or specific growth factors. The response of the endocardium to these signals is characterized by cellular hypertrophy and decreased cell-cell adhesion, followed by the upregulation of specific genes which establish the mesenchymal phenotype and promote cell migration and proliferation. (Markwald *et al.,* 1979; Potts *et al.,* 1989, 1991; Nakajima *et al.,* 1994; Huang *et al.,* 1995; Romano and Runyan, 2000).

The migration of mesenchymal cells in the endocardial cushions and other regions of the embryo involves interactions of the cells with components of the ECM which may be regulated in part by the production of specific extracellular proteinases. These enzymes may facilitate cell migration by a limited proteolysis of the ECM at cell/substrate attachment sites, or through a larger scale remodeling of the matrix, altering the regulatory information contained within the migratory pathways (i.e., guidance, migratory, and adhesive cues). One group of extracellular enzymes which are expressed and may play a critical role in this process is the matrix metalloproteinases (MMPs). The 72-kDa type IV collagenase/gelatinase, MMP-2, is expressed in a variety of tissues of the developing embryo, and has been reported to degrade a wide variety of matrix components including fibronectin, vitronectin, and types IV and V collagen (Matrisian, 1992; Aimes and Quigley, 1995; Baramova and Foidart, 1995; Imai *et al.,* 1995). Previous studies have demonstrated that MMP-2 can be localized to the surface of invasive cell types through an interaction with the $\alpha v\beta 3$ integrin (Brooks *et al.,* 1996). This interaction not only creates localized areas of controlled proteolytic activity facilitating cell invasion but also has the potential of effecting the interactions of this integrin with components of the matrix (Deryugina *et al.,* 1997). Based upon these studies it is not unreasonable to assume that appropriate levels of active MMP-2 may directly affect the ability of cells to migrate into and through the ECM.

We have previously shown that MMP-2 is present in the heart of the developing quail embryo, and is expressed specifically by the endocardium and mesenchyme of the cushion tissues (Alexander *et al.,* 1997). The level of the 72-kDa pro-form of MMP-2 remained fairly constant during the early stages of cushion tissue morphogenesis. This was in marked contrast to the levels of the lower molecular weight active species which increased significantly during the transformation of the endocardium. Coincident with the appearance of the active form of MMP-2 was an increase in the message levels for the membrane-type matrix metalloproteinase (MT-MMP), an activator of pro-MMP-2. These results suggested that a key step in the regulation of MMP-2 activity during heart development may be at the level of proenzyme activation.

The present study was designed to determine a functional role for the matrix metalloproteinases during the EMT process in the endocardial cushions. Results from this study suggest that active MMPs are critical during the EMT and the establishment of the invasive phenotype, and that specific components of the cushion extracellular matrix must be degraded in order for the mesenchyme to form normally.

MATERIALS AND METHODS

All cell culture materials were obtained from Gibco/BRL (Gaithersburg, MD) except for fetal calf serum, which was purchased from HyClone (Logan, UT). The anti-smooth muscle actin antibody Clone 1A4 was purchased from Sigma Chemical (St. Louis, MO). Anti-chicken type IV collagen antibody IIB12 was the kind gift of Dr. Thomas Linsenmayer (Tufts University School of Medicine, Boston, MA). The secondary antibody used for this study was fluorescein-isothiocyanate (FITC)-goat anti-mouse IgG Fab′2 from Jackson Laboratories (West Grove, PA). Purified extracellular matrix proteins, fibronectin, type IV collagen, laminin, and tenascin were purchased from Gibco/BRL. The matrix metalloproteinase inhibitor, BB-94, and its nonactive form, BB-1722, were supplied by British Biotech Pharmaceuticals, Inc.

Embryos and Tissue Isolation

Quail embryos (*Coturnix coturnix japonica*) were used for these studies. Fertilized eggs were obtained from B&D Game Farm (Harrah, OK) and were incubated at 38°C and 60% relative humidity. The embryos were staged according to Zacchei and used between stages 12 and 24 of development corresponding to specific developmental events within the endocardial cushions of the developing heart. At stage 12, the cushion tissues are beginning to form, and the heart is in the process of looping. The epithelial-mesenchymal transformation and the seeding of mesenchyme begins at stage 15–16. By stage 18, the cushion tissues have fully formed and the epithelial-mesenchymal transformation is well underway and a population of mesenchymal cells has invaded the cushion tissue extracellular matrix. By stage 24 of development, the cushion tissue matrix is heavily populated with mesenchyme derived from the endocardium. In some experiments, the atrioventricular canal (AVC) was separated from the outflow tract and ventricular portions of the stage 15–16 embryonic heart and used for explant culture of endocardial and mesenchymal cells as described below.

Embryo Culture and Microinjection Techniques

Live quail embryos were grown in a shell-less culture system allowing direct access to the embryos for microinjection of the metalloproteinase inhibitors (Dunn *et al.,* 1981). Following 2 days of incubation, the egg shell was opened and the egg contents were poured into a plastic sling and incubated in a humidified incubator at 38°C. When the embryos reached stage 12–14 of development, 5 μM of the appropriate material was injected with a glass micropipett directly into the ventricular portion of the heart. Injections of this type have resulted in significantly less embryonic demise compared to injections into the yolk sac vasculature and can directly target the endocardium of the cushion tissues. Following injection, the embryos were returned to the incubator and were allowed to develop until stage 16, 18, or 24 of development at which time they were collected for immunochemical, histological, or proteinase analysis.

Histological and Immunocytochemical Analysis

The extent of mesenchyme invasion into the stage 18 endocardial cushions was quantitated in control (BB-1722 or vehicle) and BB-94 inhibitor-injected embryos. Embryos were collected, fixed for 3 h in 0.1 M PO_4 buffer containing 4% paraformaldehyde, and embedded in paraffin. Serial 6-μm cross sections of the AVC portion of the heart were cut and mounted onto glass slides and coverslipped using Vectashield mounting medium with DAPI (Vector Labs, Burlingame, CA). Images were captured using a video camera mounted on a Zeiss inverted microscope and linked to a Dell computer and analyzed using the MetaMorph image analysis system. For cell counts, mesenchymal cell nuclei (i.e., those nuclei which were at least three nuclear diameters away from the endocardial and myocardial layers) were quantitated in 5–7 sections and averaged. Every other section was analyzed to avoid counting a given nucleus more that once. The data were expressed as nuclei/section for both treated and control samples.

For immunostaining, frozen sections of inhibitor-treated and control embryos were blocked in 1X TBST containing 10% normal goat serum. Immunofluorescence studies were performed using mouse monoclonal anti-smooth muscle actin antibody (1/100 dilution), or undiluted anti-type IV collagen hybridoma supernatant. The secondary antibody was a FITC-conjugated goat anti-mouse IgG (1/200). Sections with mounted using Vectashield mounting medium with DAPI to stain nuclei. The primary antibody was omitted in controls.

In Situ Hybridization

Mouse embryos (C57Bl6J) were used for *in situ* hybridization rather than quail as the available probes for type IV collagen were mouse specific. Embryos of 9.5 days gestation were fixed in 4% paraformaldehyde for 2–4 h, frozen in OCT compound, and sectioned. The heart at this stage of mouse development was roughly equivalent to that of the stage 14–15 quail (i.e., early EMT stage). Sections containing the heart were labeled with 35S-labeled sense and antisense riboprobes as described previously (Alexander *et al.*, 1997). Riboprobes were synthesized from the linearized mouse α1 type IV collagen cDNA kindly provided by Dr. Yoshi Yamada (Oberbaumer *et al.*, 1985). Following hybridization and posthybridization treatments, the sections were coated with Kodak NTB2 nuclear track emulsion and exposed for 15 days. The developed slides were examined and photographed using darkfield optics.

Proteinase Activity Measurements

The level and activation state of MMP-2 in hearts of inhibitor-treated and control embryos were examined by zymography. Hearts were collected from control and inhibitor-injected embryos at stage 18 and extracted overnight at 4°C in 0.1 M phosphate buffer containing 0.02% NaN_3 and 0.01% Triton X-100 at pH 8.0. Aliquots were removed for total protein determination using the MicroBCA assay (Pierce), and for electrophoresis in 10% polyacrylamide minigels into which gelatin (1 mg/ml) was crosslinked. Following electrophoresis, the gels were soaked for 15 min in 2.5% Triton X-100 and rinsed well with water. The gels were incubated for 48 h in LSCB buffer (100 mM Tris, 5 mM $CaCl_2$, 0.005% Brij-35, 0.001% NaN_3, pH 8.0) and stained with Coomassie. Zones of clearing corresponding to the presence of proteinase in the gel were quantitated using the Alpha Imager 2000 (Alpha Innotech Corp., San Leandro, CA). The area of the lysis zone was expressed as the integrated density value.

The effectiveness of the MMP inhibitor was determined in live tissues and cells using a H3 type IV collagen release assay. Tissue culture wells were incubated overnight at 4°C with approximately 15,000 cpm of H3 type IV collagen (0.1–1 mCi/mg, New England Nuclear, Boston MA) and rinsed extensively with PBS. Nonbound sites were blocked with BSA. Explants of the stage 15–16 AVC (described below) were placed onto the substrate and incubated overnight in the presence or absence of the MMP inhibitors. The percentage of released counts (indicating degraded type IV collagen) was determined from the total counts in the conditioned media and the counts remaining bound to the tissue culture wells. Bound counts were determined by extracting the wells with 10% SDS at the end of the incubation period. Nonspecific release (wells without AVC explants) was minimal ($\leq$1.5% of the total counts) and was subtracted from the media-derived counts. Released counts were analyzed by ultrafiltration using a Centricon-50 (Amicon, Beverly, MA) to confirm that the majority of the released counts represented small fragments of the protein.

Explant Culture and Motility Analysis

Endocardial cushion-derived cells were obtained from explants of the AVC of stage 15–16 quail embryos. The AVC was removed from intact hearts and opened longitudinally with a pair of sharpened needles and placed endocardial-side down onto the appropriate substrates and incubated in M199 medium containing 10% fetal calf serum and antibiotics. Substrates were prepared by incubating dishes overnight at 4°C with 100 μg/ml solutions of type IV collagen, fibronectin, laminin, or tenascin. Nonbound sites were blocked with a solution of 0.1% BSA at room temperature. Gelatin-coated dishes were prepared using a 0.1% solution and air-drying. For motility analysis, the explants were plated onto coated tissue culture wells and incubated at 37°C in the presence or absence of 5 μM BB-94 or BB-1722. The extent of cellular outgrowth after 16 h in culture was quantitatively analyzed using the MetaMorph image analysis system. Comparisons in the extent of cellular outgrowth from the edges of the explants were made among control (no addition), BB-1722-, and BB-94-treated cultures on the various substrates. The radial distance of migration from the edge of the explants was determined by measuring the distance at select points around the circumference of the explant (0, 45, 90, 135, 180, 225, 270, and 315°). Data were analyzed statistically using the Student *t* test and expressed as average micrometer from the edge of the explant.

Isolated cells were obtained from explants on gelatin-coated dishes. The explants were removed and the cells were passaged using trypsin (0.05%)/EDTA (0.53 mM) and grown in medium M199 containing 10% fetal calf serum and antibiotics.

Growth Factor Regulation of MMP Expression

Isolated cushion tissue cells were transferred to serum-free medium and treated with 1 or 10 ng/ml of TGFβ3 (R&D Systems) for 16 h. Total RNA was isolated, and cDNA was prepared using 1–2 μg RNA with an oligo(dT)primer and Superscript reverse transcriptase (Gibco BRL). For semiquantitative PCR, 1 μL of first strand reaction was amplified using oligonucleotide primers specific for the chicken MT-MMP, MMP-2, and GAPDH (see below). PCR conditions were optimized for each set of primers so as to be within the linear range of amplification. For MT-MMP the conditions were 94°C 1 min, 65°C 1 min, and 72°C 1 min for 30 cycles. For MMP-2 and GAPDH the PCR conditions were 94°C 1 min,

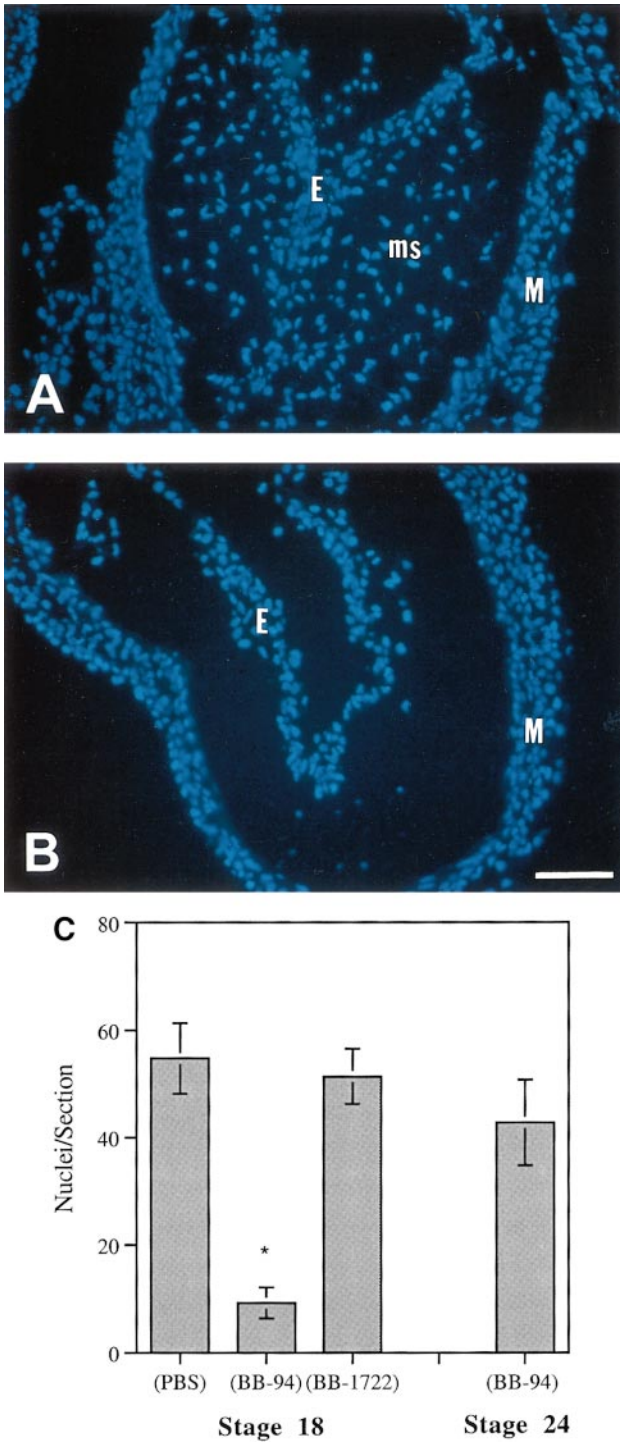

FIG. 1. Inhibition of matrix metalloproteinase activity prevents mesenchyme formation in the endocardial cushions of the AVC. Representative sections of the AVC from stage 18 quail embryos injected at stage 12 with either BB-1722 (A) or BB-94 (B). In the BB-1722-injected animals, numerous mesenchymal cells (ms) have migrated from the endocardium (E) into the surrounding cardiac jelly matrix of the cushion. This is in contrast to those animals injected with BB-94 in which there is significantly less mesenchyme formed and what is present appears to remain close to the endocardium and does not migrate into the cushion matrix. M, myocardium. Bar = 100 μm. (C) Quantitative analysis of mesenchyme formation. Values are the mean ± the SEM. *Significantly different from PBS and BB-1722-injected animals at the $P < 0.05$ level.

60°C 1 min, and 72°C 1 min for 25 cycles. Upon completion, 10 μL of the PCR was analyzed by agarose gel electrophoresis and visualized by cyber green staining. Quantitation of band density was accomplished using the Alpha Imager 2000 analysis software (Alpha Innotech Corp., San Leandro, CA). The primer sets for these analyses were as follows: chicken MT-MMP (375-bp product), 5′-AGAATCACCCCAGGGAGCCTTTGT-3′ and 5′-GATCTCACCCACTCTTGCATAG AGCGT-3′; chicken MMP-2 (413-bp product); 5′-AAATCGATATGGTCTACGAGTCCCT-3′ and 5′-GGATCGATTTCAGCAACCCAACCAGT-3′; and chicken GAPDH (350-bp product), 5′-ACCATCAAGTCCACAACACG-3′ and 5′-AAGGTC ATCCCAGAGCTGAA-3′. Data were reported as the mean integrated density value from three independent experiments. Statistical analysis was done by the Student *t* test, and values were expressed as the mean ± standard error of the mean. Tests were considered statistically significant at *P* values of ≤0.05.

RESULTS

Stage 12 quail embryos were grown in a shell-less culture system and microinjected with 50 nl (5 μM) of either BB-94 or BB-1722 in PBS/0.01% fast green. Control embryos received an injection of the equivalent volume of PBS/fast green. Injections were given directly into the ventricular portion of the heart and the embryos were returned to the incubator until stage 18 or 24 of development. The majority of injected embryos tolerated the injection well and developed further. Of those embryos that died following the injection, most appeared to do so because of excessive bleeding from the injection site.

By stage 18 of development in the quail, a significant number of mesenchymal cells are present in the extracellular matrix of the endocardial cushions in control embryos (Fig. 1A). The number of mesenchymal cells in the cushions was determined from serial sections of the AVC and expressed as the number of nuclei/section. No significant difference was found in the number of nuclei/section for embryos injected with either PBS/fast green or BB-1722 (Figs. 1A and 1C). In contrast, a significant decrease was seen in the number of mesenchymal cells present in the cushion extracellular matrix in embryos receiving an injection of the matrix metalloproteinase inhibitor BB-94 (Figs. 1B and 1C). The majority of mesenchymal cells appeared to be confined to a region very close to the endocardial lining of the AVC with a few scattered or clustered cells present in areas separated from the endocardial lining (Fig. 1B). This was in contrast to those embryos treated with either vehicle or BB-1722, which exhibited mesenchymal cells evenly distributed throughout the endocardial cushion.

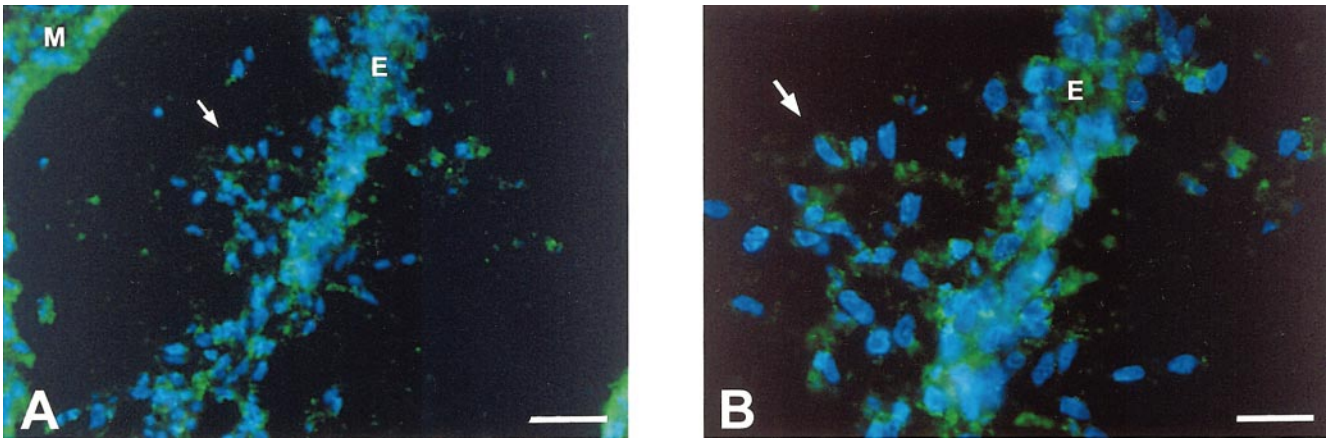

FIG. 2. BB-94 inhibition of matrix metalloproteinase activity does not disrupt the initial events of the EMT process as evidenced by the expression of smooth muscle α-actin in transforming endocardial cells and mesenchyme. (A) Immunolabeling of smooth muscle α-actin in the AVC from a stage 18 embryo injected with BB-94 at stage 12. The number of mesenchymal cells is greatly reduced; however, both the mesenchyme that has formed (arrow) and the endocardium (E) are stained positively for smooth muscle α-actin (green fluorescence). Individual cells are identified by blue nuclear staining. M, myocardium. Bar = 66 μm. (B) Higher magnification of the endocardial region in (A). Bar = 40 μm.

In some cases embryos were allowed to develop to stage 24 following the injection of inhibitor at stage 12–14. At this later stage of development in the control embryos, the AVC cushions are so densely populated with mesenchymal cells that they could not be accurately quantitated. The effect of BB-94 on the formation of AVC cushion mesenchyme appeared to be temporary as the number of cells was increased significantly in the stage 24 embryos treated with BB-94 compared to those examined at stage 18. (Fig. 1C).

To determine if BB-94 were nonspecifically inhibiting the initiation of the EMT process, embryos injected with BB-94 at stage 12–14 were fixed at stage 18 and immunostained using an antibody to smooth muscle α-actin, a marker of cells which have undergone the transformation process (Deruiter *et al.*, 1997; Nakajima *et al.*, 1999). Sections of the AVC from BB-94 injected embryos treated with this antibody showed that the endocardium had initiated the transformation process, and that the cells which were present either in the matrix or adjacent to the endocardial lining were expressing the mesenchymal cell phenotype (Fig. 2).

We have shown previously that only two members of the MMP family are detected in the heart of the developing quail embryo, MMP-2 and MT1-MMP (Alexander *et al.*, 1997). During the epithelial-mesenchymal transformation, the amount of active MMP-2 was found to increase significantly, presumably due to the action of MT-MMP on the proenzyme form of the proteinase. The hearts from embryos injected with either BB-1722 or BB-94 were analyzed at stage 18 by zymography to characterize the stage of MMP-2 within the tissue. The amount of the 64-kDa active form of MMP-2 was significantly less in BB-94-treated embryos compared to those treated with the control compound BB-1722 (Fig. 3). Those embryos injected with ve-

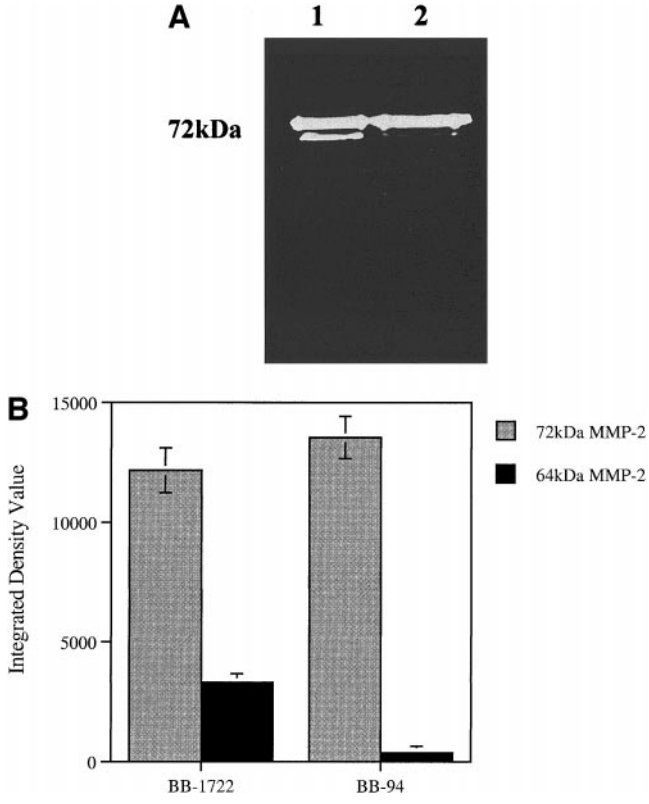

FIG. 3. BB-94 injection decreases the amount of the active form of MMP-2 in the stage 18 heart. (A) Zymographic analysis of hearts from BB-1722-injected (Lane 1) and BB-94-injected (Lane 2) embryos. The position of the 72-kDa pro-form of MMP-2 is indicated. (B) Quantitative analysis of the pro- (72 kDa) and active (64 kDa) forms of MMP-2 from zymograms indicates a significant reduction in the level of active MMP-2 in embryos injected with BB-94.

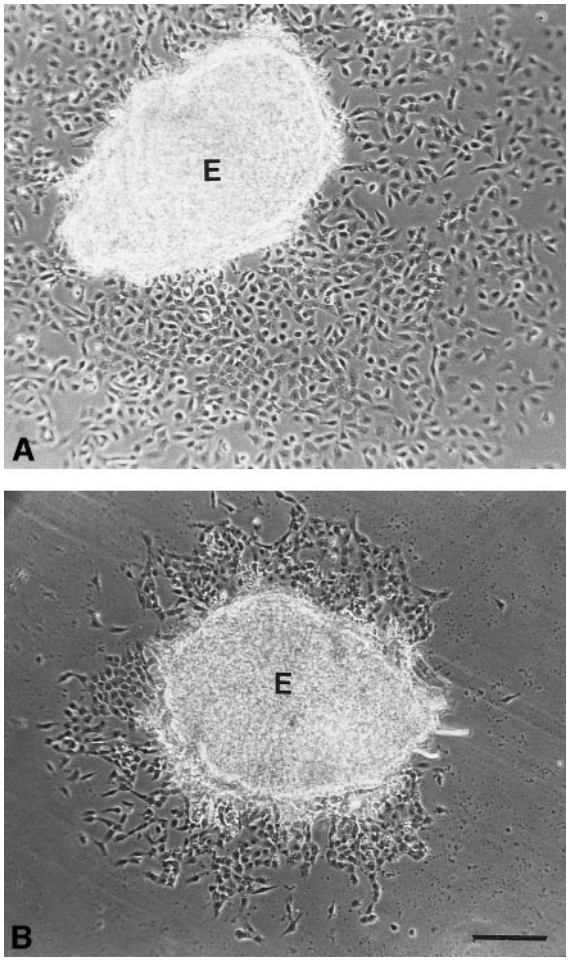

FIG. 4. BB-94 inhibits the *in vitro* outgrowth of endocardial-derived cells (endocardium and mesenchyme) from AVC explants. (A) AVC explants (E) grown on gelatin-coated substrates in the presence of 5 μM BB-1722 show an extensive outgrowth of cells after 16 h in culture. (B) AVC explant (E) grown on gelatin in the presence of BB-94 demonstrates a significant reduction in the extent of cellular outgrowth after 16 h. Bar = 300 μm.

hicle only were no different from BB-1722-injected embryos (data not shown). No changes were seen in the level of the inactive 72-kDa pro-form of the enzyme.

The degree to which the cushion becomes populated with mesenchymal cells is due in part to the migration of transformed cells from the endocardial lining, as well as to the proliferation of existing mesenchymal cells. *In vitro* explant studies were subsequently carried out to determine if the inhibition of MMP-2 activity disrupted mesenchymal cell migration. Explants of the AVC from stage 15–16 embryos were placed endocardial-side down onto a gelatin-coated substrate and incubated overnight in the presence of either BB-1722 (Fig. 4A) or BB-94 (Fig. 4B). In control cultures the endocardial-derived cells (a portion of which are transformed mesenchyme) migrated extensively from the edges of the explant. In contrast, far fewer cells migrated outward from the explants treated with the same concentration of BB-94. Quantitation of the migration distance revealed that control cells migrated approximately 500 μm from the edge of the explant on average, compared to approximately 200 μm for cells treated with BB-94 (Fig. 5).

MMP-2 may selectively regulate the ability of cushion tissue cells to interact with and migrate on specific extracellular matrix molecules. This possibility was investigated by placing AVC explants onto substrates of gelatin, fibronectin, type IV collagen, laminin, and tenascin in the presence of BB-1722 or BB-94. The explants were incubated for 16 h and the extent of cellular outgrowth was quantitated (Fig. 5). Significant inhibition of cell migration was observed for explants plated onto either gelatin or type IV collagen substrates and grown in the presence of BB-94 compared to BB-1722. No significant difference in outgrowth was seen for explants grown on fibronectin or laminin, while tenascin did not appear to provide a useful substrate for outgrowth in either case.

To confirm that the effect seen in these experiments resulted from an inhibition of MMP-2 activity, explants were plated onto a substrate of radiolabeled type IV collagen and analyzed for the release of radioactive counts. Explants grown in either the presence of BB-1722 or no additions to the media released between 20 and 22% of the total radioactivity bound to the substrate (Table 1). When explants were grown in the presence of equivalent concentrations of BB-94, the percentage of released radioactivity dropped to approximately 6%.

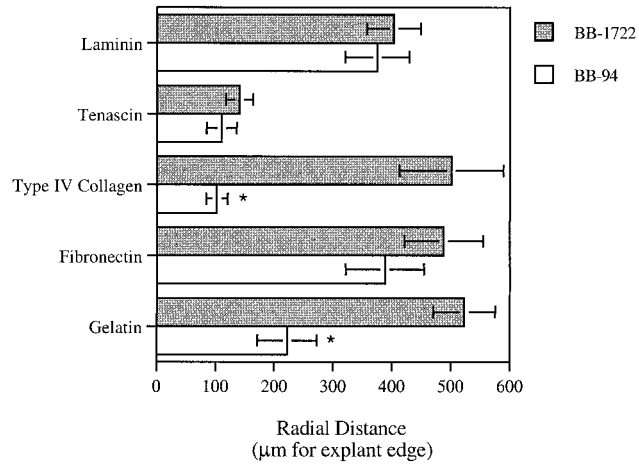

FIG. 5. Matrix metalloproteinases selectively regulate the interactions of endocardial-derived cells with components of the extracellular matrix. AVC explants were cultured on the indicated substrates for 16 h in the presence of either BB-1722 or BB-94 and the extent of outgrowth was determined. Values are the mean ± SEM from three separate experiments. *Significantly less than BB-1722-treated cultures at the $P < 0.05$ level.

TABLE 1
BB-94 Inhibits the Degradation of Type IV Collagen by Endocardial Cushion-Derived Cells

Total No of explants	Treatment	cpm media	cpm bound	% cpm released
17	No addition	1975.5 ± 103	7034 ± 334	22.0
20	BB-1722 (100 μg/ml)	1689.7 ± 175	6831 ± 212	20.0
20	BB-94 (100 μg/ml)	562.3 ± 113	8786 ± 303	6.0*

Note. Explants were plated onto H3 type IV collagen substrate and incubated in media for 16 h with the indicated additions. Media were collected and total cpm were determined. cpm remaining bound to the substrate were removed by incubation with 10% SDS. Values are the mean ± SEM for the total number of explants indicated from two experiments after subtracting the values for media only control.

*Significantly less than no addition and BB1722 at the $P \leq 0.05$ level.

The above data suggest that type IV collagen serves as a permissive substrate for endocardial/mesenchymal cell migration only if active MMP-2 and MT-MMP are present and the type IV collagen is degraded.

The site of type IV collagen synthesis in the endocardial cushions was determined by *in situ* hybridization with a probe to the α1 chain of the protein. Both the endocardium and myocardium appear to express the mRNA for this component of the basement membrane (Fig. 6). The type IV collagen protein was further localized in the cushions by immunostaining. Sections of the AVC from control, untreated embryos were immunostained with the IIb12 antibody at a stage prior to the initiation of the EMT (Stage 12). The basement membrane underlying the endocardium as well as that associated with the myocardium was intensely stained (Figs. 7A and 7B). In addition, a diffuse punctate pattern of type IV collagen staining was also evident in the cardiac jelly matrix. In control embryos during the early stages following the initiation of the EMT (stage 16), the discrete pattern of type IV collagen remained associated with the myocardium but was conspicuously absent from the endocardial lining (Figs. 7E and 7F). On occasion, some fibrillar type staining was seen associated with groups of mesenchymal cells. In embryos treated with BB-94 at stage 12–14 and examined later at stage 18, the staining associated with the endocardium and cardiac jelly appeared to persist (Figs. 7C and 7D).

A final set of experiments was carried out to determine if growth factors, known to play an important role in the regulation of the EMT, have the ability to regulate MMP expression in the cushion tissue cells. One growth factor which has been shown to influence the formation of mesenchyme in the endocardial cushions is TGFβ3 (Ramsdell and Markwald, 1997; Yamagishi *et al.*, 1999; Boyer *et al.*, 1999; Nakajima *et al.*, 2000). Isolated cushion tissue cells were incubated overnight with 1 or 10 ng/ml of TGFβ3 and analyzed by RT-PCR. The mRNA for both MT-MMP and MMP-2 were found to increase significantly in these cells in response to TGFβ3 stimulation when compared to untreated controls (Figs. 8 and 9).

DISCUSSION

This study is an extension of previous work from our laboratory which initially characterized the expression of MMPs in the endocardial cushions of the developing avian heart, and suggested that the activity of these enzymes was necessary for the migration of mesenchyme into the cardiac jelly matrix. We now provide evidence that degradation of

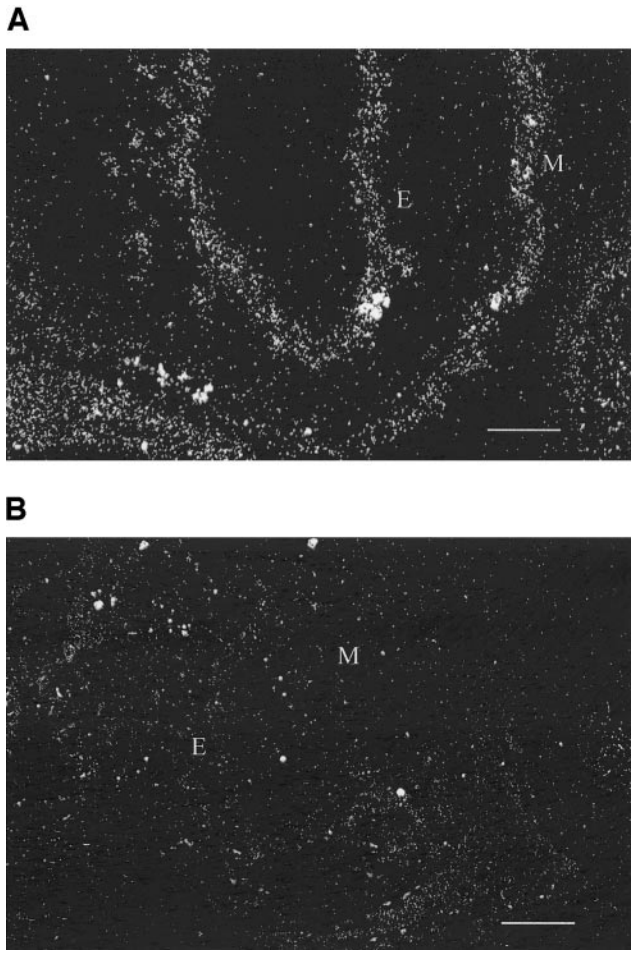

FIG. 6. *In situ* hybridization analysis of type IV collagen expression in the 9.5 day mouse embryo. Expression of type IV collagen mRNA is seen in both the endocardium (E) and the myocardium (M) of an antisense-treated section (A). Adjacent section treated with a sense probe gave no specific labeling (B). Bar = 100 μm.

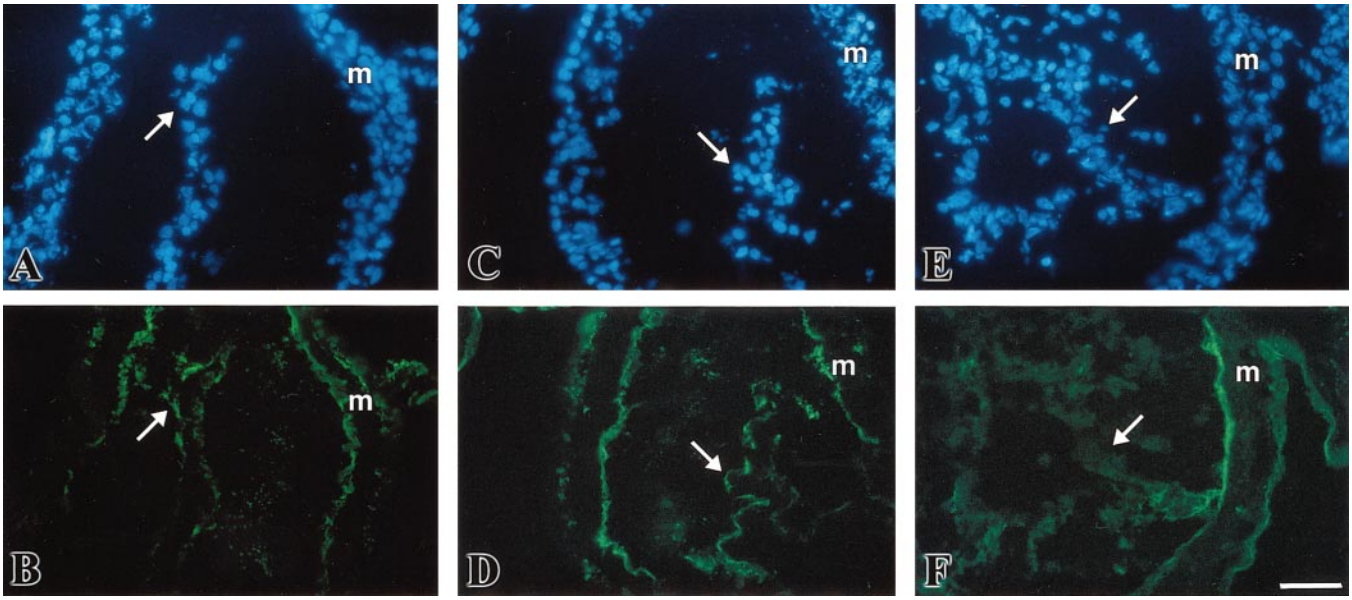

FIG. 7. Type IV collagen immunostaining in the AVC (B, D, and F). The corresponding DAPI image is shown in A, C, and E. Sections of stage 12 unmanipulated embryos (A and B) demonstrate a distinct band of type IV collagen associated with both the endocardium (arrow) and the myocardium (m). Type IV collagen staining associated with the endocardium appears to disappear following the initiation of the EMT at stage 16 (E and F). Embryos treated with BB-94 at stage 12 and examined at stage 18 demonstrate the continued presence of type IV associated with the endocardium (C and D). Bar = 50 μm.

type IV collagen in the endocardial basement membrane by MMPs is critical for mesenchyme formation during the epithelial-mesenchymal transformation process.

Embryos injected with the hydroxamic acid-based MMP inhibitor BB-94 showed significantly less mesenchyme formation than embryos receiving either an injection of vehicle alone or an injection of a nonactive form of the inhibitor (BB-1722). The BB-94 inhibitor has been shown to have broad specificity for members of the MMP family but lacks activity against a number of other metalloproteinases (Brown and Giavazzi, 1995). This inhibitor exhibits activity in the low micromolar range and has been shown to decrease angiogenesis and to inhibit tumor growth, invasion, and metastasis in a number of animal models (Taraboletti *et al.*, 1995; Watson *et al.*, 1995; Wojtowicz-Praga *et al.*, 1996). The specificity of the BB-94 effect was demonstrated by the use of BB-1722 in embryos. BB-1722 is the enantiomer of BB-94 and showed no inhibition of mesenchymal cell migration. In addition, the effect of BB-94 was shown to be partially reversible as embryos examined 2 days following a single injection demonstrated significant outgrowth of mesenchyme into the cushions. This result may have been due to a relatively short half-life of the compound in the animal. Because of the apparent short half-life and the limited life span of the cultured embryos, it would be difficult to determine if MMP inhibition and decreased mesenchyme migration have a detremental effect on subsequent septation events in the heart. Answers to this question may be obtained in studies which provide sustained delivery of these compounds to embryos *in ovo*.

The results of this study suggest that decreased levels of MMP-2 activity may negatively affect the normal EMT occurring in the developing endocardial cushions. This is in contrast, however, to reports of mice which lack MMP-2 and still apparently form mesenchyme normally (Itoh *et al.*, 1997, 1998). This difference may reflect a compensatory response in the null embryos to the lack of MMP-2 proenzyme expression involving the upregulation of other extracellular proteinases which may serve the same purpose. If this is the appropriate response, it would not be expected to occur in the embryos in this study which were injected with the inhibitor. This in fact was the case, as the level of proenzyme expression in inhibitor-treated animals remained at control levels.

In addition to inhibiting the activity of MMP-2, BB-94 would also be expected to inhibit the activity of other MMPs present in the developing heart including MT1-MMP. This was confirmed by the significant decrease in the amount of the low molecular weight active form of MMP-2 seen in the BB-94-injected embryos. Numerous studies have demonstrated that one of the primary functions of MT-MMP is the activation of proMMP-2 (Ailenberg and Silverman, 1996; Butler *et al.*, 1997; Sato *et al.*, 1997). The possibility also exists that the inhibition of MT-MMP by BB-94 also affects mesenchymal cell outgrowth independent of a loss of MMP-2 activity. Recent studies have

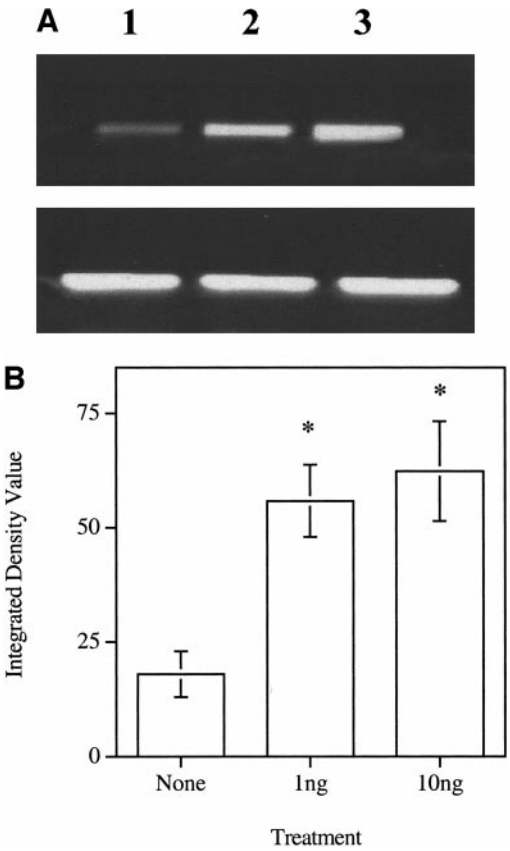

FIG. 8. RT-PCR analysis of MMP-2 expression in isolated endocardial cushion cells. (A) Representative agarose gel demonstrating the expression of MMP-2. Lane 1, no treatment; Lane 2, TGFβ3 at 1 ng/ml; Lane 3, TGFβ3 at 10 ng/ml. Cells treated with either 1 or 10 ng/ml TGFb3 showed an increased expression of MMP-2 mRNA after 16 h of stimulation (B). *Significantly greater than control. Values are the mean ± SEM.

shown that the MT-MMPs have the ability to directly degrade many components of the ECM including fibronectin, tenascin, nidogen, aggrecan, perlecan, and types I and III collagen (D'Ortho *et al.*, 1997; Ohuchi *et al.*, 1997). In contrast to the MMP-2 knockout, loss of MT1-MMP in mice resulted in significant phenotypic changes associated with the skeletal system related to an inability to remodel matrices containing high amounts of interstitial collagen. This is likely due in large part to the role of MT1-MMP in the activation of procollagenase. Since the heart and other organs apparently developed normally in these animals, this again suggests that other proteolytic pathways compensated for the loss of MT1-MMP function, or that the activation of proMMP-2 may not be exclusively via MT1-MMP (Holmbeck *et al.*, 1999; Mazzieri *et al.*, 1997; Zucker *et al.*, 1995).

The extracellular matrix composition of the endocardial cushions has been well characterized and the migration of mesenchymal cells into the cardiac jelly matrix likely involves structural alterations of some of these matrix components. Using an *in vivo* culture system we have shown that the ability of cushion-derived cells to migrate on specific substrates is dependent upon the activity of MMP-2 and MT1-MMP. In particular, type IV collagen did not act as a suitable migratory substrate when MMP activity was inhibited. This suggests that the intact type IV collagen molecule facilitates the strong attachment of cells, but that the protein must be degraded to some extent in order for the cells to demonstrate migratory activity. A similar process has been reported in the migration of breast epithelial cells on laminin-5 substrates (Giannelli *et al.*, 1997). Cleavage of a portion of the laminin-5 protein by MMP-2 produced fragments with pro-migratory activity for this cell type. Numerous ECM proteins have been shown to contain such "cryptic" information which effects both ECM organization as well as cell-ECM interactions (Davis,

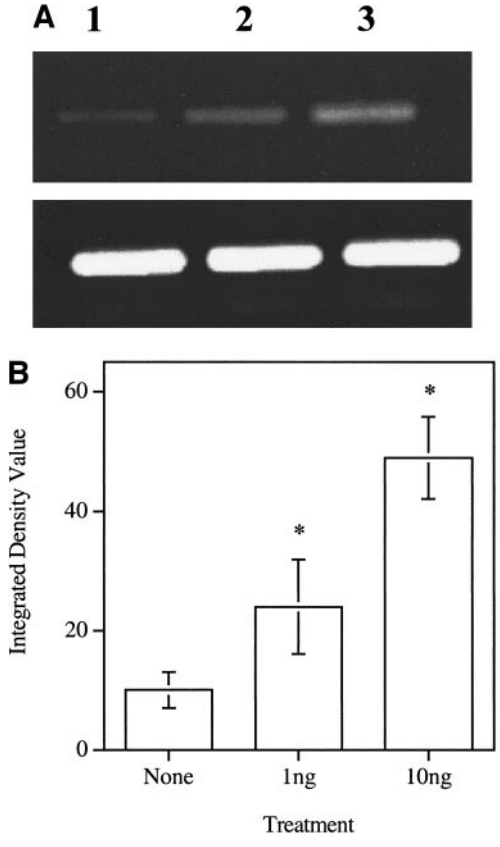

FIG. 9. RT-PCR analysis of MT-MMP expression in isolated endocardial cushion cells. (A) Representative agarose gel demonstrating the expression of MT-MMP. Lane 1, no treatment; Lane 2, TGFβ3 at 1 ng/ml; Lane 3, TGFβ3 at 10 ng/ml. Cells treated with either 1 or 10 ng/ml TGFb3 showed an increased expression of MMP-2 mRNA after 16 h of stimulation (B). *Significantly greater than control. Values are the mean ± SEM.

1992; Montgomery *et al.*, 1994; Fukai *et al.*, 1995, 1997; Barshavit *et al.*, 1995; Smith *et al.*, 1996, 1997; Zhang *et al.*, 1998). Although not reported, cryptic sites may also exist within type IV collagen which provide positive migratory cues or substrates for cells. These data suggest that the proteolytic modification of the matrix not only removes physical barriers to cell migration, but may also create new information previously embedded in the matrix with which the cell can interact.

The proteolytic alteration of type IV collagen, and perhaps other basement membrane components, appears to be a prerequisite for the migration of transformed cells from the endocardial layer into the surrounding cardiac jelly matrix. Previous immunohistochemical studies have shown that type IV collagen is present as a continuous layer beneath the endocardial lining in the AVC and becomes more intermittent as development progresses and the endocardium undergoes the EMT (Kitten *et al.*, 1987; Nakajima *et al.*, 1997). We have utilized an antibody to chicken type IV collagen (IIB12) which recognizes an epitope close to the 7S region of the protein and adjacent to the proteolytic cleavage site. This antibody is useful for identifying areas in the tissue in which the IIB12 epitope is lost due to the degradation of type IV collagen (Ryan *et al.*, 1999). Treatment of embryos with BB-94 prior to the onset of the EMT demonstrated maintenance of the endocardial-associated type IV collagen and the lack of mesenchymal cell migration. Together with the *in situ* hybridization data, this would suggest that the endocardium initially produces a barrier to cell migration which requires a subsequent signal from the myocardium or other sources in order for the EMT to occur normally.

The EMT which occurs in the developing endocardial cushions involves a discrete series of steps which are regulated by identified growth factor signals, produced by both the myocardium and the endocardium. A recent report demonstrates that TGFβ2 is primarily involved in the early phases of the EMT by regulating the induction of Slug expression (Romano and Runyan, 2000). This transcription factor has been shown to play an important role in facilitating the loss of cell-cell adhesion through the disruption of desmosomes between adjacent cells (Savagner *et al.*, 1997). The later steps of the EMT involve the migration of endocardial cells into the underlying cardiac jelly matrix and appear to be regulated by a different set of growth factors including TGFβ3, BMP, and HGF (Romano and Runyan, 2000).

The migration of cushion mesenchyme is facilitated by the production of extracellular proteinases, and the identified growth factors are likely to be involved in the regulation of their expression and activation. We have previously shown that HGF is produced by the myocardium and regulates urokinase expression in the endocardium (Song *et al.*, 1999), and other reports demonstrate a positive effect of this growth factor on MMP expression (Wang and Keiser, 2000; Hamasuna *et al.*, 1999). Results from the present study also demonstrate the ability of TGFβ3 to regulate the expression of both MMP2 and MT1-MMP in endocardial-derived cells. This suggests that abnormalities in cardiac cushion development as a result of alterations in growth factor expression and/or activity may be partially mediated through disrupted proteinase expression in endocardial and/or mesenchyme cells.

In summary, we have presented data to suggest an important role for MMP-2 and MT-MMP in facilitating the migration of endocardial-derived mesenchyme during the EMT. The persistence of type IV collagen in the endocardial basement membrane, and the lack of migration of transformed cells in embryos treated with a synthetic matrix metalloproteinase inhibitor, suggests that type IV collagen may present an initial barrier which must be overcome by the forming mesenchymal cells. This proteolytic event may play a role in establishing a matrix-induced signaling pathway, dependent upon the generation of ECM protein fragments, required for normal cell migration. These data further suggest that the expression and activation of specific matrix metalloproteinases are necessary for the normal morphogenesis of the heart, and that alterations in the levels of active enzymes may be a mechanism leading to the development of specific developmental defects associated with this organ.

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