Ectopic bone formation by composites of BMP and metal implants in rats

Gerhard Herr, Carl-Heinz Hartwig, Cornelia Boll and Wolfgang Küsswetter

Disc-shaped implants of titanium alloy (Ti-6Al-4V) were treated on one side by corundum-blasting (CB) or by coating with hydroxyapatite (HA) or pure titanium (Ti) using plasma spraying. Half of the implants were additionally coated with purified swine BMP-3. The composites and the uncoated controls were implanted into abdominal wall-muscle pouches of rats. 25 days after implantation, ectopic bone formation could be observed macroscopically and histologically in a high frequency in all 3 groups of BMP-coated implants, whereas the controls were constantly inactive. The volumes of induced bone were similar for BMP-3-coated pure Ti and HA implants, while CB implants were significantly less active. Our findings indicate that the bone formation process is influenced by the chemical composition and by the structure of the implant surface.

University of Tuebingen, Department of Orthopedics, Experimental Section, Pulvermuehlstr. 5, D-72070 Tuebingen, Germany. Tel +49 7071-2986045. Fax -45041. Correspondence: Dr. G. Herr, Labor für Exp. Unfallchirurgie, Kerkraderstr. 9, D-35394 Giessen, Germany. Tel +49-641-4994159. Fax -4994115.
Submitted 96-02-10. Accepted 96-08-27

The extracellular matrix of bone contains a variety of polypeptides with different biological activities (Goldring and Goldring 1996). Among them the bone morphogenetic proteins (BMP) have attracted interest because of their osteoinductive activity (Cook and Rueger 1996, Riley et al. 1996). The successful combination of such osteoinductive factors with bone graft substitute materials and prosthetic implants may have therapeutic applications (Glass et al. 1989, Damien et al. 1990, Horisaka et al. 1991, Herr et al. 1993, Urist et al. 1984, 1987, Kawai et al. 1993). We examined the suitability of metal implants as carriers of BMP and how the osteogenic activity of such composite implants is influenced by the chemical composition and structure of the implant surface.

Material and methods

Implants

All implant cores were machined from commercially available Ti alloy (Ti-6Al-4V) into discs of 5 mm diameter and 2 mm thickness. Subsequently, different surface characteristics were created on a single implant surface either by grit-blasting with corundum-particles (CB) or by coating with hydroxyapatite (HA, Osprovit) or pure titanium (Ti) powder, using plasma spraying.

Surface structure and topography were examined by SEM and elemental dispersive x-ray analysis (EDX). The surface roughness was determined by profilometry and the surface area was approximately calculated from profile length.

Preparation of BMP

A semi-purified BMP-3 preparation was obtained by extraction of swine cortical bone with 4 mol/L guanidine-HCl and subsequent purification was done by differential precipitation, affinity chromatography, gel filtration and reversed-phase-chromatography, according to Wang et al. (1988) and Sampath et al. (1987), with some modifications. Its osteoinductive activity was examined in a rat bioassay by intramuscular implantation of 25 mg portions of inactive rat bone matrix particles coated with 50 µg BMP-3 fraction. The metallic implants were disinfected with 70% alcohol and dried in a stream of sterile air under a laminar flow hood. For coating with BMP, a 10 µL aliquot of a BMP-3 solution (7 mg/mL in 50% acetonitrile/0.1% trifluoroacetic acid) was pipetted onto the center of the implant surfaces. Homogeneous wetting of the implant surfaces was achieved by rapid self-spreading of the BMP solution across the dry porous surfaces by capillary forces. Finally, the implants were dried in a gentle stream of sterile air under a laminar flow hood. Control implants remained uncoated.

Bioassay

The osteogenic activity of the implants was determined by intramuscular implantation into abdominal wall-muscle pouches of 22 adult Wistar rats. 3 pairs

Table 1. Physico-chemical characteristics of the different implant surfaces. Values are mean (SD)

	Thickness (μm)	Ra (μm)	Perimeter (mm)	Implant surface area [a] (mm^2)
CB	–	2.5 (0.2)	5.3 (0.02)	22 (0.12)
HA	60	8 (0.9)	5.7 (0.05)	25 (0.54)
Ti	200	28 (1.0)	6.1 (0.07)	29 (0.7)

HA hydroxyapatite-coated, Ti titanium-coated, CB corundum-blasted, Ra mean roughness.
[a] Surface area of plane implant 19.6 mm^2

of muscle pouches were prepared by blunt dissection between the m. obliquus abdominalis externus and internus along the abdominal wall. Each animal received a BMP-coated and an uncoated control implant from each material group, thus allowing for blocked comparison of all types of implants. The animals were killed 25 days after implantation and the implants were excised together with surrounding muscle tissue and examined under a surgical microscope. 2 randomly selected explants from each group were fixed in 4% formaldehyde for histology. The remaining 20 explants in each group were dissected free of muscle adjacent to the connective tissue capsule enveloping the implants, using a stereo microscope and deep-frozen for subsequent alkaline phosphatase (AP) determinations.

Histology

Explants were dehydrated and embedded in expoxy resin according to standard histologic procedures. 150 μm sections were cut on a diamond band saw and ground to approximately 50 μm thickness by hand using a rough glass plate. Staining was done with toluidine O (Plenk 1989).

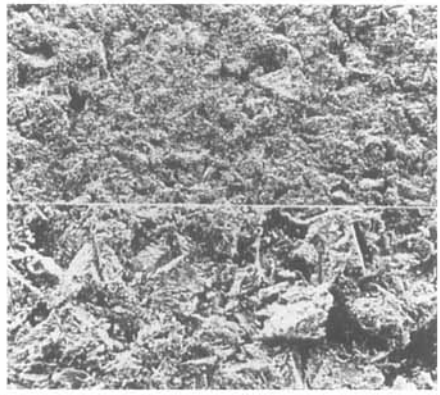
AP determination

After thawing, the explants were chemically extracted with cold 0.1 mol/L Tris-HCl, 1% (v/v) Triton-X-100, pH 7.5, for 24 hrs at 4 °C and undissolved tissue debris was removed by centrifugation. The AP activity in the clear supernatant was determined spectrophotometrically at 405 nm, using p-nitrophenylphosphate as substrate.

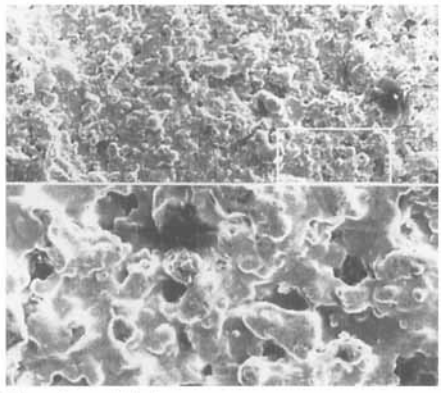
Statistics

According to the block design used in the bioassay, the AP activities obtained from the different groups of implant materials were compared, using the modified Friedman rank test, followed by paired comparison, according to Wilcoxon and Wilcox (Sachs 1992, Wittkowski 1992).

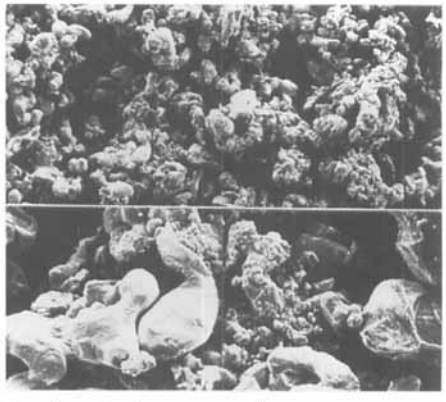
Figure 1. SEM photomicrographs of surfaces (×100, bottom ×400).



CB surface showing low roughness.



HA surface of distinct roughness.



Ti surface showing gross roughness.

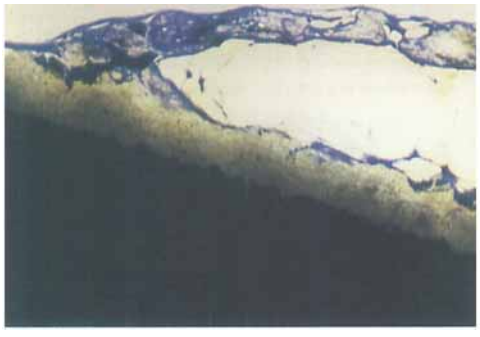
Results

Characterization of implants (Table 1 and Figure 1)

On SEM examination, CB implants revealed a non-porous surface structure of low roughness using a macroscopic scale but showed a pronounced micro-

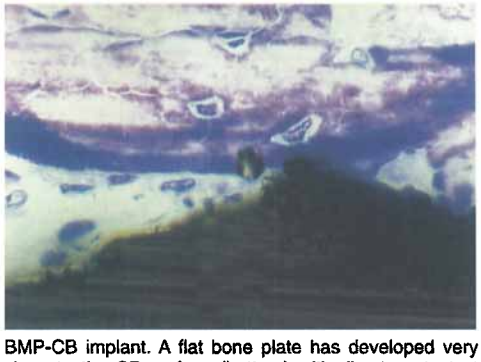
Figure 2. Grossly visible formation of bone and bone marrow on the surface of a BMP-coated HA implant 25 days after implantation (×20).

Figure 3. Photomicrographs of histologic sections of all 6 types of implants used.



BMP-HA implant. Note formation of bone and bone marrow (top) in direct contact with the HA coating (middle) on the metal implant core (bottom) (toluidine, ×150).

BMP-Ti implant. Formation of ectopic ossicles (closed triangles) including cartilage can be observed at some distance from the Ti surface (open triangle) (toluidine, ×30).



BMP-CB implant. A flat bone plate has developed very close to the CB surface (bottom) with direct contact at some sites (toluidine, ×1100).

HA control implant. Fibrous tissue in direct contact with the HA coating (toluidine, ¥900).

Ti control implant. Note fibrous tissue fibers at some distance from the porous Ti surface being infiltrated by numerous round cells (toluidine, ×150).

CB control implant. Fibrous tissue envelope with some phagocytic cells underlying it (toluidine, ×900).

Table 2. Incidence of bone formation and alkaline phosphatase activities (median and range) of BMP-coated implants and controls

Implant material	Incidence of bone formation[a]	AP activity (mU)	Ratio of implant activities[b]
CB + BMP	16 / 22	11.3 (3.3–80)	
CB control	0 / 22	0.2 (0–5.6)	60
Ti + BMP	22 / 22	72 (11–242)	
Ti control	0 / 22	0.3 (0–8.4)	276
HA + BMP	22 / 22	94 (13–168)	
HA control	0 / 22	0.1 (0–12.5)	857

[a] number of positive explants / total number of implants assayed.
[b] ratio of median activity of BMP implants/median activity of corresponding control implants.
Level of significance: CB+BMP vs Ti+BMP p = 0.0001, CB+BMP vs HA+BMP p = 0.0001, Ti+BMP vs HA+BMP p = 1.

roughness. The coating on HA implants consisted of spherical ceramic HA particles of a high degree of coalescence resulting in a macro- and microporous structure of distinct macroscopic roughness. The coating on Ti implants was composed of spherical superficially oxidized Ti particles with a lower degree of coalescence than the HA implants. They revealed a highly macro- and microporous surface structure of great roughness on the macro- and microscopic scale.

Macroscopy (Figure 2)

On explantation, all implants seemed to be well tolerated and no signs of inflammation or infection were seen macroscopically. Grossly visible bone formation and bone marrow were noted as a superficial layer of hard tissue on the BMP-coated surface of some implants, especially in the HA BMP group. The control implants were enveloped by a soft fibrous tissue capsule.

Histology (Figure 3)

Microscopic examination of the histological sections revealed direct deposition of bone on BMP-coated HA surfaces accompanied by bone marrow formation. The bone marrow cavity had direct contact with the HA surface in some cases. BMP-coated pure Ti-implants showed osteogenesis at some distance from the surface. The spherical ossicles formed consisted of an outer shell of woven bone enclosing a central area of cartilage. The ossicles were separated from the rough Ti-surface by fibrous tissue. Mononucleated cells were found near the Ti surface. The surface of CB implants was covered with flat bone plates mainly at a short distance from the surface. In-between, a thin layer of fibrous tissue and mononuclear cells was present. The induced bone was partly in direct contact with the metallic surface. All types of control implants were consistently encapsulated by a thin layer of fibrous tissue. Phagocytes could be observed near the surface of CB and Ti implants, whereas HA implants were directly covered by fibrous tissue.

AP activity

The AP activity of BMP-coated implants was increased by several orders of magnitude, compared to controls, and was highest in the group of BMP-HA implants (Table 2). The activities of BMP-HA and BMP-Ti composite implants were not statistically significantly different from each other, but were increased compared to BMP-CB implants ($p = 0.0001$). The incidence of bone formation was high in each group of BMP implants, ranging from 73% for BMP-CB to 100% (BMP-Ti and BMP-HA).

Discussion

The incidence of bone formation was high in each group of BMP-coated implants ranging from 73% to 100%, whereas controls were uniformly inactive in terms of bone formation. This demonstrates the high reproducibility and specificity of the osteoinductive activity of BMP, both on metallic carrier materials and ceramic carrier materials (Hotz and Herr 1994). The induced bone volumes, assessed by the AP activities of the explants, were not significantly different for BMP coated HA- and Ti-implants, whereas BMP-coated CB-implants induced significantly lower amounts of new bone (Table 2). This difference in osteogenic response can be explained by the larger adsorptive surface area available on the rough HA- and Ti-surfaces (Table 1), allowing more responding mesenchymal cells to bind to the active substance by their BMP receptors. This can be deduced by comparing BMP-coated CB-implants with Ti-implants. The chemical binding of BMP to both metallic surfaces can be assumed to be identical because the main constituent element is titanium. Thus the 8-fold increase in activity between these two metallic implants types must be attributed to the greatly enlarged surface area on Ti-implants. A similar dependence of activity on the adsorptive implant surface was described for BMP-coated HA ceramics of different porosities (Herr et al. 1993).

From the result that BMP-coated HA and pure Ti-implants show similar activity two conclusions can be drawn. First, due to the known hydrophobic chemical nature of BMP it can be bound effectively to metal surfaces, resulting in a slow release of BMP comparable to HA where BMP is bound mainly by ionic for-

ces. Secondly, BMP-HA-implants demonstrated the highest mean activity, but had a lower surface area than Ti-implants. Thus the ceramic HA coating itself, known to exhibit very good biocompatibility and affinity to bone (Jarcho 1981, Rawlings 1993, Willmann 1993), seems to enhance additionally the BMP-induced bone formation. This may be achieved by binding of endogenous factors acting synergistically, such as IGFs or TGFs and/or accelerated adhesion of responding mesenchymal cells.

We conclude that by coating surfaces of metallic and ceramic implants with BMP, such composites can mediate osteoinduction. This bone formation process is influenced by the chemical nature of the implant surface and, for a given type of surface material, depends on its surface area. Whether such implants exhibit the same activity in higher mammals remains to be demonstrated in subsequent animal studies, since there seems to be a difference in osteogenic competence between the various species (Aspenberg and Turek 1996, Schwarz et al. 1991).

Acknowledgement

The CB- and HA-implants were prepared by the Cerasiv Company (Plochingen, Germany) and the Ti-implants by the Aesculap Company (Tuttlingen, Germany).

References

Aspenberg P, Turek T. BMP-2 for intramuscular bone induction. Acta Orthop Scand 1996; 67: 3-6.

Cook S D, Rueger D C. Osteogenic protein-1. Clin Orthop 1996; 324: 29-38.

Damien C J, Parsons J R, Benedict J J, Weisman D S. Investigation of a hydroxyapatite and calcium sulfate composite supplemented with an osteoinductive factor. J Biomed Mater Res 1990; 24: 639-54.

Glass D A, Mellonig J T, Towle H J. Histologic evaluation of bone inductive proteins complexed with coralline hydroxylapatite in an extraskeletal site of the rat. J Periodontol 1989; 60: 121-6.

Goldring S R, Goldring M B. Cytokines and skeletal physiology. Clin Orthop 1996; 324: 13-23.

Herr G, Wahl D, Küsswetter W. Osteogenic activity of bone orphogenetic protein and hydroxyapatite composite implants. Ann Chir Gynecol 1993; 82: 99-108.

Horisaka Y, Okamoto Y, Matsumoto N, Yoshimura Y, Kawada J, Yamashita K, Tagaki T. Subperiosteal implantation of bone morphogenetic protein adsorbed to hydroxyapatite. Clin Orthop 1991; 268: 303-12.

Hotz G, Herr G. Bone substitute with osteoinductive biomaterials- current and future clinical applications. Int J Oral Maxillofac Surg 1994; 23: 413-7.

Jarcho M. Calcium phosphate ceramics as hard tissue prosthetics. Clin Orthop 1981; 157: 259-78.

Kawai T, Mieki A, Ohno Y, Umemura M, Kataoka H, Kurita S, Koie M, Jinde T, Hasegawa J, Urist M R. Osteoinductive activity of composites of bone morphogenetic protein and pure titanium. Clin Orthop 1993; 290: 296-305.

Plenk H. Knochengewebe und Zähne. In: Romeis Mikroskopische Technik (Ed. Böck P). Verlag Urban und Schwarzenberg, München, Wien, Baltimore 1989, 527-66.

Rawlings C E. Modern bone substitutes with emphasis on calcium phosphate ceramics and osteoinductors. Neurosugery 1993; 33: 935-8.

Riley E H, Lane J M, Urist M R, Lyons K M, Lieberman J R. Bone morphogenetic protein-2. Clin Orthop 1996; 324: 39-46.

Sachs L. Angewandte Statistik. Springer Verlag, Berlin 1992.

Sampath T K, Muthukumaran N, Reddi A H. Isolation of osteogenin, an extracellular matrix-associated, bone-inductive protein, by heparin affinity chromatography. Proc Natl Acad Sci USA 1987; 84: 7109-13.

Schwarz N, Schlag G, Turner M, Eschberger J, Dinges HP, Redl H. Fresh autogenic, frozen allogeneic and decalcified allogeneic bone grafts in dogs. J Bone Joint Surg (Br) 1991; 73: 787-90.

Urist M R, Lietze A, Dawson E. ß-tricalcium phosphate delivery system for bone morphogenetic protein. Clin Orthop 1984; 187: 277-80.

Urist M R, Nilsson O, Rasmussen J, Hirota W, Lovell T, Schmalzried T, Finerman G A M. Bone regeneration under the influence of a bone morphogenetic protein (BMP) beta tricalcium phosphate (TCP) composite in skull trephine defects in dogs. Clin Orthop 1987; 214: 295-304.

Wang E A, Rosen V, Cordes P, Hewick R M, Kriz M J, Luxenberg D P, Sibley B S, Wozney J M. Purification and characterization of other distinct bone-inducing factors. Proc Natl Acad Sci USA 1988; 85: 9484-8.

Willmann G. Material properties of hydroxylapatite ceramics. Interceram 1993; 42: 206-8.

Wittkowski K M. An extension to Wittkowski. J Am Statist Assoc 1992; 87: 258.