Bone graft proteins influence osteoconduction

A titanium chamber study in rats

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Although it is often emphasized that the matrix of bone grafts contains several growth factors, it is not known if these factors become activated and play a role in bone grafting. We therefore compared ground defatted bone which had or had not been deproteinized by heating with water to 270 °C at an autogenic pressure of 55 bar. This is a careful ceramic procedure which leaves the mineral unchanged. Deproteinized and non-deproteinized bone granulae derived from cortical rat bone were placed in titanium bone conduction chambers implanted bilaterally in rat tibiae. Ingrowing bone could enter the cylindrical interior of the chamber only at one end. It then penetrated the material in the chamber, but due to the length of the cylinder, it never reached the other end. The mean distance which the ingrown bone had reached in the material was then measured on histological slides. The bone formation activity was measured by TcMDP scintimetry. With the protein-containing granulae, the mean bone ingrowth distance and the scintimetric activity were increased by 41% and 31%, respectively ($p < 0.01$). We conclude that there is a limited favorable effect of proteins in a graft. Our grounded material had a large fracture surface area with no osteoid lining. The leakage of growth factors from such areas may explain the extraordinarily good clinical incorporation of morselized compacted allografts.

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The bone matrix contains large quantities of a variety of growth factors, such as BMPs and TGF-ßs, believed to play a role in bone remodeling and fracture repair (Hauschka et al. 1986). The biological significance of these factors in a nondemineralized bone graft is unknown. Certainly, if the biological activity of these growth factors is not activated in a bone graft, this would imply that a bone allograft has no biological advantages over a bone graft which has been "processed", so that these proteins are destroyed, or over a synthetic bone replacement material. Processed bone grafts may have practical advantages and diminish the risk of HIV transfection (Simonds et al. 1992). A common procedure is to extract lipids by the means of solvents such as diethyl ether. Defatted grafts are better incorporated (Aspenberg and Thoren 1990). However, molecules such as BMPs or TGF-ßs may still be active after lipid extraction (Urist 1980).

Another reason to wonder about the possible role of growth factors in bone allografts has to do with the astonishing success of the use of impacted morselized bone allografts in hip revision arthroplasty (Gie et al. 1993). The morselized grafts seem, at least in the short term, superior to structural grafts. One hypothetical explanation could be that the large total fracture area created by morselization releases biologically active substances which are otherwise trapped within the mineralized matrix under the osteoid which covers all other bone surfaces.

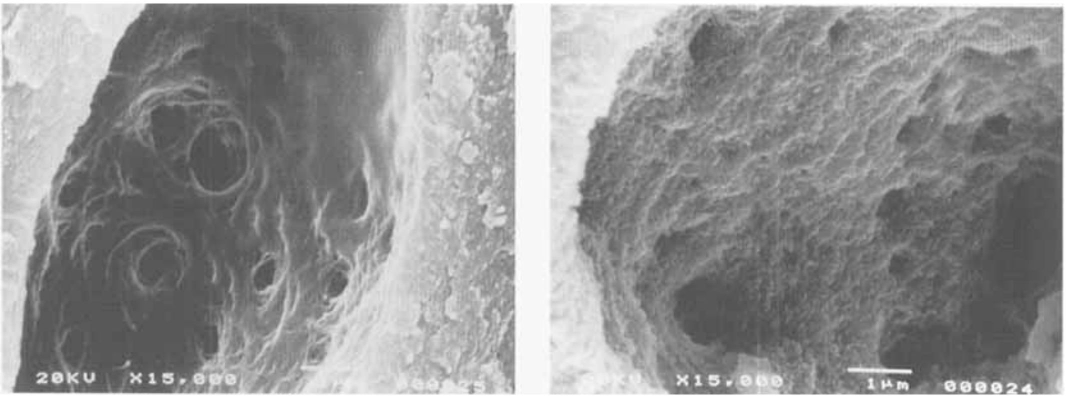
We have previously compared a defatted cancellous bone graft and porous hydroxyapatite by measuring the bone ingrowth distance into the material at 6 weeks in a titanium rat chamber model (Aspenberg and Wang 1993). In that comparison, the bone graft was superior. It could not be decided, however, whether this effect was due to the biological properties of the graft or simply to "non-biological" factors, like pore geometry. In the present study, we therefore measured bone ingrowth into a defect containing bone granulae which had, or had not been, deproteinized. The deproteinization was performed in such a way that the mineral phase of the bone remained unchanged.

Animals and methods

Graft material

Femoral and tibial diaphyses were collected from female 200 g Sprague-Dawley rats and immediately cleansed of periosteum and marrow. They were washed in water, defatted in chloroform methanol 1:1 for 20 minutes, rinsed several times in methanol, air dried, ground and sieved to a particle size of 0.35–

Figure 1. Interior of osteocyte lacunae in frozen defatted rat bone (SEM × 15000).



With no further treatment, the smooth surface represents the osteoid lining covering the mineralized bone.

In the deproteinized graft, the uncovered mineral appears as a granular surface.

0.65 mm. A portion of this powder was then heated with water to 270 °C at an autogenic pressure of 55 bar for 4 h. The material was washed in water, acetone and ethanol and then dried. Scanning electron microscopy of the powder showed no obvious difference after this deproteinization, except at high magnification (× 15 000). At this resolution, the interior of the osteocyte lacunae showed a smooth osteoid surface, whereas after deproteinization this surface had disappeared to make place for a granular surface, representing the remaining mineral (Figure 1). The degree of deproteinization was measured by ashing deproteinized bone at 900 °C. This induced a weight loss of less than 3%.

Bone conduction chamber (BCC)

The bone conduction chamber (Aspenberg and Wang 1993) consists of a threaded titanium cylinder, formed from two half-cylinders held together by a hexagonal closed screw cap. One end of the implant is screwed into the bone. The bone ingrowth chamber inside the implant has a diameter of 2 mm and is 7 mm long. There are two bone ingrowth openings at one end of the chamber (Figure 2).

Surgical procedure

18 male Sprague-Dawley rats served as graft recipients (35–381 grams; Møllegaard, Copenhagen, Denmark). They were kept in our animal facilities for 1 week before the experiments started (22 °C; 2 rats in each cage, free access to food pellets and water). The rats were anesthetized with peritoneal injections of 0.6–0.7 mL of a solution containing 1mL pentobarbital (60mg/mL), 2 mL diazepam (5mg/mL) and 1 mL saline, and were killed with an overdose of Mebumal.

Under aseptic conditions, longitudinal incisions were made bilaterally over the anteromedial aspect of the proximal tibial metaphyses. After incising and raising the periosteum, the medial and posterior lateral cortices were pierced with a 1.0 mm spike just anterior to the insertion of the medial collateral ligament. The hole created in the medial cortex was manually enlarged with a 2.7 mm drill. The bone powder was slightly moistened with saline and packed loosely into the chambers, which were then screwed into position so that the bone ingrowth holes were placed at the level of the cortical bone and the pointed end of the implant was engaged through the opposite cortical bone. Each animal received a chamber containing a defatted

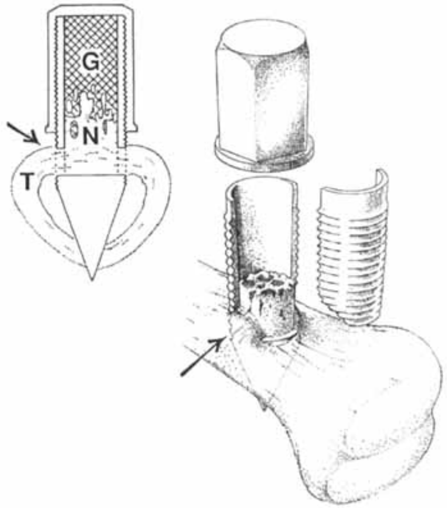


Figure 2. The chamber. Implant in position at the proximal tibial metaphysis. The ingrowth entrances (arrow) are situated in bone. Inset illustrates new bone ingrowth (N) and allograft bone (G). (Reproduced by permission of European Journal of Experimental Musculoskeletal Research).

bone graft in one leg and a defatted and deproteinized graft in the other. The wound was closed in layers.

5 days before termination of the experiment, the chamber position was checked. All chambers were correctly positioned with their ingrowth openings embedded in bone. 2 rats in the first group died from the anesthesia during this radiography. The histology from these rats was included. There were no infections.

Evaluation

The rats were killed after 6 weeks. An intravenous injection of 2.3 MBq ^{99m}Tc-MDP was given 3 hours beforehand, and the scintimetric activity of the harvested tissue was measured in a well counter. The activity was corrected for time-dependent decay.

The specimens were fixed in 4% formalin, decalcified and embedded in paraffin. They were cut parallel to the long axis of the chamber with a microtome and stained with hematoxylin and eosin. 3 sections from the middle of the specimens, each at 300 µm distance from the other, were used for histology and histomorphometry. This was done blindly, so that each specimen was given a code number, and all slides were investigated in random order. The area of the new ingrown bone was measured by using the Videoplan™ equipment at 40 × screen magnification. This area includes marrow cavities and graft remnants which had been surrounded by new bone.

The mean bone ingrowth distance in each slide was calculated by dividing the new bone area by the distance between the walls of the chamber—i.e., the width of the specimen (Aspenberg and Wang 1993). In all cases, fibrous tissue had penetrated further into the chamber ahead of the bone. The total tissue ingrowth distance, which is the distance from the ingrowth end of the chamber to the fibrous ingrowth frontier, was measured in the same way.

Thereafter, the specimens were organized in blinded experiment/control pairs and compared regarding the "density" of the fibrous tissue in the most distal part of the specimens.

The results of the 2 ingrowth distance parameters and scintimetry were tested for significance, using Student's paired t-test. The "density" of the distal fibrous tissue was tested with the sign test.

Results

Macroscopy

The part of the harvested tissue that was furthest away from the bone ingrowth openings had a different color on the deproteinized side and a greater tendency to fall apart (so that some implant particles came loose). This was also a blinded observation.

Figure 3. Specimen from bone conduction chamber 6 weeks after implantation of protein-containing bone granulae. The granulae are held together by fibrous tissue which has filled the entire chamber. Bone has grown in from the bottom and penetrated one third of the specimen (Specimen dimension 2 × 7 mm, H & E).

Histology

At the proximal ingrowth end of the cylinder, close to the bone ingrowth openings, new bone had formed a marrow cavity. Further distally, there was an irregular "bone ingrowth front" where a gradual transition from fibrous tissue to bone could be seen. Distal to this front, the fibrous tissue appeared dense with a pinkish matrix. Further distally, there was only loose connective tissue between the implanted granulae (Figures 3 and 4). There was no evidence of an inflammatory reaction to the two materials and they seemed unresorbed. The distal fibrous tissue was classified as denser in the non-deproteinized bone in 13 pairs and equal in 3 (p 0.004).

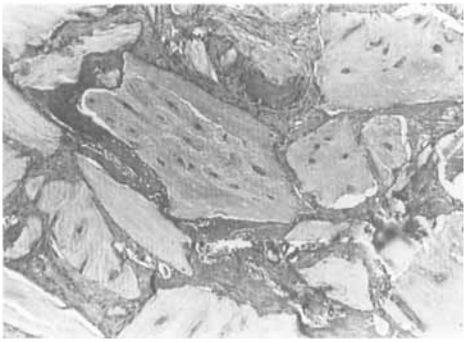
Histomorphometry

The total tissue ingrowth distance appeared unaffected by deproteinization: the difference was 2%. The mean bone ingrowth distance in each specimen (≈ new bone volume) was increased by 41% in the protein-containing powder (Table 1).

Scintimetry

The scintimetric activity was 31% higher in the protein-containing specimens (Table 1).

Figure 4. Specimen from the same rat as in Figure 3, chamber with deproteinized bone granulae.



Detail showing bone ingrowth frontier (× 100).

Fibrous tissue ingrowth has not been sufficient to keep the entire specimen together: the granulae have fallen apart at the far end from the ingrowth openings at the bottom. Bone ingrowth is less than in Figure 3. (Same magnification as Figure 3; H & E).

Table 1. Tissue ingrowth and Tc-MDP at 6 weeks in protein-containing (P) and deproteinized (D) bone grafts

	P	D	P–D	95% CI^a	P
Total tissue ingrowth^b	3.6	3.5	0.1	-0.3–0.4	0.6
Bone ingrowth^b	2.0	1.4	0.6	0.2–1.0	0.008
Scintimetric acitivty^c	5.7	4.4	1.4	0.5–2.2	0.004

[a] 95% confidence interval of the difference P–D.
[b] Mean distance (mm)
[c] Mean (cpm x 1000)

Discussion

Our experiment was designed to study osteoconduction, i.e., the distance which the ingrowing bone "front" had advanced at 6 weeks. With this definition of osteoconduction, we found a moderate increase when bone proteins were present in the osteoconductive material. Thus, it appears that bone matrix proteins in bone allografts have some effect on the graft's performance as a trellis for bone ingrowth. To our knowledge, this has not previously been demonstrated, although the presence of growth factors in bone grafts is often emphasized (Friedlaender 1987, Huo et al. 1992, Czitrom 1994). We saw no sign of osteoinduction—i.e., there was no cartilage or bone differentiation in the mesenchymal or fibrous tissue ahead of the bone ingrowth front. The effect that we observed may also have reasons other than the presence of growth factors, e.g., different conditions for cell attachment. However, the ingrowth distance for soft tissue was not affected by deproteinization, whereas the quality ("density") of this tissue was different. This may be an indication of a growth factor effect, rather than merely an effect of better conditions for cell attachment on the particles' surfaces.

The new method of using morselized compacted cancellous allografts for hip revision surgery shows results which dramatically differ from other kinds of allografting. In structural cancellous allografts, bone ingrowth is usually limited to 2–3 mm (Enneking and Mindell 1991), whereas the morselized compacted grafts seem to be totally remodeled in several cases (Gie et al. 1993). There could be two explanations of this astonishing clinical success: the production of a large fracture surface area by microfracturing the bone during morselization, or mechanical stimulation due to the compaction of the graft and the early mechanical load. A fresh fracture surface exposes the bone matrix to the surrounding tissues without a protective lining cell and osteoid layer. Perhaps the fracture surface created by morselization could have a growth-promoting effect by enabling the release of BMP or other growth factors. The BMP present in bone is considered not to be activated in experimental implants unless the bone is demineralized (Urist 1965). However, as described in Burwell's classical work on bone grafting (Burwell 1964), some new bone formation may be induced also from non-demineralized allografts. TGF-ß is present in a latent form and is known to be activated by osteoclasts (Oursler 1992). If these bone matrix-bound factors are activated during fracture healing, it seems likely that the fracture surface would play a role (Hulth et al. 1988).

"Osteoconduction" usually means the ability of a bone graft or a bone replacement material to favor bone formation in a skeletal defect by conducting and possibly stimulating trauma-induced bone formation at the resection ends. The mechanism by which this occurs is usually described as the material acting as a "trellis" for new bone formation. This would mean a rather simple mechanical function by which a porous material could protect a preosteoblastic tissue from deformation which would disturb its differentiation into bone (Aspenberg et al. 1992). However, bone conduction is often described as a surface phenomenon, which should indicate that the surfaces of an osteoconductive material selectively favor the anchorage of bone-forming cells.

It appears that one should differentiate between, on the one hand, "true" conductive or trellis effects, and on the other, bone stimulatory effects, e.g., those supposedly elicited by growth factors others than BMPs. When the terms osteoinduction (BMP effects) and osteoconduction were first defined, they were thought of as the only important phenomena in this field. Now, they appear as two extremes, between which there is room for other hypothetical bone stimulatory mechanisms. Further, the term "osteoconduction" seems unsatisfactory. The cells that invade various porous materials in our model consistently appear as mesenchymal cells, that will differentiate into fibrous tissue or bone, depending on local conditions, such as the distance from the ingrowth openings. It would seem more correct to use the term "mesenchymal tissue conduction" rather than bone conduction. In our study, deproteinization appeared to reduce the proportion of the ingrown tissue that differentiates to bone, and to influence the quality of the fibrous tissue, whereas the total ingrowth distance remained unchanged.

The effect of deproteinization was also underlined by the decrease in scintimetric activity. The scintimetric activity probably reflects the bone formation rate, rather than differences in MDP adsorption to the graft because, when these materials were implanted intramuscularly, ther scintimetric difference was in the opposite direction, with higher activity in the deproteinized grafts (Thorén and Aspenberg 1995).

The defatting procedure definitely denatured the proteins of the graft material, but not to an extent that makes it a disadvantage. We used a defatting procedure that has been standard for producing demineralized bone matrix (DBM), and that does not appear to destroy—e.g., BMP activity. Non-defatted DBM is less inductive (Aspenberg, unpublished). Cancellous allografts, defatted with this procedure, are better incorporated than conventionally frozen grafts, partly due to the removal of alloantigens. (Thorén et al. 1995).

After all, the effects of the bone proteins were small. Exogenous growth factors can be added to both defatted bone and pure porous hydroxyapatite, and in both cases the stimulatory effects are greater than those observed in this study (Wang and Aspenberg 1994, Wang 1996).

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