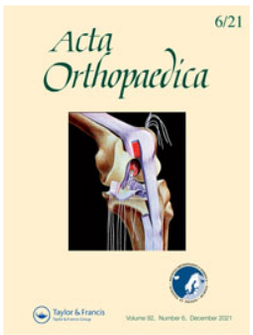

Acta Orthopaedica Scandinavica

ISSN: 0001-6470 (Print) (Online) Journal homepage: informahealthcare.com/journals/iort19

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To cite this article: Per Aspenberg, Elizabeth Wang & Karl-Göran Wang (1992) Bone morphogenetic protein induces bone in the squirrel monkey, but bone matrix does not, Acta Orthopaedica Scandinavica, 63:6, 619-622, DOI: 10.1080/17453679209169721

To link to this article: https://doi.org/10.1080/17453679209169721

Bone morphogenetic protein induces bone in the squirrel monkey, but bone matrix does not

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Demineralized bone matrix (DBM) reproducibly induces extraskeletal bone formation in rodents, but its effects in dogs and primates are negative or uncertain. In previous studies on the squirrel monkey, DBM did not induce bone, although the same implants were effective in nude rats. In the present study, the DBM was augmented with recombinant human bone morphogenetic protein-2 (BMP-2). Bone was formed in 10 of 12 monkeys, as verified by histology and calcium content. However, in 4 monkeys, the induced bone mass appeared smaller than the original implant. DBM controls induced microscopic amounts of bone in 2 out of 10 monkeys. In the nude rats, all DBM controls and augmented implants induced bone. The difficulties in achieving bone induction in higher animals may be overcome, at least partially, by using a higher concentration of the inductive protein than is present in DBM.

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Submitted 91-12-31. Accepted 92-05-17

Although demineralized bone matrix (DBM) reproducibly induces bone formation in rodents, its effects in dogs and primates are uncertain (Aspenberg 1988, Lindholm et al. 1988, Delloye 1990, Ripamonti 1991, Schwartz et al. 1991). In order to demonstrate bone induction, as it was defined by Urist (1965), it is necessary to do experiments in extraskeletal sites, so that bone inductive effects are not mixed up with non-specific stimulatory effects on bone healing. Such effects can be due not only to Bone Morphogenic Proteins (BMPs) but also to other growth factors present in bone matrix (Jingushi et al. 1990, Aspenberg et al. 1991b). However, the clinical challenge for bone induction is not to speed up skeletal healing, but to make the body produce bone where it otherwise would be producing scar tissue, as in an extraskeletal site.

Few extraskeletal experiments have been performed in man, and some in various monkey species. It has been possible to induce limited amounts of bone formation in baboons (Ripamonti 1991) and rhesus monkeys (Hosney and Sharawy 1985) using adult monkey DBM, but not in squirrel monkeys (Aspenberg et al. 1990). Occasionally, DBM from fetal squirrel monkeys have evoked a strong bone-forming response (Aspenberg 1988). Failures to induce bone in monkeys or dogs have been suggested to be caused by improper preparation of the DBM. However, those implants which did not induce bone in squirrel monkeys, regularly did so in nude rats, although with less bone formation than did rat bone matrix (Aspenberg et al. 1991a).

The reason for the failure in squirrel monkeys cannot be a total lack of BMP in the matrix, since it was effective in nude rats. It has been suggested that higher animals have a relative lack of inducible cells outside the skeleton (Lindholm et al. 1988). Further, monkeys may be more sensitive to the disturbance of bone formation caused by the immune response to allogeneic DBM. However, autogeneic DBM did not work in squirrel monkeys either (Aspenberg et al. 1991a).

We now tested the hypothesis that squirrel monkeys require more BMP in order to respond with bone formation.

Material and methods

Implant preparation

From 4 adult squirrel monkeys (Saimiri sciureus sciureus), the diaphyses of all long bones were harvested and prepared immediately. The periosteum was torn off, the bones were fragmented and the marrow was washed and scraped away manually. The specimens were deposited in chloroform methanol for two hours, then rinsed with methanol, dried and milled in a Cemotec 1090 Sample mill (Tecator, Helsingborg, Sweden) in liquid nitrogen. The bone powder was sieved to 125–350-μm particle size, and then demineralized with 0.6N HCl for 48 hours, rinsed in sterile water and lyophilized.

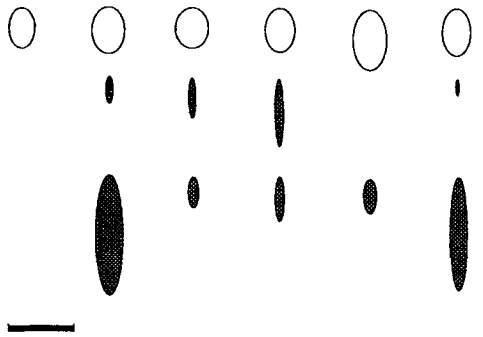

Figure 1. Dimensions of recovered hard tissues 6 weeks after implantation of DBM control (upper row) and DBM with BMP 5 µg (middle row) and 50 µg (lower row). Scale drawing based on maximum length and width, measured with a caliper. White areas: hard whitish fibrous tissue. Shaded areas: bone. Horizontal bar: 10 mm.

Samples of the powder, 25 mg each, were placed in vacuo 24 h, then 5.0 and 50.0 µg of sterile recombinant human BMP-2 in 0.1 percent trifluoroacetic acid, 35 percent acetonitrile was added. Buffer alone was added to the controls. Recombinant BMP-2 was purified as described (Wang et al. 1990).

Operation

The monkeys were operated on at two different occasions: 6 in a first, and 6 in a second group. The operations did not differ. All 12 monkeys received implants in superficial pouches in their left vastus lateralis muscle. The implanted powder samples measured roughly about 2 × 5 × 7 mm. The 3 types of implants (control, 5 µg and 50 µg) were allocated from distal to proximal in different orders in each animal (6 permutations possible). One animal did not receive a control implant because at the operation it appeared that the plastic test tube in which it was kept had a crack, entailing a risk for bacterial contamination.

6 nude rats received implants in their abdominal wall muscles, control and BMP 5 µg in separate pouches on the right side and BMP 50 µg on the left.

Evaluation

All implants were harvested at 6 weeks. The harvested tissues from the last 6 monkeys were studied under a dissection microscope before fixation, and the dimensions of any hard parts were measured with a caliper. All harvested tissues were fixed in buffered formalin and decalcified for one week in 10 mL of Parengy's solution (chrome trioxide 0.15 percent, nitric acid 4.3 percent, ethanol 27 percent).

The resulting calcium content of the decalcificating solution was then measured with atomic absorption spectrophotometry. The specimens were next sectioned throughout and stained with HE. All sections were studied. Completeness of demineralization was checked with von Kossa stain.

Results

Monkeys

Macroscopic findings. All control implants appeared as hard, whitish buttons of the same size and shape as when implanted. One control implant appeared to have been "invaded" by bone from its neighboring BMP 50 µg implant. This implant and the control implant had united into one rod-shaped ossicle, so the "control" here represents only a part of that ossicle and must be excluded.

The BMP implants, unlike the controls, were never recovered as "buttons". They had either disappeared, leaving a small scar, or been replaced by a needle- or rod-shaped piece of bone. From the first group of 6 monkeys, three showed 5–15-mm bone rods in 5- and 50-µg implants. In two animals, the BMP implants were only partially resorbed, and no striking new bone was observed grossly. In the last monkey, only small scars were found.

From the second group of 6 monkeys, all controls appeared unchanged. In one monkey, the BMP implants were totally resorbed. In the remaining 5 monkeys, bone was formed. The bone rods, always oriented parallel to the surrounding muscle fibers, were often longer and always narrower than the original implant (Figure 1).

Histological findings. Bone was observed in 10/12 BMP 50-µg implants and in 8/12 BMP 5-µg implants. Although the sizes differed, the bone tissue always consisted of a thick shell of dense, woven bone, surrounding a marrow cavity with a network of woven bone trabecula. The bone was delineated from the surrounding muscle by a thin layer of fibrous tissue. Sometimes there was an area of scar tissue adjacent to one side of the bone (Figure 2). There were no remnants of implanted matrix within the bone, but in some cases martix particles were seen outside.

The 10 control implants were surrounded by a fibrous tissue layer and the implanted granulae were not resorbed (Figure 3). In 2 implants a small marrow cavity was observed, with some bone lamellae on adjacent matrix particles. In one implant there were some bone cells adjacent to a few granulae. These cells were not numerous enough to form either areas of woven bone, or ossicles with bone marrow, and the result is classified as histologically negative.

Figure 2. Intramuscular ossicle induced by DBM with 50 μg BMP in the squirrel monkey. Transverse section of one of the smaller ossicles. A large portion of the implanted DBM has been replaced by scar tissue, × 16.

Figure 3. DBM implant without BMP in the squirrel monkey. No bone and no resorption × 8.

Histologic ossicle formation in response to BMP 50-μg (10 monkeys out of 12) differed from controls (2 out of 10); P 0.005 (Fischer's exact test).

Calcium. In 10 monkeys, the BMP 50 μg implants contained more than 2 μg calcium per mg implanted dry matrix. Values above 2.0 μg have previously been considered to indicate at least "trace amounts" of bone (Aspenberg et al. 1991a). Of the 10 controls, 8 implants contained less than 2 μg calcium (Table 1). Implants with 50 μg of BMP differed from controls ($P < 0.01$; Wilcoxon's signed rank test). The grossly measured hard tissue area of the BMP implants (length x width) correlated with the calcium content (r 0.9). Controls in monkeys differed from controls in nude rats ($P < 0.001$; Mann-Whitney test).

Nude rats

All implants in nude rats, with or without BMP, contained bone. The calcium content was higher than in the monkeys (Table 2).

Histological examinations showed that the control implants all contained several little ossicles inside the larger volume of the remaining implant particles. In contrast, all BMP implants consisted of just one big bone shell with no surrounding implanted particles. Unlike in the monkeys, the shell was very thin, and inside there was a marrow cavity containing non-resorbed implant particles, lined with new bone.

Table 1. Calcium yield (μg/mg) as an indicator of bone formation in 12 consecutively numbered adult squirrel monkeys, receiving implants of monkey bone matrix (control), and human recombinant BMP-augmented monkey bone matrix (BMP 5μg and BMP 50μg). The values are μg of Ca per mg of implanted dry matrix. Histologic bone indicated

Monkey	Control		BMP 5 μg		BMP 50 μg	
1	0.4		2.6	bone	22	bone
2	a		0.2		0.2	
3	0.1		26	bone	66	bone
4	0.3		22	bone	2.8	bone
5	b		5.0	bone	40	bone
6	0.2		0.2		3.0	bone
7	8.7	bone	4.1	bone	84	bone
8	0.2		35	bone	9.4	bone
9	0.2		0.1		3.7	bone
10	0.1		0.1		0.1	
11	2.0	bone	11	bone	6.1	bone
12	0.1		4.4	bone	284	bone

[a] Not implanted
[b] Excluded due to apparent overgrowth from neighbor implant

Table 2. Same as Table 1, but implanted in nude rats. Histologic bone in all

Rat	Control	BMP 5 μg	BMP 50 μg
1	94	104	44
2	44	78	92
3	36	98	106
4	96	112	70
5	32	112	110
6	36	112	82

Discussion

Our finding clearly indicates that extraskeletal bone induction is possible in the squirrel monkey. Inducible

cells may be present outside the skeleton in sufficient numbers, but in contrast to rodents, they seem to need a higher BMP concentration than was supplied by DBM only.

The varying or negative results with DBM in other primates and in dogs may have the same explanation, i.e., these animals require a higher dose of BMP than rodents. Sometimes, in some laboratories, the implant preparation, size and shape may have been sufficient to produce the necessary BMP stimulus, but certainly never with the same ease and reproducibility as in rodents. Experiments in intraskeletal sites are not taken in account, as any procedure may imply a variety of stimulatory effects on bone healing other than bone induction.

In 6 monkeys there was macroscopic bone of about the same volume as the implanted matrix, or more. This could be taken as an indication that BMP-augmented DBM might become clinically useful. However, clinical use requires a much higher success rate than was achieved in this study. We have no explanation why the response was smaller in 4 and absent in 2 monkeys. In contrast to the rats, the matrix was often resorbed in the monkeys. In baboons, Ripamonti (1991) lost 11 out of 47 partially demineralized bone implants due to total resorption. It is possible that the immunologic response to allogenic bone unpredictably disturbs bone induction in monkeys, just as it can abolish bone induction by xenogeneic DBM in rats (Aspenberg et al. 1988). Due to this and other shortcomings of DBM as a carrier, another carrier material for the BMP may become necessary in order to obtain enough bone in all monkeys (and in man). Many other factors are present in the DBM and may influence bone induction (Hauschka et al. 1986, Aspenberg et al. 1991b). In the rat, a DBM carrier is not necessary for bone induction by BMP-2 (EW unpublished), and the DBM with its auxiliary factors can probably be omitted in primates, too.

We were not able to demonstrate a difference between the two BMP doses. In the rats this may be due to the fact that the total volume of the BMP implants was occupied by new bone, or mostly bone marrow, at six weeks. These results are in agreement with previous findings in rats, where a dose difference was seen only after less than 2–3 weeks, whereas later no difference was seen between 0.5 or 115 μg of BMP (Wang et al. 1990). In the monkeys, the explanation may be the same, although it is possible that the number of inductive cells has become the limiting factor, once their lower sensitivity to BMP has been overcome by a sufficient BMP concentration. However, with an adequate delivery system for BMP, it may become possible to induce controlled bone formation in man.

Acknowledgements

Bodil Wüeggertz took care of the monkeys. Inger Mårtensson and Carina Kristensson operated on the rats and made the histologic preparations. Grants were received from the Swedish Medical Research Council, project 2031, the Medical Faculty of Lund, the Swedish Society for Medical Research, Trygg-Hansa, the Alfred Österlund and the Tore Nilson foundations.

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