BMP-2 can inhibit bone healing

Bone-chamber study in rabbits

Charlotte Jeppsson and Per Aspenberg

We came across a bone growing situation in which BMP-2 inhibits bone formation. Collagen sponges with BMP-2 were inserted in titanium bone chambers in rabbits. In this model, bone formation occurs spontaneously. With 2.4 and 0.12 μg BMP-2 per mm^3 of the chamber volume, bone ingrowth was reduced to 16% and 42% of collagen controls, respectively. Sponges with 2.4 μg BMP-2 per mm^3 that were placed in a similar titanium chamber in an intramuscular subfascial site induced bone formation in 4 cases of 5. The BMP-2 concentrations used are considered optimal for extraskeletal bone induction, and intramuscular sponges in rats all induced bone. The inhibitory effect is most likely model-related, but indicates unknown effects of BMP-2.

Department of Orthopedics, Lund University Hospital, S-221 85 Lund, Sweden. Tel +46 46-17 15 26. Fax -13 07 32
Correspondence: Dr. P. Aspenberg
Submitted 96-02-05. Accepted 96-09-03

There are high expectations concerning the clinical usefulness of BMPs for stimulation of bone production. Many experiments have proven their efficiency for healing of critical size defects in rodents, but also in non-human primates (Tourimi et al. 1991, Yasko et al. 1992). In all these experiments or models, skeletal healing is impaired because of the size of the defect. In 1992, we addressed the question whether BMP could also be used to accelerate the healing of small skeletal defects, which already had a potential for spontaneous healing. We therefore implanted BMP-2 in a hyaluronate carrier gel in bone harvest chambers. The bone harvest chamber is a standardized small skeletal defect inside a titanium implant in rabbits. The defect heals spontaneously within 3–4 weeks (Thoren et al. 1995). Much to our surprise, we found a strong inhibitory effect of the BMP-2 preparation used at that time, although it induced bone formation intramuscularly in rats. As this negative effect was so unexpected, we thought that we had prepared the implants incorrectly. Due to the limited amount of BMP-2 available, no further experiments could be done and we did not publish our findings. Recently, however, there have been reports of cyst formation in the bone callus induced by high amounts of BMP-2 (Sciadini et al. 1995). It is possible that the lack of bone formation in the old pilot experiment in reality reflected the same reaction as these cysts. This leads us to believe that the findings of the old study may have been correct. We have now repeated the previous experiments, this time using a BMP-2 collagen composite entirely produced by the BMP-2 manufacturer (Genetics Institute) and known to function well in other models (Turek T, personal communication).

Methods and animals

Bone Harvest Chamber and titanium tubes

The Bone Harvest Chamber (BHC) is a 7 mm high and 6 mm wide titanium cylinder, which is threaded so that it can be screwed into the proximal medial tibial metaphysis of adult rabbits (Albrektsson et al. 1983). The cylinder contains a piston-like core with a 1 × 1 × 5 mm groove facing the bottom of the cylinder. This groove is co-axial, with holes in the outer cylinder providing a continuous canal for tissue ingrowth through the entire device. The cylinder will osseointegrate with the surrounding bone in a period of 6 weeks, but one end sticks out of the bone. From this end,which can be reached through a cutaneous incision, the core can be pulled out, thus exposing the tissue in the ingrowth canal.

The tissue can be harvested without disturbing the surrounding bone. The chamber can be used for repeated harvesting, and bone formation in the chamber has been shown to be quite sensitive to various forms of disturbance (Goodman 1994), yet bone ingrowth remains stable over as many as 20 consecutive harvests (Thoren et al. 1995).

A 5 mm long titanium tube, open at both ends and with an inner diameter of 1 mm, was designed to mimic the ingrowth canal of the BHC, and was used for subfascial implantation. The tubes had 2 external

circumferential grooves enabling them to be sutured to a specific site.

Surgical procedure

Implantation of the BHC was carried out by exposing the proximal, medial tibial metaphysis of the rabbit, just anterior to the medial collateral ligament. A 6.5 mm cortical window was excised with a hollow drill. The cylinder of the BHC was screwed into the bone with a special wrench so that the ingrowth openings were located at the level of the tibial cortex. After 6 weeks of osseointegration, the contents of the BHC were harvested and discarded. The experiments then started.

Bilateral subfascial tubes were implanted in the back of 5 rabbits via one cutaneous incision just cranial to the sacrum and a minor incision in the fascia bilaterally. Each tube was attached to the under-surface of the fascia by a 6–0 nonresorbable suture. The tubes were harvested by removal en bloc, together with a surrounding 5 mm soft tissue layer.

Animals and BMP-2 implants

We started with 5 adult rabbits, a crossbreed between lop-eared and white native breed. After 6 weeks, the chambers were harvested every second week, including 2-week rest periods with no treatment following each 2-week period with active substances. As 1 rabbit became infected, its series of implantations was continued in a new animal (Table 1).

The implants were a gift from Genetics Institute (Cambridge, MA, USA). They were delivered as 200 mm^3 lyophilized discs of highly purified bovine collagen type I, and kept in a –80 °C freezer. Recombinant human BMP-2, formulated in a cryoprotectant buffer system had been lyophilized onto the discs, so that they contained 0 (control), 10 and 200 µg per disc. Discs from the same batch regularly induced bone in a dose-dependent manner when intramuscularly implanted in rats (Aspenberg and Turek 1996). We cut the discs into pieces corresponding to a total dose of 0 µg, 0.6 µg, or 12 µg per piece. These pieces fitted precisely into the chambers.

For the periods with active substances, collagen with BMP-2 was introduced into one chamber in each animal, and the collagen control into the other. In the first experiment, the rabbits received 12 µg per chamber (2.4 µg per mm^3) on the active side. After a control period with no implant in the chambers, a second experiment, with 0.6 µg per chamber (0.12 µg per mm^3), and a new period with no implants followed.

Subfascial tubes were implanted in 5 animals. They were filled with collagen 12 µg BMP-2 on one side of the back, and the contralateral tube served as a control, containing the collagen carrier in one case, but otherwise empty. In one rabbit, a control tube was not inserted. The tubes were harvested after 4 weeks and the specimens were fixed in formalin before the tissue was mechanically detached from the tubes.

Evaluation

3 hours before harvesting the BHC and tubes, the rabbits were given a known dose of 99-technetium methylene diphosphonate (99Tc MDP, approximately 45 MBq) as a bolus intravenous injection. After harvesting, the gamma emission of the specimen was measured and corrected for time-dependent decay.

All specimens were fixed in 4% buffered formalin, decalcified in Parengy's solution, embedded in paraffin and cut into 5 µm sections, enabling the entire length of the specimen to be visualized on one slide. The sections were stained with hematoxylin and eosin.

Histomorphometry was performed with a microscope connected to a computerized video digital table system at a screen magnification of ×125. The entire specimen and the bone areas were circumscribed with a pen on the digitizing table and the bone area was expressed as a percentage of the total area of the section. All measurements were performed by one person, with the slides numbered at random and the identity tags covered.

The specimens from the subfascial tubes were evaluated by qualitative histology.

Statistics

Statistical analysis was done by one-way ANOVA, followed by Fishers PLSD test, with a significance level of 0.02. 8 treatment groups (corresponding to the figures in Table 1) were entered in the ANOVA.

Results

With 12 µg BMP-2, the specimens usually consisted of a fibrous string on the bottom of the chamber canal, while with 0.6 µg BMP-2, the specimens usually filled the whole canal, forming a rectangular cord of tissue. On the collagen side, the specimens formed a cord filling the whole canal, and usually macroscopic bone was formed in both ends of the specimens. This was also the result when the chambers were harvested after 2 weeks without implants. With 12 and 0.6 µg BMP-2, the percentage of the section consisting of bone was reduced to 16% and 42% of the collagen controls (Table 1). The percentage of bone in the section and the ^{99}Tc activity correlated ($r = 0.8$; $p = 0.0001$). With 12 µg BMP-2, both the percentage of

Table 1. Bone in harvested specimens (%) after implantation of collagen with 12 or 0.6 µg BMP-2 (BMP 12, BMP 0.6), collagen with BMP-2 (exp), collagen without BMP-2 (collagen) or after rest periods with no implants (after exp, after c)

	BMP 12				BMP 0.6			
Rabbit	Exp	Collagen	After exp	After c	Exp	Collagen	After exp	After c
1	0.9	19	18	14				
2	2.5	23	16	14	14	18	13	20
3	0	20	38	26	3.1	20	23	25
4	2.1	16	20	7.2	3.5	20	28	10
5	8.1	6.4	16	17	13	17	18	24
6					4.9	18	14	20
Mean	2.7	17	22	16	7.8	18	19	20

Table 2. ^{99}Tc activity of harvested specimens after implantation of collagen with 12 or 0.6 µg BMP-2 (BMP 12, BMP 0.6), collagen with BMP-2 (exp), collagen without BMP-2 (collagen) or after rest periods with no implants (after exp, after c)

	BMP 12				BMP 0.6			
Rabbit	Exp	Collagen	After exp	After c	Exp	Collagen	After exp	After c
1	1.2	16	15	21				
2	1	17	20	21	8.3	17	12	15
3	0.9	20	23	18	6.9	18	16	16
4	1.7	8.8	11	6.9	2	5.6	15	9.3
5	4.7	11	12	16	11	14	8.5	12
6					3.3	17	10	17
Mean	1.9	14	16	17	6.3	14	12	14

bone and the ^{99}Tc activity were lower than all 6 control treatments (i.e., contralateral collagen sponge, contralateral collagen sponge of 0.6 µg experiment and chambers with no implants of both sides, following both experiments; Tables 1 and 2). With 0.6 µg BMP-2, the percentage of bone and the ^{99}Tc activity were lower than in the contralateral collagen controls; the percentage of bone was also lower than in both sides of the consecutive controls with no implants. Neither the percentage of bone, nor the ^{99}Tc differed between the high and low BMP-doses. The 6 control groups (2 collagen and 6 groups without implants) appeared equal ($p > 0.05$). All mentioned differences were statistically significant ($p < 0.02$).

The subfascially implanted tubes showed bone production in or around the tubes in 4 cases of 5. In 2 cases, the BMP-2-containing tubes were surrounded by bone, in 1 case bone was found at the tube entrances, and in 1 case there were only microscopic amounts in a few histologic sections, which also contained a large amount of inflammatory cells. The last specimen and the controls contained only a little connective tissue, without signs of inflammation.

Discussion

There is no doubt that the implants contained active BMP-2: other implants from the same batch regularly induced marked bone formation intramuscularly in rats (Aspenberg and Turek 1996) as did most of the extraskeletal titanium implants in the rabbits. The inhibitory effect on bone healing in the chambers was obvious. Not only was bone formation impaired, but tissue ingrowth into the defect was generally diminished. The concentration of BMP-2 in the collagen sponges was the same as that used in other series and recommended by the producer. However, as the half-life of BMP-2 in the body is probably very short because of inactivation and diffusion loss, the situation in the titanium chambers may be markedly different from the situations for which this concentration is recommended. Thus, in this chamber, diffusion losses may be less, since the material is surrounded by metal, except for the 2 bone ingrowth openings which contained compact bone. Further, it is possible that the inflammatory reaction in an intramuscular site has a large area for attacking the implant, whereas in the chamber the BMP-2 is protected. Such protection has

been thought to diminish BMP-2 effects when a collagen carrier is used and the implant is partly surrounded by a porous membrane (Linde and Hedner 1995). Therefore the BMP-2 concentration in the chamber at a critical time point may have been much higher than in a corresponding intramuscular implant. The local conditions in the chamber, however, could parallel the center of a large induced callus and this is also the location where cysts have been found (Sciadini et al. 1995). The negative effect of BMP-2 could have been a result of an unphysiologically high concentration, causing inhibition of proliferation, or perhaps even toxicity. Another possibility would be that the differentiation signal given by the BMP-2 made the bone at the resection ends mature so completely that no further proliferation into the chamber canal was achieved. In the present model, we have not been able to study the bone surrounding the implant, but only the tissue which has formed where the original implant was placed (the canal).

The collagen carrier was not responsible for the inhibition of bone ingrowth, because collagen controls showed large amounts of bone in the defect, with no difference from untreated controls. One cause of toxicity could be minimal amounts of contaminants to the BMP-2, which are harmless if not enclosed in a volume like the chamber or in the center of a big callus. It remains to be clarified whether the present negative effect is a general problem or strictly a problem related to model or species. As spontaneous healing is rapid in this model, increased bone production may be hard to achieve with any growth factor treatment. However, increased bone production has been demonstrated in similar models by electrical stimulation (Buch et al. 1984). The next step, of course, would be to repeat the present experiments with lower doses.

Added in proof

It has recently been shown that BMPs induce programmed cell death (apoptosis) during embryonal development: a negative type I BMP receptor, which specifically binds BMP-2 and BMP-4, blocked interdigital apoptosis in chicken, leading to webbed feet (Zou and Niswander 1996). In our chambers, the overall decrease in the amount of tissue following BMP 2 treatment may result from BMP-induced apoptosis.

Acknowledgements

We thank Inger Mårtensson and Carina Forslund for technical assistance. This study was financially supported by the Swedish Medical Research Council (project 2031), the Medical Faculty of Lund University, the Kung Gustaf V Jubileumsfond, the Greta och Johan Kocks, Alfred Österlund, Trygg-Hansa and Tore Nilsson Foundations.

References

Albrektsson T, Jacobsson M, Kälebo P. The harvest chamber—a newly developed implant for analysis of bone remodelling in situ. In: Biomaterials and biomechanics. 1983 (Eds. Ducheyne P, Van der Perre G, Ubert A, El). Elsevier Sciences Publ, Amsterdam 1984; 283-8.

Aspenberg P and Turek T. BMP-2 for intramuscular bone induction. Effect in squirrel monkeys is dependent on implantation site. Acta Orthop Scand 1996; 67 (1): 3-6.

Buch F, Albrektsson T, Herbst E. Direct current influence on bone formation in titanium implants. Biomat 1984; 5: 341-6.

Goodman S B. The effects of micromotion and particulate materials on tissue differentiation. Bone chamber studies in rabbits. Acta Orthop Scand (Suppl 258) 1994; 65.

Linde A, Hedner E. Recombinant bone morphogenic protein-2 enhances bone healing, guided by oosteopromotive e-PTFE membranes: An experimental study in rats. Calcif Tissue Int 1995; 56: 549-53.

Sciadini M F, Dawson J M, Berman L M, Johnson K D. Dose response characteristics of recombinant human bone morphogenetic protein-2 (rh-BMP-2) in a canine segmental defect model (Abstract). Trans Orthop Res Soc 1995; 20: 594.

Thoren K, Aspenberg P, Thorngren K-G. Lipid-extracted bank bone. Clin Orthop 1995; (311): 232-46.

Tourimi D M, Kotler H S, Luxenberg D P, Holtrop M E, Wang E A. Mandibular reconstruction with a recombinant bone-inducing factor. Arch Otolaryngol Head Neck Surg 1991; 117: 1101-12.

Yasko A W, Lane J M, Fellinger E J, Rosen V, Wozney J M, Wang E A. The healing of segmental bone defects induced by recombinant human bone morphogenic protein (rhBMP-2). J Bone Joint Surg (Am) 1992; 74: 659-70.

Zou H, Niswander L. Requirement for BMP signalling in interdigital apoptosis and scale formation. Science 1996; 272: 738-41.