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Role of TGFβs and BMPs as signals controlling the position of the digits and the areas of interdigital cell death in the developing chick limb autopod

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SUMMARY

The establishment of the digital rays and the interdigital spaces in the developing limb autopod is accompanied by the occurrence of corresponding domains of expression of TGFβs and BMPs. This study analyzes whether these coincident events are functionally correlated. The experiments consisted of local administration of TGFβ-1, TGFβ-2 or BMP-4 by means of heparin or Affi-gel blue beads to the chick limb autopod in the stages preceding the onset of interdigital cell death. When beads bearing either TGFβ-1 or -2 were implanted in the interdigits, the mesodermal cells were diverted from the death program forming ectopic cartilages or extra digits in a dose- and stage-dependent fashion. This change in the interdigital phenotype was preceded by a precocious ectopic expression of *ck-erg* gene around the bead accompanied by down-regulation of *bmp-4*, *msx-1* and *msx-2* gene expression. When BMP-beads were implanted in the interdigital spaces, programmed cell death and the freeing of the digits were both accelerated. Implantation of beads bearing BMP-4 at the tip of the growing digits was followed by digit bifurcation, accompanied by the formation of an ectopic area of cell death resembling an extra interdigit, both morphologically and molecularly. The death-inducing effect of the BMP beads and the chondrogenic-inducing effect of the TGFβ beads were antagonized by the implantation of an additional bead preabsorbed with FGF-2, which constitutes a signal characteristic of the progress zone. It is concluded that the spatial distribution of digital rays and interdigital spaces might be controlled by a patterned distribution of TGFβs and BMPs in the mesoderm subjacent to the progress zone.

Key words: TGFβ, apoptosis, growth factor, Msx-1, Msx-2, ck-erg, BMP-4, cell death, chick, limb bud, autopod

INTRODUCTION

The developing limb bud is a relatively simple structure consisting of mesoderm covered by ectoderm, which grows at the lateral surface of the embryonic body wall. Outgrowth of the limb is controlled by the apical ectodermal ridge (AER), a specialized thickened region of the ectoderm along the rim of the limb bud. The AER maintains the underlying mesoderm in an undifferentiated and proliferating state, and formation of the skeletal prechondrogenic condensations occurs when these undifferentiated mesodermal cells leave the zone of influence of the AER (Progress zone; Summerbell et al., 1973).

In the last few years knowledge of the molecular basis for the regional specification of the early limb bud tissues has advanced dramatically. However, little is known about how the positional signals are later translated into particular skeletal morphologies (see Tickle, 1995). The formation of the digital rays in the latest stages of limb morphogenesis is a good example of this problem. By these stages, positional specification has been established and in the distal region of the limb (autopod) the cells leaving the progress zone have two possible fates. In the digit-forming regions, the mesenchymal cells undergo chondrogenic differentiation and form the cartilaginous skeleton of the digits. In contrast, the interdigital mesenchyme is maintained for some time in an undifferentiated state and eventually undergoes massive cell death by apoptosis (Garcia-Martinez et al., 1993). Despite these different fates, in vivo and in vitro experimental analyses have revealed that prior to cell death the interdigital mesoderm has the potential to form extra digits (Hurle et al., 1989, 1991; Lee et al., 1994). The potential of the interdigits to form digits under experimental conditions indicates the existence of signals in the autopod that control the position of the digital rays and the alternating pattern of digits and interdigits.

Members of the transforming growth factor β superfamily are good putative candidate signals for the specification of the digital and interdigital regions, as can be deduced from their pattern of expression in the developing limb and from their biological effects when administered exogenously to the embryo or to embryonic cell cultures. Several components of the TGFβ family (one of the groups of the TGFβ superfamily), which are highly homologous, show specific patterns of expression in the prechondrogenic condensations, including the digital rays, of mouse (TGFβ-2; Millan et al., 1991) and chick (TGFβ-3;

Roark and Greer, 1994) limb buds, and in high density chick limb mesodermal cell cultures (TGFβ-1-3; Leonard et al., 1991; Roark and Greer, 1994). Furthermore, these growth factors are intensely chondrogenic when added to limb mesenchymal cell cultures (Kulyk et al., 1989; Schofield and Wolpert, 1990; Leonard et al., 1991; Roark and Greer, 1994) and cause skeletal malformations when they are administered exogenously to early chick limb buds (Hayamizu et al., 1991).

In contrast to the digital rays, the interdigital spaces exhibit a precise pattern of expression of members of the BMP family of growth factors (also belonging to the TGFβ superfamily). The expression of BMP-2, BMP-4 (Lyons et al., 1990; Francis et al., 1994; Wozney et al., 1993) and OP-1 (BMP-7; Helder et al., 1995; Luo et al., 1995) has been demonstrated in the interdigital spaces of the developing autopod preceding the establishment of the areas of interdigital cell death (INZ). Furthermore, at least one of these growth factors (BMP-4) triggers apoptosis in other models of embryonic programmed cell death (Graham et al., 1994).

These observations have led us to hypothesize that the formation of the digital and interdigital regions might be established by the influence of the proximal mesoderm on the cells leaving the progress zone, mediated by TGFβs and by BMPs, respectively. To test this hypothesis we have analyzed the effect of implanting beads, soaked either in phosphate-buffered saline (PBS) or in solutions of the different growth factors, under the progress zone of the developing chick autopod. Our results show that beads soaked in either TGFβ-1 or TGFβ-2 and implanted in the interdigital region inhibit cell death and lead to the formation of interdigital extra digits. In contrast, beads soaked in BMP-4 accelerate interdigital cell death when implanted in the interdigital region, and when implanted at the tip of the growing digits lead to digit bifurcation accompanied by the formation of an ectopic area of cell death. The effects of these growth factors were inhibited by implanting an additional bead soaked in FGF-2. Changes in the pattern of expression of *Msx*, *bmp-4* and *ck-erg* genes following implantation of the beads were also consistent with a role for these growth factors in the specification of the digital and interdigital autopodial regions.

MATERIALS AND METHODS

This study was carried out on Rhode Island chick embryos ranging from day 5 to day 7 of incubation (stages 27-31, Hamburger and Hamilton, 1951).

Experimental manipulation and morphological analysis of the limbs

The eggs were windowed at the desired stages and the right leg bud was exposed. Heparin or Affi-gel blue beads soaked in PBS, or in the appropriate growth factor solution as described below, were implanted into the mesenchyme subjacent to the progress zone of the third interdigital space or at the tips of digits III or IV. The embryos were then returned to the incubator and killed at different intervals.

The morphology of the limbs was studied in specimens fixed in 5% trichloroacetic acid and stained for cartilage with alcian green.

Preparation of beads

Affi-gel blue beads (Bio-Rad), diameter 50-80 μm, or heparin acrylic beads (Sigma, H5263), diameter 100-160 μm, were used. The beads were washed in PBS and then incubated for 1 hour at room temperature (20°C) in different concentrations of growth factor. Recombinant human TGFβ-1 and TGFβ-2 (both from R&D Systems) diluted in PBS were used at concentrations of 2, 10 and 50 μg/ml, using either Affi-gel blue or heparin beads as carriers. Recombinant human BMP-4 (from Cambridge Genetic Institute, MA) diluted in PBS was used at concentrations of 10, 100 and 160 μg/ml. Recombinant human FGF-2 (R&D Systems) diluted in PBS at 1 μg/μl was administered in Affi-gel blue beads. Control beads were incubated in PBS.

Detection of cell death

The distribution of cell death was analyzed by vital staining with neutral red or by TdT-mediated dUTP nick end labeling (TUNEL).

For neutral red vital staining, the limbs were excised from the embryos and 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 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 in paraffin sections of paraformaldehyde-fixed specimens using the in situ cell death detection fluorescein kit, according to the directions of the manufacturers (Boehringer).

Measurement of cell proliferation

Cell proliferation in the subridge mesoderm was analyzed by anti-bromodeoxyuridine immunolabeling at 8, 20 and 30 hour intervals after implantation of TGFβ beads. For this purpose 100 μl of bromodeoxyuridine (BrdU) solution (100 μg/μl) was pipetted directly over the limb. After 30 minutes of further incubation, the embryos were fixed in 70% ethanol. The autopod was then dissected free, dehydrated and embedded in paraffin wax. Immunocytochemistry to detect BrdU incorporation into DNA was carried out in tissue sections according to the directions of the manufacturers (Becton Dickinson) using anti-BrdU and fluorescein-conjugated secondary antibody.

In situ hybridization

Changes in the expression of genes in relation to limb mesenchyme differentiation were explored by in situ hybridization. Hybridization with *Msx1* (obtained from B. Robert), *Msx2* (obtained from A. Kuroiwa), *ck-erg* (Dhordain et al., 1995) and *bmp-4* (obtained from J.C. Izpisua-Belmonte) probes was performed in paraffin-embedded tissue sections 8, 20,30 and 48 hours after the implantation of the beads, as described by Ros et al. (1994).

RESULTS

Effects of implanting control PBS beads

Limbs implanted with control heparin or PBS beads provided evidence for an influence of the distal differentiating mesoderm on the fate of the cells leaving the progress zone. Implantation of heparin beads in the interdigital spaces was followed in all cases (n=150) by normal development of the autopod (Fig. 1A). However, implantation of control heparin beads at the digital tips was followed in 80% of the cases by a variable degree of digit bifurcation (n=67). These digital alterations ranged from a thickening or duplication of the digit skeleton that was restricted to the zone located immediately distal to the bead (Fig. 1B), which was observed in 76% of the altered limbs, to a full duplication of the digital ray distal to the bead (Fig. 1C,D), observed in 24% of the altered limbs.

TGFβ beads induce a stage- and dose-dependent chondrogenic response in the interdigital mesoderm

Implantation of beads soaked in TGFβ-1 or TGFβ-2 in the third

interdigit of chick leg buds was followed by ectopic interdigital chondrogenesis. The results were identical for TGFβ-1 and TGFβ-2, using both Affi-gel blue and heparin beads as carriers for these growth factors. The extent and the pattern of the chondrogenic response was stage- and dose-dependent. The most significant results were obtained when the beads were implanted at stage 29. This stage precedes the onset of programmed cell death by 24 hours and constitutes a period of maximum development of the interdigital space.

At stage 29, implantation of a bead soaked in 50 μg/ml TGFβ was followed by truncation of digits III and IV and massive interdigital chondrogenesis in 100% of the experimental embryos (n=44). Fig. 1E illustrates the most characteristic appearance of the autopod 3 or 4 days after bead implantation. Digits III and IV appeared truncated, with only two or three recognizable proximal phalanges, and were fused distally with a cartilaginous rod encompassing the most marginal segment of the interdigital space. In addition to this abnormal digital morphology, a large mass of cartilaginous tissue lacking any identifiable morphology filled the distal part of the interdigit. Similar results were obtained in experiments performed at stages 27 and 28, although the truncation of the digits was more proximal. Implantation of the TGFβ beads later than stage 29 had no effect on the development of the adjacent digits and the incidence of interdigital chondrogenesis dropped dramatically compared to experiments performed at earlier stages. Ectopic chondrogenesis was found in 9 out of 21 (42%) of the experimental embryos treated at stage 30, and in only 2 out of 20 (10%) at stage 31. In all these cases the ectopic cartilage was small and appeared isolated in the interdigital space (Fig. 1F).

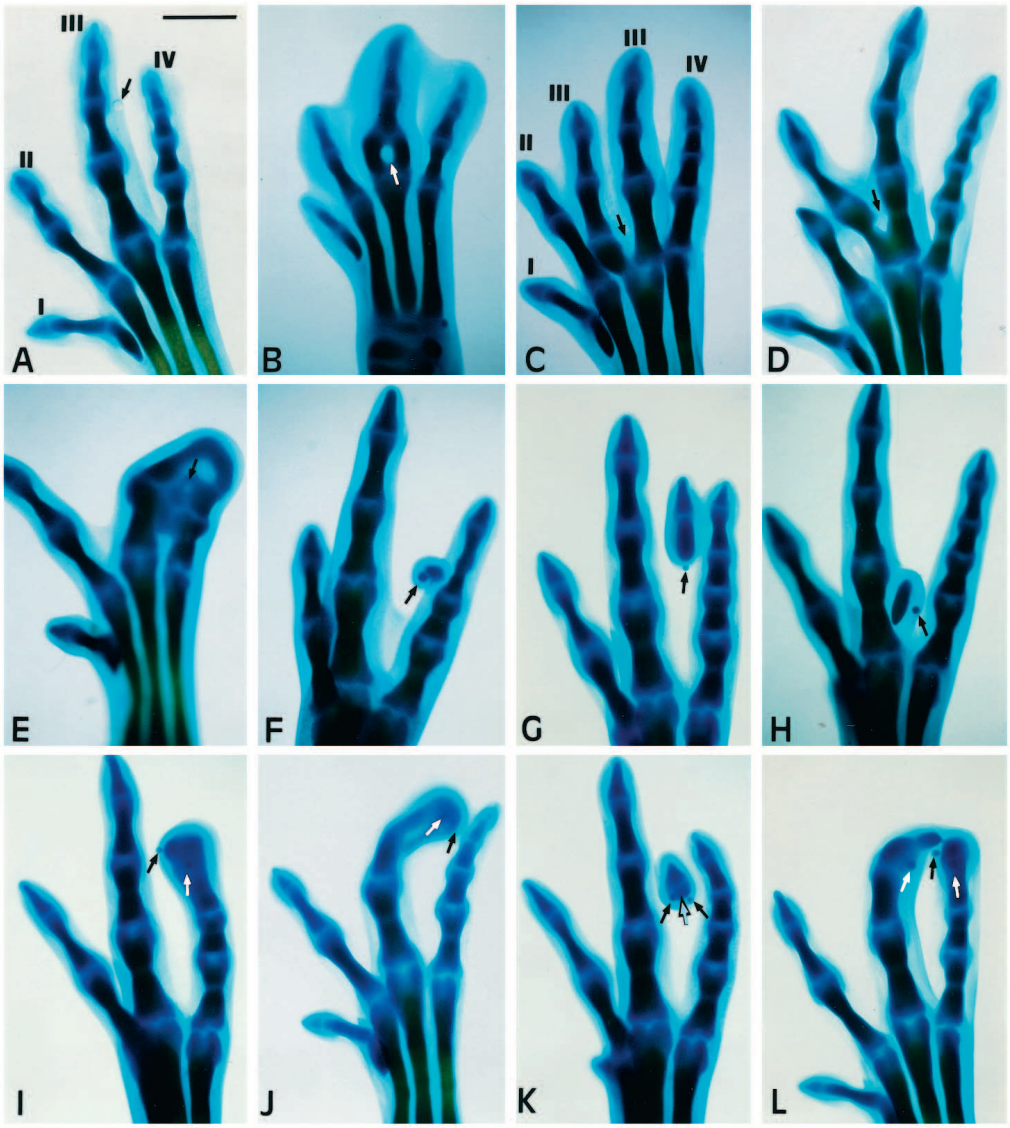


Fig. 1. Whole-mount cartilage-stained limb autopods at days 7.5-9 of incubation, illustrating the skeletal pattern of the digits after the different experimental manipulations performed in this study. Arrows show the location of the beads, although in some cases they are out of the plane of focus of the photographs. The digit number is indicated in A and C, for orientation. Bar, 1 mm. (A-D) Experiments using heparin acrylic beads as barriers implanted under the progress zone at stage 27-28. In (A), the bead was implanted in the third interdigit and the foot morphology is normal. In (B-D), the bead was implanted at the tip of digit III. (B) A duplication restricted to the first phalange. (C) A full duplication of digit III. (D) A duplication of digit III from the first phalange. (E) The typical appearance of the autopod after implanting a 50 μg/ml TGFβ bead in the third interdigit at stage 29. Note the intense ectopic chondrogenesis in the interdigit around the bead and the fusion of digits III and IV. (F) The appearance of a small ectopic interdigital cartilage after implanting a 50 μg/ml TGFβ bead at stage 31. (G) The formation of an ectopic extra digit after implantation of a 10 μg/ml TGFβ bead in the third interdigit at stage 29. (H) The formation of a proximal elongated interdigital cartilage after implantation of a 10 μg/ml TGFβ bead in the third interdigit at stage 27. (I-L) Autopodial alterations after implanting 50 μg/ml TGFβ beads and 1 μg/μl FGF-2 beads side by side in the third interdigit at stage 29. The locations of TGFβ beads are indicated with white arrows, and those of the FGF beads with black arrows. Comparison of the morphology of these limbs with those shown in E indicates the modifications caused by the addition of FGF beads. (I) A TGFβ bead was implanted close to digit IV and the FGF bead close to digit III and only digit IV shows excess of chondrogenesis. (J) The TGFβ bead was implanted close to digit III, and the FGF bead close to digit IV, and again the digit altered is only the one close to the TGFβ bead. (K) FGF beads were implanted close to each digit and a TGFβ bead was implanted midway in the interdigit. In this case a small extra digit is formed and digits III and IV are normal. (L) TGFβ beads were implanted close to digits III and IV and the FGF bead was implanted midway in the interdigit. In this case both digits are altered, but excessive chondrogenesis is arrested in the middle zone of the interdigit surrounding the FGF bead.

At the lower concentration of 10 μg/ml TGFβ, the incidence of interdigital chondrogenesis in the experimental embryos was still very high (77%; n=22) when the bead was implanted at stage 29. However, at this concentration of growth factor, instead of truncation of digits III and IV and massive interdigital chondrogenesis, the implantation of the bead was followed in all cases by the formation of an extra digit consisting of two distal phalangeal elements (Fig. 1G). This digit-forming response of the interdigit was also stage-dependent. When the beads were implanted at stage 28, ectopic chondrogenesis was found in 83% of the embryos but an extra digit was only observed in half of these embryos. In the other half, the ectopic cartilage appeared as an elongated rod occupying a proximal position in the interdigit, thus resembling an extra digit truncated distally (Fig. 1H). At stage 27, the incidence of ectopic chondrogenesis was 66%, and the ectopic cartilages always appeared as elongated rods located in the most proximal part of the interdigit.

At concentrations of 2 μg/ml TGFβ, the incidence of chondrogenesis at stage 29 dropped to 50% and only half of the ectopic cartilages had a recognizable digit-like appearance.

FGF beads restrict the chondrogenic response of the interdigital mesoderm induced by TGFβ beads

Possible opposing effects of FGF and TGFβ on the interdigital mesenchyme of the chick were explored by implanting different combinations of FGF beads and TGFβ beads in the third interdigital space at stage 29. In all these experiments, the beads were implanted side by side in the distal mesenchyme of the interdigit. FGF beads were always preabsorbed with 1 μg/μl FGF-2. TGFβ beads were preabsorbed with TGFβ-1 at the different concentrations described above. The results of these experiments showed that the FGF reduced the extent and incidence of interdigital chondrogenesis and inhibited the truncation of the adjacent digits induced by high concentrations of TGFβs. This inhibition was never seen in controls where the FGF bead was replaced by a bead soaked only in PBS, thus ruling out a possible nonspecific effect of the implantation of additional beads.

The most indicative results were obtained when FGF beads were implanted in combination with TGFβ beads preabsorbed with 50 μg/ml of growth factor. Implantation of two beads, one TGFβ bead and one FGF bead, was always followed by abnormal development of the zone located close to the TGFβ bead and normal development of the zone located close to the FGF bead. As can be seen in Fig. 1I,J, the digit close to the TGFβ bead appeared truncated and its distal phalangeal element was abnormally thickened due to the spreading of chondrogenesis towards the adjacent interdigital mesenchyme. In contrast, the digit located close to the FGF bead followed normal development. Implantation of one central TGFβ bead and two lateral FGF beads was followed by normal development of digits III and IV and by formation of an ectopic extra digit in the central zone of the interdigital mesenchyme (Fig. 1K). Implantation of one central FGF bead and two lateral TGFβ beads was followed by alteration of both digits, but chondrogenesis of the interdigital mesenchyme was inhibited in the central zone of the interdigit, which was subjected to the influence of the FGF bead (Fig. 1L).

Inhibition of cell proliferation and interdigital cell death (INZ) by TGFβ beads

Cell proliferation as detected by immunolabeling of BrdU incorporation appeared inhibited in the mesenchymal tissue surrounding the bead 8 hours after its implantation (Fig. 2).

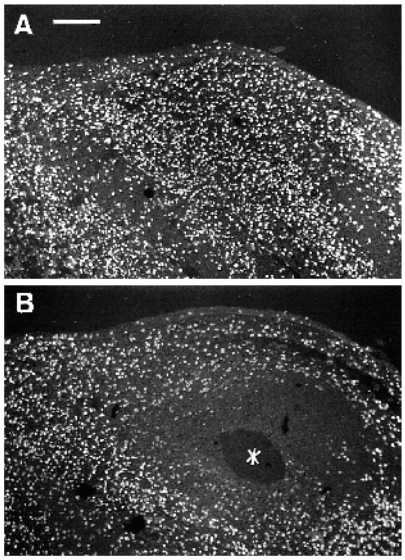


Fig. 2. Horizontal sections of control (A) and experimental (B) interdigits 8 hr after the implantation of a TGFβ bead at stage 29, showing the pattern of proliferation by immunofluorescent localization of Brdu incorporation. Note the absence of immunolabeled cells around the bead (*). Bar, 100 μm.

Analysis of the pattern of cell death by means of neutral red vital staining also revealed an intense inhibition of the INZ using both low (10 μg/ml) and high (50 μg/ml) concentrations of TGFβ-1 or -2. Fig. 3A,B shows the absence of cell death from the third interdigit at day 7.5 of incubation after implantation of a TGFβ bead at day 6 (stage 29). By day 8.5, when the zone of chondrogenesis associated with the bead was fully identifiable, a new region of cell death appeared in the mesenchymal cells surrounding the ectopic cartilage. This delayed cell death was not observed in the untreated contralateral limbs and was particularly intense in the mesenchyme located distally to the ectopic cartilage (Fig. 3C).

Changes in the expression of *ck-erg*, *bmp-4* and *Msx* genes precede inhibition of interdigital cell death and ectopic chondrogenesis induced by TGFβ beads

Changes in the expression of *ck-erg*, *bmp-4* and *Msx* genes preceded the inhibition of programmed cell death and the ectopic chondrogenesis of the interdigital tissue after administration of TGFβs.

Ck-erg is a member of the *ets* gene superfamily, which are transcriptional modulators (Dhordain et al., 1995). Expression of this gene in the embryonic chick has been studied up to day 6 of development, showing a precise domain of expression in the limb prechondrogenic aggregates (Dhordain et al., 1995). In this study we confirmed the presence of a strong signal for this gene in the prechondrogenic condensations of the developing digits (Fig. 4A). However, the observation of older embryos revealed that this domain of expression is transitory. When the epiphysis and the diaphysis of the phalangeal cartilages are established *ck-erg* expression becomes restricted to the developing joints and, with less intensity, to the perichon-

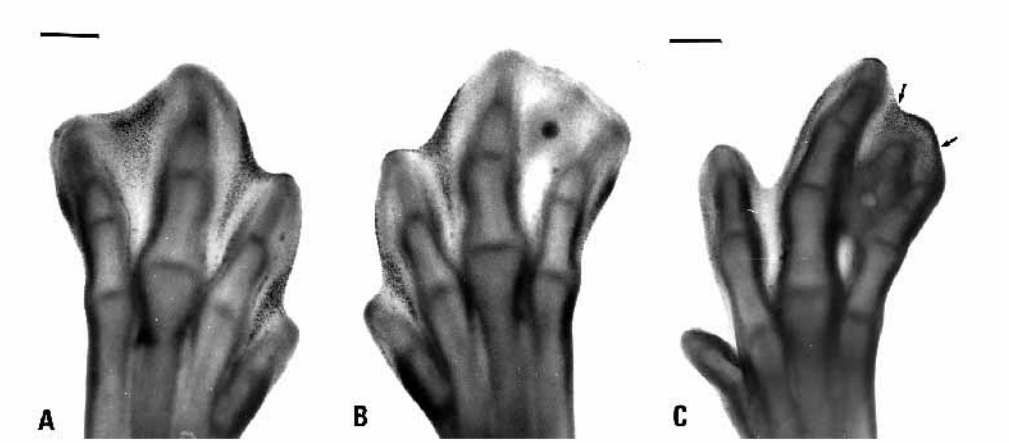
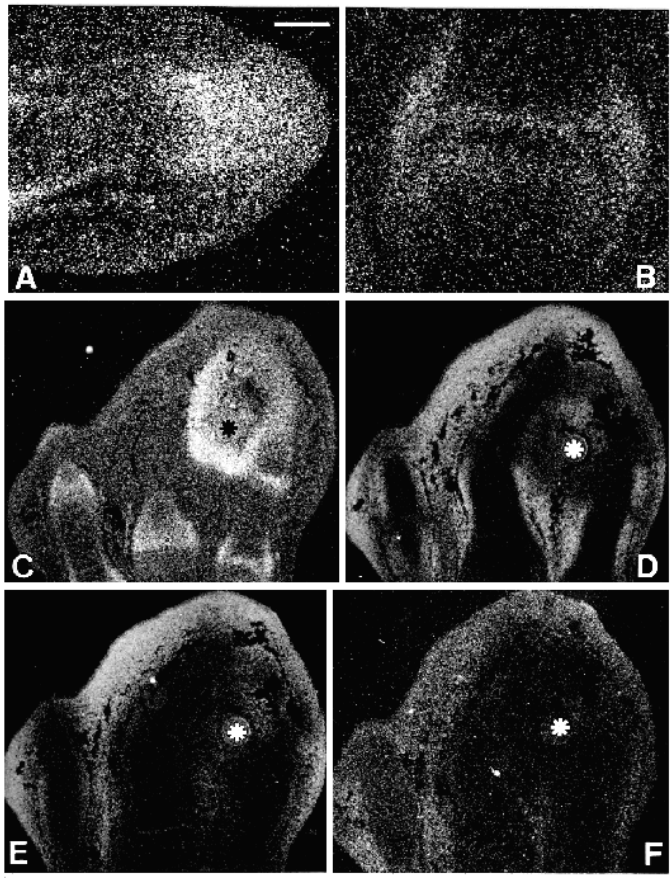


Fig. 3. Micrographs showing the pattern of interdigital cell death by means of vital staining with neutral red. (A and B) The left (control, A) and the right (experimental, B) limbs of the same embryo at day 7.5 of incubation. Note the absence of cell death in the third interdigit of the experimental limb (B) after implantation of a TGFβ bead at stage 29. (C) The pattern of cell death at day 8.5 of incubation in the limbs subjected to TGFβ treatment. Note the presence of an intense area of cell death (arrows) distally to the zone of ectopic chondrogenesis. At this stage, as can be seen for the first and second interdigits, the normal interdigital tissue has been almost removed in the normal limbs. Bar, 500 μm.

drium (Fig. 4A,B). In the experimental embryos a very intense area of *ck-erg* expression was detected in the interdigital mesenchyme 8 hours after implantation of the TGFβ beads (Fig. 4C). This domain of expression was maintained 20 and 30 hours after bead implantation, when the ectopic chondrogenic aggregate induced by TGFβs became detectable by alcian green staining.

Msx and *bmp-4* gene expression in the autopod has been described in detail in previous studies (see Ros et al., 1994 and Francis et al., 1994). At the stages studied here, *bmp-4*, *Msx-1* and *Msx-2* all exhibit a characteristic domain of expression in the undifferentiated distal mesoderm and in the interdigital spaces of the autopod. Implantation of TGFβ beads was followed by down-regulation of the expression of these genes in the interdigital mesenchyme surrounding the bead, which was detectable 8 hours after the implantation of the bead. As can be seen in Fig. 4D-F, the zone of inhibition of *bmp-4* and *Msx* gene expression closely paralleled the zone of ectopic *ck-erg* expression. It is also interesting to note that expression of these genes was maintained in the distal mesenchyme of the interdigit, corresponding with the zone of cell death present in the experimental limbs at day 8.5 of incubation.



BMP beads induce cell death by apoptosis in the undifferentiated mesoderm of the autopod

Implantation of beads preabsorbed with BMP-4 was followed by mesodermal cell death, whereas implantation of control beads soaked only in PBS was not. This death process was only induced in the undifferentiated mesodermal cells. The differentiating chondrogenic cells were always healthy even at the stage of prechondrogenic condensation, regardless of whether cells were very close to the bead or if the beads had been soaked in high concentrations of BMP-4. As in the normal areas of interdigital cell death, the dying cells induced by BMP administration showed intense positivity by TUNEL fluorescent labeling (Fig. 5).

Fig. 4. Expression pattern of *ck-erg* (A-C), *Msx-1* (D), *Msx-2* (E) and *bmp-4* (F) in tissue sections of the autopod. (A,B) The normal pattern of *ck-erg* expression at an advanced stage of digit development. A is a horizontal section of digit IV at day 8 of incubation (stage 33), showing positivity in the distal-growing phalange and in the perichondrium of the penultimate differentiating phalange. B illustrates the precise pattern of *ck-erg* expression in the articular surfaces and developing capsule of a metatarso-phalangeal joint at day 7.5 of incubation. Bar, 100 μm. (C-F) Consecutive sections of a experimental limb 8 hours after implantation of a TGFβ bead at stage 29, showing the expression of *ck-erg* (C), *Msx-1* (D), *Msx-2* (E) and *bmp-4* (F). Note the intense positivity for *ck-erg* in the interdigital mesenchyme surrounding the bead (*) and the decrease in *Msx-1*, *Msx-2* and *bmp-4* expression in comparison with the normal pattern of expression of these genes observed in the second interdigit (delimitated by digits II and III). Bar, 300 μm.

When the BMP beads were implanted in the interdigital position, a small area of cell death was clearly detected around the bead 10 hours after implantation of the bead (Fig. 6A,B). 20 hours after implantation of the bead, the area of cell death induced by this procedure occupied almost the entire interdigital region (Fig. 6C), resembling the normal distribution of the INZ but preceding it by up to 24 hours. As can be seen in Fig. 6C, the freeing of the digits was also accelerated in these experimental limbs.

When the BMP beads were implanted at the tip of the growing digits, cell death was induced in the mesoderm immediately distal to the bead in a fashion resembling an ectopic interdigit (Fig. 6D). The mesoderm proximal to the bead corresponding to the differentiating cells of the digital ray was never affected and in the course of development these always appeared bifurcated (Fig. 6E).

Implantation in the same interdigit of an FGF bead together with the BMP bead caused an intense reduction of the zone of cell death induced by the BMP bead (Fig. 6F). In accordance with this reduction in the extent of cell death, the regression of the interdigital tissue was also found to be partially arrested 3 days after the implantation of the beads (not shown).

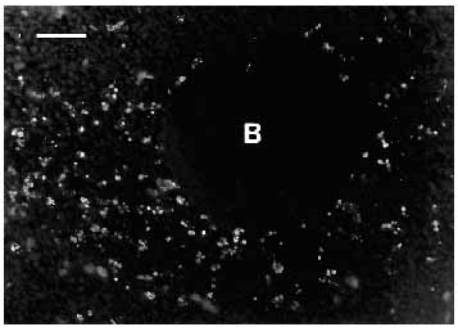


Fig. 5. Transverse section of an experimental interdigit 24 hours after implantation of a BMP bead (B), showing the presence of dying cells positive for fluorescent TUNEL labeling. Bar, 50 μm.

Expression of *Msx* genes after implantation of BMP beads

No significant modifications in the pattern of expresssion of *Msx* genes were found in the first 10 hours following the implantation of BMP beads. After this period changes in the spatial distribution of *Msx-1* and *Msx-2* genes were detected when the beads were implanted at the tip of the growing digits. The initial modification consisted of an increase in thickness of the zone of gene expression distal to the digit tip. In the subsequent period this region of *Msx* gene expression took on a wedge appearance, again resembling the morphology of an extra interdigit (Fig. 7).

DISCUSSION

The main conclusion of this study is that the spatial distribution of the digital rays and the interdigital spaces in the developing autopod might be controlled by a patterned distribution of TGFβs and BMPs in the mesoderm subjacent to the progress zone.

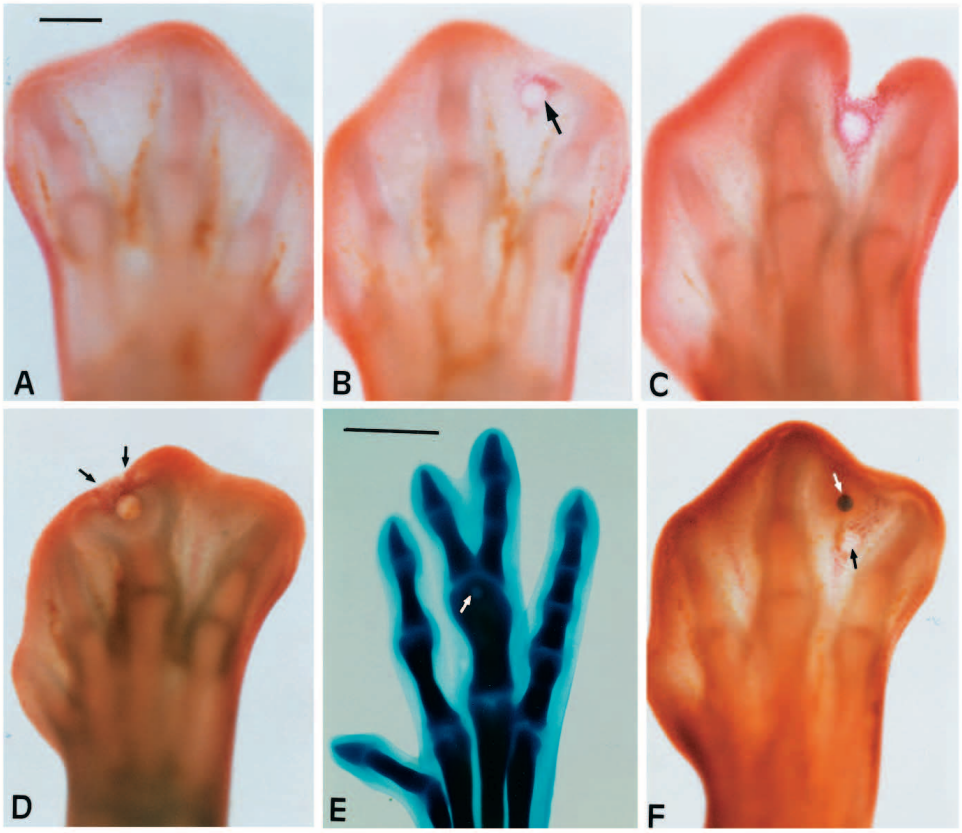


Fig. 6. Chick leg autopods treated with BMP-4 beads. (A,B) The left (control, A) and right (experimental, B) limbs vital-stained for cell death with neutral red 10 hours after implanting a BMP bead at stage 29. Arrow shows the inital appearance of an area of cell death around the bead. (C) The pattern of cell death in the third interdigit 20 hours after implanting a BMP bead. Note that physiological cell death has not yet started in the untreated interdigits. Note also the prominent indentation in the experimental interdigit. (D) The pattern of cell death 20 hours after implanting a BMP bead at the tip of digit III (arrows). Note the initial bifurcation of the digit caused by the bead. (E) The characteristic digit bifurcation in an experimental autopod 4 days after implanting a BMP bead at the tip of digit III. The location of the bead is indicated by the arrow. (F) An experimental autopod vital-stained for cell death 20 hours after implanting a BMP bead (black arrow) and a FGF bead (white arrow). Note the inhibition of cell death in the zone close to the FGF bead, and the intensity of cell death compared with that shown in C. Bar in A-D and F, 300 μm; in E, 1 mm.

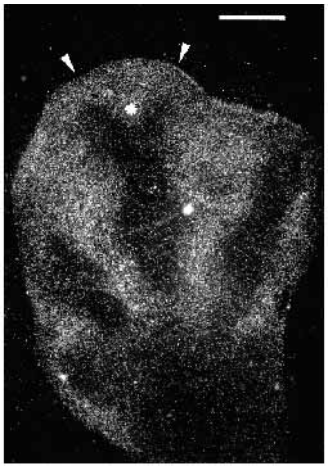


Fig. 7. Section of an experimental autopod 20 hours after implantation of a BMP bead at the tip of digit III, showing the expression pattern of *msx-1*. Digit III exhibits an early stage of bifurcation encompassing an ectopic region of *msx-1* gene expression (delineated by arrowheads), which resembles an extra interdigit. Bar, 500 μm.

TGFβs and the formation of the digits

Distal outgrowth of the limb skeleton is due to the progressive incorporation and subsequent differentiation of the most proximal cells of the progress zone as they become displaced proximally from the influence of the AER (Summerbell et al., 1973). In this study the occurrence of digit duplications after implanting control heparin beads at the tip of the differentiating digits suggests that the condensing cartilages generate a signal which promotes the distal progression of the condensation. Our interpretation is that the heparin beads act as biological or mechanical barriers, causing a splitting of this signal. The absence of alterations when the beads were implanted in the interdigital regions rules out a direct effect of the beads on the cells of the progress zone.

The formation of ectopic cartilages following the implantation in the interdigits of beads preabsorbed with TGFβ-1 or TGFβ-2 provides support for a role of these and/or other homologous members of this family of growth factors as the hypothetical signal originated in the prechondrogenic condensation. Genes of the TGFβ family are expressed in the limb chondrogenic aggregates in avian (Roark and Greer, 1994) and mammalian embryos (Millan et al., 1991), even prior to the appearance of any morphological distinction between the chondrogenic aggregate and the adjacent mesenchyme. Furthermore, as observed here in in vivo conditions, TGFβs are potent chondrogenic stimuli for the limb mesoderm in vitro (Kulyk et al., 1989; Schofield and Wolpert, 1990; Leonard et al., 1991; Roark and Greer, 1994).

The formation of the ectopic cartilages by TGFβs was preceded by the induction of an ectopic domain of *ck-erg* in the interdigit. It has been found that *ck-erg* expression constitutes a precocious molecular marker of chondrogenic differentiation of the limb mesoderm (Dhordain et al., 1995). In our study the interdigital cells exhibited very abundant *ck-erg* RNA transcripts 8 hours after the implantation of the beads. This short period of time contrasts with the 15 hour time lapse required to detect positivity with peanut-lectin labeling used in previous studies of interdigital ectopic chondrogenesis in vivo (Hurle et al., 1989), a procedure that was considered to be the earliest specific marker for prechondrogenic cell differentiation (Aulthouse and Solursh, 1987). In accordance with these facts, our present observation supports the role of *ck-erg* in the initiation of chondrogenesis, also indicating a role for TGFβs in the activation of this gene.

The precise correlationships between the pattern of interdigital chondrogenesis and the doses and stage of TGFβ administration are also consistent with the hypothetical physiological role of these growth factors in skeletogenesis. It is particularly noteworthy that beads soaked in 10 μg/ml TGFβ and implanted at stage 29 are able to change the fate of the interdigit from cell death to digit formation. It might be argued that similar extra digits can be induced by a variety of experimental manipulations of the autopod (Hurle and Gañan, 1987; Hurle et al., 1989; Gañan et al., 1994). However, all the procedures so far reported to induce interdigital extra digits involved the surgical wounding of the limb ectoderm, and this manipulation has been found to induce an intense and rapid (1-3 hour) increase of TGFβ-1 transcripts in the mesoderm subjacent to the wound (Martin et al., 1993). This finding, and our present observations, suggest that all surgical procedures reported to induce extra digits may share a common molecular mechanism involving the release of TGFβs.

The results obtained in the experiments involving the implantation of combinations of FGF beads and TGFβ beads further support a role for TFGβs in skeletogenesis. The maintenance of the progress zone has been found to be dependent on the release of a FGF by the AER (Fallon et al., 1994; Niswander et al., 1993; Mahmood et al., 1995). In this study, combined implantations of FGF-2 beads and TGFβ beads revealed an antagonistic influence of the two growth factors on mesoderm chondrogenesis. This finding contrasts with results obtained in vitro (Schofield and Wolpert, 1990; Knudson et al., 1995), and is again compatible with a role for TGFβs as signals for cell differentiation acting on the progress zone in a proximodistal direction, the opposite of the influence of FGFs. A noteworthy feature of these experiments is that combinations of TGFβ beads and FGF beads were translated into parallel morphological patterns of interdigital chondrogenesis.

BMPs and the establishment of the areas of interdigital cell death (INZ)

Our present observations show that BMP-4 beads induce cell death by apoptosis in the undifferentiated limb mesoderm. When the beads are implanted in the interdigits, cell death resembles the normal spatial distribution of INZ but follows a precocious pattern of temporal distribution. Implantation of the BMP beads at the digital tips potentiates the barrier effects of control heparin beads, as deduced by the constant presence of digit bifurcation, and induces the formation of an ectopic area of cell death resembling an extra-interdigit. These results, along with the normal expression of *Bmp-4* in the interdigital spaces (Wozney et al., 1993; Francis et al., 1994), suggest a role for BMP-4 as a signal for programmed cell death. Our preliminary observations reveal similar results for BMP-2 and OP-1, which are other members of the BMP family that are also expressed in the interdigits (Lyons et al., 1990; Francis et al., 1994; Helder et al., 1995; Luo et al., 1995).

The participation of members of the BMP family in pro-

grammed cell death has been documented for rhombencephalic neural crest cells by Graham et al. (1994). On the basis of their results these authors suggested that cell death depends on the coincident presence of BMP-4 and *Msx-2* gene expression. In the interdigital mesoderm, *Bmps* and *Msx* genes are also expressed in the cells destined to die, and here we show that formation of an ectopic area of cell death following the implantation of a BMP bead at the tip of the growing digits is also accompanied by a localized extension of the domain of *Msx* gene expression. However, it is unlikely that the temporally coincident expression of *Bmps* and *Msx* genes in the limb bud is enough to trigger cell death, since these genes are also expressed in the subridge mesoderm where the cells are maintained alive and proliferating (Francis et al., 1994; Coelho et al., 1991, 1992; Suzuki et al., 1991). In this study we found that implantation of beads soaked in FGF-2, which is one of the signals accounting for the maintenance of the progress zone (Fallon et al., 1994), antagonizes partially or totally the death-inducing effects of the BMP beads. This observation complements previous studies showing that FGF beads inhibit INZ (Macias et al., 1996) and ectopic cell death in the progress zone secondary to AER removal (Fallon et al., 1994). Thus, the triggering of interdigital cell death in situ might be a complex process, dependent not only on the presence of *Bmps* and *Msx* gene expression but also on the absence of FGFs. These observations suggest that embryonic cell death is controlled by the balance of death and survival signals mediated by different growth factors.

The molecular changes observed here, which precede the inhibition of INZ by TGFβ beads, fits in nicely with our interpretation of TGFβs and BMPs as signals controlling the position of the digits and interdigits in the autopod. Cell death inhibition by TGFβs was preceded by down-regulation of *Msx* and *Bmp-4* gene expression and by the induction of an ectopic domain of *ck-erg* expression in the experimental interdigits. All these changes parallel closely the sequence of events observed under normal conditions during the formation of digits, where the cells of the progress zone express *Bmp-4*, *Msx-1* and *Msx-2* until they enter into the distal tip of the digital rays, when they become negative for these genes and positive for *ck-erg*.

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