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ORIGINAL STUDY

Bone morphogenetic protein signaling in the murine distraction osteogenesis model

Johan LAMMENS, Zhendong LIU, Frank LUYTEN

From the Department of Orthopaedic Surgery, UZ Pellenberg, the Laboratory for Skeletal Development and Joint Disorders, UZ Gasthuisberg, and Prometheus, Division of Skeletal Tissue Engineering, Katholieke Universiteit Leuven, Belgium

A murine distraction osteogenesis model was standardized to allow analysis of the molecular pathways associated with postnatal *de novo* bone formation. The authors examined the presence and expression of Bone Morphogenetic Proteins (BMPs) -2,-3,-4,-6 and -7, and the BMP receptors Alk3 and Alk6 at different stages. Strong signals were detected for BMP-4 at the end of the distraction period and for BMP-6 during the entire experimental period. Signals for BMP-7 (Osteogenic Protein-1) were very low, suggesting a less important role during the normal process of distraction bone healing. Immunohistochemical staining revealed the presence of BMP-4 in the early chondroblasts, while BMP-6 was detected in the more mature cartilage cells. The data indicate a BMP molecular profile reminiscent of the embryonic maturation process in endochondral bone formation.

Keywords : distraction osteogenesis ; murine model ; Bone Morphogenetic Protein signaling.

INTRODUCTION

The skeletal repair process appears to be of a well ordered sequence of cellular and molecular events. To some extent these events are mimicking embryonic tissue formation. A better understanding of the molecular processes which direct skeletal formation is of clinical relevance as it will help to identify targeted therapeutical strategies for skeletal injuries (*4*). One of the clearest examples of skeletal repair is bone lengthening where, through a process of gradual distraction osteogenesis, massive cylinders of new bone can be regenerated (*9*). From these observations it is obvious that distraction osteogenesis may be a powerful model for the study of bone regeneration and its underlying mechanisms (*11,12*). Although a lot of investigations have been conducted on this topic, the knowledge on the hierarchy of the signaling pathways involved, from the stage of undifferentiated mesenchymal cells towards osteoblasts secreting the osteoid matrix leading to full bone maturation and remodeling, remains incomplete (*5,17,22*). The murine model

■ Johan Lammens, MD, Professor of Orthopaedic Surgery.
Department of Orthopaedic Surgery, UZ Pellenberg, Katholieke Universiteit Leuven, and Prometheus, Division of Skeletal Tissue Engineering, Katholieke Universiteit Leuven, Belgium.
■ Zhendong Liu, Research Fellow.
■ Frank Luyten, MD, Professor.
Laboratory for Skeletal Development and Joint Disorders, UZ Gasthuisberg, and Prometheus, Division of Skeletal Tissue Engineering, Katholieke Universiteit Leuven, Belgium.
Correspondence : Johan Lammens, UZ, Weligerveld 1, B-3212 Pellenberg, Belgium.
E-mail : johan.lammens@uz.kuleuven.ac.be

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is one of the most favored models to study mammalian embryology and biology, predominantly because of its strong genetics. Analysis of the role of specific signaling pathways can be done successfully using genetic models such as knockout mice. Therefore, a mouse distraction model was further refined, allowing to study at the molecular level the gradual process of bone formation and maturation during tibial lengthening. This complex phenomenon of bone repair is triggered by numerous molecules acting through different pathways, necessitating the study of different ligands, receptors and intracellular signaling pathways. This study focuses on the analysis of the bone morphogenetic proteins, because of their well documented role in embryonic skeletal formation and their well known biological activity in the ectopic induction of endochondral bone formation (*3,8,24*).

MATERIALS AND METHODS

Establishment of the model

Analogous to circular external fixation used for limb distraction in humans and large animal models, a custom-made mini ring frame was developed for lengthening of the mouse tibia, a procedure already described previously (*10, 22*).

The device was composed of one proximal and one distal 4 mm thick radiolucent Ertalyte PETP ring (Spronken Orthopedie® Belgium), 4 mm wide and with an inner diameter of 12 mm. The rings were connected with two 16 mm guide rods. Two stainless steel screws with a 0.5 millimeter thread allowed gradual separation of the rings. The tibia was fixed to the rings with one pair of 0.45 mm (27 Gauge) needles proximally and one pair of 0.40 mm (29 Gauge) needles distally, which fitted exactly in premade holes. These holes were drilled slightly eccentrically to allow the tibia to be centered in the anteromedial quadrant of the rings, leaving enough space for the more abundant posterolateral soft tissue structures, and resulting in a central position of the entire limb (fig 1).

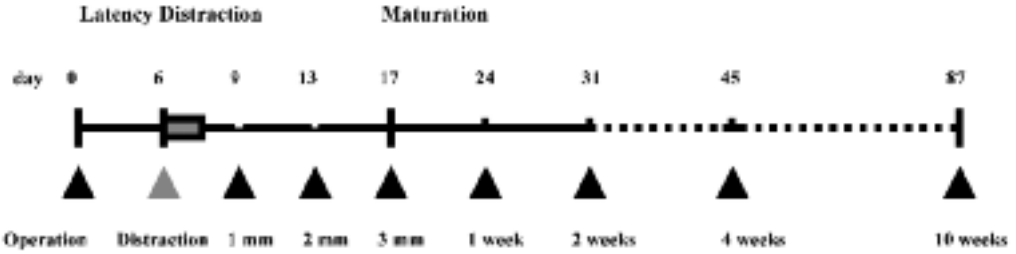
Surgical procedure

All procedures were performed according to the ethical guidelines of the institute on 52 (table I) 10 weeks old male CD-1 mice, using intraperitoneal barbiturate anaesthesia. After assembling the fixator on the limb, a fibulotomy was performed with a pair of scissors through a small lateral approach. For the tibia an anterior incision was made in the upper third. After predrilling three 0.5 mm holes, the osteotomy was completed with a very small cutting forceps. After wound closure with Prolène 5/0 an anteroposterior and a lateral radiograph were taken to confirm good alignment according to both axes.

Table I. — Experimental design for distraction osteogenesis in the mouse model

The number of animals for the experiments

	Fracture	Distraction			Maturation			
	5 days	4 days	8 days	12 days	1 week	2 weeks	4 weeks	10 weeks
Histology	2	2	2	2	2	2	2	2
RT-PCR	2	2	2	2	2	2		
IHC	2	2	2	2	2	2		
ISH	2	2	2	2	2	2		



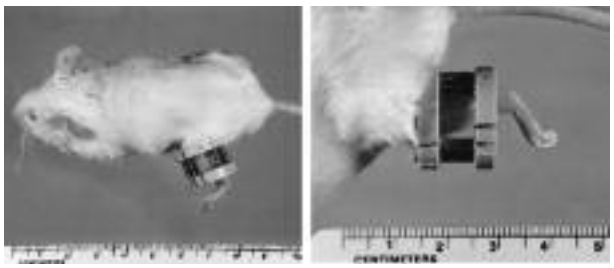


Fig. 1. — View of minifixator applied to the tibia of a mouse

Distraction protocol

After a "resting" period of 5 days, distraction was started at a rythm of 0.250 mm/day with 0.125 mm increments twice a day. The lengthening was continued for 12 days, allowing an increase in length of 3 mm, being about 20% of the initial length of the adult male mouse tibia. Then the fixator was kept in a neutral position till a maximum maturation period of 10 weeks (table I). During distraction, radiographs were taken at 3 day intervals and after neutralization at 1 week intervals.

Analysis

To prepare samples for histologic and *in situ* hybridization analysis, groups of 2 mice were sacrified at different time intervals during the distraction or neutralization period. The lengthened tibia was disarticulated and stored in 4% paraformaldehyde in PBS for 24 hours. Subsequently the fixator was removed and the tibia cleaned from soft tissue. The specimens were then decalcified in 20% EDTA in PBS for 6 days and imbedded in paraffin.

Histology

For histologic analysis 5 µm longitudinal sections were prepared and mounted on Silane-Prep™ slides (Sigma). Haematoxylin-eosin and Masson's trichome staining were performed.

RNA extraction and semi-quantitative RT-PCR

For total RNA extraction, calluses were freshly dissected and separated at different time points. The RNA isolation was performed using Trizol Reagent (GIBCO/BRL). Using thermoscript (Life Technologies) first strand cDNAs were obtained by reverse transcription of 1µg of total RNA with oligo $(dT)_{20}$ as primer. Polymerase chain reaction (PCR) was performed in a volume of 10 µl. The cDNAs were added to the following PCR mixture : 0.5 units Taq polymerase (Eurogentec, Seraing, Belgium), 0.2 m*M* dNTPs, 0.5 µ*M* specific primers, 1.5 m*M* $MgCl_2$. Negative controls were either RT without enzyme or PCR with Milli-Q water instead of cDNA. PCR reactions were carried out in a Perkin Elmer thermal cycler 9600 (Applied Biosystems, Lennik, Belgium). After 1 minute denaturation at 95°C, cycles (19 for β-actin, 33 for type II collagen, 34 for osteocalcin, and 30 for the other genes) were 10 seconds at 94°C, 10 seconds at the optimal annealing temperature, and 30 seconds at 72°C. Cycling was followed by 10 minute elongation at 72°C. Primer pairs were designed using Vector NTI software (InforMax, North Bethesda, Maryland, USA). The specific prime sequences of oligonucleotides used for PCR have been published elsewhere (*6*). The primers for the mouse beta-actin gene were used to equalize the DNA aliquots of each sample.

PCR products were electrophoresed in 1.5% agarose gel in TBE (Trisborate/EDTA) electrophoresis buffer, stained with ethidium bromide, visualized by UV transillumination, and analyzed by densitometry using the Image Master software (Amersham Pharmacia Biotech, Roosendaal, The Netherlands).

In situ hybridization

In situ hybridization techniques were carried out to demonstrate the expression pattern of type II collagen and osteocalcin in the distracted gap. S^{35} radio-active labeled single-strand cRNA probes were prepared as described previously (*7*). Briefly, after a short prehybridization, sections were incubated during 16 hours at 55°C in 50% formamide, 10% dextran sulfate, 4 × SSC, 10 mM dithiotreitol, 1 × Denhardt's solution, 500 µg/ml freshly denatured salmon sperm DNA and yeast tRNA with 0.2-0.4 ng/ml 35 S-labeled riboprobe (1×10^9 cpm/µg) in a humidified chamber. After hybridization the sections were washed to a final stringency of 0.1 SSC, 50°C for 15 minutes. After dehydration in a graded ethanol series containing 0.3 M ammonium acetate, slides were covered with NTB-2 emulsion (Kodak) and exposed for 1-3 weeks. Hybridization of type II collagen and osteocalcin was performed at 55°C for 16 hours and the signals detected with the nucleic acid detection kit (Boehringer Mannheim Biochemica, Mannheim, Germany). For control, hybridization with the sense probes was performed.

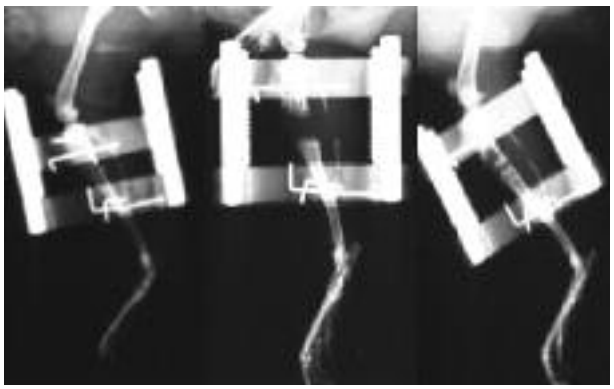


Fig. 2. — Radiographs of mouse tibia : immediately after osteotomy (left), at the end of the distraction (middle) and at 8 weeks postoperatively (right), still showing the central radiolucent band.

Immunohistochemistry

Immunohistochemistry was done to analyze the presence of BMP-4 and -6 in the distracted callus. After deparaffinization and hydration, endogenous peroxidase was quenched with 3% hydrogen peroxidase for 5 minutes. Nonspecific binding was blocked by normal donkey serum (1:5 dilution in Tris buffered saline, pH 7,5) for 20 minutes. For immunostaining, commercially available polyclonal goat anti-BMP-4 and -6 peptide antibodies were used (Santa Cruz Biotechnology Inc.). Sections were incubated with these antibodies overnight at 4°C in a humidified chamber. For negative controls the purified goat IgG was used. A peroxidase-conjugated donkey anti-goat antibody (Jackson Immuno Research Laboratories, Inc.) was used as secondary antibody. Sections were stained using the avidin-biotin complex method and 3.3'-diaminobenzidine tetrachloride (Sigma). Finally, the sections were slightly counterstained with haematoxylin and mounted.

RESULTS

Radiographic and histological analysis

During distraction the lengthened area was clearly marked radiographically as a radiolucent band with however the very first signs of bone formation at the edge of both fragments as early as one week postoperatively. Progressively during the consolidation period, bone seemed to grow from both ends leaving an initial lucent interzone. Between 6 and 8 weeks this central gap started to be bridged, resulting in a homogeneous callus with signs of differentiation between cortex and medulla (fig 2).

These findings were confirmed by histologic analysis using Masson's trichrome staining. Between the two bony fragments an initial very cell-dense area was observed, surrounded by external cartilaginous callus. Progressively the gap was filled with bone through a process of intramembranous ossification, finally resulting in a bony union of the two distracted fragments, with signs of remodelling at 10 weeks postoperatively (fig 3). However, close to the periosteum areas of endochondral ossification were noticed, and confirmed with toluidine blue staining (fig 6b, top left).

Presence and Expression of Bone Morphogenetic Proteins

Using RT-PCR, BMP-3 and -4 were noticed as faint signals at the start of the distraction, and became clearly present at the end of the distraction period (17 days postoperatively), and after the first week of consolidation (24 days postoperatively) (fig 4). A faint signal for BMP-6 was already detected at 5 days after the osteotomy, strongly increased at the beginning of the distraction (9 days postoperatively) and remained present till 2 weeks of maturation (day 31 postoperatively). Signals of BMP-2 were present from the beginning of the distraction but decreased progressively. No clear signal could be detected for BMP-7 until the end of distraction. It was present after a seven day consolidation period but disappeared by week 2 of consolidation. The BMP receptors Alk3 (BMP R-IA) and Alk6 (BMP R-IB) as well as COL II and X were clearly present during the entire distraction period till the start of the maturation. The transcription factor Cbfa 1 and mature bone matrix marker osteocalcin progressively appeared during the lengthening, and remained present during the consolidation phase.

In situ hybridization (ISH)

In situ hybridization for collagen type II confirmed its secretion by active chondroblasts indicating endochondral bone formation, most

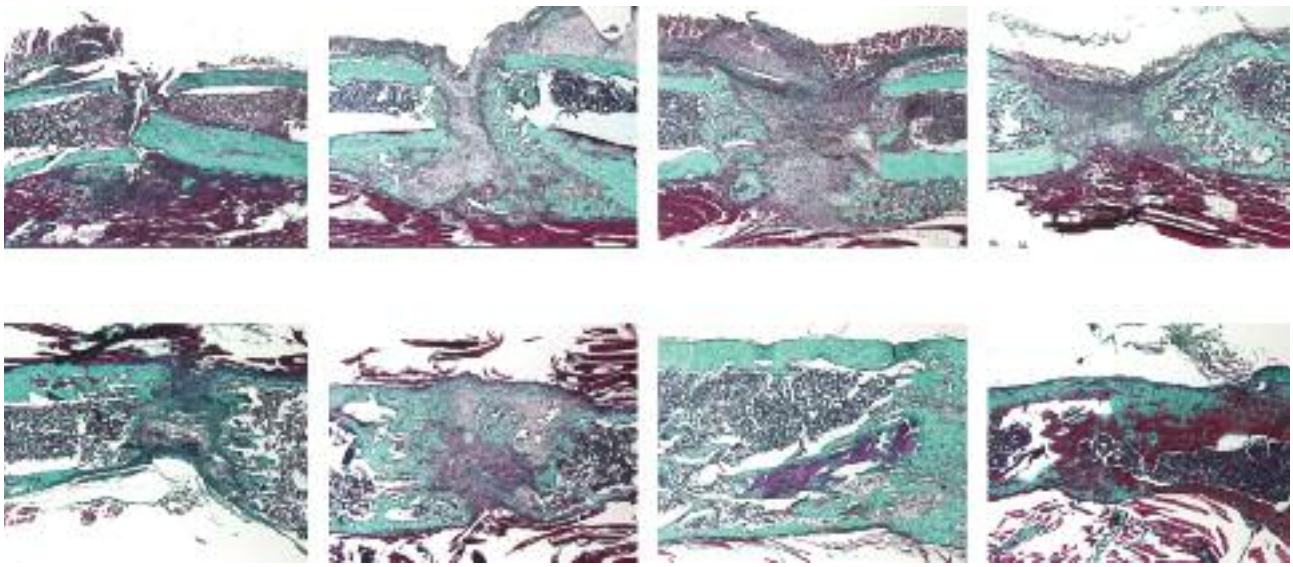


Fig. 3. — Histological representation of the distracted mouse tibia at different time intervals postoperatively, using Masson's trichrome staining.

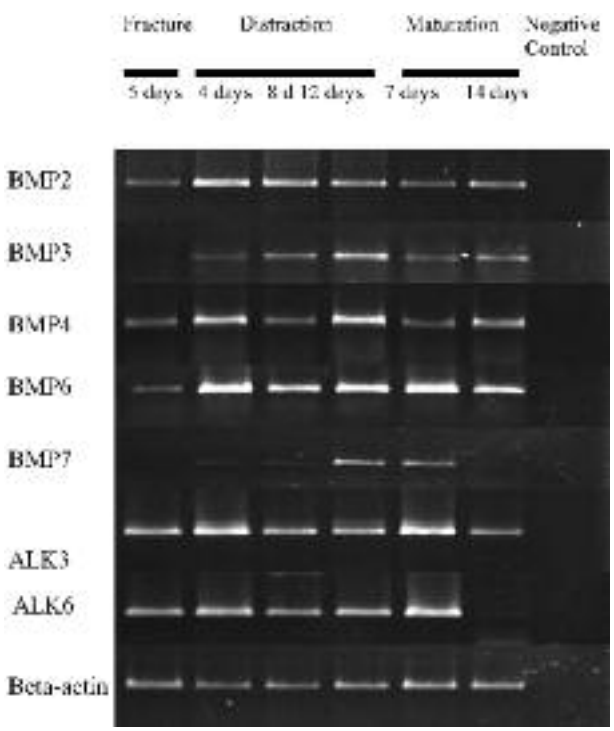


Fig. 4. — Reverse Transcriptase Polymerase Chain Reaction indicating expression of BMP-signals during distraction osteogenesis.

abundant close to the periosteum. The presence of osteocalcin secreting osteoblasts mainly inside the distracted gap was also documented by ISH (fig 5 D,E,F).

Immunohistochemistry

Immunostaining at the central gap was not convincing for BMP-4, throughout the experiment, but was clearly positive for BMP-6 especially at the start of the distraction. Close to the periosteum, in the areas of endochondral bone formation staining was positive both for BMP-4 in the chondroblasts and BMP-6 in the more mature chondrocytes (fig 6b).

DISCUSSION

Among growth factors, BMPs are clearly interesting candidates for therapeutical intervention in bone healing processes (*15,18,23*). It is obvious that BMPs, by stimulating differentiation of mesenchymal cells into osteoblastic and chondroblastic lineage, may contribute to the intramembranous

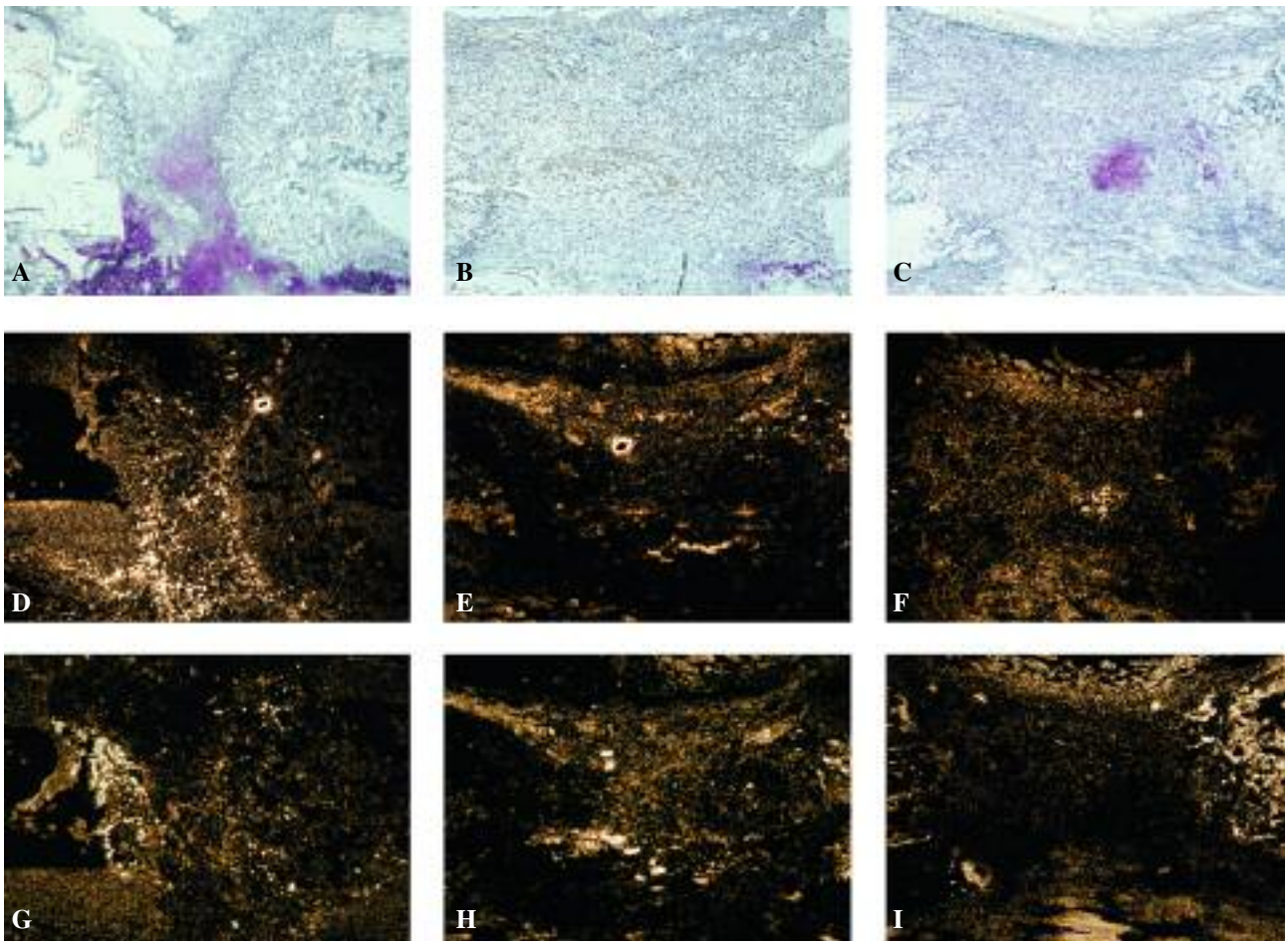


Fig. 5. — In situ hybridization (ISH) showing clear expression of osteocalcin by osteoblasts, and collagen II by chondroblasts in areas of enchondral bone formation. The distraction gap is documented by toluidine blue staining at 1 mm of distraction (A), 2 mm of distraction (B) and 3 mm of distraction (C) respectively.
D, E, and F show the corresponding ISH for osteocalcin. G, H, and I show the corresponding ISH for collagen II.

bone formation and endochondral ossification in fracture callus. Their exact role in distraction osteogenesis however still has to be defined, as the translations from mechanical stimuli to biological signals are not clearly understood (*2,16,21*). Moreover the temporal and spatial expression of several different BMPs might be totally different during bone lengthening, as this has to be considered as a particular form of fracture healing (*12*).

The bone healing process during distraction can be divided in three stages : the latent stage or lag phase, which is an early stage of a normal bone healing process, the distraction stage during which the newly forming tissue is subjected to tension stress, and finally the consolidation period which is mainly a maturation process towards mineralized bone (*14*).

During the *latent stage* from day 0-5, histologically characterized by granulation tissue replacing the haematoma, only the receptors and a faint signal for BMP-6 were detected, as well as Col II and X corresponding with cartilage formation and maturation. BMP-2, -4 and -7 were not detected at this stage. Without tension stress there seemed to be no clear induction of BMPs unless for BMP-6. These findings partially correspond with the rat model described by Sato *et al* (*19*). Although they could detect some initial BMP-2 and -4 at 4 days post-

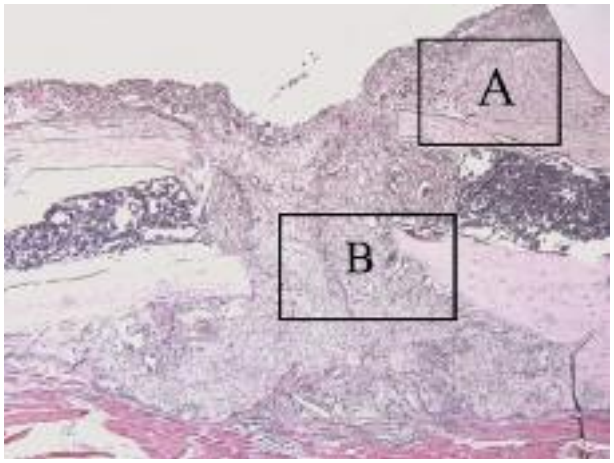


Fig. 6a. — Toluidine blue staining (A : subperiosteal area, B : gap area). The areas which were selected here, are reproduced in fig 6b.

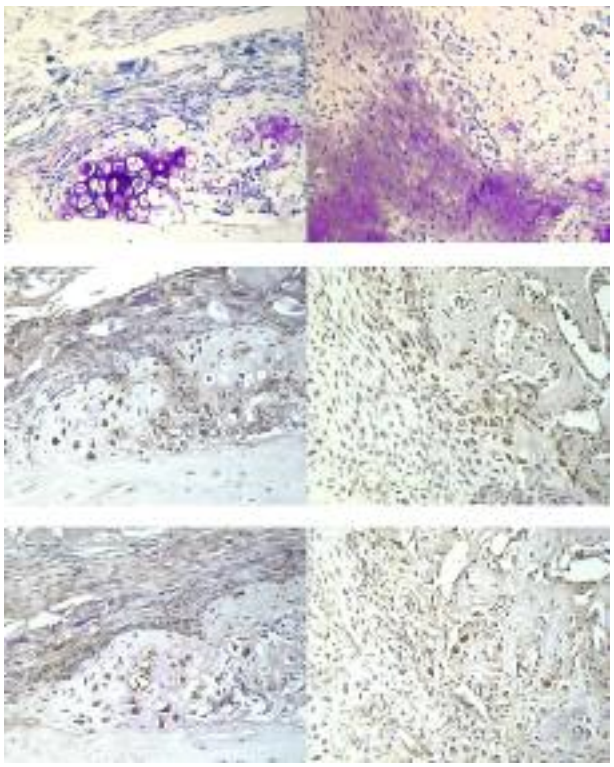


Fig. 6b. — Top : Toluidine blue stain. Left (A) : subperiosteal area ; right (B) : gap area. These areas are reproduced below. Middle left and bottom left : subperiosteal area : immunohistochemistry showing positive staining in chondroblasts and chondrocytes for both BMP-4 and BMP-6, with endochondral bone formation. Middle right and bottom right : gap area : immunohistochemistry showing very weak BMP-4 signal but stronger BMP-6 staining.

operatively, a progressive decline was noticed, whereas the signals for BMP-6 and GDF-5 became stronger. For BMP-7 no signal was detected in their model. However both these data and the findings in our murine model are not in accordance with the rabbit model described by Rauch *et al* (*17*) who detected BMP-2, -4 and -7 at the end of the lag phase.

During the *distraction* mRNA for collagen II and X remained expressed, indicating the endochondral bone forming process going on. Moreover, mRNA for Cbfal appeared as soon as the distraction was started, whereas transcripts for osteocalcin were delayed till 8 days after the distraction was initiated. That protein expression paralleled the RNA exprimation was shown by the ISH for both collagen II and osteocalcin. The collagen II clearly appeared in areas of endochondral bone formation, whereas osteocalcin was present at areas of ossification, both in the distraction gap and subperiostally. These data partially correspond with the analysis by Tay *et al* (*22*) in the murine model, where the distraction gap stained positive for alkaline phosphatase, indicating osteoblast activity. Collagen II was also detected in their analysis, but only at subperiosteal sites (*22*). Findings in the rat model by Sato *et al* (*20*) showed collagen II expression in the early distraction phase, with clear presence of osteocalcin in osteoblasts and prechondrocytes, but not in differentiated chondrocytes (*20*). In the *maturation period* the signals for BMP-2, -3 and -4 tended to become weak, whereas the expression of BMP-6 remained strong. The weak expression of BMP-7, already present at the end of the distraction, continued till 7 days after the distraction, but then completely disappeared.

The continuous expression of BMP receptors Alk3 and Alk6 documents the susceptibility of chondroblasts/osteoblasts for BMPs during the entire healing process, but at present it is still unclear what the exact temporal and spatial expression pattern is for each of the separate BMPs. Although the overall presence of BMP-6 during distraction and maturation might suggest that this is one of the most important BMPs during the phenomenon of bone distraction, it should be kept in mind that BMP-6 is found in chondrogenic cells, as

supported by the study of Sato *et al* (*20*), where BMP-6 was exclusively found in areas of endochondral ossification. In cases of decreased stability such as in very small external fixators, and typically seen in the small animal models, enchondral bone formation is enhanced, and this might explain the more abundant chondrogenesis and concomitant BMP-6 expression. This finding is supported by the ISH for BMP-6 from this study where staining for BMP-6 was only clearly positive at subperiosteal areas of chondrogenesis.

The investigation of other BMPs in different distraction models documents that their expression is not linked to a single cell type. In their rabbit model Li *et al* (*13*) reported the presence of BMP-4 in the fibroblasts of the central fibrous area, the bone surface cells and osteoblasts of newly-formed woven bone, but also in the chondrocytes in the developing cartilage region, as well as the proliferating periosteum overlying the distraction gap and the muscles surrounding the distracted bone region. In the same animal model Rauch *et al* (*17*) detected strong signals for BMP-2, -4 and -7, their expression being not by mature osteoblasts, but by preosteoblasts and early osteoblasts. In the rat model of Sato *et al* (*19*) BMP-2 and -4 expression was very strong in the fibrous interzone, where elongated chondrocytes and spindle shaped fibroblast like cells, clearly undergoing the effect of mechanical tension stress, stained positive. Chondrocytes and their precursors were positive for BMP-2, -4, -6 and GDF-5, whereas, during advanced distraction, osteoblasts and preosteoblast had the strongest signals for BMP-2 and -4.

Taken together all these data suggest an expression of BMPs in areas of cell differentiation and progression of maturation. Especially BMP-2 and -4 are expressed during osteogenesis, whereas BMP-6 appears to be more associated with chondrocyte maturation. The significance of BMP-7 remains less clear as it was neither detected in Sato's (*20*) rat model, nor very obvious in the present murine distraction model, whereas strong signals in preosteoblasts and osteoblasts were documented in the rabbit model.

That the mechanical tension stress triggers the expression for BMPs was clearly shown in Sato *et al*'s experiments (*20*). Osteotomized but not distracted rat femora showed no expression of BMP-2 and -4 and only a short temporal expression of BMP-6 and GDF-5 between 17 and 28 days since the operation (*19*). Another study on a femoral fracture model in the rat showed only a weak staining for BMP-2 and -4 during early stages of fracture healing and an increase with positive staining osteoblasts during the formation of woven bone (*1*).

The exact translation of mechanical factors into biological signals is not understood, but parameters such as distraction rate or rhythm might play a role, as demonstrated in the experiments of Farhadieh *et al* (*5*) showing a stronger expression of TGF-β, IGF-I and bFGF with increased distraction speed in the ovine mandible. These findings suggest that different mechanical conditions may lead to variations in the upregulation of protein expression, making it even more difficult to elucidate the exact sequences of temporal and spatial secretions of BMPs and other growth factors under the influence of gradual distraction.

CONCLUSION

It is obvious from this study that gene expression of several BMPs is induced by the mechanical tension stress due to the gradual bone distraction. The translation of mechanical stresses into molecular signals remains unclear, making more detailed studies mandatory. An increasing knowledge of the signals involved in the cascade of cellular events of tissue regeneration and repair might finally lead to more targeted therapeutical strategies in treating bone healing problems.

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