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# Osteoinductive, Morphologic, and Biomechanical Properties of Autolyzed, Antigen-Extracted, Allogeneic Human Bone

NORBERT KÜBLER, MD, DMD,* JÜRGEN REUTHER, MD, DMD,†
THOMAS KIRCHNER, MD,‡ BERND PRIESSNITZ, MS,§
AND WALTER SEBALD, PHD||

Autolyzed, antigen-extracted, allogeneic (AAA) bone was prepared from human cortical bone and its morphologic, biomechanical, and osteoinductive properties were compared with untreated (frozen) as well as lyophilized human bone. Scanning electron microscopy revealed removal of inorganic calcium phosphates and persistence of shrunken collagen fibrils on the surface of AAA bone matrix. Biomechanical testing of differently prepared bone samples showed that lyophilization increased both the modulus of elasticity ($P < .00001$) and the compressive strength ($P < .00001$). Depending on the depth of decalcification in the preparation of AAA bone, both measured values decreased in rehydrated AAA bone compared with untreated bone ($P < .00001$). Completely demineralized and rehydrated AAA bone was soft, flexible, and showed very little compressive strength. Differences in biomechanical behavior between samples drilled longitudinally or perpendicularly to the diaphyseal bone axis were observed. Xenogeneic human bone samples were implanted in muscle pouches of Sprague-Dawley rats for 6 weeks. AAA bone implants showed chondrogenesis and osteogenesis in 50% of the cases, while untreated or lyophilized bone implants induced no new cartilage or bone formation. As decalcification exposed xenogeneic organic matrix components, AAA bone implants provoked the highest inflammatory reaction. When AAA bone samples were implanted in immunosuppressed rats, the inflammatory reaction was suppressed and 94% of the implants showed endochondral bone formation. The chondroinductivity of the bone samples also was tested in vitro using neonatal rat muscle tissue to avoid interference with inflammatory cells and secreted cytokines. In this assay, 68% of AAA bone samples induced chondroneogenesis, while untreated as well as lyophilized bone samples failed to induce any cartilage formation. The results clearly demonstrate that AAA bone has osteoinductive properties. Biomechanical stability of AAA bone implants depends on the degree of demineralization. Thus, they can be prepared in an appropriate manner for different indications in oral and maxillofacial surgery.

Received from the University of Würzburg, Würzburg, Germany.
* Resident, Department of Oral and Maxillofacial Surgery, and Bone Research Laboratory.
† Professor and Head, Department of Oral and Maxillofacial Surgery.
‡ Assistant Professor, Department of Pathology.
§ Physicist and Research Assistant, Department of Experimental Dentistry.
|| Professor and Head, Department of Physiological Chemistry (II).
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Address correspondence and reprint requests to Dr Kübler: Department of Oral and Maxillofacial Surgery, Bayerische Julius-Maximilians-Universität, Pleicherwall 2, D-97070 Würzburg, Germany.

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In 1965 Marshall Urist first described the osteoinductive potential of demineralized bone after intramuscular implantation in animal experiments.[1] After an implantation period between 4 and 16 weeks he observed heterotopic ossicle formation, including central bone marrow formation in 50% to 98% of the animals.[1,2] Several years later the same group showed that chemically altered and decalcified bone matrix yields a higher amount of new bone formation than exclusively demineralized bone.[3,4] The product of their extraction and alteration procedures was called bone matrix gelatin.

Further investigations revealed that osteoinductivity of bone implants could be destroyed either by extrac-

tion with 4 mol/L guanidine hydrochloride (GuHCl) and 6 to 8 mol/L urea, respectively, or by incubation in neutral phosphate buffer.[3-7] Explanations for these findings were that 1) GuHCl or urea extracted osteoinductive bone matrix-derived proteins (bone morphogenetic proteins, BMPs), which were responsible for induction of new bone formation by diffusion from the bone matrix,[7,8] and 2) neutral-buffered solutions activated endogenous enzymes in bone matrix, which degraded and metabolized BMPs.[5,6] Nowadays, seven different BMPs and other bone-derived osteoinductive proteins have been isolated and even have been expressed by recombinant DNA technology.[9-13] However, these factors only show osteoinductive properties in combination with inactivated (GuHCl extracted) bone matrix. Thus, they cannot yet be used as bone inducers or bone substitutes. Incubation of partly or completely demineralized bone matrix in neutral phosphate-buffered solutions led to autolytic digestion of cellular components in bone. Subsequent extraction of bone matrix with lithium chloride, calcium chloride, and chloroform-methanol showed that bone cell-bound antigenicity could be lowered.[14-18] At present it is generally agreed that the major antigens responsible for bone allograft reactivity are cell-surface glycoprotein molecules encoded in the major histocompatibility complex (class I antigens: HLA-A, HLA-B, and HLA-C; class II antigens: HLA-D).[19,20] Thereby, class I antigens function as targets for cytotoxic (CD8+) T lymphocytes,[21] while class II antigens are key elements in the control of T-cell response to antigen and function as part of the antigen complex recognized by the T-cell receptor of regulatory (CD4+) T lymphocytes.[22,23]

The finding that phosphate buffer-mediated activation of endogenous bone enzymes that degrade osteoinductive proteins could be inhibited by different enzyme inhibitors ($NaN_3$, iodoacetic acid, iodoacetamide, N-ethylmaleimide, phenylmethyl sulfonly fluoride, benzamidine-hydrochloric acid, thimersol, p-chloromercuribenzoate),[3,5,6,24,25] while autolysis of bone cells was not influenced, led to the preparation procedure of autolyzed, antigen-extracted, allogeneic bone (AAA bone).[14-18,26,27] Thus AAA bone has full osteoinductive properties, while its antigenicity is highly reduced.[14,16,18] Thereby, the cascade of chemical extraction and alteration is very similar to the preparation of highly osteoinductive bone matrix gelatin.[3,14,17]

Because alloplastic bone replacement materials (hydroxylapatite, tri-calcium-phosphate, etc) and conventionally prepared bone allografts (frozen, freeze-dried, etc) only have an osteoconductive effect, and autogeneic bone grafts often cause postoperative complaints at the donor sites, we have been looking for an allogeneic, low-antigenic bone graft that has osteoinductive as well as osteoconductive characteristics in clinical situations where a recipient bed with strong regenerative capacity exists (no acute infection, no prior irradiation, etc). This article describes basic investigations on the osteoinductive, morphologic, and biomechanical properties of human AAA bone that have been performed before its clinical applications in oral and maxillofacial surgery.[28,29] In order not to confuse the nomenclature, the term "AAA bone" was used in this article although human AAA bone was used as a xenogeneic implant in the animal and tissue culture experiments. The term 'untreated bone' also was used to distinguish it from other preservation and preparation methods, although all bone preparations were made from frozen bone.

## Materials and Methods

### Preparation of AAA Bone

Bone donors must meet guidelines of the Deutsche Bundesärztekammer (German Federal Physicians Chamber) when explants are used in humans.[30] Human diaphyseal cortical bone of femur, tibia, and humerus were procured from donors under nonsterile conditions within 6 hours after death and subsequently stored at −80°C. Although this low temperature minimizes enzymatic destruction, and thus preserves osteoinductive activity for more than 12 months (unpublished data), it is advisable to process the bone within 6 months. After storage, frozen bone was thawed in distilled water containing 2 mmol/L sodium azide ($NaH_3$) (Aldrich, Steinheim, Germany) at 4°C. The $NaH_3$ acts as an enzyme inhibitor to prevent digestion of osteoinductive bone matrix proteins.[14] Afterward, diaphyseal bone was scraped free of surrounding soft tissue and the remaining bone marrow was removed. If storage was required during this process, the bone was stored in distilled water, containing 2 mmol/L $NaH_3$, 2 mmol/L N-ethylmaleimide and 0.1 mmol/L benzamidine-hydrochloric acid at 4°C (all: Aldrich, Steinheim, Germany) to inhibit endogenous enzymatic activity.

At this step of the preparation, bone cylinders used in the biomechanical investigations, as well as in the in vivo and in vitro studies, were drilled as described later. All bone samples used in this study were treated equally until this point. Thereafter, demineralization was performed in 0.6 mol/L hydrochloric acid (Merck, Darmstadt, Germany) at 4°C (the amount of demineralization is a function of time and the relation between mineral weight and volume of hydrochloric acid). Depending on the purpose, bone was either totally decalcified for 30 hours or only surface-demineralized (surface demineralization was checked by cutting crevices with a scalpel blade). In addition, hydrochloric acid also extracts acid-soluble matrix proteins (bone sialoprotein, osteopontin, osteonectin, osteocalcin (bone gla-protein), and thrombospon-

din).[3,14,17] The purpose of decalcification is to enable BMPs to diffuse in the recipient bed after implantation and to facilitate osteoconduction and resorption by macrophages and osteoclasts.[8,18,31]

After demineralization, the bone was again scraped to remove remaining soft tissue on the top layer of the bone and subsequently it was washed in distilled water at 4°C for 30 minutes. Autolytic digestion of bone cells was performed by incubation in 0.1 mol/L phosphate buffer (Aldrich), pH 7.4, containing 3 mmol/L N-ethylmaleimide and 10 mmol/L $NaH_3$ (for preservation of osteoinductive matrix proteins) at 37°C in a shaking water bath for 3 days. The buffer solution was changed on the second day.[3,14,27] Thereafter, the bone was washed in stirred deionized water for 2 to 4 hours at 4°C. The water was changed twice during the procedure.

The next step comprised shrinking of collagen fibrils and extraction of high-molecular-weight protein polysaccharides (proteoglycans) by 6 mol/L lithium chloride (Aldrich) and extraction of low-molecular-weight protein polysaccharides (biglycan, decorin, fibromodulin, etc) by 0.3 mol/L calcium chloride (Merck).[3,18] The solution also contained 3 mmol/L $NaH_3$ and the extraction was performed for 24 hours at 4°C. The shrinkage of collagen fibrils also facilitates bone resorption and osteoconduction, both associated in orthotopic implantation site (important for clinical use in humans).

Afterward, the bone was washed in stirred distilled water for 12 hours at 4°C, with the water changed several times. Lipids, as well as cell membrane lipoproteins, were extracted by a mixture of chloroform-methanol 1:1 (Baker, Deventer, the Netherlands; and Merck) for 24 hours at room temperature. In addition, chloroform-methanol inhibits or extracts endogenous BMP degrading enzymes.[3,14,17,18] After the chloroform-methanol was decanted, the bone was air dried. Finally the bone was washed again in deionized water at 4°C for 4 hours, deep frozen, and subsequently lyophilized for 7 days.

### Preparation of Untreated and Lyophilized Bone Samples

Untreated bone samples were drilled from thawed human bone that had been stored at −80°C (see Preparation of AAA Bone section). After drilling, part of the bone samples were frozen at −80°C and lyophilized for 7 days to prepare lyophilized bone samples.

### Sterilization

Sterilization of bone samples was performed in a chloroform gas chamber at room temperature for 12 hours prior to their implantation in vivo or their use in vitro.

### Biomechanical Studies

Cortical bone cylinders with a diameter of 4 mm and a length of 6 mm were drilled longitudinally as well as perpendicular to the axis of an untreated human femur that had been stored at −80°C. To examine the influence of different steps in the preparation of AAA bone on its biomechanical properties, the cylinders were either 1) not treated further, 2) lyophilized without later resuspension (rehydration), 3) lyophilized and thereafter resuspended in Ringer's solution, 4) surface-demineralized (AAA bone treated and lyophilized without later resuspension), 5) surface-demineralized (AAA bone treated, lyophilized, and thereafter resuspended), 6) totally demineralized (AAA bone treated and lyophilized without later resuspension), or 7) totally demineralized (AAA bone treated, lyophilized, and thereafter resuspended). None of the samples was sterilized.

Surface demineralization was only carried out longitudinal to the long axis of the cylinder (the top and bottom of the cylinders were covered by a thin disc of wax to prevent decalcification). The depth of surface-demineralization was approximately 1 mm and thus was equivalent to a demineralization of about 75% of the total bone cylinder volume.

Modulus of elasticity and compressive strength of bone cylinders were determined at room temperature by using a material testing device (type 1445, Zwick, Ulm, Germany). Preload at the begin of testing was 1 N and compression speed was 1 mm per minute. Testing was terminated at 60% of maximum force or deformation of more than 3 mm, respectively. Data were calculated as mean ± standard deviation of 10 specimens.

### Animal Experiments

Human cortical bone cylinders with a diameter of 4 mm and a length of 6 mm were drilled in the middle to form hollow cylinders with a central cavity of 1.5 mm in diameter. Untreated, lyophilized, and AAA bone-treated (totally demineralized) sterilized hollow bone cylinders were implanted in muscle pouches in the abdominal wall of adult Sprague-Dawley rats for 6 weeks. For immunosuppression experiments, Sprague-Dawley rats were fed 20 mg cyclosporin A (Sandimmun, Sandoz, Nürnberg, Germany) per kg body weight 1 day prior to implantation and until the end of the implantation period. After 6 weeks the animals were killed and the samples were examined radiologically as well as histologically.

### IN VITRO EXPERIMENTS

The middle part of the m. triceps humeri from neonatal Sprague-Dawley rats was excised and minced in a drop of culture medium to a particle size smaller than .5 mm$^3$. Thereafter the minced tissue was placed on sterilized hollow hemicylinders (see Animal Experiments section) of either untreated or lyophilized human cortical bone or totally demineralized AAA bone-treated human cortical bone that were rehydrated in culture medium and cut almost halfway across perpendicular to their longitudinal axis to make crevices. Twenty microliters of fibronection (Gibco, Berlin, Germany) connected the tissue to the bone samples, which were placed on a wire grid in organ culture dishes (Becton Dickson, Heidelberg, Germany). CMRL 1066 (Gibco) was used as the culture medium and supplemented with 15% fetal calf serum, 100 U/mL penicillin, 100 $\mu$g/mL streptomycin and sodiumbicarbonate (all: Gibco) to an adjusted pH of 7.2. The medium was changed every second day and incubation was done in an atmosphere of 5% carbon dioxide in air. After an incubation period of 14 days, incubated tissue was examined histologically.[32-36]

### RADIOLOGY

Excised abdominal muscle walls of Sprague-Dawley rats with implanted samples were examined radiologically using the Mammomat x-ray unit (Siemens, München, Germany) with an exposure of 28 keV, 16 mA. Cronex 10 S (Du Pont, Bad Homburg, Germany) was the x-ray film and Fast Detail (Du Pont) was used as an intensifying screen.

### HISTOLOGY

In vivo and in vitro bone samples were fixed in 10% neutral formalin, decalcified in formic acid if necessary, embedded in paraffin, sectioned, and stained with hematoxylin-eosin or alcian blue.

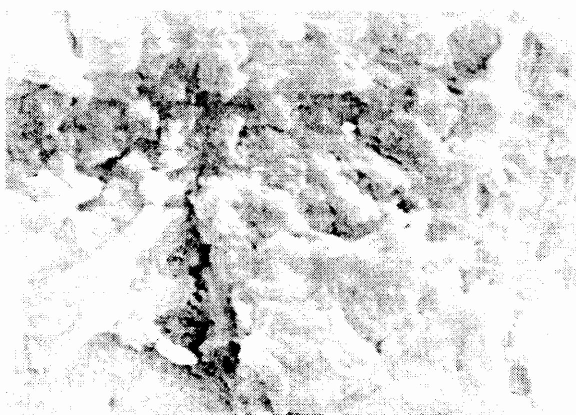

FIGURE 1. Scanning electron micrograph of untreated human bone. Note the inorganic surface structure consisting of calcium phosphates (original magnification ×5,000).

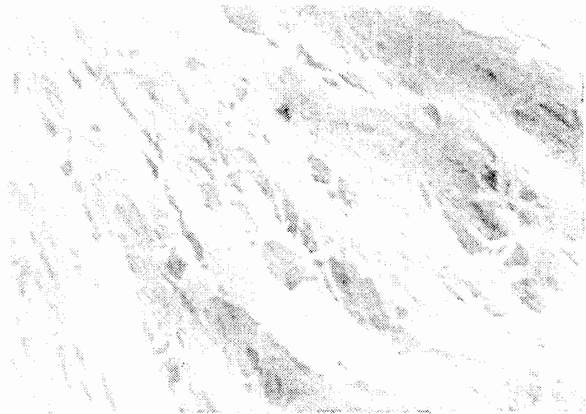

FIGURE 2. Scanning electron micrograph of human AAA bone. Note the matrix structure consisting of shrunken collagen fibers (original magnification ×5,000).

### STATISTICS

Student's *t* test was applied for statistical calculations of independent random samples.

### SCANNING ELECTRON MICROSCOPY

Specimens of untreated bone and resuspended AAA bone were fixed in 6.25% glutaraldehyde buffered with .1 mol/L sodium phosphate, pH 7.2, for 18 hours. Samples were stepwise dehydrated in acetone followed by critical point drying in carbon dioxide.[37] Mounted specimens were sputtered with 30 nm gold and examined in the Zeiss scanning electron microscope (DSM 962).

## Results

### SCANNING ELECTRON MICROSCOPY

Specimens of untreated human bone, as well as human AAA bone, were examined in the scanning electron microscope at various magnifications. The surface of untreated bone showed a structure mainly consisting of calcium phosphates, especially hydroxylapatite (Fig 1). In contrast, the demineralized AAA bone surface lacked inorganic structures and instead exposed a network of shrunken collagen fibrils (Fig 2).

### BIOMECHANICAL STUDIES

The biomechanical properties of differently treated human bone cylinders drilled longitudinally to the di-

**Table 1. Biomechanical Properties of Differently Treated Human Bone Cylinders (Length: 6 mm, Diameter: 4 mm) Drilled Longitudinally to the Diaphyseal Axis of the Femur**

| Sample | n | Modulus of Elasticity ± SD (MPa) | Compressive Strength ± SD (MPa) |
|---|---|---|---|
| Untreated bone | 10 | 2,103.2 ± 314.1 | 105.5 ± 7.4 |
| Lyophilized bone | 10 | 2,805.5 ± 135.9 | 243.5 ± 34.4 |
| Lyophilized, rehydrated bone | 10 | 1,735.3 ± 377.1 | 117.8 ± 12.6 |
| Surface-demineralized,* lyophilized AAA bone | 10 | 1,841.3 ± 50.2 | 111.5 ± 10.3 |
| Surface-demineralized,* lyophilized, rehydrated AAA bone | 10 | 1,009.6 ± 87.6 | 57.7 ± 7.3 |
| Completely demineralized, lyophilized AAA bone | 10 | 896.2 ± 108.8 | † |
| Completely demineralized, lyophilized, rehydrated AAA bone | 10 | 36.3 ± 4.8 | 7.2 ± 0.9 |

* Approximately 1 mm depth of demineralization.
† Not determinable.

aphyseal bone axis are shown in Table 1 and those of cylinders drilled perpendicularly to the bone axis are shown in Table 2. Measured values of the modulus of elasticity ($P < .003$ to $P < .00001$) as well as of compressive strength ($P < .005$ to $P < .00002$) were higher in bone samples longitudinally drilled than in samples drilled perpendicular to the diaphyseal bone axis, irrespective of the method of preparation (with one exception: the modulus of elasticity of completely demineralized, rehydrated AAA bone samples was higher in samples perpendicularly drilled [$P < .03$]). No statistically significant differences in compressive strength were seen between differently drilled samples of untreated bone or of completely demineralized, rehydrated AAA bone).

Lyophilization increased the modulus of elasticity (33% to 65%, $P < .00001$) as well as the compressive strength (81% to 131%, $P < .00001$) of bone. After rehydration the mean value of both measures decreased below the respective values of untreated (frozen) bone samples ($P < .03$ to $P < .0004$) (with one exception: the compressive strength of longitudinally drilled cylinders of rehydrated, lyophilized bone was higher than that of untreated bone [$P < .02$]).

Demineralization decreased both the modulus of elasticity and the compressive strength. Measured values depended on the degree of decalcification. In the lyophilized state, surface-demineralized AAA bone samples showed a decrease of 12% to 13% for the elastic modulus ($P < .02$ to $P < .04$) and approximately equal values (no statistically significant differences) for compressive strength compared with untreated bone samples. Comparison of measured values of rehydrated surface-demineralized AAA bone samples with those of untreated bone samples revealed a decrease of 52% to 70% for the elastic modulus ($P < .00001$) and of 45% to 60% for the compressive strength ($P < .00001$). Completely demineralized and resuspended AAA bone showed the lowest modulus of elasticity as well as the lowest compressive strength. The values of elastic modulus decreased between 97% and 98% ($P < .00001$) and those of compressive strength decreased between 93% and 94% ($P < .00001$) compared with untreated bone samples. Compressive strength of completely demineralized, lyophilized AAA bone was not determinable because the samples did not show a real fracture behavior, but instead showed continuous microfractures.

**Table 2. Biomechanical Properties of Differently Treated Human Bone Cylinders (Length: 6 mm, Diameter: 4 mm) Drilled Perpendicularly to the Diaphyseal Axis of the Femur**

| Sample | n | Modulus of Elasticity ± SD (MPa) | Compressive Strength ± SD (MPa) |
|---|---|---|---|
| Untreated bone | 10 | 1,533.6 ± 237.4 | 101.1 ± 9.2 |
| Lyophilized bone | 10 | 2,529.7 ± 200.5 | 183.3 ± 27.6 |
| Lyophilized, rehydrated bone | 10 | 1,146.4 ± 147.8 | 93.6 ± 4.4 |
| Surface-demineralized,* lyophilized AAA bone | 10 | 1,327.6 ± 178.8 | 98.4 ± 5.4 |
| Surface-demineralized,* lyophilized, rehydrated AAA bone | 10 | 456.8 ± 75.0 | 40.6 ± 6.6 |
| Completely demineralized, lyophilized AAA bone | 10 | 452.4 ± 51.9 | † |
| Completely demineralized, lyophilized, rehydrated AAA bone | 10 | 42.9 ± 6.8 | 6.4 ± 1.1 |

* Approximately 1 mm depth of demineralization.
† Not determinable.

**Table 3. Induced Chondroneogenesis and Osteogenesis After Implantation of Differently Treated Human Bone Hollow Cylinders in Muscle Pouches of Sprague-Dawley Rats' Abdominal Walls for 6 Weeks**

| Sample | Immunosuppression | n | Chondroneogenesis and Osteoneogenesis |
|---|---|---|---|
| Untreated bone | − | 15 | 0 |
| Lyophilized bone | − | 14 | 0 |
| Completely demineralized, AAA bone | − | 18 | 9 |
| Completely demineralized, AAA bone | + | 18 | 17 |

## ANIMAL EXPERIMENTS

Samples of untreated human bone, lyophilized human bone, and totally demineralized human AAA bone were intramuscularly implanted in rats for 6 weeks (Table 3). Radiologic and histologic examinations revealed no new bone formation after implantation of untreated human bone hollow cylinders (Fig 3). The bone implants were surrounded by inflammatory infiltrates composed of lymphocytes and plasma cells. Fibrous tissue was found in direct contact with the bone surface, where single macrophages and isolated osteoclasts had started bone resorption. No chondrocytes could be detected.

Implantation of lyophilized human bone samples also showed no heterotopic bone induction after an implantation period of 6 weeks (Fig 4). The cellular inflammatory reaction was comparable with that with untreated bone. However, when human AAA bone hollow cylinders were implanted, half of the samples showed either chondroneogenesis or osteoneogenesis (Fig 5). The lacunae of the implants were enlarged and partly or completely evacuated by incubation of the samples in the neutral buffer solution used in the preparation of AAA bone. Some Haversian systems were repopulated either by fibrous tissue, partly in combination with macrophages and osteoclasts, or by induced chondrocytes. Moreover, areas of cartilage that were characterized by large, hypertrophied chondrocytes were observed in crevices and cavities of the AAA bone matrix (Fig 6). Newly formed woven bone was deposited on the wall of resorption tunnels on the surface of the human AAA bone implants (Fig 7). In most of the cases newly formed bone was observed in close proximity to cartilage. Thus, the mechanism for osteogenesis probably was endochondral bone formation. Cement lines separated acellular human AAA bone from appositional deposits of new, immature cellular rat bone of the recipient. As with the other xenogeneic human implants, the AAA bone samples also were surrounded by small round cells. However, this inflammatory reaction was more distinct than around untreated or lyophilized human bone implants.

When human AAA bone hollow cylinders were im-

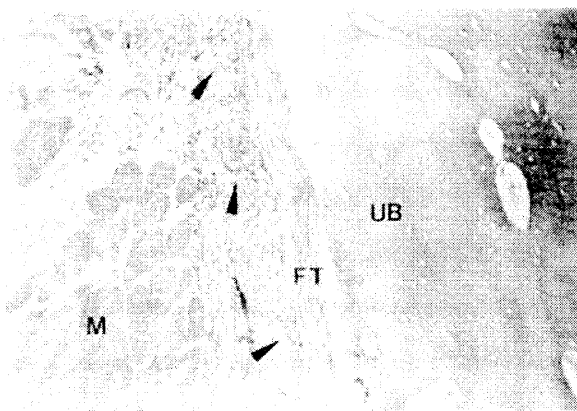


FIGURE 3. Photomicrograph of untreated human bone (UB) implanted in a muscle (M) pouch of a Sprague-Dawley rat for 6 weeks. The implant is surrounded by fibrous tissue (FT) and inflammatory cells (*arrows*). No bone or cartilage induction is visible (hematoxylin-eosin stain, original magnification ×27).

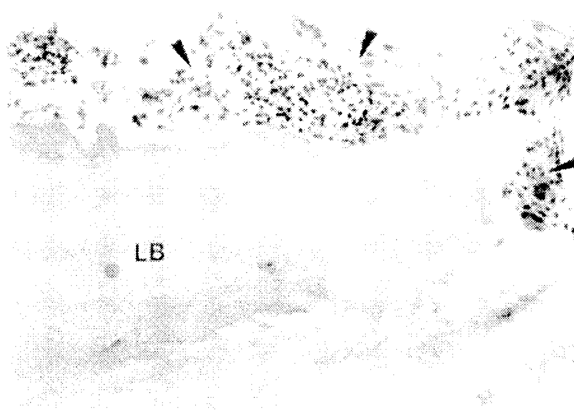


FIGURE 4. Photomicrograph of lyophilized human bone (LB) implanted in a muscle pouch of a Sprague-Dawley rat for 6 weeks. No chondroneogenesis or osteoneogenesis has occurred. A cellular inflammatory reaction is observed next to the bone implant (*arrows*) (hematoxylin-eosin stain, original magnification ×54).

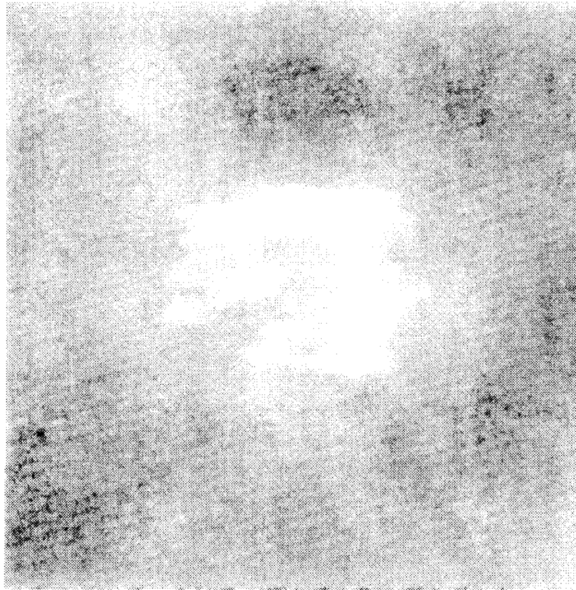

FIGURE 5. Radiograph of human AAA bone implanted in a muscle pouch of a Sprague-Dawley rat for 6 weeks. Note recalcification of the implant that was previously completely demineralized.

planted in immunosuppressed rats, 17 of 18 implants showed chondroneogenesis as well as osteoneogenesis. Not only the induction rate, but also the amount of induced cartilage and bone per implant was much higher than in nonimmunosuppressed rats. Histologic observations were similar to those with human AAA bone implants in nonimmunosuppressed rats; however, no inflammatory reaction was observed around the human AAA bone implants.

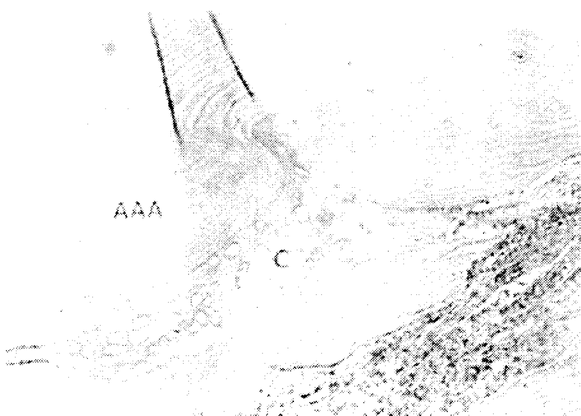


FIGURE 6. Photomicrograph of human AAA bone (AAA) implanted in a muscle pouch of a Sprague-Dawley rat for 6 weeks. New cartilage formation (C) is induced within crevices in the implant, which is surrounded with small round inflammatory cells and fibrous tissue (hematoxylin-eosin stain, original magnification ×38).

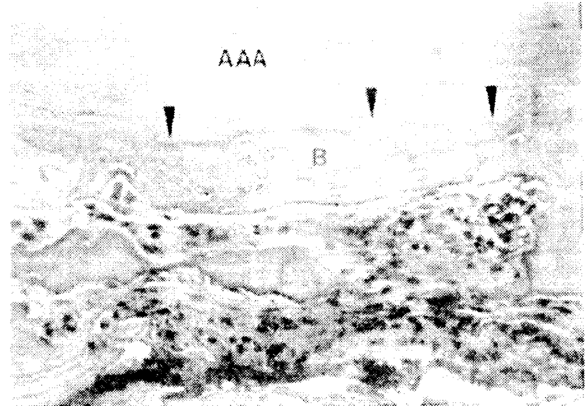


FIGURE 7. Photomicrograph of human AAA bone (AAA) implanted in a muscle pouch of a Sprague-Dawley rat for 6 weeks. Induced new bone formation (B) is separated from the acellular AAA bone matrix by a cement line (*arrows*) (hematoxylin-eosin stain, original magnification ×76).

### In Vitro Experiments

Muscle tissue of neonatal Sprague-Dawley rats was incubated on hollow hemicylinders of either untreated human bone, lyophilized human bone, or completely demineralized human AAA bone for 2 weeks (Table 4). Neither untreated nor lyophilized human bone induced chondrogenesis. Incubation of minced muscle tissue resulted in the outgrowth of fibroblasts that covered all exposed bone surfaces, including the crevices (Fig 8). In contrast, muscle tissue incubated on human AAA bone showed cartilage formation in more than two-thirds of the explants (Fig 9). Chondrocytes were found in apposition to exposed human AAA bone matrix and had a strong tendency to penetrate the crevices of the samples. None of the samples showed signs of cell damage or necrosis.

## Discussion

Fresh cortical bone consists mainly of hydroxylapatite, amorphous calcium phosphates, collagen, water, and a small amount of noncollagenous organic components.[38] While lyophilized bone lacks only the water content, and hydroxylapatite or tricalcium phosphates

**Table 4. Induced Chondrogenesis in Tissue Culture of Neonatal Rat Muscle Explants on Differently Treated Human Bone Hollow Hemicylinders After 2 Weeks**

| Sample | n | Chondroneogenesis |
|---|---|---|
| Untreated bone | 20 | 0 |
| Lyophilized bone | 26 | 0 |
| Completely demineralized AAA bone | 38 | 26 |

as bone substitutes consist only of mineral, AAA bone is partly or completely demineralized to expose the organic bone matrix to the recipient bed. The different top layer structure, with shrunken collagen fibrils on the surface of decalcified AAA-bone matrix, was seen in the scanning electron micrographs.

The superficial removal of the mineral content of bone is necessary to make possible the diffusion of the osteoinductive proteins (BMPs) and their possible cofactors bound tightly to the collagen polypeptide structure of the bone matrix.[8,18,31] Sequential extraction of human bone matrix prepared as AAA bone with various chemicals and solvents leads to the isolation of hBMPs in association with a mixture of noncollagenous proteins (hBMP/NCP).[18,39-43] The implantation of this semipurified human protein complex induces ossicle formation in 98% of mice and less than 10% new heterotopic bone formation in rats (unpublished data). While highly purified bone matrix-derived and recombinant human osteoinductive proteins fail to induce bone or cartilage formation without organic bone matrix as a carrier in any animal model,[9,44-47] heterotopic implantation of human AAA bone showed in 50% of the cases chondroneogenesis as well as osteoneogenesis in nonimmunosuppressed rats. Thus, human AAA bone contains all inductive factors necessary for the transformation of migratory mesenchymal cells into chondroblasts and osteoblasts. In contrast, none of the samples consisting of untreated or lyophilized human bone showed heterotopic intramuscular cartilage or bone formation in rats. These results are in accordance with increased bone formation after orthotopic implantation of allogeneic AAA bone in rodents and pigs.[48-50]

To study osteoinductivity of bone implants, however, it is important to use heterotopic (intramuscular

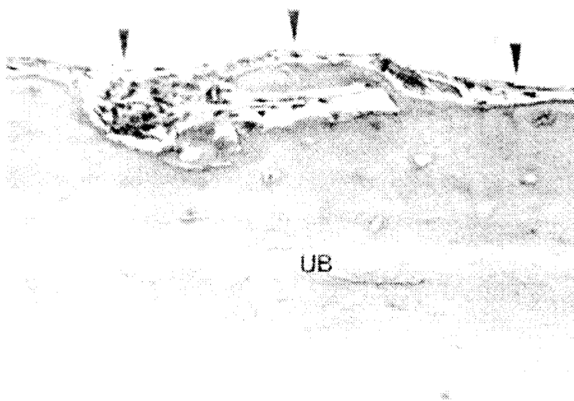


FIGURE 8. Photomicrograph of untreated human bone (UB) incubated with neonatal rat muscle tissue in vitro for 2 weeks. The explant is covered with fibrous tissue (*arrows*). No cartilage formation is visible (hematoxylin-eosin stain, original magnification ×58).

FIGURE 9. Photomicrograph of human AAA-bone (AAA) incubated with neonatal rat muscle tissue in vitro for 2 weeks. Chondroneogenesis (*arrows*) has been induced in crevices of the explant (alcian blue stain, original magnification ×28).

or subcutaneous) implantation sites because other effects (ie, osteoconduction, creeping substitution, etc) besides inductive properties of the implants are involved in orthotopic bone formation. There are also numerous observations that indicate chondroneogenesis and osteoneogenesis after heterotopic implantation of allogeneic AAA bone in rodents.[48,51-54] In addition, there are reports that indicate that allogeneic demineralized bone matrix or allogeneic AAA bone matrix induce heterotopic osteoneogenesis in nonhuman primates.[55,56] Contrary to these observations, other investigators deny de novo bone formation in heterotopic sites after implantation of allogeneic demineralized bone in monkeys,[57] or after implantation of demineralized human and monkey bone matrix in rats.[58] Never before has human AAA bone been shown to induce heterotopic bone formation. Although a xenogeneic animal model was used in this investigation, it clearly demonstrated the osteoinductive capacity of human AAA bone. Besides this study, there exists only one additional report about implantation of human AAA bone in an animal model. It indicates increased

bone formation after orthotopic implantation of human AAA bone in nonhuman primates.[59]

The majority of cell-bound antigens are extracted by autolytic digestion in the protocol for the preparation of AAA bone.[14-18] Histologic examinations of AAA bone implants revealed empty lacunae. Thus, the enzyme inhibitors preserve the osteoinductive matrix-derived proteins, which otherwise would have been degraded.[14-18,26,27] The results showing more than 68% induced chondrogenesis in neonatal muscle tissue cultures also clearly demonstrate the preservation of inductive proteins in human AAA bone. While these morphogens could diffuse from the AAA bone matrix to transform mesenchymal cells that are present in a high percentage in neonatal rat muscle tissue into chondroblasts, this did not occur when untreated or lyophilized bone was used. Muscle tissue in vitro lacks an immune response; however, human AAA bone was also used as a xenogeneic implant in rats. While cell surface glycoprotein molecules are the major antigens for an immunogenic allogenic response,[14,19,20] matrix components (collagen, proteoglycans, γ-carboxylated proteins, sialoproteins, glycoproteins, growth factors, BMPs, and immunoglobulins) may be most significant for a xenogeneic immune response.[58] Organic bone matrix components, however, are still present in AAA bone. By demineralizing the bone surface, they become even more exposed as antigens to the recipient's immune cells. This might be the reason for the distinct inflammatory reaction around human AAA bone implants in contrast to the minimal inflammation around untreated or lyophilized human bone implants in nonimmunosuppressed rats. This explanation is further supported by the finding that implantation of demineralized allogeneic rat bone matrix gelatin in rats shows almost no inflammatory reaction.[60,61] The distinct inflammation around xenogeneic human AAA bone implants in nonimmunosuppressed rats may have lowered their osteoinductive properties by preventing direct contact between the mesenchymal cells and the inductive substrate.[2] The latter explanation is supported by the observation that xenogeneic human AAA bone samples implanted in immunosuppressed rats induced endochondral bone formation in 94% of the animals. Thus, the immune response was almost completely suppressed. Moreover, semipurified rat BMP/NCP induces heterotopic bone formation in 100% of allogeneic rats,[61] while semipurified hBMP/NCP fails in more than 90% of the animals (unpublished data). In addition, others report that demineralized human or monkey bone matrix implanted in rats induces no new bone formation, whereas demineralized allogeneic rat bone matrix has a strong capacity to induce bone.[58] However, high purification of matrix-derived human or monkey osteoinductive proteins, followed by reconstitution of formerly extracted allogeneic bone matrix, results in osteoinduction in rats.[58] These observations demonstrate that species specifity of xenogeneic extracellular bone matrix is due to the immunogenic organic components in bone matrix.[58] After implantation of allogeneic bone matrix, cytokines secreted by host cells lead to osteoclastic bone resorption and increase the activity of osteoblasts formerly induced by osteoinductive matrix-derived proteins.[62] The excessive inflammatory reaction after implantation of xenogeneic human AAA bone in nonimmunosuppressed rats may have impaired these cytokine-mediated cellular interactions, a situation that is not likely to occur following allogeneic implantation of AAA bone in humans.

Demineralization also had a major influence on the bone's biomechanical properties. Untreated bone has viscoelastic properties.[63,64] The initial elastic response is followed by a creeping behavior, and plastic deformation is possible. Lyophilized bone is more rigid.[64] Thus, its plastic behavior is less pronounced and the elastic limit is near the fracture stress. While lyophilization significantly increased the modulus of elasticity as well as the compressive strength of bone, both values decreased after rehydration. This is in accordance with previous reports.[63-67] Compared with lyophilized bone, demineralized, lyophilized AAA bone showed significantly decreased moduli of elasticity as well as significantly decreased values of compressive strength, depending on the degree of decalcification. When demineralized AAA bone was rehydrated, the moduli of elasticity and the values of compressive strength decreased significantly compared with untreated bone. The latter finding has also been reported by others.[68] Thus, the decrease of the modulus of elasticity as well as of compressive strength correlated with the depth and the degree of decalcification. Completely demineralized, lyophilized, and rehydrated AAA bone showed 97% to 98% lower moduli of elasticity and 93% to 94% less compressive strength than untreated controls. The reason for this observation is that collagen is a fiber with a low modulus of elasticity, a strong traction power, and a weak resistance against compression load.[64] Therefore, totally demineralized bone is soft, rubber-like, flexible, and only resists traction. Hydroxylapatite represents a rigid material with a high resistance against compressive load. The combination of collagen and apatite leads to an anisotrophic material that can resist traction and compression, as well as torsion.[64] Because bone is an anisotropic material, values for the modulus of elasticity as well as for the compressive strength measured along the osteonal direction of the fibers in most cases differed from values measured crosswise, a fact also observed by others.[64,67]

Because elasticity of AAA bone implants depends on the absolute amount of mineralized tissue, it is possible to prepare implants with different biomechanical properties (ie, when bending is necessary demineral-

ization should be as complete as possible, while in cases when biomechanical stability is not required, eg, filling of bone cysts with AAA bone powder, demineralization should be complete because osteoinduction as well as resorption are improved). In contrast, demineralization should only be superficial, enough to allow osteoinductive matrix proteins to diffuse, when biomechanical stability is desirable.

Originally AAA bone was not sterilized further because the chemical treatment was believed to destroy all microorganisms and AAA bone was regarded as chemosterilized.[14-17,56] Recently, however, there occurred one case of transmission of human immunodeficiency virus (HIV) through transplantation of sterile collected, untreated bone.[69,70] Although it was also reported that demineralization itself inactivates HIV in bone,[71,72] it may be advisable to perform a final sterilization after the preparation of AAA bone prior to its clinical application. Currently we are studying different sterilization methods that ensure sterility of AAA bone while preserving the largest part of its osteoinductive properties. Nevertheless, the different steps in the preparation of AAA bone guarantee a higher safety level against transmission of infectious diseases than the grafting of untreated or lyophilized bone because freezing or freeze-drying alone do not destroy HIV.[69,70,73]

We conclude that human AAA bone is osteoinductive in contrast to lyophilized or untreated human bone. Human AAA bone includes all morphogens and cofactors necessary for the cascade to induce new bone formation. Through its partial (superficial) or complete demineralization, human AAA bone has more distinct osteoconductive properties than untreated or lyophilized human bone grafts. Human AAA bone also is safer with regard to the transmission of infectious diseases, including HIV, because its preparation destroys microorganisms within the bone matrix.

Lyophilized and sterilized human AAA bone can be stored at room temperature in a container for several years without losing its osteoinductive properties (unpublished data). Thus, the right shape of a required bone graft can be chosen from a collection of partly or completely demineralized human AAA bone segments and completely demineralized human AAA bone powders in the operation room. The biomechanical stability of human AAA bone depends on the degree of decalcification. Surface-demineralized, full-thickness cortical bone has almost the same mechanical strength as untreated bone. However, completely demineralized, thin cortical AAA bone discs are bendable and can be used, for example, as a substitute for the floor of the orbit. Finally, resuspension of lyophilized human AAA bone segments and powders in an antibiotic solution allows prophylactic local antibiosis, especially in case of an intraoral operative procedure.

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