BMP-2 for intramuscular bone induction

Effect in squirrel monkeys is dependent on implantation site

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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 rats. DBM implants augmented with recombinant human bone morphogenetic protein-2 (rhBMP-2) induced intramuscular bone formation in squirrel monkeys. However, the amount of induced bone was often minimal and sometimes absent. One explanation of this weak and unpredictable effect could be reactions to cellular components of the allogeneic DBM that was used as carrier. Therefore, we now repeated the experiment, using bovine type I collagen as carrier. 48 collagen discs (10 mm diameter), containing 0, 10, 40 or 200 µg rhBMP-2, were implanted in 6 monkeys and 6 rats. The BMP-2 implants induced dose-dependent amounts of intramuscular bone in rats whereas, in squirrel monkeys, macroscopic bone induction occurred in less than half of the BMP-2 implants. There was no dose-dependency. Whether bone was formed or not was significantly influenced by individual variation among the monkeys, and by implant location within the muscle. Implants close to the muscle aponeurosis more often induced bone than did purely intramuscular ones. In this small series, we could not demonstrate a significant effect of BMP-2, as compared to control implants. Presumably there were too few cells expressing BMP-2 receptors in the only minimally traumatized muscle of these monkeys.

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Demineralized bone matrix (DBM) reproducibly induces extraskeletal bone formation in rodents, but its effects in dogs and primates are uncertain (Aspenberg et al. 1988a, Delloye 1990, Schwartz et al. 1991). In previous studies on the squirrel monkey, allogenous or autogenous DBM did not induce bone, although the same implants were effective in nude rats (Aspenberg et al. 1991). When DBM implants were augmented with recombinant human bone morphogenetic protein-2 (BMP-2) they induced intramuscular bone formation in squirrel monkeys. However, the amount of induced bone was often minimal and sometimes absent (Aspenberg et al. 1992). Immune reactions can inhibit bone induction by DBM (Aspenberg et al. 1988b). We hypothesized that the weak and unpredictable effect of DBM augmented with BMP-2 could be due to immunological reactions to cellular components of the carrier. Therefore, we now repeated the experiment, using bovine collagen as carrier. In order to separate bone induction from less specific stimulation of a normal skeletal healing response, only extraskeletal sites were used.

Material and methods

The implants were formulated at the Genetics Institute, Cambridge, Massachusetts, USA. Implantation and evaluation were performed at the Department of Orthopedics, Lund University Hospital, Lund, Sweden.

Implants and animals

The implants consisted of 200 mm^3 lyophilized discs of bovine type I, tendon-derived collagen, cross-linked through exposure to formaldehyde. The disc diameter was 10 mm. Recombinant human BMP-2, formulated in a cryoprotectant buffer system was lyophilized onto the discs, so that these contained 0 (control), 10, 40 or 200 µg per implant. The recipient animals were 6 adult Bolivian strain squirrel monkeys of both sexes, aged more than 4 years, operated on using Alphaxalone anesthesia, and 6 female 200 g Sprague-Dawley rats that were anesthetized with a mixture of pentobarbital and diazepam.

Operations

The monkeys were operated on, using ordinary operating room routines for asepsis. A longitudinal inci-

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Table 1. Calcium yield (µg/implant) from collagen implants with different doses of rhBMP-2, implanted in rats

Rat	rhBMP-2 dose µg			
	0	10	40	200
1	54	520 [a]	620 [a]	11
2	9	540 [a]	5	1500 [a]
3	11	550 [a]	1100 [a]	1400 [a]
4	8	1300 [a]	1300 [a]	1600 [a]
5	31	330 [a]	1400 [a]	2300 [a]
6	31	2000 [a]	1300 [a]	2600 [a]
Mean	24	880	1130	1870
SD	18	660	320	530

[a] macroscopic bone

Table 2. Calcium yield (µg/impl.) from collagen implants with different doses of rhBMP-2, implanted in squirrel monkeys

Monkey	rhBMP-2 dose µg			
	0	10	40	200
1	1	1	1	1
2	1	71 [b]	17 [b]	1
3	1	4200 [a]	15000 [a]	15000 [a]
4	2	4	13	110 [a]
5	5000 [a]	5	54 [a]	160 [b]
6	5	1300 [a]	1100 [a]	7

[a] macroscopic bone
[b] microscopic bone only

sion through the skin and fascia lata was made on the lateral side of the left thigh. 2 superficial muscle pouches were created in the vastus lateralis. The proximal part of the vastus lateralis has a thin translucent fibrous aponeurosis. This aponeurosis partly formed the cover of the proximal pocket, whereas the distal pocket was purely intramuscular. The discs were inserted flat in their pockets and only the skin was sutured. The monkey was then turned on the other side, washed and draped again and a similar procedure was performed on the right thigh. Each animal received one of each type of discs and these were evenly allocated to proximal and distal sites. At every procedure, both during implantation and harvest, the identity of each implant and implantation site was checked by two persons. After implantation, the discs were visible through the very thin covering muscle layer. The animals recovered well and moved without any sign of discomfort on the day after the operation. After 6 weeks, the monkeys were anesthetized again and a similar operation procedure was performed, which now consisted of harvesting the implants and a thin layer of surrounding muscle.

The rats received their discs in the abdominal muscle pouches created between the 2 oblique layers lateral to the rectus sheath on both sides. Here the implants were also evenly distributed in cranial and caudal sites. The rats were killed after 6 weeks and their implants were harvested.

Evaluation

The harvested tissues from the 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 1 week in 10 mL of Parengy's solution (chrometrioxide 0.15%, nitric acid 4.3%, ethanol 27%). The resulting calcium content of the decalcifying solution was measured with atomic absorption spectrophotometry. The specimens were then serially sectioned all through and stained with HE. The completeness of demineralization was checked with the van Kossa stain.

The occurrence of bone was tested for significance, using Fischer's exact test, and the calcium yield was checked by the Kruskal Wallis test.

Results

In the rats, none of the control implants induced bone formation, whereas macroscopic bone was present in 16 of the 18 implants containing BMP (Table 1). The amount of calcium in these 16 samples was dependant on the BMP dose (r 0.6, $p < 0.01$).

2 of the monkeys showed no macroscopic bone formation in any of the implants. 1 monkey had formed a big ossicle along its entire vastus lateralis, incorporating the implantation sites for the 2 higher BMP doses, and also a considerable ossicle on the contralateral side at the site for the lowest BMP dose. In the last 3 monkeys, macroscopic bone was seen in 4 of the 9 BMP implants and in 1 of the control implants (Table 2). The bone pieces were flat, usually only 1 mm thick. Their longest diameter was parallel to that of the muscle fibers, sometimes exceeding that of the collagen implant. The dimensions of the bone samples (length by width by thickness) correlated to the calcium content (r 0.9, $p < 0.0001$). 3 samples without macroscopic bone showed bone histologically. All 3 were BMP-implants.

We found that the occurrence of macro- or microscopic bone in response to BMP, had no statistical significance. There was no dose dependency in the calcium yield. Regardless of BMP content, the proximally-placed implants more often yielded bone (p 0.001; Table 3). Only 1 out of 9 BMP implants in the distal site induced macroscopic bone formation,

Table 3. Number of implants yielding macroscopic bone formation per total number of implants in proximal versus distal implant sites. Implants in the proximal vastus lateralis are close to the aponeurosis

Site	rhBMP-2 dose µg				Total
	0	10	40	200	
Prox.	1/3	2/3	3/4	1/2	7/12
Dist.	0/3	0/3	0/2	1/4	1/12

p 0.001

whereas 6 of the 9 proximal BMP implants did so (p 0.02). This difference was also significant for microscopic bone (p 0.01). There was a significant (p 0.04) difference in calcium yield between individual animals, but not between the 4 types of implants.

On histological examination, classical ossicles were observed, with a 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. However, the ossicles were usually flat in the plane of the original implant, so that 2 bone layers were seen with marrow in between. In some cases, areas of woven bone had not yet organized into an ossicle.

In cases with no bone formation, we found only minimal scar tissue.

Discussion

Urist (1965) demonstrated extraskeletal bone induction by demineralized bone matrix and postulated the existence of a bone morphogenetic protein. Since then, a large volume of experimental work has been performed, both on laboratory animals and patients. However, it remains uncertain whether DBM has bone-inductive effects in man (Aspenberg et al. 1988a). In squirrel monkeys, DBM is ineffective (Aspenberg et al. 1991), whereas in rhesus monkeys, DBM induced intramuscular bone formation to a limited degree, and only after a delay of over 40 days (Hosny and Sharawy 1985).

BMP-2 on an inert carrier induces bone formation extra- and intraskeletally in rodents (Wang et al. 1990, Yasko et al. 1992) and at least intraskeletally in dogs (Toriumi et al. 1991). BMP-2 is superior to DBM, also in rats (Marden et al. 1994). The BMP-2 and collagen device used in this study has proven efficient in critical-sized long bone and mandibular defects in rodents, dogs and monkeys (manuscripts in preparation). Studies on extraskeletal sites in primates are rare. Ripamonti and coworkers have demonstrated extraskeletal bone induction following implantation of BMP-3 (osteogenin) with a collagenous bone matrix carrier in baboons (Ripamonti et al. 1992a). With a hydroxyapatite carrier, the BMP also seemed active. However, even the carrier alone caused bone formation, and the role of the BMP was uncertain (Ripamonti et al. 1992b). It appears that extraskeletal bone induction in baboons is rather easily achieved. In crab-eating monkeys, partially purified BMP induced intramuscular bone formation by a transfilter technique, which leaves no doubt about the effect of the BMP preparation (Miyamoto et al. 1993). In our previous experiments with BMP-2-augmented DBM in squirrel monkeys, there was clear bone induction, but not in all cases, and the amount of bone was sometimes small (Aspenberg et al. 1992).

In the present study, there was a dose-dependent effect of the BMP implants in the rats, and the result closely resembled a similar dose study at the Genetics Institute, using long Evans rats and a subcutaneous ventral thorax implant site (data not shown). Therefore, the explanation of the weak BMP-response in the monkeys is not faulty implants. The avoidance of the supposedly immunogenic DBM-carrier did not enhance bone formation in monkeys. The most striking findings are the variation between individual monkeys and the difference in response between different sites the muscle. The higher likelihood of bone formation in the proximal part of the thigh close to the aponeurosis could be caused by a higher number of cells with BMP receptors in fibrous tissue than in muscle. It seems important to study the expression of BMP receptors in various tissues in different species. The significant BMP effect in our previous study using the same model, but a DBM carrier, could perhaps be explained by the effects of auxiliary factors in the DBM, or that the inflammatory reaction to cell debris, etc, facilitated the response to BMP.

The expression of BMP genes does not necessarily lead to bone formation. For example, BMPs are expressed in benign prostatic cells (Bentley et al. 1992). The role of matrix-derived BMPs in fracture healing is unknown, but it seems likely that they participate in the process at some stage, and BMPs are produced within the callus. It is also possible that the production or release of growth factors in fracture healing is already optimized by nature, so that an extra supply by implants or injections is at best harmless. Under pathological conditions, BMPs could perhaps be useful—for example, in a defect which is too big for spontaneous healing. However, it is unclear

whether the middle of such a defect should not be regarded as an extraskeletal site, and so we get back to the question whether BMP receptors are expressed in sufficient amounts.

Probably, BMPs will become powerful medical tools, but more work seems required before we get there than previously anticipated. Not only does the development of good carriers seem to be a problem, but also it cannot be taken for granted that all mesenchymal-derived tissues in humans respond to BMP treatment with bone formation.

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