Expression of BMP-4 mRNA during distraction osteogenesis in rabbits

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We characterized the presence and localization of bone morphogenetic protein 4 (BMP-4) mRNA in the regenerating tissues produced by distraction osteogenesis following tibial osteotomy in the rabbit. The findings are consistent with previous reports on the localization of BMP-4 mRNA expression during fracture repair. The BMP-4 gene is expressed by less differentiated osteoprogenitor cells (fibroblastic mesenchymal cells and preosteoblasts), and not by fully differentiated osteoblasts. BMP-4 gene expression is localized in callus-forming tissue (muscle, periosteum) during callus formation.

Our observations suggest that the BMP-4 gene product is one of the local contributing factors in regulating bone and cartilage formation in distraction osteogenesis.

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Bone formation in distraction osteogenesis resembles aspects of both normal fracture healing and embryonic limb development (Aronson et al. 1989, Ilizarov 1989). Shared features include a stimulus for proliferation of mesenchymal cells and an induction of the mature osteoblasts. Urist et al. (1982) have suggested that a common morphogen (bone morphogenic protein, BMP) exists in fracture healing, bone graft incorporation and heterotopic bone formation.

Although many cytokines, growth factors, hormones and extracellular matrix components are capable of regulating specific aspects of bone regeneration and remodelling during bone growth and repair, BMPs are the most potent osteoinductive proteins and the only factors known hitherto that can induce bone formation in vivo (Urist 1965, Campbell and Kaplan 1992, Wozney 1992). Bone morphogenetic proteins have also been considered potential transducers of the mechanobiological influence on bone development, making the proteins appropriate candidates for mediation of the influence of mechanical forces (Van der Meulen et al. 1993, Mikic et al. 1995). The histological (Schenk and Gachter 1994) and clinical features (Ilizarov 1990) of distraction osteogenesis have been well documented. However, little is known about the presence of BMPs during distraction osteogenesis.

BMP-4 is a member of the BMPs and TGF-ß superfamily, it plays an important role in normal embryogenesis, inducing proliferation and differentiation of mesodermal tissues (Rosen and Thies 1992, Hogan 1996). BMP-4 can induce primitive mesenchymal cells to form bone by a direct or by an endochondral process in vitro and in vivo (Wozney 1992, Hughes et al. 1995). We hypothesize that BMP-4 may act as a mediator of induction of bone formation in distraction osteogenesis. To test this hypothesis, we examined the temporal and spatial patterns of BMP-4 mRNA expression using in-situ hybridization methods in an established rabbit model of leg-lengthening.

Animals and methods

Animal model of leg-lengthening

6 New Zealand white rabbits (age 6 months, body weight 3.0–3.5 kg) had mid-tibial osteotomies performed in the left tibiae and were stabilised with external fixators (Orthofix M100) under general anaesthesia, as previously described (Kojimoto et al. 1988). After a 7-day latency period, twice daily distraction was initiated at a rate of 0.7 mm/day for 28 days. During the distraction period, weekly radiographs were taken to confirm the lengthening. The animals were killed when lengthening to 20% (approximately 2 cm) of the original length of the tibia was achieved. The experimental protocol was performed under an Animal License granted by the British Home Office.

Tissue preparation and histological examination

The animals were killed by an overdose of anaesthestic and the lengthened tibiae were surgically excised, sliced longitudinally into 2–3 pieces and fixed in 4% paraformaldehyde (pH 7.4) for 48 hours. The samples were then decalcified for 14–28 days in 14.5% (w/v) EDTA (pH 7.4), which was changed weekly, and completeness of decalcification was determined radiographically. The tissue was dehydrated in graded ethanols, cleared in xylene and embedded in paraffin wax. Sections (5 µm) were cut and mounted on poly-L-lysine (Sigma, Poole, Dorset, U.K.)-coated slides for histology or on 3-aminopropyltriethoxy silane (APES) (Sigma, Poole, Dorset, U.K.)-coated slides for in-situ hybridization. For histological examination, the sections were stained with haematoxylin and eosin (HE).

Cell culture of rabbit and human bone marrow cells and human osteosarcoma cells

Human bone marrow cells were collected from trabecular bone specimens obtained during surgery at the Nuffield Orthopaedic Centre, Oxford. Only bone which would otherwise be discarded was used, with the approval of the Hospital Medical Staff Committee. Rabbit marrow stromal cells were obtained from the non-operated femurs of New Zealand rabbits. Primary cultures of bone marrow cells were established as previously described (Green et al. 1986). Saos2 cells were thawed from a frozen glycerol stock (gift from Clarke Anderson, University of Kansas Medical Center, Kansas City, USA) and resuspended in Essential Medium, alpha modification (Life Technologies, Paisley, U.K.). Saos2 cells are human osteosarcoma cells, which are known to express human BMP-4 mRNA constituitively (Anderson et al. 1995). Rabbit marrow cells and Saos2 cells were plated in T-175 cm^2 flasks at a concentration of 10^7 cells/flask. All cells were cultured in Alpha minimum essential medium (Sigma, Poole, U.K.) supplemented with 10% (v/v) foetal calf serum, and re-fed every 3 days with fresh medium. Cultured cells were harvested when they were confluent.

Preparation and use of of probes for bmp-4 in northern blot analysis and in-situ hybridization

A single-stranded RNA probe (riboprobe) for human BMP-4 was made from a cDNA fragment inserted into a plasmid, which contains a 301 base pair region of the human BMP-4 gene, corresponding to the carboxy-terminal coding region (Gift from J. Wozney, Genetics Institute, Cambridge, MA). This region has the most specific (least homologous) coding domain for the human BMP-4 gene (Rosen et al. 1989). For northern blotting, to generate the antisense probe, the human BMP-4 template was linearized with Hind III (Promega, Madison, WI, USA) and incubated at 37 °C with SP6 RNA Polymerase (Boehringer Mannheim, East Sussex, U.K.). The riboprobe was labelled with [^{32}P], using a standard kit (Boehringer-Mannheim, East Sussex, U.K.). For in-situ hybridization on paraffin sections, the human BMP-4 cDNA fragment was subcloned into a PBS vector from a Pst vector, in order to facilitate transcription of a sense probe for use as a negative control. The plasmid was linearized with Hind III and transcribed with T7 for the antisense probe or linearized with EcoR1 and transcribed with T3 for the sense probe. The riboprobes were labelled with digoxygenin, using a standard kit, according to the protocols provided by the manufacturer (Boehringer-Mannheim, East Sussex, U.K.).

The sensitivity and cross-reaction of the human BMP-4 probe to human and rabbit tissues were examined by northern blotting. The human BMP-4 probe was hybridized with mRNAs isolated from human osteosarcoma cells, rabbit and human bone marrow cells. Total RNA was isolated from cells, using acid guanidine thiocyanate-phenol-chloroform, as described previously (Chomczynski and Sacchi 1987). mRNAs were purified from the extracted total RNA using oligo (dT)-coated magnetic microspheres (Dynabeads, Dynal IK Ltd., Wirral, U.K.), according to the manufacturer's instructions. Equal amounts of purified mRNAs were then separated by electrophoresis on 1% agarose gels, before transfer to a Biodyne nylon membrane (Pall Ultrafine Filtration Corporation) under alkaline conditions. Membranes were prehybridized and hybridized in a buffer of 50% (v/v) deionized formamide, 1% (v/v) SDS, 4 x SSPE (0.18M NaCL, 0.01M NaH_2PO_4, 0.001M Na_2EDTA, pH 7.7), 0.05% (w/v) non-fat dried milk and 300 mg/mL denatured salmon sperm DNA. [^{32}P]-labelled riboprobe was used at > 1×10^6 cpm/mL for hybridization. Hybridization was performed overnight at 55 °C. Post-hybridization washes were at 55 °C in 2 × SSC with 0.1% SDS, followed by 1 × SSC with 0.1% SDS. The signal was obtained on autoradiographs by exposing the blot to pre-sensitized Kodak X-OMAT film (Kodak Ltd., Hemel Hempstead, U.K.) at –70 °C for 4–24 hours.

For in-situ hybridization on paraffin sections, a standard procedure was used (Sandberg et al. 1993). The pretreatments were (1) 0.2 N hydrochloric acid, (2) 6% hydrogen peroxide, (3) 20 µg/mL proteinase K (Sigma, Poole, U.K.) in Tris-EDTA buffer, pH 8.0, for 10 minutes at 37 °C, (4) 4% paraformaldehyde, pH

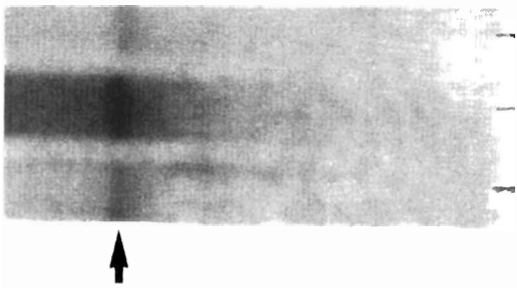


Figure 1. Northern blot analysis showing identical patterns of human and rabbit bone marrow cells, and human osteosarcoma cells (SaOs2) when hybridized with the human BMP-4 antisense riboprobe.

7.4 for 20 min, (5) 0.1 M glycine (2 × 10 min) and (6) 0.25% acetic anhydride for 10 minutes. The sections were dehydrated through graded ethanols and 40 µL hybridization solution was applied per section. The hybridization solution contained 50% formamide, 10mM Tris-HCl, pH 7.4, 1.0 mM EDTA, 0.02% polyvinyl and Ficoll, 0.02% bovine serum albumin, 0.5% sodium dodecyl sulphate, 300 mM NaCl, 10% dextran sulphate, 0.5 mg/mL yeast or E. coli tRNA and the labelled antisense, or sense, probe, as appropriate. Hybridization was for 18 hrs at 55 °C in a humid chamber. After hybridization, the sections were treated with 20 µg/mL RNAse in Tris-EDTA-NaCl buffer, pH 8.0. The sections were washed initially with standard saline citrate (SSC), followed by 0.5 ×, then 0.1 × SSC; all washes were at 55 °C, except the final wash was at room temperature. The digoxygenin-label was detected using the Boehringer kit (Boehringer Mannheim, East Sussex, U.K.). NBT and X-phosphate (Boehringer-Mannheim, East Sussex, U.K.) were used as substrates to visualise the antibody according to the manufacturer's instructions, and the sections were subsequently mounted in glycerine jelly for microscopic observation.

Results

Northern blotting

Northern blotting, using human BMP-4 probes on equal amounts of mRNAs of Saos2 cells, human and rabbit bone marrow cells showed that the hybridization patterns of Saos2 cells and human bone marrow cells were identical to those of rabbit bone marrow cells (Figure 1). This indicates cross-reaction of the human BMP-4 probe to rabbit BMP-4 mRNA and validates the use of the human BMP-4 probe in this study.

In-situ hybridization on paraffin sections

At the end of the distraction period (28 days), at the distraction rate of 0.7 mm/day, histological investigation of the distraction regenerates of all animals showed that new bone had developed from the cut surfaces with a central area of fibrous tissue and areas of cartilage between the central fibrous region and the new bone. Small areas of cartilage were also found adjacent to the periosteum (Figure 2). BMP-4 mRNA was detected in the fibroblasts of the central fibrous region, the bone surface cells and osteoblasts of newly-formed woven bone, the chondrocytes in the developing cartilage region, the proliferating periosteum surrounding the distraction gap and the surrounding muscles. BMP-4 mRNA was not detected in most of the osteocytes in the newly formed bone or in the normal cortical bones. With hypertrophy of the chondrocytes, the expression of BMP-4 mRNA decreased (Figure 3). Osteoblasts on the surfaces of the developing trabecular bone exhibited strong BMP-4 mRNA expression, but as lamellar (mature) bone replaced this primitive woven bone, the intensity of the expression decreased and eventually diminished (Figure 4). All control sections hybridized with sense probes showed no specific staining (Figure 5).

Discussion

Our findings show that BMP-4 mRNA is expressed in specific regions of the regenerating tissues during the distraction period. This mRNA expression pattern is distinct from that described in a mouse model of rib

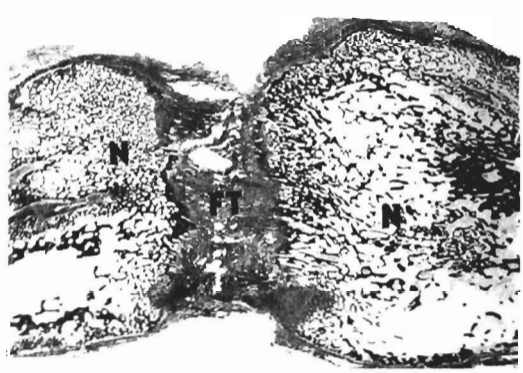
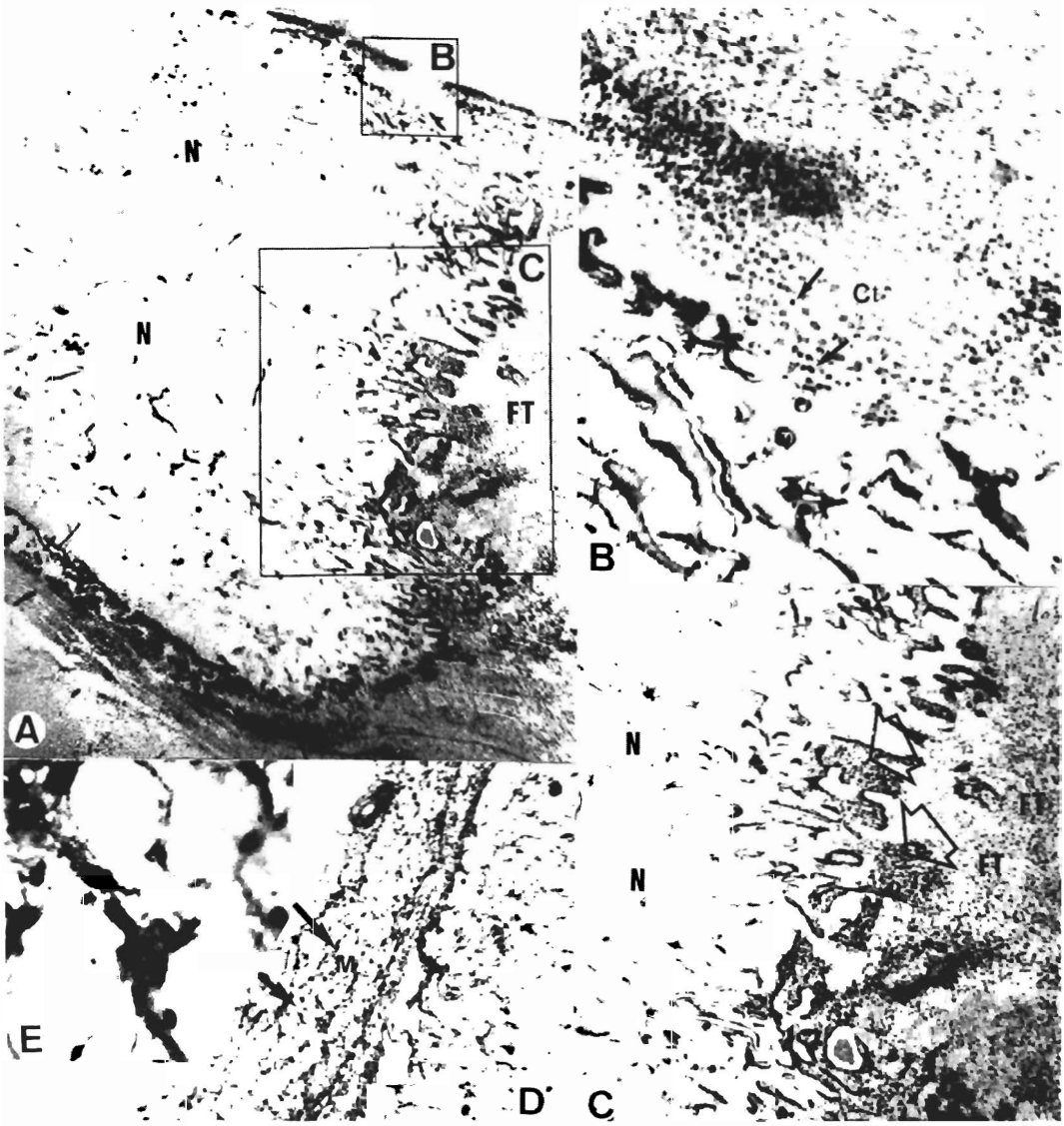


Figure 2. The histological appearance of the distraction regenerate. New bone (N) has been formed, and there is a central area of fibrous tissue (FT). HE ×8.

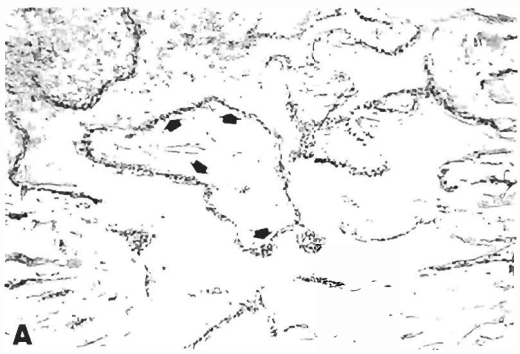
Figure 3. In-situ hybridization with BMP-4 antisense mRNA.



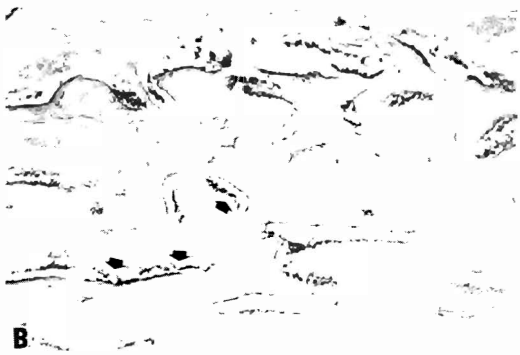
A. Showing half of the regenerate sectioned longitudinally. N: new bone; FT: fibrous tissue. Areas B and C are shown in higher power as Figures 3B and C.
B. Chondrocytes in the cartilage developing area from the central fibrous tissues showing the presence of BMP-4 mRNA (arrows), Ct: cartilage tissue.
C. Positive signal in fibroblasts of the central fibrous tissue (arrows). N: new bone, FT: fibrous tissue.
D. Skeletal muscles adjacent to the distraction regenerate showing intensive labelling (arrows), M: muscle.
E. High power view of muscle shown in Figure 3D. Original magnification, A ×8; B ×20; C ×12; D ×20; E ×400.

fractures (Nakase et al. 1994), in which there was a transient expression of BMP-4 mRNA from 12–72 hours after fracture, as detected by in-situ hybridization, prior to the appearance of differentiated osteoblasts at the fracture site. Normal fracture healing in mammals occurs in a series of overlapping phases, which may broadly be considered as inflammatory, regenerative and remodelling (McKibbin 1978). The early phases of fracture healing involve a recruitment of primitive mesenchymal cells, proliferation of osteoprogenitor cells and induction of a differentiated osteoblastic phenotype. During distraction osteogenesis, these early phases are prolonged in the regenerating callus, creating a continuous proliferation and induction, similar to those in the early fracture period. This extended induction of new bone formation is

Figure 4. In-situ hybridization using BMP-4 antisense mRNA probe, ×100.



A. The osteoblastic cells on bone surfaces of primitive woven bone exhibited strong BMP-4 mRNA expression (arrows).



B. As bone becomes mature, the intensity of BMP-4 mRNA expression decreases (arrows), and eventually diminishes.

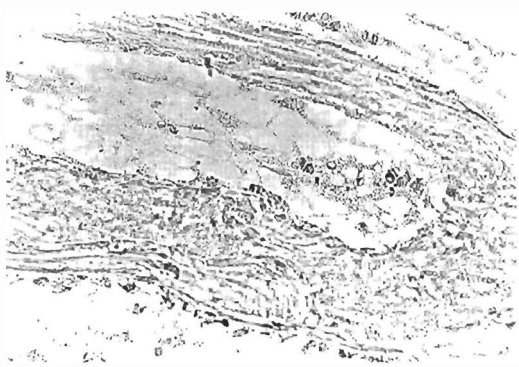


Figure 5. In-situ hybridization using BMP-4 sense mRNA probe showing absence of staining, ×100.

paralleled by the observed longer expression (35–40 days after osteotomy) of BMP-4 in distraction osteogenesis compared to normal fracture healing.

The spatial pattern of BMP-4 mRNA expression during distraction osteogenesis is similar to the pattern demonstrated in fracture models by in-situ hybridization (Hirakawa et al. 1994, Nakase et al. 1994), and by immunohistochemistry (Yan et al. 1994, Bostrom et al. 1995). The intense staining of the inner cambium layer of the periosteum in all models suggests that BMP-4 plays a role in the proliferation and differention of mesenchymal tissue. The presence of cartilage in our specimens may be due to relative instability by unilateral external fixation, as models using circular fixation have described solely intramembranous osteogenesis (Aronson et al. 1989, Ilizarov 1989). The finding of cartilage in our model allows for an extension of our comparison of distraction osteogenesis with mechanisms of fracture healing, as the endochondral sequence is observed in the fractures. In this study, BMP-4 mRNA expression was found in peripheral, immature chondrocytes, with less staining of central, histologically mature chondrocytes in the cartilagenous callus. This pattern is similar to that reported by using immunohistochemical localizations (Bostrom et al. 1995). The source of chondroprogenitor cells appears to be in the active periosteum, abutting the cartilagenous portion of the callus, in the same layer of the periosteum that seems to give rise to primitive osteoblasts elsewhere, showing that the primitive mesenchymal cells are pluripotent, and can differentiate along either chondroblastic or osteoblastic lineages.

BMP-4 mRNA expression was also localized in the surrounding skeletal muscle in the region of the regenerate in this model of distraction osteogenesis, and similarly demonstrated in the muscle region close to the callus in the mouse rib fracture model by in situ hybridization (Nakase et al. 1994). The ability of bone morphogenetic proteins to induce osteogenesis in intramuscular sites was an essential feature of the early assays of BMPs activity (Urist 1965), and this indicates that cells in muscle can form bone. However, in the present study it is unlikely that multipotential cells from skeletal muscle migrate and differentiate into bone-forming cells and thus contribute to the bone-forming tissue in the regenerate during distraction osteogenesis.

As mRNA expression does not necessarily equate with protein synthesis, the present study would be strengthened considerably if BMP-4 immunohistochemistry were included, to determine in which regions transcription was accompanied by translation. However, antibodies to rabbit BMPs are not available at present and immunohistochemical studies await future availability of a suitable antiserum.

In conclusion, our studies indicate that BMP-4 may play an important role in the control of osteoprogenitor cell differentiation and proliferation, but is probably less important in the function of mature osteo-

blasts. The results suggest that the BMP-4 gene product is one of the local factors regulating bone and cartilage formation. These findings, together with previous studies on the function of BMPs, support the suggestion that application of BMPs may have potiential value for treatment of inadequate bone formation during fracture healing, bone transport or limb-lengthening.

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