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Diminished Matrix Metalloproteinase 2 (MMP-2) in Ectomesenchyme-Derived Tissues of the *Patch* Mutant Mouse: Regulation of MMP-2 by PDGF and Effects on Mesenchymal Cell Migration

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Platelet-derived growth factors (PDGF) regulate cell proliferation, survival, morphology, and migration, as well as deposition and turnover of the extracellular matrix. Important roles for the A form of PDGF (PDGF-A) during connective tissue morphogenesis have been highlighted by the murine *Patch* mutation, which includes a deletion of the α subunit of the PDGF receptor. Homozygous (*Ph/Ph*) embryos exhibit multiple connective tissue defects including cleft face (involving the first branchial arch and frontonasal processes), incomplete heart septation, and heart valve abnormalities before they die *in utero*. Analyses of the cell biology underlying the defects in *Ph/Ph* embryos have revealed a deficit in a matrix metalloproteinase (MMP-2) and one of its activators (MT-MMP) that are likely to be involved in cell migration and tissue remodeling, two processes necessary for normal cardiac and craniofacial development. Morphogenesis of these structures requires infiltration of ectomesenchymal precursors and their subsequent deposition and remodeling of extracellular matrix components. First branchial arch and heart tissue from E10.5 embryos were examined by gelatin zymography and RT-PCR in order to characterize the expression of MMPs in these tissues. Of the MMPs examined, only MMP-2 and one of its activators, MT-MMP, were expressed in the first arch and heart at this stage of development. Tissues from *Ph/Ph* embryos exhibited a significant decrease in both MMP-2 and MT-MMP compared to tissues from normal embryos of the same developmental stage. In order to assess whether this decrease affects the motile activity of mesenchymal cells, cell migration from *Ph/Ph* branchial arch explants was compared to migration from normal arch tissue and found to be significantly less. In addition, the migratory ability of branchial arch cells from normal explants could be reduced in a similar manner using a specific MMP inhibitor. Although it is still unclear whether the MMP-2 reduction is a direct result of the absence of response of *Ph/Ph* cells to PDGF-A treatment of normal branchial arch cells *in vitro* with recombinant PDGF-AA significantly upregulated MMP-2 protein. Together, these results suggest that PDGF-A regulates MMP-2 expression and activation during normal development and that faulty proteinase expression may be at least partially responsible for the developmental defects exhibited by *Ph/Ph* embryos.

Key Words: **PDGF; PDGF-α receptor; *Patch* mice; mouse embryos; MMP-2; cranial neural crest; heart development; craniofacial development; connective tissue; cell migration; ectomesenchyme.**

INTRODUCTION

During early vertebrate embryogenesis, neuroepithelial cells from the cranial neural folds (i.e., the cranial neural crest) undergo an epithelial-to-mesenchymal transition and migrate into the developing face and heart. Like those derived from trunk neural crest, cranial neural crest cells localize at sites where they form neurons and glia of the peripheral nervous system in addition to a wide variety of other derivatives. However, a subset of cranial crest cells has the unique ability to differentiate into mesenchyme (i.e.

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ectomesenchyme) which later forms cartilage, bone, and other connective tissues of the head and neck, as well as discrete structures of the heart (Kirby, 1983; Nichols, 1986; Kontges and Lumsden, 1996). In particular, we are interested in those neural crest cells that (1) migrate into the first branchial arch, where they participate in morphogenesis of the mandible and maxilla, and (2) enter the outflow tract of the heart where they contribute to formation of the aorticopulmonary septum. The critical events involved in these processes include effective migration of the cells from the dorsal aspect of the neural tube to appropriate sites, adequate cell proliferation to ensure tissue growth, synthesis and secretion of extracellular matrix (ECM) molecules that contribute to specific connective tissues, and degradation of these ECM molecules as the structures in question grow and change composition. Very little is known, however, about the intrinsic factors that may regulate these events. Gaining such knowledge is important not only for elucidating the course of normal development, but also for understanding the etiology of relatively common human craniofacial and cardiac birth defects.

An intriguing way of studying regulation of specific developmental events is with animals carrying naturally occurring or targeted gene deletions. One such animal is the *Patch* mutant mouse, which carries a deletion of the entire coding region of the α subunit of the platelet-derived growth factor (PDGF) receptor (PDGFRα; Gruneborg and Truslove, 1960; Stephenson *et al.*, 1991). PDGFRα binds both the A and the B chains of PDGF, and the ability to respond to the A form of PDGF is completely absent in homozygous recessive (*Ph/Ph*) embryos. Although these embryos can respond to PDGF-B when the β receptor subunit begins to be expressed at embryonic day (E; days postcoitum) 7.5 (Mercola *et al.*, 1990), this form of the growth factor appears to be involved in later developmental events (Morrison-Graham *et al.*, 1992). *Ph/Ph* embryos exhibit numerous defects of craniofacial, heart, and axial connective tissue, including connective tissues derived from ectomesenchyme, before they die *in utero* (Morrison-Graham *et al.*, 1992; Orr-Urtreger *et al.*, 1992; Schatteman *et al.*, 1992). Although the *Patch* deletion is quite large (Stephenson *et al.*, 1991), the cause of connective tissue defects in *Ph/Ph* embryos (i.e., absence of the PDGFRα) has been confirmed in PDGFRα-null mice (Soriano, 1997). The defects on which we focus here are the cleft face, small mandible, and defective aorticopulmonary septum, all of which may result from faulty migration and/or differentiation of specific populations of cranial neural crest cells which normally express elevated levels of PDGFRα. Importantly, similar defects also arise in human embryos. These include clefts of the lip and palate, which result from failure of specific facial processes to develop properly and fuse; mandibular dysostosis, which involves hypoplasia of the musculoskeletal derivatives of the first and second branchial arches; and abnormalities of cardiac septa (Van Mierp *et al.*, 1962; Poswillo, 1988). Although the defects of *Ph/Ph* embryos have been described in detail, the cell behaviors underlying them remain obscure. Thus, *Ph/Ph* embryos provide an excellent opportunity to study the behavior of specific populations of cells in the absence of a response to PDGF-A and to determine whether this leads to alterations in expression of other molecules which may also contribute to the observed defects.

Because it seems likely that migration of cranial neural crest cells, and/or the ability of their mesenchymal derivatives to remodel developing structures, is affected by the *Patch* mutation, we have asked whether expression or activity of extracellular proteases is altered in *Ph/Ph* embryos and whether such an alteration could account, at least in part, for the facial and heart defects in these embryos. Regulated expression of extracellular proteases is required for cell movements through ECM, for cell shape changes during differentiation, and for cellular remodeling of ECM as development progresses (Hewitt *et al.*, 1996; Kinoh *et al.*, 1996). The matrix metalloproteinases (MMPs) are a family of such enzymes that can selectively degrade at least one ECM component (Parsons *et al.*, 1997). MMPs are secreted in a latent form and their activity is controlled by specific activators and inhibitors (Woessner, 1991). They may be sequestered in the ECM or bound to cell surfaces and are suspected to play critical roles in any physiological event that requires cell migration or tissue remodeling (e.g., wound healing, tumor cell invasion, bone resorption, and morphogenesis). We have found that two MMPs, MMP-2 and MT-MMP (an activator of MMP-2), are selectively underexpressed in branchial arch and heart tissue from *Ph/Ph* embryos. This deficit may result in impaired motile activity of mesenchymal cells as suggested by the failure of cells in *Ph/Ph* branchial arches to migrate on gelatin substrata as their wild-type counterparts do. Our results provide the first definitive insights into the mechanisms by which the developmental abnormalities in *Ph/Ph* embryos may arise and raise approachable questions concerning the functional relationships among molecules required for these events to occur normally.

MATERIALS AND METHODS

Embryos

An inbred colony of heterozygous (*Ph*/+) *Patch* mice (C57BL/6 background) was established and maintained through a continuous regimen of sibling matings (a generous gift from Dr. James Weston, University of Oregon). Members of this colony were mated with Balb/cGn mice to obtain an outbred generation. Siblings of this mating were, in turn, mated to produce the timed-pregnant females from which homozygous (*Ph/Ph*) and normal embryos were obtained for this study. Additional normal embryos were obtained by mating wild-type (+/+) outbred hybrid siblings, producing embryos which were of the same genetic background as the *Patch* embryos. Embryonic day (E) 0 was judged to be 12 h prior to the morning on which a vaginal plug was observed. Tissues used in this study (first branchial arch, heart, and tail) were dissected from embryos, frozen on dry ice, and stored at −70°C until needed for further analysis.

Genotyping

Mutant embryos were identified by the absence of the PDGFRα gene by PCR analysis and were designated (*Ph/Ph*). All others were designated "normal" and included both wildtype (+/+) and heterozygotes (*Ph*/+) as described above. Frozen tail tissue was thawed in 50 μl of a DNA extraction buffer of 10 mM Tris, pH 7.4, 2.0 mM EDTA, and 0.2% Triton X-100. Proteinase K (Boehringer Mannheim, Indianapolis, IN) was added to a final concentration of 200 μg/ml, and the samples were heated to 55°C for 3 h. Finally, the samples were boiled at 100°C for 5 min. Two microliters of each sample was used in a standard PCR at 94°C for 1 min, 60°C for 1 min, 72°C for 1 min for 27 cycles. The following primers for mouse PDGFRα were used: forward, ACC TCC TTT CGG ACG ATG AC, reverse, ATC ACT TCA GAA TGG CTC CA. Reactions were analyzed using ethidium bromide-stained agarose gels.

RT-PCR

Total RNA was prepared from the first branchial arch, including the maxillary and mandibular processes, and from the entire heart, including the inflow and outflow tracts. The frozen tissue was thawed in 500 μl Trizol reagent (Gibco BRL, Gaithersburg, MD) and total RNA prepared utilizing the manufacturer's instructions. cDNA was prepared from 1–2 μg of total RNA using an oligo(dT) primer and Superscript reverse transcriptase (Gibco BRL). One microliter of each first-strand reaction was amplified using a standard PCR (see above) using the primers for MMP-2 (forward, ATC TAC TTG CTG GAC ATC AGG GGG, reverse, TGG CTC GAA ATT CAC AAG GTC C), for MMP-3 (forward, TGT ACC CAG TCT ACA AGT CCT CCA, reverse, CTG CGA AGA TCC ACT GAA GAA GTA G), for MMP-7 (forward, ACT GGA AAA CTC TCC CCC TAC ATC, reverse, TGC AGA CCG TTT CTG TGA TCT G), for MMP-9 (forward, TGC GAC CAC ATC GAA CTT CG, reverse, CCA GAG AAG AAG AAA ACC CTC TTG G), and for MT-MMP (forward, GTG ATG GAT GGA TAC CCA ATG C, reverse, GAA CGC TGG CAG TAA AGC AGT C). Primers for 18S rRNA were purchased from Ambion (Austin, TX). The MMP primers were designed from the full-length mouse MMP sequences using the MacVector Sequence Analysis Program 5.0 (Oxford Molecular Group PLC, 1996). The primers were tested utilizing positive control RNA isolated from postpartum mouse uterine tissue, which expresses all five of the MMPs analyzed in this study (Rudolph-Owen *et al.*, 1977). In preliminary experiments, the conditions used for PCR amplification were determined to be within the linear range of product amplification by quantitating the amount of product produced at every fifth cycle between 5 and 35 cycles. Quantitation of ethidium bromide-stained bands in agarose gels was accomplished by digitizing the gels and then quantitating the signal intensity of individual bands utilizing NIH Image. Values for expression of each of the genes were normalized to the level of signal intensity for 18S to control for differences in the amount of starting RNA in each sample. Analyses were repeated twice on separate RNA isolates.

Zymography

Individual branchial arches and hearts were extracted in 50 μl of a 0.1 M phosphate buffer, pH 7.4, containing 0.1% Triton X-100 and extracted overnight at 4°C. Aliquots of each sample were subjected to electrophoresis in 10% SDS–polyacrylamide gels into which gelatin (1 mg/ml) had been crosslinked. Following electrophoresis the gels were soaked for 15 min in 2.5% Triton X-100 and rinsed with water. The gels were incubated for 48 h in LSCB buffer (50 mM Tris, pH 7.6, 0.2 M NaCl, 5 mM $CaCl_2$ 0.02% Brij 35, and 0.02% NaN_3) at 37°C. Following incubation, the gels were stained for 1 h with 0.125% Coomassie blue and destained with 10% acetic acid. The stained gels were digitized, and the zones of proteolysis, corresponding to the presence of proteases in the gel, were quantitated using NIH Image. Identification of the 72- and 62-kDa forms of MMP-2 was determined by comparison of the migratory position of the bands with known MMP-2 standards (Zucker *et al.*, 1994). DNA contents of each extract were obtained using a spectrofluorimeter, and values for MMP-2 were normalized to the DNA content to control for discrepancies in the amount of tissue in each sample and to determine the amount of MMP-2/cell number.

Explant Cultures

First branchial arch tissue from E10.5 embryos was minced into small (≤1 mm^2) pieces and explant cultures were established by culturing the pieces on gelatin-coated coverslips for motility analysis or in 35-mm^2 gelatin-coated culture dishes for the PDGF addition experiments. The motility analysis was carried out by maintaining the tissue in F12 medium supplemented with 10% FCS and 1000 U/ml Pen/Strep (Gibco BRL). Inhibitor experiments were carried out by preparing a 10 mM stock solution of the matrix metalloproteinase inhibitor (KB8301; a nonselective inhibitor of all metalloproteinases [4-(*N*-hydroxyamino)-2*R*-isobutyl-3*S*-methylsuccinyl]-L-3-(5,6,7,8-tetrahydro-1-naphyhyl)alanine-*N*-methylamide; Pharmingen, San Diego, CA) in DMSO. The inhibitor was added to the cultures to a final concentration of 10, 50, or 200 μM, and equivalent concentrations of DMSO not containing KB3801 were added to some cultures. Following a 24-h culture period, images of the explant and associated cells were obtained and quantitated for the extent of cellular outgrowth using the Metamorph Image Analysis System (Universal Imaging, West Chester, PA). The extent of cell migration was determined by measuring the radial distance from the edge of the explant to the farthest cells at six consistent points around the circumference of the explant. Data were expressed as average micrometers from the edge of the explant and analyzed statistically using a Student *t* test.

PDGF addition experiments were carried out in cultures maintained in F12 medium containing 1% ITS+ and 1000 U/ml Pen/Strep. A 1 μg/ml stock solution of recombinant human PDGF-AA (R & D Systems, Minneapolis, MN) was prepared in PBS containing 1% (w/v) BSA. The growth factor was added to the cultures to a final concentration of 10 or 100 ng/ml. Twenty-four hours later, the tissue pieces were washed three times with PBS and then extracted in 50 μl of a 0.1 M phosphate buffer, pH 7.4, containing 0.1% Triton X-100. The extracts were analyzed by gelatin zymography (see above). Unless otherwise mentioned, all reagents used in this study were purchased from Sigma Chemical Company (St. Louis, MO).

RESULTS

MMP Expression and Activity in Normal Mouse Embryos

In order to determine which MMPs are present in mouse first branchial arch and heart tissues during a relevant period of embryogenesis, both tissues were examined by gelatin zymography at E10.5 (Fig. 1A). Extracts were pre-

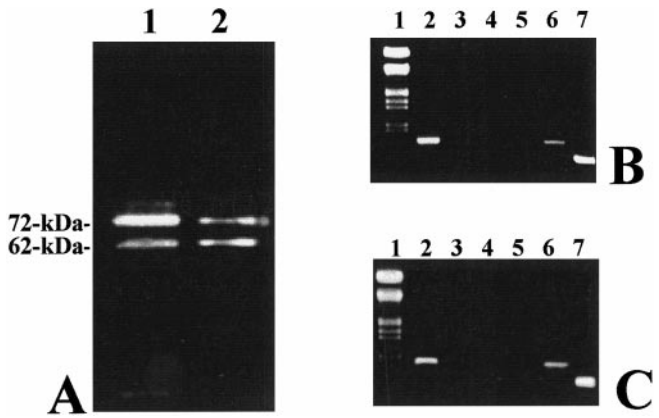

FIG. 1. Gelatinase activity and MMP expression in first branchial arch and heart tissues. (A) Representative gelatin zymography of tissue extracts from E10.5 normal (+/+) embryos. Lane 1, extract of first branchial arch; lane 2, extract of whole heart. The positions of the 72-kDa pro- and 62-kDa active forms of MMP-2 are indicated at the left. Confirmation of these bands as the pro- and active forms of MMP-2 was established by their comigration with known MMP-2 standards (see Materials and Methods). (B and C) Representative RT-PCR analysis of MMP expression in branchial arch and heart, respectively. RNA was prepared from first branchial arch (B) and whole heart (C) from E10.5 normal (+/+) embryos and used in a reverse transcription reaction. First-strand cDNA was subsequently amplified by PCR with the following primers: MMP-2 (lane 2), MMP-3 (lane 3), MMP-7 (lane 4), MMP-9 (lane 5), MT-MMP (lane 6) and 18S rRNA (lane 7). (Lane 1) DNA ladder. All of the primers used were tested with positive control RNA isolated from postpartum mouse uterine tissue, which expresses all five of the MMPs analyzed in this study.

pared from the first branchial arch, including both the maxillary and the mandibular processes, and from whole heart, including the inflow and outflow tracts. In both tissues, MMP-2 (gelatinase A) was the only MMP detected, being present in the inactive 72-kDa pro-form and the proteolytically activated 62-kDa form. Other MMPs which can also be detected with this analysis, including MMP-9 (gelatinase B), were not observed in either tissue at this stage of development.

RT-PCR was used to determine which MMP genes are expressed in arch and heart tissue at this developmental stage (Figs. 1B and 1C). Total RNA prepared from each tissue was transcribed into cDNA and subsequently used in PCR utilizing primers for MMP-2, 3, 7, and 9 and MT-MMP (the membrane-bound MMP which proteolytically activates MMP-2). In both tissues, expression of MMP-2 and MT-MMP was easily detectable. Expression of MMP-3, 7, and 9, however, was not observed in either tissue.

MMP-2 Activity and Expression Are Reduced in Ph/Ph Branchial Arch and Heart

All embryos used in the experiments reported were genotyped by PCR utilizing genomic DNA and primers for PDGFRα. Extracts of the first branchial arch and heart from *Ph/Ph* and normal (*Ph*/+ and +/+) embryos were analyzed by gelatin zymography. Figure 2 is a representative zymogram of heart tissue extracts from a single litter of E10.5 embryos and demonstrates significantly diminished levels of MMP-2 in the *Ph/Ph* embryos compared to the normal littermates. Normalized values for MMP-2 were then compared between these two groups to quantify the apparently altered levels of MMP-2 seen in the *Ph/Ph* embryos. In *Ph/Ph* embryos, the pro- and active forms of MMP-2 in branchial arch tissue were significantly reduced by 60 and 40%, respectively, compared to normal embryos (Fig. 3A). Analysis of MMP expression in branchial arch tissue by RT-PCR showed a similar reduction in the level of MMP-2 mRNA (Fig. 3B). Even more striking was a 95% reduction in the level of expression of MT-MMP, the activator of MMP-2, in *Ph/Ph* embryos compared to normal embryos.

Heart tissue from *Ph/Ph* embryos showed a similar reduction in the level of the 72- and 62-kDa forms of MMP-2 compared to the same tissue in normal embryos (Fig. 3C). Analysis of MMP-2 and MT-MMP expression by RT-PCR analysis also showed significant reductions in the level of expression of both proteinases in *Ph/Ph* embryos (Fig. 3D).

Cells from Ph/Ph Branchial Arch Exhibit Impaired Migratory Ability

In light of the critical role MMPs are believed to play in cell migration, studies were performed to determine whether crest-derived mesenchymal cell migration was impaired in *Ph/Ph* embryos. Branchial arch tissue from normal and *Ph/Ph* E10.5 embryos was minced into small (<1 mm^2) pieces and cultured on gelatin-coated coverslips for a period of 24 h. Tissue pieces from individual embryos were cultured separately and the remainder of the embryos were subsequently genotyped. Arch tissue from normal animals adhered well to the gelatin-coated substratum, and after 16 to 24 h a substantial number of polygonal-shaped

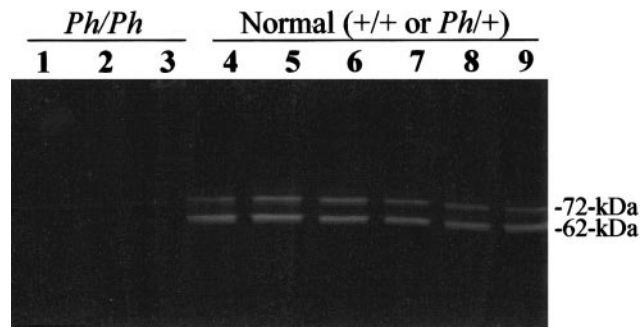

FIG. 2. MMP-2 is reduced in the first branchial arch of *Ph/Ph* embryos. Representative zymogram of first branchial arch extracts from a litter of E10.5 embryos. A significant reduction in the amount of MMP-2, both pro- and active forms, can be appreciated in homozygous (*Ph/Ph*) embryos compared to normal littermates.

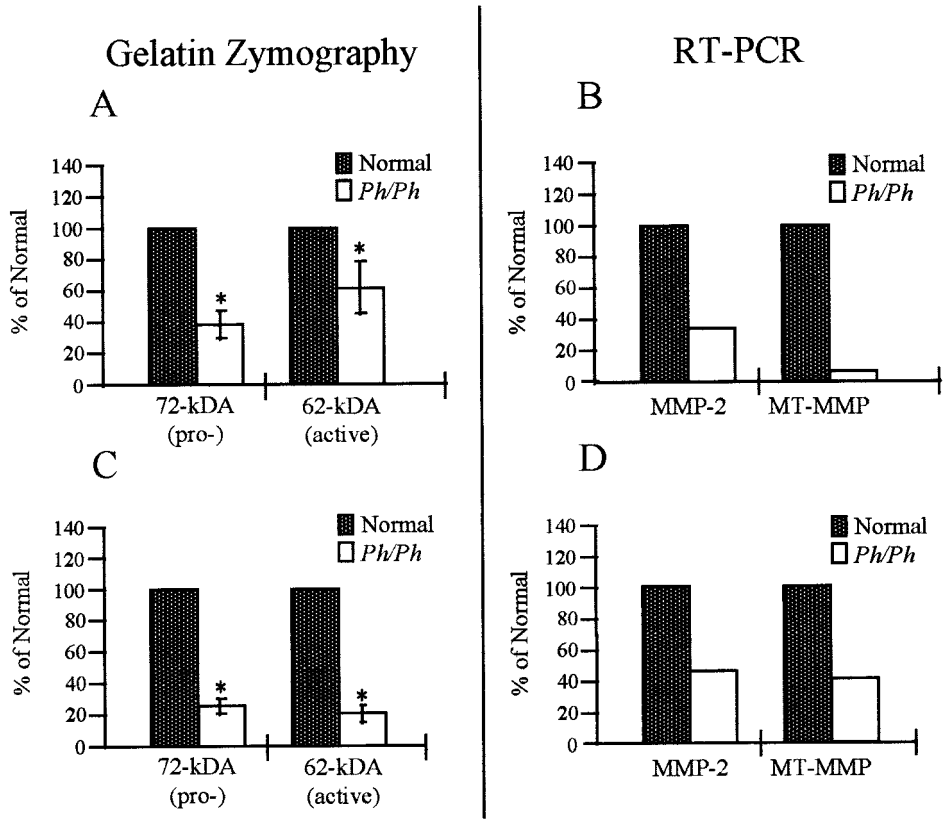

FIG. 3. MMP-2 activity and expression in branchial arch and heart tissue from normal and *Ph/Ph* embryos. (A and C) Quantification of MMP-2 activity in extracts of first branchial arch and heart tissue, respectively, from E10.5 *Ph/Ph* and normal (*Ph*/+ or +/+) littermates. Values represent the mean areas of the lysis zones and are expressed as the percentage of control, ± SEM, for $n = 7$ control and $n = 7$ normal embryos. *Significantly different at the $P \leq 0.05$ level by Student's t test. (B and D) Semiquantitative RT-PCR analysis of MMP-2 and MT-MMP expression in arch and heart tissue, respectively, from *Ph/Ph* and normal (*Ph*/+ or +/+) littermates. Normalized values represent intensities of ethidium bromide-stained bands expressed as a percentage of control.

cells had migrated out from the tissue and formed large "halos" around the explants (Fig. 4A). The pattern of outgrowth from *Ph/Ph* explants was markedly different (Fig. 4B). While the tissue from the *Ph/Ph* embryos adhered well to the gelatin-coated surface, many fewer cells migrated out of the explant. The extent of migration was quantified by measuring the average distance the cells moved from the edge of the explant in a 24-h period. As seen in Table 1, branchial arch cells from normal embryos migrated nearly four times farther on the culture substratum compared to those from *Ph/Ph* mutant embryos. In addition, the presence of DMSO in the cultures had no apparent adverse affect on the migratory ability of the normal cells.

In order to assess whether the impaired migratory ability of *Ph/Ph* cells was due to inadequate MMP-2 activity, explants of branchial arch tissue from normal wild-type embryos were cultured in the presence of the synthetic metalloproteinase inhibitor, KB3801. Addition of KB3801 to the cultures resulted in a dose-dependent decrease in cell migration, and when it was added to a final concentration of 200 μM, the average distance of migration was reduced by nearly 10-fold (Fig. 4C and Table 1). The inhibition of MMP activity in the explant cultures by KB3801 was confirmed by analyzing treated and untreated explants by zymography. Explants incubated for 24 h with increasing concentrations of KB3801 demonstrated a dose-dependent reduction in the level of activated (62 kDa) MMP-2 (Fig. 5). No significant change was seen in the level of the proenzyme in response to the addition of inhibitor. This result suggests that MT-MMP was sensitive to the effects of KB3801, in addition to the active form of MMP-2.

PDGF-AA Stimulates Increased MMP-2 Synthesis in Explants of Normal Arch Tissue

The next experiments addressed whether MMP-2 levels in the branchial arch could be regulated by PDGF-A, thus providing a link between loss of PDGFRα signaling and decreased levels of MMP-2 in *Ph/Ph* embryos. Branchial

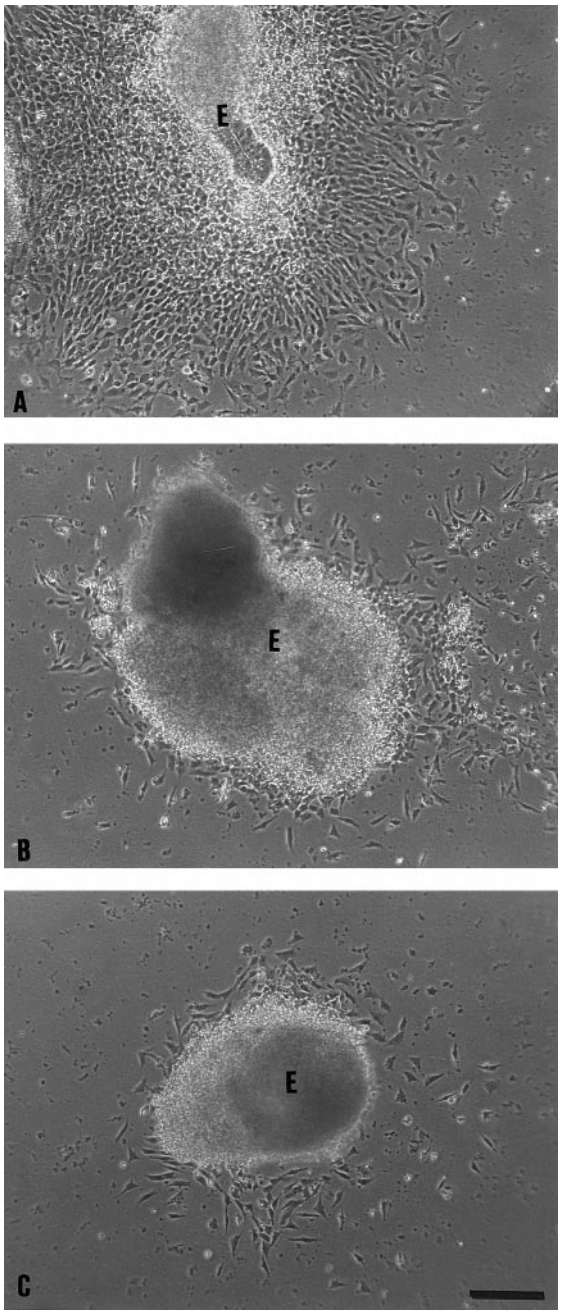

FIG. 4. Mesenchymal cell migration from branchial arch explants is reduced in *Ph/Ph* embryos and is dependent upon MMP-2 activity. Explants of first branchial arch tissue from normal (+/+) embryos (A), from *Ph/Ph* embryos (B), and from normal (+/+) embryos cultured in the presence of the synthetic MMP inhibitor KB3801 (200 μM) (C). E, explant. Bar, 100 μm.

arch explants from normal embryos were treated with either 10 or 100 ng/ml recombinant PDGF-AA for 24 h and the tissue was subsequently analyzed by zymography. Significant increases were seen in both the pro- and the active forms of MMP-2 in branchial arch tissue treated with 100 ng/ml PDGF (Fig. 6). While these experiments showed that MMP-2 protein levels were elevated by PDGF-AA, RT-PCR analysis did not show a similar increase of MMP-2 mRNA. Following treatment at either concentration of PDGF-AA, significant differences in MMP-2 mRNA were not observed (data not shown). These data suggest that PDGF-A may regulate MMP-2 synthesis at a posttranscriptional level or that the growth factor stimulates a transient increase in MMP-2 mRNA which is no longer detectable at 24 h.

TABLE 1

Animal treatment condition	Avg. dist. (μm) of cell migration from edge of explant (± SEM)
Normal (w/o DMSO)	287 (46)
Ph/Ph	75 (5)*
Normal (+200 μM DMSO)	238 (39.6)
10 μM KB3801	78.3 (5.8)*
50 μM KB3801	66.8 (11.6)*
200 μM KB3801	29.0 (9.35)*

* Statistical significance at a level $\leq$0.5, Student's *t* test. For normal and *Ph/Ph* samples, $n = 7$ embryos. For normal samples and samples with added KB3801, $n \geq 4$ explants.

DISCUSSION

The dramatic reduction in MMP-2 and one of its activators, MT-MMP, in defective tissues of *Ph/Ph* embryos strongly suggests a role for this extracellular proteinase in normal morphogenesis. The fact that cells in these embryos cannot respond to PDGF-A raises the possibility that there is normally a link between this response and MMP-2

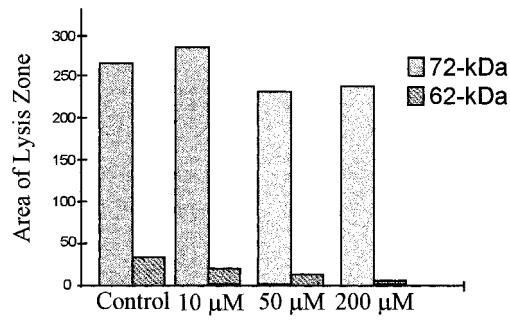

FIG. 5. Activation of MMP-2 is inhibited in branchial arch tissue by the matrix metalloproteinase inhibitor KB3801. Explants of first branchial arch tissue from normal (+/+) control, E10.5 embryos were incubated with increasing concentrations of KB3801 and analyzed by zymography. Values are the areas of the lysis zones for individual cultures.

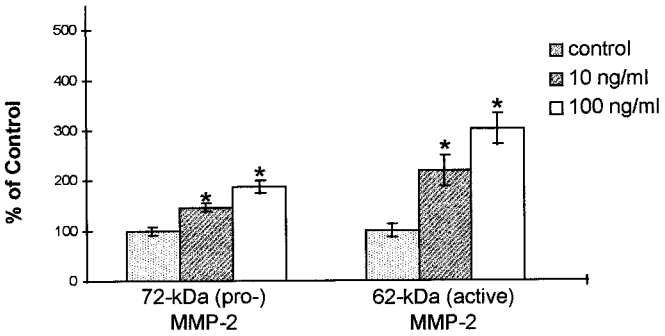

FIG. 6. PDGF-A regulates MMP-2 levels in branchial arch explants. Explants of first branchial arch tissue from normal (+/+) control, E10.5 embryos were treated with 10 or 100 ng/ml recombinant PDGF-AA for a period of 24 h. Levels of MMP-2 within the tissue were analyzed by gelatin zymography and normalized to the DNA content of the explants to control for sample-to-sample variability in the amount of tissue used in the analysis. Values represent mean areas of lysis zones expressed as a percentage of control values ± SEM, for $n = 6$ explants. *Significantly different at the $P \leq 0.05$ level by Student's t test.

expression, and this is supported by our *in vitro* studies demonstrating that PDGF-A can, indeed, regulate MMP-2. We have focused our analyses on the craniofacial and heart defects in the mutant embryos, due to our interest in understanding the mechanisms underlying migration and differentiation of cranial neural crest-derived mesenchymal cells (ectomesenchyme). In normal embryos, derivatives of this subpopulation of crest cells express high levels of PDGFRα, including branchial arch mesenchyme and cushion tissue of the heart (Morrison-Graham *et al.*, 1992; Orr-Urtreger *et al.*, 1992; Schatteman *et al.*, 1995), and the facial and heart defects of *Ph/Ph* mice suggest that PDGF-A is involved in initial localization of cranial crest cells and/or their subsequent expression of a mesenchymal phenotype. The reduction in MMP-2 in *Ph/Ph* mice could at least partially account for perturbations in either of these events.

A population of neuroepithelial cells of the cranial neural folds undergoes an epithelial-to-mesenchymal transition and then follows stereotyped pathways into the branchial arches, outflow tract of the heart, and other sites at which they differentiate (e.g., LeDouarin, 1982; Kirby *et al.*, 1983; Tan and Morriss-Kay, 1985). Neural crest cells express MMPs and other proteinases *in vitro* (Valinsky and LeDouarin, 1985; Duong and Erickson, 1995; Agrawal and Brauer, 1996), and it seems likely that these molecules are involved in their ability to both emigrate from the neural tube and invade adjacent mesenchyme *in vivo*, as has been shown for other cell types (Matrisian, 1992; Ray and Stetler-Stevenson, 1994; Chin and Werb, 1997). We and others (Reponen *et al.*, 1992) have shown that MMP-2 appears to be the only gelatinase expressed in mouse embryos during the period of crest migration into the branchial arches and heart, and it seems likely that the broad substrate specificity of this enzyme allows crest cells to migrate through a variety of tissues (Duong and Erickson, 1995). In this study, we demonstrate that crest-derived mesenchymal cells from *Ph/Ph* embryos do not migrate normally *in vitro* and that *normal* cells treated with an MMP inhibitor exhibit similarly diminished migratory ability. Taken together, these results support the idea that crest migration may be perturbed in *Ph/Ph* embryos, leading to insufficient numbers of cells at critical locations. An important point in this regard is that the neuronal and glial crest derivatives in *Ph/Ph* mice appear to be normal (Morrison-Graham *et al.*, 1992), which is not surprising because these cells in normal animals do not express the PDGFRα. Therefore, migration of ectomesenchyme precursors is likely to be affected selectively. Although there may be consequences of the PDGFRα defect in addition to perturbation of migration (discussed below), it is of interest that PDGF is known to have chemotactic effects on some cell types (Raines *et al.*, 1990), and the recognition of its ability to regulate MMPs may have revealed a mechanism for this phenomenon.

The inability of *Ph/Ph* mesenchymal cells to migrate *in vitro* is somewhat puzzling as MMP-2, although reduced in amount, was nevertheless present in the explant cultures. However, we have not yet determined the overall level of MMP-2 *activity* (i.e., activity in the presence of specific endogenous tissue inhibitors) in either the embryos or the cultures, and this may be the critical deficit in the mutant tissues. Regulation of proteolytic activity in tissues is mediated through interactions between active enzymes and their specific inhibitors such as TIMP-2 (tissue inhibitor of metalloproteinase-2). TIMP-2 is a specific inhibitor of MMP-2 and it is expressed in the first branchial arch prior to and during crest migration (Chin and Werb, 1997). Therefore, assessment of enzyme activity levels and inhibitor expression will be a crucial next step in evaluating the impact of diminished MMP-2 in *Ph/Ph* embryos.

Once ectomesenchyme precursors have arrived in the branchial arches and heart they contribute to the formation of connective tissue. ECM molecules are synthesized and secreted by differentiating crest cells, and the ECM must undergo changes in its structure and composition as the face and heart enlarge and change shape. During such tissue remodeling, matrix metalloproteinases, including MMP-2, have been shown to be critical to these alterations as morphogenesis proceeds (Parsons *et al.*, 1997; Chin and Werb, 1997). They may function to degrade existing ECM components and also to regulate cell–ECM interactions as ECM structure changes. Just as inhibition of MMP activity in the embryonic mouse mandible disrupts its development (Chin and Werb, 1997), underexpression of MMP-2 may contribute to the observed defects of *Ph/Ph* embryos. A study by Iamaroon *et al.* (1996) demonstrated intense staining for MMP-2 at the zone of fusion of the facial prominences in the mouse embryo, suggesting a role for MMP-2 in this process. Reduction of MMP-2 in the *Patch* mouse may affect remodeling of the facial primordia resulting in

the characteristic cleft face seen in the homozygous animals.

Although our studies and the others cited above indicate that MMP-2 has important roles in normal development, knockout mice which do not express MMP-2 develop to term and produce fertile offspring (Itoh *et al.*, 1997). This discrepancy might arise from critical differences in the responses of cells in *Ph/Ph* and MMP-2-null embryos to diminished or absent MMP-2. It remains unknown whether the knockout mice compensate for the complete absence of MMP-2 through upregulation of other extracellular proteinase genes, such as urokinase or other metalloproteinases. This response would not be as likely to occur in *Ph/Ph* embryos, in which at least some MMP-2 is present. In addition, the MMP-2-null and *Ph/Ph* mice might be distinguished by perturbation in *Ph/Ph* of additional molecules that act in concert with MMP-2, such as MT-MMP and/or TIMPs. The balance of, and interactions among, such molecules are likely to be more important than the absolute amount in which each is present.

To our knowledge, this is the first report that MMP-2 synthesis and activity by embryonic cells can be regulated by PDGF-A. Results from this study also suggest that the regulation of MMP-2 by PDGF may occur at a posttranscriptional level, as no changes were seen in MMP-2 mRNA in response to PDGF stimulation. A previous study by Overall *et al.* (1991) has demonstrated that another growth factor, TGFβ, alters the level of MMP-2 by stabilizing the mRNA for this protein. Whether this is the mechanism by which PDGF increases the level of MMP-2 in the embryonic branchial arch tissue remains to be determined.

In another study, Kenagy *et al.* (1997) demonstrated a dependence of PDGF-induced migration on MMPs in cultures of vascular smooth muscle cells and found that MMP-2 levels in these cultures were decreased by blocking antibodies to bFGF and PDGF. The results of that study directly support our findings, although the origins of the cell types in each study were different (i.e., embryonic vs adult, mesenchymal vs smooth muscle). Additional studies addressing the relationship between PDGF and MMPs have shown that both the A and the B chains of PDGF can stimulate synthesis of MMP-1 and TIMP-1 by a variety of cell types *in vitro* (Fabummi *et al.*, 1996; Yang *et al.*, 1989; Alexander *et al.*, 1989; Alvares *et al.*, 1995). However, demonstration that one role for PDGF during embryogenesis may be regulation of MMPs is particularly important because PDGFRα is expressed by cells throughout mesenchyme (including ectomesenchyme), and mesenchymal cells must interact with ECM during morphogenesis in a variety of ways that require such proteinases. Coexpression of PDGFRα and MMP-2 during mouse development (Morrison-Graham *et al.*, 1992; Reponen *et al.*, 1992) certainly supports this idea.

Although we hypothesize that regulation of MMP-2 is a critical response of ectomesenchyme to PDGF-A, we recognize that this growth factor has other critically important effects on embryonic cell behavior. The potent mitotic action of PDGF has been widely known for many years (Raines *et al.*, 1990), and expression of PDGFRα in embryonic mesenchyme certainly suggests that it may regulate cell proliferation in those locations. While there did not appear to be a deficit in proliferation of cells in defective structures of PDGFRα-null embryos, increased apoptosis in the branchial arches was noted (Soriano, 1997), although its extent is difficult to assess. We plan to examine this issue closely in *Ph/Ph* embryos, as alterations in either of these processes could contribute to the observed craniofacial and heart defects. In addition, there have been limited reports of PDGF regulation of ECM synthesis (Raines *et al.*, 1990), a question that we are currently addressing in our laboratories. Thus, PDGF might regulate aspects of ECM deposition during growth and remodeling of the branchial arches and heart and/or its degradation and turnover via effects on MMP expression or activity.

In summary, we suggest that regulation of MMP-2 is one important effect of PDGF-A on migrating and/or differentiating mesenchymal cells during normal development and that perturbation of this effect in *Ph/Ph* embryos, which are unable to signal through the α receptor for this growth factor, contributes to the extensive connective tissue abnormalities that they exhibit. We have focused on ectomesenchymal cells that arise from the cranial neural folds and contribute to structures of the developing face and heart, as these cells must migrate to appropriate sites at which their derivatives express and remodel ECM. Having demonstrated a dramatic reduction in MMP-2 levels in the first branchial arch and heart, we must now determine precisely which aspects of crest cell behavior this deficit affects *in vivo*. These questions are of paramount interest in elucidating the bases of human craniofacial and heart defects, several of which are closely analogous to those exhibited by *Ph/Ph* embryos (Van Mierop *et al.*, 1962; Poswillo, 1988).

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