Role of BMP-2 and OP-1 (BMP-7) in programmed cell death and skeletogenesis during chick limb development

D. Macias1, Y. Gañan1, T. K. Sampath2, M. E. Piedra3, M. A. Ros3 and J. M. Hurle3,*

1Departamento de Ciencias Morfológicas, Universidad de Extremadura, E-06071 Badajoz, Spain
2Creative Biomolecules, Hopkinton, MA 01748, USA
3Departamento de Anatomia y Biología Celular, Facultad de Medicina, Universidad de Cantabria, E-39011 Santander, Spain

*Author for correspondence (e-mail: hurlej@galeno.medi.unican.es)

INTRODUCTION

The Bone Morphogenetic Proteins (BMPs) family of secreted signaling molecules belongs to the transforming growth factor beta superfamily. The initial discovery of BMPs was made based on the ability of purified bone extracts to promote ectopic cartilage and bone formation when implanted under the skin or into the muscle of adult rats (Urist et al., 1979; Sampath and Reddi, 1981). However, it is now evident that the BMP family consists of a large number of genetically related molecules, conserved in evolution, with key roles in development (Wozney et al., 1988; Kingsley, 1994; Hogan, 1996).

BMP-2 and OP-1 (BMP-7) exhibit relatively similar temporal and spatial patterns of expression in the growing limb bud, which suggests common roles in the early stages of limb morphogenesis. Prior to the formation of the cartilaginous condensations, these BMPs are expressed in the AER and in the posterior mesenchyme of the limb (Lyons et al., 1990; Jones et al., 1991; Francis et al., 1994; Francis-West et al., 1995; Luo et al., 1995; Helder et al., 1995). This early pattern of expression has been related to the establishment of the limb axes (Francis et al., 1994; Francis-West et al., 1995; Duprez et al., 1996c). In addition, BMPs and OP-1 are expressed in the interdigital regions of the developing autopod (Lyons et al., 1990; Francis et al., 1994; Luo et al., 1995) where they may control programmed cell death (Zou and Niswander, 1996). However, such a role for interdigital apoptosis has only been documented for BMP-4 (Gañan et al., 1996), a member of the BMP family that is also expressed in the interdigital spaces of the developing limb (Francis et al., 1994).

The expression of Bmp-2 and Op-1 genes in the perichondrium during skeletogenesis (Lyons et al., 1989, 1990; Wozney et al., 1993; Helder et al., 1995; Hogan, 1996; Duprez et al., 1996a), and the chondrogenic promoting effect of BMP-2 in vitro (Chen et al., 1991; Roark and Greer, 1994; Duprez et al., 1996b) and in vivo (Duprez et al., 1996a), suggest additional roles for these BMPs in the morphogenesis of the appendicular skeleton. Skeletogenesis is a complex process that involves a cascade of cellular events including the formation of the prechondrogenic aggregates, the differentiation and growth of the cartilages, the establishment and differentiation of the joints, and the replacement of the cartilages by bone. While the molecular signals that control most of these events are unknown (see however, Gañan et al., 1996; Vortkamp et al., 1996b), the role of BMPs and OP-1 in controlling interdigital apoptosis suggests that these factors may play a key role in the regulation of interdigital apoptosis.

SUMMARY

Bone Morphogenetic Protein 2 (BMP-2) and Osteogenic Protein 1 (OP-1, also termed BMP-7) are members of the transforming growth factor beta superfamily. In the present study, we have analyzed the effects of administering them locally at different stages and locations of the chick limb bud using heparin beads as carriers. Our results show that these BMPs are potent apoptotic signals for the undifferentiated limb mesoderm but not for the ectoderm or the differentiating chondrogenic cells. In addition, they promote intense radial growth of the differentiating cartilages and disturb the formation of joints accompanied by alterations in the pattern of Indian hedgehog and ck-erg expression. Interestingly, the effects of these two BMPs on joint formation were found to be different. While the predominant effect of BMP-2 is alteration in joint shape, OP-1 is a potent inhibitory factor for joint formation. In situ hybridizations to check whether this finding was indicative of specific roles for these BMPs in the formation of joints revealed a distinct and complementary pattern of expression of these genes during the formation of the skeleton of the digits. While Op-1 exhibited an intense expression in the perichondrium of the developing cartilages with characteristic interruptions in the zones of joint formation, Bmp-2 expression was a positive marker for the articular interspaces. These data suggest that, in addition to the proposed role for BMP-2 and OP-1 in the establishment of the anteroposterior axis of the limb, they may also play direct roles in limb morphogenesis: (i) in regulating the amount and spatial distribution of the undifferentiated prechondrogenic mesenchyme and (ii) in controlling the location of the joints and the diaphyses of the cartilaginous primordia of the long bones once the chondrogenic aggregates are established.

Key words: apoptosis, chondrogenesis, joint formation, cartilage growth, Indian hedgehog
1996), members of the BMP family (Carrington et al., 1991; Kingsley et al., 1994; Wozney et al., 1993; Duprez et al., 1996a), including several cartilage-derived morphogenetic proteins and growth and differentiation factors (Storm et al., 1994; Chang et al., 1994; Höten et al., 1996) appear to be involved in appendicular skeletogenesis.

In this study, we have attempted to gain insights into the function of BMP-2 and OP-1 during limb development by analyzing the effect of their local administration at different stages and locations of the chick limb bud using heparin beads as carriers.

**MATERIALS AND METHODS**

Rhode Island chick embryos ranging from day 3 to day 9 of incubation (stages 19-35, Hamburger and Hamilton, 1951) were employed in this study.

**Experimental manipulation and morphological analysis of the limbs**

The eggs were windowed at the desired stages and the limb bud was exposed. Heparin-acrylic beads (diameter 100-160 μm; Sigma, H5263) soaked in PBS or incubated for 1 hour at room temperature in different solutions of human recombinant BMP-2 or human recombinant OP-1 were implanted into the limb bud mesenchyme. BMP-2 was employed at concentrations of 1, 0.5 and 0.1 mg/ml. OP-1 was employed at concentrations of 2, 0.5 or 0.1 mg/ml.

In the early stages of limb development (stages 19-25), the experiments were performed on the wing bud and the beads were implanted in the anterior margin, posterior margin, dorsal surface and distal mesoderm subjacent to the AER. At later stages of limb development (stages 27-31), the experiments were performed on the leg bud and the beads were implanted in the distal region of the third interdigital space or at the tip of digit III. After the operation, the embryos were returned to the incubator and sequentially fixed.

The skeletal morphology of the limbs was studied in whole-mount specimens fixed in 5% trichloroacetic acid and stained for cartilage with Alcian green.

**Detection of cell death**

After the implantation of the bead, cell death was analyzed at 10 and 20 hours by vital staining with neutral red, by Tdt-mediated dUTP nick end labeling (TUNEL) and by light and transmission electron microscopy (TEM).

For neutral red staining, the whole embryo or the excised limbs (depending on the stage) were immersed in a solution of 1×10⁻⁵% of neutral red in Ham F-12 medium and maintained in the incubator at 38°C. When the staining was optimal, the specimens were washed in PBS and fixed at 4°C in 4% neutral formalin for 24 hours. The specimens were then dehydrated in pure isopropilic alcohol and cleared in xylene.

TUNEL was performed on 8 μm thick paraffin sections of parafomaldehyde-fixed specimens using the ‘in situ cell death detection fluorescein kit’, according to the directions of the manufacturers (Boehringer).

For histology and TEM, the specimens were fixed in 3% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated in a graded series of acetone and propylene oxide and embedded in Araldite. Serial semithin sections were cut at 1 μm and stained with 1% toluidine blue. Ultrathin sections of selected areas were mounted on uncoated copper grids, stained with uranyl acetate and lead citrate and examined with a Zeiss EM 10C electron microscope.

**In situ hybridization**

The expression of Bmp-2 and Op-1 genes (using probes obtained from J. C. Izpisúa-Belmonte) was studied in the normal limbs at the stages of digit morphogenesis (29-34 HH). Ck-erg (using a probe obtained from M. Duterque-Couquillaud) and Indian Hedgehog (Ihh; using a probe obtained from J. C. Izpisúa-Belmonte) gene expression were studied to detect molecular alterations in the cartilage and developing joints in the experimental limbs. The autopodial regions of chick leg buds were fixed in paraformaldehyde and embedded in paraffin wax. In situ hybridizations were performed on 6 μm thick sections as described in Ros et al. (1994).

**RESULTS**

**BMPs trigger apoptosis in the undifferentiated limb mesenchyme**

Implantation of BMP-beads in different regions and stages of the developing limb was always followed by cell death in the undifferentiated limb mesenchyme. In semithin sections, dying

![Fig. 1. Illustrations showing the histological and ultrastructural features of cell death induced by BMP-beads. (A) Toluidine-blue-stained semithin section of a wing bud 20 hr after the implantation of a BMP-bead showing the abundance of dying cells and phagocytes in the mesenchymal tissue around the bead. Bar, 50 μm. (B) Low-magnification view of a section adjacent to A, showing the abundance of phagocytes in the area of cell death induced by the BMP-bead. Note that the ectoderm and the condensing mesenchyme of the central core of the limb are free of dying cells. (C) Histological section of an experimental wing bud showing the positivity for TUNEL of the dying cells around the BMP-bead (*). (D) Similar section of a control limb after implantation of a PBS-bead(*) showing the absence of dying cells. Bar, 50 μm. (E) The characteristic morphology of apoptosis of the dying cells induced by BMP-beads. Bar, 1 μm.](image-url)
cells and an extraordinary number of phagocytes were identified around the bead (Fig. 1A,B). The dying cells were positive for TUNEL (Fig. 1C,D) and exhibited the ultrastructural features of apoptosis (Fig. 1E). This cell death process was evoked using beads bearing a concentration of 0.1 mg/ml of BMP. Maximum intensity of cell death was achieved with a BMP concentration of 0.5 mg/ml. In all cases, dying cells were first detected 10 hours after the implantation of the BMP-beads and maximum intensity of the dying process was observed 20 hours after the implantation of the beads. Apoptosis was never found in the ectoderm or in the differentiating chondrogenic cells, even at higher concentrations of 1 mg/ml of OP-1 or 2 mg/ml of BMP-2.

As illustrated in Fig. 2, implantation of BMP-beads into the early wing bud (stages 19-25) was followed in all cases by the appearance of an area of cell death in the undifferentiated mesenchyme. The area of cell death spread around the bead but showed a clear boundary with the edge of the condensing skeletal blastemas. When the beads were implanted under the apical AER at stages 19-21, the area of cell death occupied almost the whole limb mesoderm (Fig. 2A,B). At later stages, the area of cell death was restricted to the progress zone mesenchyme (Fig. 2D). When the beads were implanted in the anterior or in the posterior margin of the bud, the elicited area of cell death resembled the normal anterior and posterior areas of cell death (ANZ and PNZ), but the extension and the intensity of these areas increased dramatically (Fig. 2E,F). When the beads were implanted into the dorsal mesoderm of the limb, the area of cell death occupied the dorsal surface of the limb and extended as far as the central condensed mesenchyme (Fig. 2G).

At later stages of development (27-29), the beads were implanted in the leg bud either in the third interdigit or at the distal tip of digit III (Fig. 2H-J). When the beads were implanted at the distal tip of digit III, an ectopic area of cell death resembling an extra-interdigit was always observed (Fig. 2I). When the beads were implanted in the interdigital spaces, the area of cell death was similar to the normal areas of interdigital cell death (INZ) but appeared earlier and showed a significantly greater intensity (Fig. 2J).

Skeletal alterations induced by BMP-beads

The main skeletal alterations observed in the experimental limbs 2 or 3 days after implanting the BMP-beads were (i) joint fusions, (ii) skeletal reduction defects and (iii) radial thickening of the cartilages. The pattern of distribution of these alterations showed differences according to the type and concentration of the BMP used, and to the stage and zone of implantation of the beads. At concentrations of 0.1 mg/ml of either OP-1 or BMP-2, the skeletal alterations were very mild. At concentrations of 0.5 mg/ml or more, the intensity of the alterations increased dramatically. The following description refers to the experiments using these higher concentrations of growth factor. The pattern of skeletal alteration varied with the stage of implantation of the beads, and these differences could be clearly related with the stage of chondrogenic differentiation of the limb mesoderm.

Between stages 19 and 21, the mesodermal core of the limb consisted mostly of undifferentiated mesenchyme and the predominant alteration caused by the beads was the loss of skeletal pieces in a pattern consistent with the area of cell death induced by the bead (Fig. 3B-E). When the beads were implanted in the anterior margin of the bud, the radius was totally or partially missing (Fig. 3B,C) in all the embryos treated with OP-1-beads (n=5) and in 50% of the embryos treated with BMP-2-beads (n=6). In some cases, the absence of radius was accompanied by loss of digit II (Fig. 3C) and by alterations in the morphology of the more proximal skeletal pieces of the limb. When the beads were implanted in the posterior margin of the bud, the ulna and the posterior digits were absent in 50% of the embryos treated with OP-1 (n=6) and in 33% (2 out of 6) of the experimental limbs treated with BMP-2 (Fig. 3D).
When the beads were implanted into the dorsal mesoderm of the limb, the pattern of skeletal alteration was more variable. Full truncation of the limb was observed in 4 of 6 experimental limbs treated with OP-1 and in 2 of 7 experimental limbs treated with BMP-2. The radius was missing in the remaining two embryos treated with OP-1-beads and in two embryos treated with BMP-2-beads. Implantation of the beads in the distal mesoderm of the progress zone was followed by full truncation of the limb (Fig. 3E) in all the experimental embryos whether OP-1-beads (n=4) or BMP-2-beads (n=5) were used.

Between stages 22 and 25, the prechondrogenic condensations detectable by peanut lectin labeling (Aulthouse and Solursh, 1987) are progressively formed in the core of the bud and, except when the beads were implanted in the progress zone, the whole skeleton was usually formed (see Table 1). Implantation of either OP-1 or BMP-2 -beads in the distal mesoderm of the progress zone was followed by distal truncation of the limb in a stage-dependent fashion (Fig. 3F-H). Implantation of the beads in the anterior, posterior or dorsal mesenchyme caused a relatively low incidence of joint fusions and/or local thickenings of the skeletal element located in the proximity of the bead (Table 1; Fig. 3I-L).

During stages 27-29, a well-defined cartilaginous skeleton is present in the limb, except for the most distal phalanges, which are in course of formation, and, as described below, Bmp-2 and Op-1 genes are expressed in association with the differentiating perichondrium. When the beads were implanted at these stages either in the interdigits or at the distal tip of digit III, regional thickening of the cartilages and alterations of the joints were very intense and truncation affected only the most distal phalanges of the digits in a stage-dependent fashion. The pattern of these skeletal alterations was dependent on the zone of location of the bead and showed differences with the type and dose of BMP. When beads bearing 0.5 or 2 mg/ml of BMP-2 were...
were implanted in the third interdigit, digits III and IV were always abnormal \( (n=37) \). As shown in Fig. 4B-D, the digits were characterized by the presence of phalanges with abnormal morphology accompanied by alterations in the arrangement of the joint interzone (70% of the altered digits) or by joint fusion (30% of the altered digits). The morphology of the altered interphalangeal joints was variable, including the appearance of oblique (Fig. 4D) or ‘Y’-shaped articular surfaces or the formation of a small articular cartilage intercalated in the joint (Fig. 4B). In contrast with these alterations caused by BMP-2, when OP-1-beads bearing a concentration of 1 or 0.5 mg/ml were implanted in the third interdigit, digits III and IV exhibited in all cases \( (n=30) \) a morphology characterized by the absence of interphalangeal joints (Fig. 4E). Lateral excrescences of the cartilages protruding towards the zone of location of the bead were also observed when the OP-1-beads were implanted at stages 27 or 28 (not shown).

When BMP-beads were implanted at the tip of the growing digits, the resulting altered digit bifurcated distal to the bead (Fig. 4F,G). With both BMP-2 and OP-1-beads, the phalange associated with the bead exhibited a dramatic increase in radial diameter (Fig. 4F,G). When OP-1-beads were employed, joint inhibition in the treated digit was a constant finding (Fig. 4G; \( n=20 \)) that was not observed following implantation of BMP-2-beads (Fig. 4F; \( n=16 \)). It should be mentioned that digit bifurcation without any other alteration was also observed in a reduced number of control limbs in which a PBS-bead was implanted at the distal tip of the growing digit (see details in Gañán et al., 1996).

During stages 30-31, all the phalangeal elements of the digits are formed and undergo differentiation into a central diaphyseal region formed by prehypertrophic cartilage and the epiphyses and metaphyses containing rounded and flattened chondrocytes, respectively. The intensity and the incidence of digital alterations following implantation of BMP-beads in the interdigital regions at these stages was reduced drastically (only one digit altered in 6 experimental limbs treated with BMP-2-beads at stage 31 and two out of 8 limbs treated with OP-1). This reduction was observed even implanting two beads bearing high concentration of BMPs in the same interdigit.

**In situ hybridization studies**

In view of the contrasting effects of OP-1 and BMP-2-beads on joint formation, we analyzed the expression of these genes in the course of digit morphogenesis in an attempt to find molecular evidence for the role of these BMPs in joint formation. In addition to the characteristic pattern of expression of \( Op-1 \) in the interdigital regions and subridge mesoderm, RNA transcripts of this gene were abundant in the perichondrium and developing tendons (Fig. 5). The perichondrial domain of \( Op-1 \) expression followed a proximodistal sequence and exhibited characteristic interruptions in the zones of formation of the metatarsophalangeal and interphalangeal joints (Fig. 6A). This pattern of expression contrasted with that of BMP-2. The expression of \( Bmp-2 \) in the developing chick limb bud has been previously studied up to stage 30 when the interdigital regions exhibit an intense expression of this gene (Francis et al., 1994). Here, we extended the study of \( Bmp-2 \) expression until stage 34 and, as shown in Fig. 6B, transcripts of this gene were detected in the articular interzones of the developing digits. In addition, a weak BMP-2 gene expression is also detected along the contours of the developing phalanges and at the distal tips of the digits (Fig. 6B).
The expression of ck-erg was studied in the treated digits due to its specific expression in developing joints (Gañan et al., 1996). In the course of normal development (Fig. 7A), ck-erg is first expressed in the differentiating distal tip of digits. As the prechondrogenic condensation differentiates, ck-erg is down-regulated in the phalangeal cartilage and its expression become restricted to the zones of joint formation. Following implantation of OP-1-beads in the interdigital regions (Fig. 7B), ck-erg transcripts remained identifiable at the distal tip of the growing digits and in the joints formed prior to the implantation of the bead (metatarsophalangeal), but no expression was detectable in the regions where the interphalangeal joints should have formed. In the embryos treated with BMP-2-beads, ck-erg expression in the joints was considerably reduced but the articular regions were identifiable. In addition, as can be seen in Fig. 7C, abnormalities in the articular domain of ck-erg consistent with the morphological alterations of the joints detectable by Alcian green staining were also observed.

The molecular characteristics of the abnormal cartilages induced by BMP-beads were explored analyzing the pattern of expression of Ihh in the developing digits following the implantation of the BMP-beads in the interdigital spaces or at the tip of digit III. Ihh constitutes a specific marker of the prehypertrophic cartilage with a proposed pivotal role in the control of growth of the developing long bones (Vortkamp et al., 1996). In the stages covered by this study, Ihh exhibit a short domain of expression in the middle of each developing phalange (Fig. 7D). Alterations in the pattern of expression of this gene were detected 30 hours after the implantation of the beads. Following the implantation of OP-1-beads in the interdigital spaces, the characteristic digits lacking interphalangeal joints showed a single and extended domain of Ihh expression which has its central region close to the zone of implantation of the bead (Fig. 7E). When the beads bearing either OP-1 or BMP-2 were implanted at the tip of the developing digits, the domain of Ihh of the most proximal phalanges was conserved but the distal region of the digits exhibited a single domain of Ihh expression located around the bead (Fig. 7F).

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**Fig. 6.** Illustrations showing the pattern of Op-1 (A) and Bmp-2 (B) gene expression in two consecutive sections of digits III and IV at stage 35. Note the intense expression of Op-1 in the perichondrium with discontinuities at the level of the developing joints (*). Bmp-2 expression is above background at the outer surface of the phalanges and exhibit linear domains of expression in the joint interspaces (arrows).

**Fig. 7.** Illustrations showing the pattern of expression of ck-erg (A-C) and Ihh (D-F) gene expression in the developing normal (A,D) and BMP-treated (B,C,E,F) digits. (A) The normal pattern of ck-erg expression in the distal growing region and developing joints of digit II at stage 32. (B) ck-erg expression in digits III and IV of an experimental autopod 40 after the implantation of a OP-1-bead (+) in the third interdigit. Both digits lack interphalangeal joints and their corresponding ck-erg domains. (C) Detailed view of digit IV showing a U-shaped articular domain of ck-erg expression (arrow) following implantation of a BMP-2-bead in the third interdigit at stage 29. Note the continuity between phalanges 1 and 2 laterally to the disrupted joint (the boundary of the cartilage is indicated by dashed lines). (D) Ihh expression in a consecutive section of the digit showed in A. Note the labeling distributed in the diaphysis of the developing phalanges. (E) Ihh expression in a section similar to that shown in B. Note the Ihh exhibits a single and long domain of expression in both experimental digits. The bead is indicated by (*). (F) The pattern of Ihh expression following implantation of a BMP-2-bead at the tip of digit III. Note the presence of a proximal domain of Ihh expression in diaphysis of the first phalange and a distal domain in the cartilage surrounding the bead (arrow). The boundary of the digit skeleton is indicated by dashed lines.
DISCUSSION

BMPs and programmed cell death

The occurrence of well-defined areas of mesodermal cell death is a characteristic event of the developing limb bud of amniota embryos (Hurle et al., 1995). In the chick limb bud, programmed cell death is found in the following areas: anterior and posterior necrotic zones (ANZ and PNZ), which remove the anterior and posterior marginal mesoderm of the early limb bud; the opaque patch, which removes the central mesenchyme of the limb delimited by the condensations of the two skeletal pieces of the zeugopod (tibia/fibula; ulna/radius) and the areas of interdigital cell death (INZs), which remove the undifferentiated mesenchyme located between the developing digital rays (Hinchcliffe, 1982; Hurle et al., 1996). INZs are observed in all amniotas and there is increasing evidence that they are instructively controlled by BMPs. In chick (Francis et al., 1994; Francis-West et al., 1995) and mouse embryos (Wozney et al., 1993; Lyons et al., 1995; Luo et al., 1995), Bmp-2, Bmp-4 and Op-1 genes are expressed in the interdigital mesoderm before and during cell death. Experiments designed to block BMP-signaling in chick embryos inhibit interdigital cell death and result in the formation of webbed digits (Zou and Niswander, 1996). We have previously demonstrated that exogenous BMP-4 triggers apoptosis in the interdigital mesoderm (Gañan et al., 1996). Here we extend this observation to BMP-2 and OP-1.

The present results show that both BMP-2 and OP-1 induce cell death in the undifferentiated limb mesoderm of the early limb bud. We have also observed the same effect for BMP-4 (unpublished observations). These findings are compatible with a role for these BMPs as physiological signals for ANZ and PNZ but their involvement in opaque patch is unlikely. In the case of ANZ and PNZ, the dying cells are located within the domain of expression of BMP genes, though these domains have a wider temporal and spatial distribution (Francis et al., 1994; Francis-West et al., 1995). In addition, the cell death induced by implantation of BMP-beads in the anterior and posterior margins of the limb resembled the distribution of ANZ and PNZ and had only deleterious effects on the developing skeleton when the beads were implanted prior to the formation of the skeletal condensations. In contrast, neither BMP-4 nor BMP-2 or OP-1 are expressed in the opaque patch (Francis et al., 1994; Francis-West et al., 1995) and the distribution of cell death caused by implanting beads into the dorsal mesoderm of the limb bud lacked any similarity to this area of physiological cell death. The lack of correlation between opaque patch and the area of cell death induced by BMPs is not surprising finding. The opaque patch eliminates cells of the central core of the limb that have initiated chondrogenic differentiation and it is very likely that other factors control this area of cell death (Kochhar and Agnish, 1977). This interpretation is also supported by the lack of Msx-2 gene expression in the opaque patch as distinct to the other areas of limb mesodermal cell death (Coelho et al., 1991) and by the differential effects of retinoic acid on mouse limb development which leads to syndactyly (defective INZs) and severe mesomelia secondary to a massive increase of cell death in the opaque patch (Alles and Sulik, 1989).

There is however an important question in relation to the hypothetical role for BMP-2 and OP-1 as apoptotic signals for the undifferentiated mesoderm. How can this function be reconciled with their role in the signalling pathway that controls the anteroposterior axis of the limb (Francis-West et al., 1995; Duprez et al., 1996b) and with their physiological expression in the AER of the early limb bud (Francis et al., 1994; Francis-West et al., 1995)? One likely explanation for this apparent contradiction is the functional association of BMPs in these processes with members of the FGF family, which are potent survival signals for the undifferentiated mesoderm (Macias et al., 1996). In the case of the polarizing signal pathway, Bmp-2 occupies an intermediate position between Sonic hedgehog and Fgf-4 (Duprez et al., 1996c). In the early limb bud, several members of the FGF family, including Fgf-2, -4 and -8 are coexpressed with Bmp-2 and Op-1 in the AER. According to this interpretation, the undifferentiated limb mesoderm would be under the balanced influence of death and survival signals. Outgrowth would take place when the proliferating influence of FGFs predominates over the apoptotic influence of BMPs. On the contrary, cell death would result when BMP-mediated stimuli predominate over FGF-mediate stimuli. For example, INZ appears when the AER ceases functioning and ANZ and PNZ when the undifferentiated mesoderm leaves the area of influence of the active AER. This interpretation is in agreement with previous experimental approaches on the effect of FGFs and BMPs on limb outgrowth (Niswander and Martin, 1993) and explains why the domains of expression of BMPs in the developing limb bud exceed the extension of the areas of physiological cell death. In addition, the occurrence of polydactylly in BMP-7-deficient mice (Hofmann et al., 1996) fits very well with our hypothesis.

BMPs and skeletogenesis

Our results rule out a possible function of these BMP-2 and OP-1 in the establishment of the limb chondrogenic aggregates and provide evidence for a role of these BMPs in controlling the growth and differentiation of the cartilages and the formation of the joints. These conclusions are supported both by the pattern of expression of these BMPs and by the skeletal alterations caused after the implantation of BMP-beads in the developing limb.

Although the undifferentiated chick limb mesoderm has a high chondrogenic potential under both ‘in vivo’ (Hurle et al., 1989; Gañan et al., 1996) and ‘in vitro’ conditions (Ahrens et al., 1977), BMP-beads failed to induce ectopic cartilages in the chick limb bud. This finding is in agreement with in vitro studies by Roark and Greer (1994) and contrast with the characteristic effect of BMPs in promoting ectopic chondrogenesis and bone formation in adult rats. In this regard, it is worth mentioning previous evidence suggesting that the establishment of the prechondrogenic aggregates in the chick limb is controlled by members of the TGF-β family (Gañan et al., 1996).

In contrast to the lack of a chondrogenic promoting effect on the undifferentiated mesenchyme, our study shows that the differentiating cartilages undergo dramatic radial growth under the influence of both BMP-2 and OP-1. A similar finding has recently been reported by Duprez et al. (1996a) who induced overexpression of Bmp-4 and Bmp-2 genes in the limb bud by means of retroviral vectors. These authors showed that the increase in thickness of the cartilages is accompanied by cartilage dysplasia. Here we show that the cartilage dysplasia involves alterations in pattern of expression of Ihh. In addition, we show that the ability of BMP-2 and OP-1 to induce radial
growth of the cartilage reaches its highest potential simultaneously with the onset of Bmp gene expression in the perichondrium and is reduced drastically when the cartilaginous tissue initiates differentiation into hypertrophic cartilage. Vortkamp et al. (1996) have recently found that Ihh gene is expressed in the prehypertrophic chondrocytes of the developing limb cartilages defining the future diaphysis of the bone. In the course of development, the hypertrophic cartilage differentiates in the middle of the diaphysis displacing the Ihh-expressing prehypertrophic cartilage towards the metaphyses. This process is then followed by ossification of the hypertrophic cartilage. According to Vortkamp et al. (1966), Ihh expression in the prehypertrophic cartilage in association with the parathyroid hormone-related protein (PTHrP) gene expression in the perichondrium (Lanske et al., 1996) regulate the growth of the limb skeleton controlling the differentiation of the prehypertrophic chondrocytes into hypertrophic cartilage. This mechanism provides a consistent explanation for the growth of the skeletal pieces from the period of formation of the hypertrophic cartilage and subsequent ossification. Our findings suggest that BMPs control the growth and morphogenesis of the cartilages prior to these stages promoting the differentiation of the immature cartilage into prehypertrophic cartilage. This function might account for the establishment of the epiphyses and diaphysis in the cartilaginous anlage of the long bones prior to the onset of ossification. Although Ihh is upregulated in the zones of application of BMP-beads, the 30 hours period detected between the implantation of the beads and the expression of Ihh does not rule out the possibility that this was an indirect effect associated with a primary action of BMPs on previous steps of the differentiation of the immature cartilage.

Our findings are also indicative of a role for BMP-2 and OP-1 in joint formation. In the course of skeletal development, prechondrogenic condensations appear initially as continuous and differentiation of joints develop in regions lacking signals that prior to these stages promoting the differentiation of the immature cartilage into prehypertrophic cartilage. This function might account for the establishment of the epiphyses and diaphysis in the cartilaginous anlage of the long bones prior to the onset of ossification. Although Ihh is upregulated in the zones of application of BMP-beads, the 30 hours period detected between the implantation of the beads and the expression of Ihh does not rule out the possibility that this was an indirect effect associated with a primary action of BMPs on previous steps of the differentiation of the immature cartilage.

Our findings are also indicative of a role for BMP-2 and OP-1 in joint formation. In the course of skeletal development, prechondrogenic condensations appear initially as continuous and the joints develop as a specialization of these condensations. In the avian embryo, these events are accompanied by a redistribution of the expression of the transcriptional factor ck-erg to the developing joint region following an initial expression throughout the prechondrogenic blastemas (Gafan et al., 1996). One possible explanation for these molecular events is that joints develop in regions lacking signals that promote down-regulation of ck-erg and differentiation of cartilage. The present study provides evidence for a specific role of OP-1 in this process. Op-1 gene was found to be highly expressed in the differentiating perichondrium of the cartilages from stage 29, showing characteristic interruptions in the zones of joint formation. Consistent with this pattern of expression, implantation of OP-1-beads at these stages down-regulated ck-erg gene expression in the joint region and was followed by full inhibition of joint formation. The most likely interpretation for this finding is that the exogenous protein diffusing from the bead interferes with the physiological discontinuities in Op-1 expression in the perichondrium, leading to the formation of single-boned digits.

The possible role of BMP-2 in joint formation is more obscure. In contrast with Op-1, Bmp-2 transcripts are expressed in the interarticular spaces suggesting a role in joint cavitation. In accordance with this pattern of expression, implantation of BMP-2-beads caused a high incidence (70%) of interphalangeal joints with anomalous articular interspaces. However, we have also observed that implantation of BMP-2-beads caused a relatively high incidence of joint fusion in agreement with previous experiments by Duprez et al. (1996a). These apparently contradictory findings might be a consequence of an OP-1-like effect of BMP-2 at the dose used in our experimental conditions. In fact, BMP-2 and OP-1, in addition to their high degree of homology, may interact with the same receptors (Liu et al., 1995) and form functional heterodimers (Sampath et al., 1990; Hazama et al., 1995). Alternatively, joint fusion by BMP-2 might be a side effect secondary to its growth-promoting influence on the differentiating cartilages.

A final observation of this study was the occurrence of a domain of expression of Op-1 in the developing tendons of the chick leg. In the last few years, a number of genes have been identified as being expressed in association with the formation of the long tendons of the autopod (Oliver et al., 1995; Patell et al., 1996) but their roles remain uncertain. We cannot speculate on the function of OP-1 in tendon formation since, in our study, the implantation of BMP-beads in the autopod did not cause tendon alterations.

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Note added in proof

The pattern of BRK-1, BRK-2 and BRK-3, BMP receptor gene expression during chick limb development has been published by Kawakami et al. (1996) while this manuscript was being reviewed and their findings are in agreement with our interpretation of a double role for BMPs in cell death and cartilage growth and differentiation.