Epimorphin acts extracellularly to promote cell sorting and aggregation during the condensation of vertebral cartilage

Yumiko Oka a,b, Yuki Satoc, Hokari Tsuda a, Kazunori Hanaoka d, Yohei Hirai b, Yoshiko Takahashi c,c,⁎

⁎ Corresponding author. Current address: Graduate School of Biological Sciences, Nara Institute of Science and Technology, 8196-5, Takayama, Ikoma, Nara 630-0101, Japan. Fax: +81 743 72 5559.
E-mail address: yotayota@bs.naist.jp (Y. Takahashi).

Formation of vertebrae occurs via endochondral ossification, a process involving condensation of precartilaginous cells. Here, we provide the first molecular evidence of mechanism that underlies initiation of this process by showing that the extracellular factor, Epimorphin, plays a role during early steps in vertebral cartilage condensation. Epimorphin mRNA is predominantly localized in the vertebral primordium. When provided exogenously in ovo, it causes precocious differentiation of chondrocytes, resulting in the formation of supernumerary vertebral cartilage in chicken embryos. To further analyze its mode of action, we used an in vitro co-culture system in which labeled 10T1/2 or sclerotomal prechondrogenic cells were co-cultured with unlabeled Epimorphin-producing cells. In the presence of Epimorphin, the labeled cells formed tightly packed aggregates, and sclerotomal cells displayed augmented accumulation of NCAM and other early markers of chondrocyte differentiation. Finally, we found that the Epimorphin expression is initiated during vertebrogenesis by Sonic hedgehog from the notochord mediated by Sox 9. We present a model in which successive action of Epimorphin in recruiting and stacking sclerotomal cells leads to a sequential elongation of a vertebral primordium.

© 2005 Elsevier Inc. All rights reserved.
Keywords: Vertebrogenesis; Somite; Skeletogenesis; Cartilage; Avian embryo

Introduction

The vertebral bones originate from the sclerotome, a somite derivative induced to form by the notochord mediated by Sonic hedgehog (Brand-Saberi and Christ, 2000; Dockter, 2000; Fan and Tessier-Lavigne, 1994; Stockdale et al., 2000, references therein). Subsequently, a subpopulation of sclerotomal cells located adjacent to the notochord differentiates into precartilage. This occurs concomitantly with a morphological change that causes the cells to align concentrically with the notochord. The entire precartilaginous area elongates progressively in a ventral-to-dorsolateral direction, eventually enclosing the spinal cord at the dorsal midline of the body.

Vertebrogenesis proceeds via endochondral ossification, in which a cartilage template is ultimately replaced by osteogenic cells. During endochondral processes, in general, mesenchymal cells undergo condensation to produce precartilaginous cells, and these cells subsequently differentiate into chondrocytes embedded in extracellular matrix secreted by themselves. After undergoing hypertrophy, the mature cartilage is eventually replaced by osteoblasts and osteoclasts, finalizing the ossification (de Crombrugghe et al., 2001; Hall and Miyake, 2000; Olsen et al., 2000). It is well accepted that mechanisms underlying these relatively later stages of cartilage differentiation and replacement by osteoblasts are shared among all the endochondral skeletogenesis including vertebrae, ribs, limb appendages, and cranial bones (de Crombrugghe et al., 2001; Olsen et al., 2000). In contrast,
earlier steps such as the condensation and early differentiation of chondroblasts appear to employ different mechanisms between them. For instance, whereas ectodermal signals are important for mesenchymal condensation during facial bone formation (Hall and Miyake, 1995, 2000; Takahashi et al., 1991; Taylor and Jones, 1979), it is obviously not the case for vertebral bones because the initial condensation starts around the notochord, located away from the ectoderm. In addition, the first morphological sign of vertebral chondrogenesis is the concentrically arrayed cells around the notochord (Fig. 1H), but this characteristic behavior is not seen in limb buds or facial tissues. Distinct genetic cascades have also been

Fig. 1. Cloning and expression of avian Epimorphin cDNAs. (A) Alignment of amino acid sequences predicted from cDNAs of chicken, quail, mouse, and human Epimorphins (isoform II). Identical amino acids are shown by asterisks. Colons and dots indicate strong and week similarity groups, respectively. The alignment was achieved by the CLUSTAL W multiple sequence alignment program. Red box, α-helical domain; blue box, membrane anchor domain. (B) Similarity at the amino acid level between avian Epimorphins, vertebrate homologues, and mouse syntaxin1A (msyx1A). Chicken Epimorphin, a partial fragment of ORF obtained, is compared with the corresponding region of other homologues. (C) Northern blot analysis of Epimorphin mRNA in E5 chicken embryos yielded a signal of 3.4 kb. (D–H) Epimorphin expression localized in the vertebral anlage of a chicken embryo. In situ hybridization performed in a tissue slice (200 μm) with Dig-labeled antisense (D–F) and sense (G) probes. Arrows indicate prominent domains of Epimorphin expression in forming vertebral anlage observed at E3.5 onward. (H) A standard histological section stained with hematoxylin of an E4.5 embryo trunk level. Arrows indicate concentrically aligned precartilage cells around the notochord, coinciding with the presence of Epimorphin expression. N: notochord.
reported for different types of skeletogenesis (for vertebrae, Murtaugh et al., 1999, 2001; Peters et al., 1999; Tribioli and Luukin, 1999; for limbs, Francis-West et al., 1999; for ribs, Gras et al., 1996).

The condensation step during cartilaginous formation, which is pivotal for subsequent differentiation of the chondrocytes, has long been a central subject of bone formation research (Hall and Miyake, 1995, 2000). However, the molecule(s) that initiate and trigger the condensation have remained largely unknown despite a number of studies, most of which used micromass cultures of limb mesenchymal cells or administration of factors in ovo. While the factors involved specifically in vertebral cartilaginous condensation are unknown, such factors would be expected to explain how the cells become successively recruited into condensed primordia so that a sequential elongation of the vertebral anlage is manifested in a ventral-to-dorsal direction. The aim of the present study is to clarify the molecular mechanisms by which the early patterning of vertebral cartilage is established, mainly by focusing on the condensation step controlled by an extracellularly acting signaling molecule, Epimorphin.

Epimorphin was originally discovered in the mouse as a protein that is produced in the mesenchyme to regulate morphogenesis of the overlying epithelium in lung and hair follicle (Hirai et al., 1992). Since then, the roles of Epimorphin have extensively been studied with a variety of assay systems, mainly in vitro, showing the possible importance of this molecule for tissue interactions and cell movements during formation of several organs including blood vessel, liver, mammary gland, skin, pancreas, gallbladder, and intestine (reviewed by Radisky et al., 2003). The homologues have also been reported for human and rat, which have at least three splicing variants with their extracellular domains being identical (Bennett et al., 1993; Hirai, 1993). The shortest isoform lacks the membrane-anchoring domain. Epimorphin, although lacking a signal peptide sequence at its N-terminus, was proven to be present at cell surface and/or as a secreted form, both of which exert extracellular functions (Hirai, 1994; Lehner et al., 2001). To date, however, the roles for this molecule during early embryogenesis have poorly been explored.

In the present report, we first describe the expression patterns of Epimorphin mRNA and find it predominantly localized in the vertebral cartilage anlagen. We then demonstrate the formation of extra vertebrae by providing Epimorphin ectopically in ovo. These morphogenetic effects are attributed to the extracellularly acting Epimorphin which directly promotes aggregation and sorting of chondrogenic cells during cartilage condensation. We present a model in which Epimorphin expression is initiated by the notochord, and then Epimorphin acts on adjacent sclerotomal cells to promote their aggregation. In this model, the successive action of Epimorphin emanating from the tip of forming precartilage leads to a progressive propagation of the cartilage primordium. This is the first report that shows the mechanisms at the molecular and cellular levels underlying vertebral cartilage condensation.

### Materials and methods

#### Cloning of avian Epimorphin cDNAs

Chicken fibroblast lambda cDNA library (STRATAGENE) and the quail embryonic day 5 (E5) cDNA library (Takahashi et al., 1992) were screened with the [12P]-labeled coding region of mouse Epimorphin cDNA. 2.5 × 107 plaques were screened, respectively, and cDNA fragments from the positive clones were subcloned into pBluescript vector. GenBank accession numbers: chicken Epimorphin, AB099979; quail Epimorphin, AB076670.

#### Thick section in situ hybridization

Digoxigenin (Dig)-labeled probes were prepared as previously described (Tonegawa et al., 1997). For in situ hybridization, chicken embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS). After dehydration with methanol and bleaching with 2% hydrogen peroxide in methanol, embryos were embedded in 3% agarose in PBS to be sliced by the Micro Slicer (DOSAKA EM) at a thickness of 200 μm. The procedure for hybridization was as previously described (Henrique et al., 1995) with slight modifications; sections were treated with 10 μg/mL proteinase K in PBS containing 0.1% Tween 20 for 10 min ~15 min and hybridized with Dig-labeled RNA probe in the hybridization buffer containing 1× SSC.

#### Production of Epimorphin by COS cells

Epimorphin expression vector was constructed using a pCDM8 (Invitrogen)-derived vector in which the elongation factor promoter drives inserted DNA expression (Tonegawa et al., 1997). A modified form of quail Epimorphin cDNA was constructed by removing the C-terminal hydrophobic region and by adding the interleukin-2 (IL-2) signal peptide sequence and the T7 tag at the N- and C-termini, respectively. Transfection into COS7 cells and preparation of cell aggregates were carried out as previously described (Takahashi et al., 1996; Tonegawa et al., 1997).

#### Immunoblotting and immunoprecipitation were done by standard protocols.

#### Rabbit antisera was made against untagged recombinant quail Epimorphin, prepared as previously described (Oka and Hirai, 1996), and was purified with Hi-trap protein G column (Amersham Biosciences) and dialyzed against PBS followed by sterile filtration. Whole lysate of Epimorphin-transfected COS cells was prepared by adding the SDS-PAGE sample buffer directly into a culture dish after washing with PBS. For the immunoprecipitation experiments, transfected cells were cultured under serum-free conditions for 48 h, and subsequently Epimorphin secreted into the culture medium was precipitated by anti-Quail Epimorphin antibody followed by detection with anti-T7-tag monoclonal antibody (Novagen).

#### Implantation of COS cells into chicken embryos

COS cells transfected with either Epimorphin or Shh (Shh-pMMV II; given by Dr. Watanabe, Tohoku Univ.) were implanted as a cell aggregate into a slit made between the segmental plate and neural tube of a chicken embryo at the Hamburger and Hamilton (HH) stage 13–15 (Hamburger and Hamilton, 1951).

#### Cell aggregation assay

Sclerotomal fragments were dissected from E4 chicken embryos with a sharpened tungsten needle under a dissecting microscope and treated with 0.125% Trypsin/0.01% EDTA solution for 10 min at room temperature. After the reaction was stopped by adding fetal calf serum (FCS), cells were dissociated into single cells by pipetting and cultured overnight in a medium containing 10% FCS and 1:1 mixture of DMEM and Ham F12 and (DH10). The dissociated sclerotomal cells or C3H10T1/2 cells were labeled with a fluorescence dye PKH26 (SYNAXIS) prior to the co-culture with Epimorphin-producing COS cells. The following combination was made in a co-culture for each well of 24-well dish: 1.2 × 10^3 of sclerotomal cells and 6 × 10^6 EPM/COS, 3 × 10^6 10T1/2 and 6 × 10^6 EPM/COS, 6 × 10^4 COS and 6 × 10^4 EPM/COS. The cells were plated in a well pre-coated with 1% agarose in HCMF (calcium- and
magnesium-free HEPES buffer and cultured at 37°C under gentle rotation (100 rpm). For the Western blot analysis, anti-NCAM polyclonal antibody (CHEMICON) was used at 1:800 dilution.

Skeletal preparation and histochemistry

To examine skeletal patterns of vertebral cartilages, embryos were stained with Alcian blue in toto as previously described (Tonegawa et al., 1997).

For histochemical analysis of early cartilage marker expression, operated embryos were fixed with 4% paraformaldehyde and embedded in paraffin wax and then serial histological sections were prepared. Cell aggregates cultured in vitro were embedded into O.C.T. compound (Tissue-Tek), and 10 μm cryostat sections were fixed with 4% paraformaldehyde on a slide glass. Antibody against Type II collagen (Collagen Research Center) used for immunostaining was diluted to 1:100. Biotinylated peanut agglutinin (Vector laboratories) was used at 5 μg/ml. After incubation with HRP- or Fluorescenc-conjugated secondary antibody or HRP-conjugated streptavidin (Amersham Biosciences), the sections were visualized with DAB substrate kit (Vector Laboratories) followed by counterstaining with Mayer’s hematoxylin (Wako). Differentiated cartilaginous cells were stained in 1% Alcian blue in 0.1 N HCl (pH 1.0) for 30 min at room temperature.

Infection of RCAS-Sox 9 and RT-PCR

Quail Sox 9 cDNA was inserted into a RCAS (A) vector, and Sox 9-RCAS was infected to chicken embryonic fibroblast DF1 cells. After infection as a monolayer in plastic dish for 3 days, the infected cells were treated with trypsin/EDTA to be dissociated into single cells. 1 × 106 cells were laid as a pellet onto a monolayer in plastic dish for 3 days, the infected cells were treated with trypsin/EDTA and then serial histological sections were prepared. Cell aggregates cultured in vitro were embedded into O.C.T. compound (Tissue-Tek), and 10 μm cryostat sections were fixed with 4% paraformaldehyde on a slide glass. Antibody against Type II collagen (Collagen Research Center) used for immunostaining was diluted to 1:100. Biotinylated peanut agglutinin (Vector laboratories) was used at 5 μg/ml. After incubation with HRP- or Fluorescenc-conjugated secondary antibody or HRP-conjugated streptavidin (Amersham Biosciences), the sections were visualized with DAB substrate kit (Vector Laboratories) followed by counterstaining with Mayer’s hematoxylin (Wako). Differentiated cartilaginous cells were stained in 1% Alcian blue in 0.1 N HCl (pH 1.0) for 30 min at room temperature.

Results

Cloning and expression of avian Epimorphin

We isolated avian cDNA homologues of mouse Epimorphin from cDNA libraries prepared from quail (Takahashi et al., 1992) and chicken embryos of embryonic day 5 (E5) using the coding region of mouse Epimorphin as a probe. cDNA fragments encoding the entire or partial open reading frames (ORF) were obtained for quail and chicken, respectively, with 95% identity between them (Figs. 1A, B). A predicted sequence from the quail cDNA has 286 amino acids. As in the Epimorphins identified in other species, the quail Epimorphin ORF contains a C-terminal hydrophobic region (membrane anchoring), no signal peptides, and heptad repeats in α-helix domains (Hirai et al., 1992) (Fig. 1A). The cDNA obtained in this study encodes isoform II, which differs from isoform I by several amino acids in the membrane-anchoring region (Hirai, 1993). Isoform II appears to be the most abundantly present in tissues (Hirai, unpublished). Northern blotting with this cDNA as a probe detects a single band of 3.4 kb in a specimen prepared from E5 embryos (Fig. 1C).

A low level of expression of Epimorphin mRNA precluded detection of signals by standard protocols for whole-mount in situ hybridization or direct hybridization on paraffin or frozen histological sections with a digoxigenin/radioactive probe. We therefore performed thick section (200 μm) in situ hybridization (see Materials and methods) with prolonged coloration time of the alkaline phosphatase reaction.

Among a variety of tissues displaying Epimorphin signals such as sclerotome, dorsal aorta, nephric ducts, and myotome (Fig. 1; Y.O and Y. T, unpublished), we paid particular attention to the expression in the vertebral lineage. The signal was evident around the notochord at E3.5 (Fig. 1D), and by E5, it became expanded in the medio-ventral region of the sclerotomal territory (Fig. 1E). When the cartilage primordium dorsally progressed to E6.0, the Epimorphin signal was confined to this tissue (Fig. 1F). A sense probe gave no signal over the background level (Fig. 1G). The onset of Epimorphin expression near the notochord coincides with morphological changes of sclerotomal cells during which time they become concentrically aligned around the notochord (Fig. 1H).

Ectopically administered Epimorphin in ovo affected formation of the vertebral cartilage

To study the roles of Epimorphin in verteobrogenesis, we carried out an ectopic administration of Epimorphin protein into the sclerotomal territory of chicken host embryos. We used COS cells as a vehicle for the protein administration because this method was shown to be highly efficient in synthesizing and secreting extracellularly acting proteins (Takahashi et al., 1996; Tonegawa et al., 1997; Tonegawa and Takahashi, 1998). Briefly, an aggregate of COS cells that had been transfected with Epimorphin cDNA expression vector was implanted into a presomite or young somite of a chicken host embryo (Fig. 2B).

Epimorphin secreted by the COS cells

To facilitate the secretion of Epimorphin out from the transfected COS cells, the quail Epimorphin cDNA was engineered by adding signal peptides of IL-2 to its N-terminus and also by deleting the membrane-anchoring region, the form that has previously been used in a number of in vitro studies (Hirai, 2001; Hirai et al., 2001; Radisky et al., 2003) and also in some in vivo studies (Bascou et al., 2005) (see also Discussion). As shown below in more details, this modified form of Epimorphin exhibited essentially the same activity with slightly higher efficiency compared to the native form. After transfection of Epimorphin cDNA tagged with T7 into COS cells, production of the protein was assayed by Western blotting using anti-quail Epimorphin antibody (see Materials and methods) and also anti-T7 antibody. Epimorphin proteins of 31 kDa and 34 kDa, with the latter presumably being glycosylated, were efficiently produced in the cells (Fig. 2A). Secretion of the protein into the culture medium was further confirmed by immunoprecipitation with anti-Epimorphin antibody followed by detection with anti-T7 antibody (Fig.
Fig. 2. In ovo implantation of Epimorphin-producing COS cells caused malformation of a vertebral cartilage. (A) Immunoblot analysis detected Epimorphin protein produced by transfected COS cells. Recombinant Epimorphin of 31 kDa and glycosylated 34 kDa was present in the cell lysate of Epimorphin/COS cells (left panel). CBB; Coomassie Brilliant Blue staining. Immunoprecipitation by anti-qua Epimorphin polyclonal antibody showed Epimorphin protein secreted into the culture medium detected with anti-T7 monoclonal antibody (right panel). (B) A diagram showing an experimental design of Epimorphin/COS implantation. A cell aggregate of Epimorphin/COS was implanted in between the neural tube and segmental plate of an E2 chicken embryo. Manipulated embryos were allowed to develop until E5 or E8 when assessed for bone formation. (C) Skeletal specimens of E8 embryos. Epimorphin caused supernumerary formation of the ventral (vertebral body) and lateral (neural arch) portions of the vertebral column (red arrows). A vertebral body of successive segments was fused (C, C'; ventral view of the same specimen with higher magnification in C''). Lateral view highlights an extra cartilaginous element spanning two adjacent segments at the neural arch level (C''). (D) Implantation of Epimorphin/COS resulted in precocious differentiation of chondrocytes revealed by augmented accumulation of type II collagen in adjacent precartilage cells at E5 (arrowhead). Dotted lines encircle the area of implanted COS cells. A paraffin section was stained for type II collagen (left) followed by hematoxylin staining (right).
2A). Mild treatment of the transfected cells with trypsin, which digests only cell surface proteins, resulted in a reduction of signal (data not shown), suggesting that a portion of Epimorphin produced by COS cells is also present near the cell surface.

In ovo implantation of EPM/COS caused supernumerary cartilage

Chicken host embryos implanted with an aggregate of Epimorphin-transfected COS cells (EPM/COS) were allowed to develop until E8. In skeletal specimens of these embryos, we observed supernumerary cartilages associated with vertebral components. In most cases, the ectopic cartilages straddled successive segments at the levels of vertebral body and neural arch along the dorso-ventral axis (Fig. 2C, n = 9), whereas these effects were not observed in control COS cells (n = 7). The site of extra-cartilage formation was usually confined to one segment along the antero-posterior (A–P) axis. It was unclear if Epimorphin also exhibited specific effects on the dorsal portion (i.e. spinous process) since control COS also gave rise to similar abnormal morphology. These observations suggest that, during normal development, Epimorphin expressed in the ventral cartilage primordium appears to participate in subsequent morphogenesis of the vertebra as a signaling molecule.

To more carefully examine the effects by EPM/COS in vertebral formation, we looked at earlier stages after the implantation. In E5 embryos, type II collagen, an early marker for the cartilage differentiation, was localized adjacent to EPM/COS. Staining was more intense toward the host notochord, showing precocious differentiation of the cartilaginous cells (Fig. 2D). Control/COS cells gave no effect. We did not detect an appreciable change of Pax-1 expression in embryos implanted with EPM/COS (data not shown), consistent with the expression of Pax 1 preceding that of Epimorphin in the ventral cartilage primordium in normal embryos (not shown). Thus, the effects elicited by EPM/COS seen in ventral vertebrae can be traced back to a stage as early as E5, suggesting a role of Epimorphin in promotion of cartilaginous differentiation during vertebrogenesis.

Fig. 3. Epimorphin promotes sorting/aggregation of 10T1/2 cells in vitro. (A) 10T1/2 cells labeled with fluorescent dye (PKH26) were co-cultured with Epimorphin/COS or control/COS cells under rotating condition for 24 h. (B) Labeled 10T1/2 cells were visualized under fluorescence microscopy. 10T1/2 cells segregated from Epimorphin/COS and aggregated between themselves, whereas they were mingled with control/COS cells. Dotted lines show the border of aggregates. Distribution profile of labeled 10T1/2 cells in an aggregate is shown by cell aggregation index: a ratio between a scattered range of labeled cells (diameter X) and the diameter of the aggregate (diameter Y), assessed for a random sampling of 15 aggregates in three independent experiments. P values between control/COS and native or modified Epimorphin/COS were <0.05. The cell-sorting/aggregation-promoting effect was abolished by anti-quail Epimorphin antibody in a dose-dependent manner. N-EPM, native form Epimorphin; M-EPM, modified form Epimorphin; α-EPM, anti-Epimorphin antibody; α-cont, control preimmune antibody; Scale bar, 100 μm. (C) Labeled COS cells did not segregate from Epimorphin/COS. (D,E) Growth of COS or 10T1/2 cells was not significantly affected by Epimorphin (P > 0.05), as assessed by three independent experiments, where the number of cells before and after the 24 h culture were compared. (D) COS cells transfected with or without Epimorphin; (E) 10T1/2 co-cultured with control/COS or Epimorphin/COS.
Epimorphin promotes sorting/aggregation of 10T1/2 cells

How does Epimorphin contribute to the promotion of vertebral cartilage differentiation? To address this question, we performed an in vitro culture to directly investigate the behavior of mesenchymal cells exposed to Epimorphin. First, we chose murine mesenchymal cell line 10T1/2 (Campbell et al., 1987; Taylor and Jones, 1979) since these cells are known to possess an ability to differentiate into chondrocytes and also to react to Epimorphin (Hirai, 1994). 10T1/2 cells were labeled with fluorescein and co-cultured with unlabeled EPM/COS under the rotating condition for 24 h (Fig. 3A). Although the average diameter of cell aggregates containing 10T1/2 and EPM/COS did not significantly differ from that of control (not shown), behavior of labeled 10T1/2 cells in the aggregate was profoundly different: 10T1/2 cells became tightly packed at the center of an aggregate and segregated from EPM/COS, contrasting with the control aggregate in which 10T1/2 cell were scattered and intermingled with normal COS cells (Fig. 3B). This aggregation-promoting effect elicited by COS cells transfected with the modified form of Epimorphin as described above was also observed for the native form of this protein (Fig. 3B). The segregation of 10T1/2 cells out from EMP/COS was not due to the change in cell aggregation of EMP/COS since EMP/COS did not affect on normal COS cells (Fig. 3C). To further investigate whether the sorting/aggregation of 10T1/2 cells was caused by specific action of Epimorphin, we added anti-Epimorphin antibody (polyclonal; see Materials and methods) into the culture medium. The aggregation-promoting effect by EPM/COS was extinguished by the antibody, yielding a pattern of cell behavior similar to the control, whereas this sorting-out effect was retained when preimmune serum was used as a control. The neutralizing effect by the anti-Epimorphin antibody was dose-dependent (Fig. 3B). Finally, the cell-sorting/aggregation-promoting effects by Epimorphin were not brought about by a gross change in cell proliferation since Epimorphin transfection did not alter the growth of COS or 10T1/2 cells (Figs. 3D, E).

Epimorphin promotes sorting/aggregation of vertebral prechondrogenic cells

We reasoned that the condensation-promoting effect by Epimorphin on 10T1/2 cells would reflect enhanced differentiation of the vertebral cartilage caused by EPM/COS implantation in ovo as shown earlier. To test this, we performed a similar aggregation assay, but this time we used sclerotomal cells taken from E4 chicken embryos. A strip of sclerotome-containing tissue was dissected from an embryo and was confirmed to be positive for Pax 1 expression (Fig. 4A). A notochord was then removed from this piece to obtain the sclerotomal population. The dissected prechondrogenic/sclerotomal cells were dissociated and labeled with fluorescein prior to the rotation co-culture with unlabeled EPM/COS. After 24 h of culture, the labeled sclerotomal cells were condensed at the center of an aggregate and clearly segregated from EPM/COS (Fig. 4B). When co-cultured with control/COS, in contrast, the labeled cells were sparsely distributed in an aggregate (Fig. 4B). Thus, Epimorphin triggers and promotes the sorting/aggregation of the embryonic sclerotomal cells.

Promoted aggregation of sclerotomal cells by Epimorphin resulted in precocious differentiation of cartilage

We further studied a consequence of the aggregated sclerotome caused by Epimorphin. We examined expression of NCAM, a cell–cell adhesion molecule, and also type II collagen and chondroitin sulfate (by Alcian blue staining) as
relatively late markers for cartilage differentiation. NCAM has been known to mediate cell–cell interaction during limb chondrogenesis (Widelitz et al., 1993). In the sclerotomal cells co-cultured with Epmorphin/COS for 2 days, NCAM production was upregulated compared to the control (Fig. 5A). The signal for NCAM detected in this experiment was specifically derived from the chicken sclerotome because neither control/COS nor EPM/COS yielded the signal. Sclerotomal cells co-cultured for 2 days with EPM/COS also displayed precocious expression of type II collagen and Alcian-blue-positive signal (Fig. 5B). When sclerotomal cells were cultured alone, they formed a loose cluster and exhibited a very faint signal for type II collagen and Alcian blue staining (Fig. 5B, insets). These observations suggest that Epimorphin promotes chondrogenesis in sclerotomal cells. We did not observe significant difference in expression of Epimorphin mRNA or N-cadherin between cultures with EPM/COS and control/COS (data not shown).

Taken together, we conclude that, during normal development, extracellularly acting Epimorphin influences the
condensation step of vertebral precartilage by triggering and promoting sorting/aggregation of the sclerotomal prechondrogenic cells. The enhanced condensation leads to subsequent differentiation of the vertebral cartilage. This notion is consistent with the fact that Epimorphin-positive region around the notochord includes the type-II-collagen-positive area wherein concentrically aligned chondrocytes are recognized and also the area being condensed seen by the binding of peanut aggrutinin (PNA) (Bagnall and Sanders, 1989; Hall and Miyake, 1992) (Fig. 5D). Therefore, it is reasonable to hypothesize that Epimorphin, produced near the notochord, successively acts on adjacent mesenchymal sclerotome to cause them to undergo condensation, leading to a sequential elongation of the vertebral cartilage template in a ventral-to-dorsal direction (see also a model in Fig. 7 and Discussion).

Fig. 6. Epimorphin is regulated by the notochord, Shh, and Sox 9. (A, B) An ectopic notochord (asterisk) induced Epimorphin expression in the adjacent sclerotome. Three days after a notochord was ectopically transplanted into the anterior segmental plate of a host, an embryo was subjected to the thick section (200 μm) in situ hybridization. One such specimen shown in (B) received the notochord next to the host one, resulting in enhanced expression of Epimorphin between them (arrowheads). (C) A slice of an embryo operated in the same way as (A, B) was hybridized for Pax 1, showing successful transplantation of the notochord, confirmed by Pax 1 induction. (D–G) Shh/COS cells (dotted line) were implanted into the segmental plate, resulting in ectopic expression of Epimorphin (D) and Sox 9 (F) detected by thick section in situ hybridization (arrows). D–F and E–G are neighboring thick sections of the same embryo, respectively. (H–K) DF1 cells infected with RCAS-Sox 9 or RCAS-GFP were cultured on a nuclepore filter as pellets. After cultured for 72 h, Sox 9-RCAS-infected cells formed aggregates (arrows in I). (J) RT-PCR revealed upregulation of Epimorphin and Aggrecan by Sox 9 infection. (K) Relative change in mRNA level of Epimorphin and Aggrecan after Sox 9 infection, quantified in three different cultures.
Regulation of Epimorphin expression during vertebrogenesis

Last, we studied how the expression of Epimorphin is regulated during vertebrogenesis. Since the onset of Epimorphin mRNA expression is seen adjacent to the notochord, we started to examine the effect of this tissue by ectopic transplantation. The thick section in situ hybridization revealed that Epimorphin mRNA expression was induced by the grafted notochord (Figs. 6A, B). One such specimen, in particular, received a notochord adjacent to the host one, and the signal of Epimorphin was enhanced by the two notochords (Fig. 6B). A similar phenomenon was also observed when Pax 1 was used as a control (Fig. 6C), suggesting synergistic effects by the notochords acting on these genes. We also implanted COS cells expressing Sonic hedgehog (Shh), a factor secreted by the notochord, into a host and observed upregulation of Epimorphin mRNA in the vicinity of the implant. Thus, the notochord triggers the expression of Epimorphin mRNA, mediated at least by Shh.

We further extended our study to learn any relationship between Epimorphin and Sox 9, the latter reported to be involved in cartilaginous condensation during vertebrogenesis. Sox 9 expression is known to be initiated by the notochord/Shh (Zeng et al., 2002) (also confirmed in Figs. 6F, G), suggesting a similarity to Epimorphin. We therefore examined whether Epimorphin would be affected by Sox 9 by overexpressing Sox 9 into chicken embryonic fibroblast cell line, DF1, using retrovirus technique. After culture for 72 h as a pellet on a nucpore filter, Sox 9-infected cells, but not the control cells, formed aggregates (Fig. 6I) that exhibited a markedly augmented level of expression of Epimorphin mRNA (Figs. 6J, K). The relative rate of Epimorphin upregulation was similar to that of Aggrecan mRNA, used as a control (Zeng et al., 2002) (Figs. 6J, K). Intriguingly, when Sox 9 was infected into cells that were cultured as a monolayer in plastic dish, we did not find appreciable effects for cell condensation or upregulation of Epimorphin and aggrecan (data not shown), implying that three-dimensional environment is important for triggering the condensation, mechanisms of which remain unknown. These results suggest that Sox 9 acts upstream of Epimorphin and also that Epimorphin appears to be a close target of Sox 9 during vertebrogenesis.

Discussion

We have shown in the present study that Epimorphin, previously known to be a morphogenetic factor acting primarily on epithelial morphogenesis, plays important roles in vertebrogenesis by promoting condensation of prechondrogenic cells. This is the first report demonstrating that the initiation of vertebral-cartilaginous condensation is controlled by an extracellular molecule.

Epimorphin is known to play distinct roles depending on its membrane topology either extracellularly or intracellularly. It is thought that extracellularly presented Epimorphin elicits morphogenic programs in a wide variety of cell types shown mainly by in vitro studies (Hirai et al., 2005; Hirose et al., 1996; Koshida and Hirai, 1997; Mori and Miyazaki, 2000; Oka and Hirai, 1996; Simian et al., 2001; Takebe et al., 2003; Terasaki et al., 2005; Watanabe et al., 1998). Supporting this, a soluble form of Epimorphin is detected in a medium in which pancreatic cells or chicken primary fibroblasts are cultured (Lehnert et al., 2001) and also in murine milk (Hirai et al., 2001). Our results reported here further substantiated an importance of extracellular action of Epimorphin.

Roles of Epimorphin in prechondrogenic condensation during vertebrogenesis

We have demonstrated that Epimorphin causes precocious differentiation of vertebral cartilage when ectopically administered in vivo. These effects are brought about by the activity of this molecule in promoting precartilage condensation, revealed by the in vitro cell aggregation assay that allowed us to directly trace the behavior of prechondrogenic cells influenced by Epimorphin. Thus, this report shows evidence that Epimorphin is a condensation-initiating (or -promoting) factor in vertebrogenesis, which has long been awaited. Given these facts, we propose a model as shown in Fig. 7 that shows how Epimorphin contributes to vertebrogenesis during normal development. Soon after sclerotomal cells are segregated from the somite, Epimorphin is induced by the notochord to be expressed in its adjacent cells, being regulated by Shh and Sox 9. Epimorphin protein produced by these cells then acts on neighboring cells to change their morphological behavior to be concentrically aligned and form a perinotochordal tube. The tip of the precartilage that has undergone condensation, in turn, recruits and stacks the surrounding mesenchyme into the forming primordium, mediated by Epimorphin. This successive action of Epimorphin leads to a progressive elongation of the vertebral cartilage primordium in a ventral-to-dorsal direction. This model does not exclude the possibility that Epimorphin acts in an autocrine fashion. Reciprocal interactions mediated by Epimorphin between neighboring sclerotomal/prechondrogenic cells as proposed here are consistent with the previously reported observations that sclerotomal tissues taken from E4–E6 could form normal cartilage only when placed into an age-matched environment (Epimorphin-positive), but not in an E2 sclerotome (Epimorphin-negative) (Dockter and Ordahl, 1998).

In this model, Epimorphin initially acts radially around the notochord and later unidirectionally (dorsal) at the bifurcated tips of the vertebral body anlage. It is conceivable that the perichondrium, which demarcates the cartilage primordium, and/or some inhibitory factors present in the surrounding mesenchyme confine the direction of Epimorphin action. It was reported in limb bud skeletogenesis that GDF11 (Gamer et al., 2001) acts as an inhibitory factor for the cartilaginous formation and also that tenascin and syndecan inhibit growth of a condensed cell mass (Brand-Saberi and Christ, 2000; Christ et al., 2000; Hall and Miyake, 2000).
Epimorphin regulates the cell sorting/aggregation step during cartilage condensation

The condensation during cartilage formation is an output of multiple morphogenetic processes: cell sorting among prechondrogenic mesenchyme, aggregation and increased cell density, and cell growth and survival. A large number of reports using micromass cultures of limb bud mesenchyme and cell lines with chondrogenic potentials such as C2C12 cells (Ahrens et al., 1977; Daniels et al., 1996; Solursh et al., 1982) showed that several factors including TGFβ and FGF family molecules are involved in cartilage condensation processes. These studies, however, have not been successful in defining how the factors promote the condensation since in most cases condensation was assessed by the consequent differentiation of chondrocytes using relatively late markers (i.e. Alcian blue staining). In the present study, we carried out the cell aggregation assay in which we could directly assess the behavior of cells affected by Epimorphin. In particular, 10T1/2 cells manifested aggregation promoted by Epimorphin while their cell proliferation rate remained unaffected. The cell-sorting/aggregation-promoting effect by Epimorphin was even more pronounced on embryonic sclerotome/prechondrogenic cells taken from E4. We therefore concluded that Epimorphin plays a principle role in the initial step of condensation during vertebrogenesis, probably by regulating other extracellular molecules including N-CAM, which are involved in cell–cell adhesion and cell–substrate adhesion.

Regulatory cascade leading to vertebro-cartilagenous condensation

Our findings that Epimorphin expression is upregulated by the notochord, Shh, and Sox 9 have allowed to depict a molecular cascade at early stages in vertebrogenesis, in which the notochord initiates the expression of Sox 9 mediated by Shh, which in turn triggers the expression of Epimorphin. It has previously been proposed mainly by genetic or tissue combination studies that Sox 9 is regulated by Pax 1, Pax 9, and Bapx 1/Nkx 3.2 (Akiyama et al., 2002; Bi et al., 1999; Furumoto et al., 1999; Iida et al., 1997; Murtaugh et al., 2001; Peters et al., 1999; Rodrigo et al., 2003; Tribioli and Lufkin, 1999; Zeng et al., 2002). Among these vertebrogenetic genes, Sox 9 seems to be most relevant to the cartilage condensation,
whereas others are rather involved in growth/survival of early sclerotomal cells (Akiyama et al., 2002; Bi et al., 1999).

The regulation of cell condensation controlled by Sox 9/Epimorphin appears to be complex since, although overexpression of Sox 9 resulted in markedly enhanced aggregation of cells cultured in three-dimensional environment (Fig. 6), such effects were not observed in cells cultured as a monolayer in plastic dish (data not shown). Coincidentally, Epimorphin was not upregulated in such cases (data not shown). The regulation between Epimorphin and Sox 9 might require other factors that are affected by environment in which cells reside. It has recently been reported that Sox 9 acts in concert with other factors including the homeobox transcription factor Barx2 and peroxisome proliferator-activated receptor γ co-activator 1α (PGC-1α) (Kawakami et al., 2005; Meech et al., 2005). In this study, we observed that Epimorphin enhanced N-CAM expression, but not Epimorphin itself (data not shown), suggestive of another factor(s) involved in upregulation of Epimorphin/Sox 9 in progressively elongating vertebral cartilage.

It is of interest to understand to what extent the condensation/aggregation-promoting effects by Epimorphin are shared between vertebrogenetic and other endochondrogenetic phenomena. Our findings also open a way to study mechanisms by which the condensation of non-chondrogenic mesenchyme is controlled in a variety of organogenesis, including cutaneous structures (hair, feathers, mammary glands), gut organs (liver, pancreas), lungs, and kidneys, in which Epimorphin has been shown to be a mesenchymal factor that acts on their respective epitheliogenesis (Fritsch et al., 2002; Hirai et al., 1992, 1998, 2001; Hirose et al., 1996; Watanabe et al., 1998).

Acknowledgments

We express special thanks to Dr. S. F. Gilbert for his intensive discussion. We are grateful to Dr. S. Nakagawa (instruction for RCAS experiments), Dr. Y. Wakamatsu (Sox 9-expression vector), and Dr. Y. Watanabe (Shh-pMiw II), and also Drs. K. Yasuda, N. Funayama, and A. Tonegawa for helpful comments. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References


Murata, Y., Fujita, Y., 1996. BMP-4 mediates interacting signals between the neural tube and skin along the dorsal midline. Genes Cells 1, 237–244.


