Implantation of Natural Hydroxyapatite from Porcine Bone into Soft Tissues in Mice*

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The natural origin of hydroxyapatite (HA) derived from pig bones (Polish patent No.P-359 960 pending from 5th May 2003) was histologically examined for its biocompatibility following implantation into mouse muscles. The implanted ceramic was encapsulated by well vascularized connective tissue and very slowly resorbed by multinucleated cells. This material did not elicit an immune reaction and adjacent bones were unaffected. This ceramic could be safely used as a filling material alone, or as a composite graft.

Key words: Hydroxyapatite, implantation, symplasts, multinuclear cells, biocompatibility, porcine.

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Many type of ceramics based on tricalcium phosphate, hydroxyapatite, calcium aluminate or glass ceramics are widely used in dental, craniomaxillofacial and orthopedic surgery as a filling material and carrier vehicle for cells and osteoinductive substances (for a review see DAMIEN & PARSON 1991; BIENIEK et al. 1988; URIST et al. 1984). Hydroxyapatite (HA), with good biocompatibility and bioaffinity, is one of the most commonly used ceramics to this end. It is commercially available, but its composition as well as manufacturing process varies. For example HA produced by Electromet (Poland) and Mitsubishi (Japan) completely lack carbonate groups and magnesium oxide, while Bio-Oss (Geistlich) HA derived from bovine bone contains magnesium.

The naturally occurring mineral component of bone has a nominal composition of $Ca_{10}(PO_4)_6(OH)_2$ and Ca/P ratio of 1.67 (DAMIEN & PARSON 1991). Other authors reported that in HA derived from bones this ratio is 1.71-1.73, a value slightly lower than for synthetic HA (1.66-1.68) (HABERKO *et al.* 2004).

Ceramics have osteoconductive properties, i.e. they allow an ingrowth into their pores of bone tissue from the bone bed, but are devoid of any osteoinductive capacity, i.e. are unable to generate signals to non-bony cells to differentiate into an osteogenic lineage. The only exception is a coralline hydroxyapatite ceramic Interpore made by the conversion of the calcium carbonate skeletal structures of corals. According to RIPAMONT (1996), these corals have osteoinductive potential in primates, but not in other species (rats and dogs), commonly used as experimental animals. Such ceramics, combined with osteogenic bone marrow cells support osteoblastic differentiation (OKU-MARA et al. 1997; YOSHIKA et al. 1997). When implanted into human periodontal defects, they are colonized by vascularized connective tissue, leading to ossification (KENNEY et al. 1986). Other ceramics, such as biphasic calcium phosphate and aluminum-coated HA, when coated with fresh bone marrow cells (TAKAOKA et al. 1996) or cultured human marrow stromal cells (TOQUET et al.

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1999), on implantation develop bone and express and conserve an osteoblastic phenotype. Grafted alone, without osteogenic cells, they do not show osteogenesis (TAKAOKA *et al.* 1996).

Synthetic HA, similar in composition to naturally occurring bone mineral and calcium phosphate ceramics, are biocompatible when examined by in vivo implantation. The results show a lack of local and systemic toxicity, no inflammation and no foreign body response (JARHO 1981). Porous bioceramic material containing aluminum oxide also satisfies the requirements for biomedical materials. In experiments on rabbits conducted by KOTZ *et al.* (1988 a,b) and BIENIEK *et al.* (1988), morphological and histochemical analysis of tissues, exposed to this material, revealed a lack of immune response, good tolerance and osteoconductive properties of this material.

To meet the need for biocompatibile ceramics, in our country, a team of the Faculty of Material Science and Ceramics, University of Science and Technology AGH in Kraków, has manufactured HA derived from pig bones. The detailed chemical and crystallographic characteristics of this ceramic, which has been patented (Polish patent P-359 960 pending from 5th May 2003), is reported elsewhere (HABERKO *et al.* 2003, 2005). Here it is sufficient to indicate that the applied material differs from the synthetic one. It contains carbonate groups and the Ca/P ratio is higher than in stechiometric hydroxyapatite. Table 1 shows the chemical analysis of the material.

In the present paper the histological analysis of the intramuscular implantation site of this ceramic as well as the reaction of nearby lymphatics and femoral bone are given.

Material and Methods

HA granules (ca 10 mg) were inserted into a pocket made into the right thigh muscles of an inbred strain of female BALB/c mice, aged 4-5 months.

The animals were anaesthetized, and the skin of the hind leg shaved. The shank skin was incised and a pocket in the femoral muscles made with ophthalmic scissors, and the material was inserted there. The muscles and overlaying skin was closed with 3-0 Dexon "S" Polyghlotic absorbable sutures. The wound was washed with 70% ethanol.

The animals were housed in the animal facility of the Department of Histology, fed on standard chow and had *ad lib*. access to water.

Animals were used in accordance with the Warsaw Medical Academy Ethics Committee guidelines for the care and use of laboratory animals.

Ceramic was inserted into 28 recipient mice. Ten, 20, 24, 31, 38, 46, 52, 60, 66, 90, 131, 150 and 180 days post implantation two animals were sacrificed by cervical dislocation, the popliteal lymph nodes from both legs were excised, freed from the adjacent adipose tissue and weighed accurately to 0.1 mg. This procedure allowed the assessment of the local immune response to the implants. The implants together with surrounding soft tissues were removed, fixed in Bouin fixative, decalcified in saturated EDTA solution, embedded in paraffin, and serially cut at 8 microns. Sections were stained with haematoxylin+eosin or toluidine blue and examined by light microscopy.

Spleen weights were measured to ascertain the strength of the immune response.

The femoral bones of both legs were excised, hydrolyzed overnight in 0.1 M NaOH at 64°C and the isolated bones were washed several times in distilled water, dried thoroughly for 24 hrs at 64°C and weighed accurately. Weights were constant, providing information as to the effects of implantation on adjacent bones (loss or weight increase).

The implanted material was not integrated into femoral bones, the skin wounds were healed completely within 7 days without evident pathology.

Results

Implanted HA particles evoked a very mild immune response. In the connective tissue encapsulating the implanted material, large, multinucleated cells (symplasts) were present on day 10 onward (Figs 1, 2). These cells were located within the connective tissue surrounding the implanted HA particles and later in the experiment their numbers

Table 1

Chemical analysis of the hydroxyapatite sample (percent of the mass – wt%). The proportion of Ca/P is higher than in stechiometric material. Specific surface area (m^2/g) of the examined hydroxyapatite was determined by nitrogen absorption

CaO, wt%	MgO, wt%	P ₂ O ₅ , wt%	CO_3^{-2} , wt%	Ca/P	Specific surface area, m ² /g
51.37	0.62	37.76	5.3	1.72	71.4

Porcine Hydroxyapatite is Slowly Resorbed by Multinucleated Cells



Fig. 1. HA granules 10 days post implantation. Grafted particles are surrounded by connective tissue without signs of inflammatory reaction. Toluidine-blue staining, x 100.



Fig. 2. Numerous polykaryons (symplasts) (arrows) in the area of HA implantation, 20 days post implantation. Hematoxylin+eosin (H+E) staining, x 400.

diminished, but up to day 60, they were still present. These cells lacked a ruffled border and were rarely in contact with the ceramic surface, which indicates that they were modified inflammatory cells rather than the typical osteoclasts (Figs 2, 3, 4). From 90-180 days the implanted material was very slowly resorbed, but was present until day 180. The spaces within were enlarged and the material was less intensively stained, but the shape of implanted particles remained basically unchanged. The internal crevices of implanted material were slightly colonized by connective tissue as early as day 20.

The connective tissue that encapsulated the implanted granules and separated them from the surrounding muscles was very well vascularised. The inflammatory reaction, produced by the implants, was negligible. Later, connective tissue surrounding the implant was more cellular and became granulation tissue, rich in epithelial-like cells. No mast cells were found amongst this encapsulating tissue (Fig. 1).



Fig. 3. Bone-derived HA particles 38 days post grafting. Persisting osteocytic lacunae are enlarged, indicating slow resorption. The encapsulating connective tissue is cellular, the multinucleated cells are present, no evidence of inflammatory reaction. H+E staining, x 100.



Fig. 4. HA particles eroded by polykaryons 52 days post implantation. Connective tissue encapsulating HE particles is rich in blood vessels. No signs of inflammatory reaction. H+E staining, x 400.

During the whole observation period, i.e. until day 180, no bone formation at the implantation site was found.

No immunostimulatory effect following implantation of HA granules was observed. The mean weight of popliteal lymph nodes of the implant bearing leg against the untreated contralateral control was 3.43 ± 1.04 and 3.41 ± 1.18 mg, respectively. Histological examination of popliteal lymph nodes from the implant-draining area did not reveal any sign of activity.

Also, the spleen weight did not change during the experiment and was 143 mg \pm 22. Collectively this data indicates that implanted particles did not evoke local or generalized lymphatic activity.

Implantation of HA particles had no effect on adjacent femoral bones (as revealed by dry bone mass measurement). The mean femoral weight of the implant-bearing and contralateral leg was, accordingly, 37.7 ± 3.7 mg and 38.1 ± 3.8 mg, the difference being statistically insignificant.

Discussion

Bovine bone-derived HA was very well tolerated as a biostatic material. The host reaction to this material was merely encapsulation by well vascularised connective tissue and was characterized by the presence of large polykaryons (foreign body cells). These giant cells, usually located at the edges of the particles, slowly resorbed them. Our results are in line with the study of JARHO et al. (1981) on biocompatibility to synthetic HA, similar in composition to naturally occurring bone mineral. In contrast, a foreign body response was observed in the present study. The lack of protein in the implanted granules removed from bovine bones by extraction with hot NaOH solution (HAB-ERKO et al. 2003), explains the only negligible inflammatory reaction, observed at the earlier stages of implantation. This mild inflammatory reaction, not observed at later stages, was not specific and could be explained by the surgical injury of muscles.

The resorption of HA particles proceeded very slowly and until day 180 the material was still present at the implantation site. The very slow resorption of hydroxyapatite granules and of coral, implanted into rats, was also reported earlier by BEGLEY *et al.* (1995).

The connective tissue response had no influence on adjacent bones.

The material examined could be safely used as a filling material alone, or as a composite graft.

Material tested here can be colonized by human osteosacroma cells CAL-72 as shown before (HABERKO et al. 2005). In another experiment performed in vitro on HeLa cells (data not presented), it was demonstrated that this material did not inhibit the proliferation of cells and does not have a toxic effect on HeLa cells when exposed for three days to this ceramic. Although this material is not osteoinductive, its lack of immune response and good vascularisation of reactive connective tissue makes it a good conducting material. Whether this ceramic used as an adjuvant to guided tissue regeneration for the treatment of bone defects will not arrest bone formation as some bioactive glass or some deproteinized bovine bone do (STRAVOPOULOS et al. 2003), remains to be examined. These studies are now in progress.

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