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The biodegradation of hydroxyapatite bone graft substitutes *in vivo*

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Hydroxyapatite (HA) ceramics are widely used for bone reconstruction. They are osteoconductive and serve as structural scaffolds for the deposition of new bone. Generally, scaffold materials should be degradable as they affect the mechanical properties of the reconstructed bone negatively. Degradation by osteoclasts during the bone remodelling process is desirable but often does not take place. In the current study we analysed by light microscopy the degradation of two granular HA implants in critically sized defects in the mandibula of Goettingen mini-pigs five weeks after implantation. Bio-Oss® consists of sintered bovine bone and NanoBone® is a synthetic HA produced in a sol-gel process in the presence of SiO_2. We found that both biomaterials were degraded by osteoclasts with ruffled borders and acid phosphatase activity. The osteoclasts created resorption lacunae and resorptive trails and contained mineral particles. Frequently, resorption surfaces were in direct contact with bone formative surfaces on one granule. Granules, especially of NanoBone®, were also covered by osteoclasts if located in vascularised connective tissue distant from bone tissue. However, this usually occurred without the creation of resorption lacunae. The former defect margins consisted of newly formed bone often without remnants of bone substitutes. Our results show that the degradation of both biomaterials corresponds to the natural bone degradation processes and suggest the possibility of complete resorption during bone remodelling.

Key words: osteoclasts, bone defects, Goettingen miniature pig, mandibula

INTRODUCTION

The high incidence of morbidity during autogenous bone grafting has been the rationale for the increase in applications of bone substitute materials. Hydroxyapatite (HA) ceramics, produced synthetically or by processing biological substrates, have been widely and successfully used for bone reconstruction. They are available as granules, as blocks with pores of different sizes or as injectable material. In any case, owing to its osteoconductive capacity, HA serves as a structural scaffold for the building of new bone tissue. The scaffold itself should be completely degradable, as remnants could affect the mechanical properties of reconstructed bone negatively and,

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as non-self, may induce inflammation. It is desirable for scaffold degradation to take place during the regular bone remodelling processes [28]. The extent and distribution of the remodelling of bone substitutes are influenced by the quality of the host site and the local mechanical environment [2]. Particles of HA ceramics are often detectable in patients even years after implantation.

The biodegradation of HA scaffolds may take place by dissolution or fragmentation with subsequent phagocytosis by macrophages [8] but also by the activity of osteoclasts [6, 25, 34]. The latter mechanism of biodegradation is favourable [30], because mimicry of the physiological bone processes should create optimal surfaces for colonisation with osteoblasts and vascular tissue. The degree of osteoclast activity on HA scaffolds depends on material qualities such as crystal size [6, 23, 25] and surface roughness [14]. Macrocrystalline HA is not degradable by osteoclasts [24, 32], but microcrystalline HA may also escape resorption, if the biomaterials were sintered during production [26].

We studied the cellular biodegradation of two granular microcrystalline HA implants in critically sized defects in the mandibula of Goettingen miniature pigs. Bio-Oss® is an HA ceramic produced by sintering bovine spongious bone and is extensively used as a bone substitute. NanoBone® is a synthetic HA with nano- and micropores manufactured in a sol-gel process in the presence of SiO_2 [12]. It has been available commercially since 2005. Using light microscopy we made a comparative analysis of the distribution and activities of osteoclastic cells on these biomaterials five weeks after implantation.

MATERIAL AND METHODS

Animals

Six adult Goettingen miniature pigs (Ellgaard, Dalmose, Denmark) of 25–30 kg body weight were used in this study. The animals were kept free in an appropriate facility. Standard mini-pig diet and tap water were available *ad libitum*. The animals were handled in agreement with the European Communities Council Directive of November 24, 1986. The experiments were performed with the permission of the local ethics committee.

Grafting materials

Bio-Oss® (Geistlich-Pharma, Wolhusen, Switzerland) is a sintered HA ceramic derived from deproteinised bovine spongious bone and was used as granules of 1–2 mm diameter. NanoBone® (Artoss GmbH, Rostock, Germany) contains 74% unsintered HA and 24% SiO_2 and is manufactured in a sol-gel process. It was used as granules measuring about 0.6×2 mm.

Critically sized defects and insertion of biomaterials

Bone perforating critically sized defects (about $3 \times 1.5 \times 1.3$ cm; > 5 cm^3) were placed bilaterally in the anterior part of the corpus mandibulae basal to the teeth, leaving the lingual periosteum intact. The defects were filled with the biomaterial mixed with blood (N = 3 animals per tested material) and closed with the vestibular periosteum and skin. For further details and for handling of the animals during surgery see Henkel et al. [16].

The animals were killed by an intracardial injection of pentobarbital five weeks *post operationem*.

Histological analysis

The defects were fixated in toto in 4% buffered formaldehyde for a minimum of 7 days before cutting transversally into 4 mm thick blocks. Undecalcified blocks were embedded in methacrylate resins (Technovit 7200 VLC, Technovit 9100^{NEW}, Kultzer & Co, Wehrhein, Germany) for the sawing-grinding technique [9] and the preparation of thin sections. Samples decalcificated with EDTA were embedded in paraffin. Sections were stained with haematoxylin & eosin (HE), toluidine blue, Giemsa, Goldner's trichrome or silver nitrite (von Kossa stain). Evaluation of tartrate-resistant acid phosphatase (TRAP) activity was made with naphthol AS-BI phosphate and pararosaniline.

For the microscopic evaluation we used a photomicroscope (Axioplan, Zeiss, Germany) and the software Analysis® (Soft Imaging Systems, Münster, Germany).

RESULTS

General findings

With one exception, there was a complete bridging of the lingual wall of the defects by newly formed bone tissue surrounding granules of the biomaterials (Fig. 1A). The new bone was compact, but locally cancellous. Remodelling was evident as young osteons with large vascular channels embedded in woven bone. The peripheral parts of the former defects consisted of new bone with only minimal biomaterial remnants or none at all (Fig. 1B).

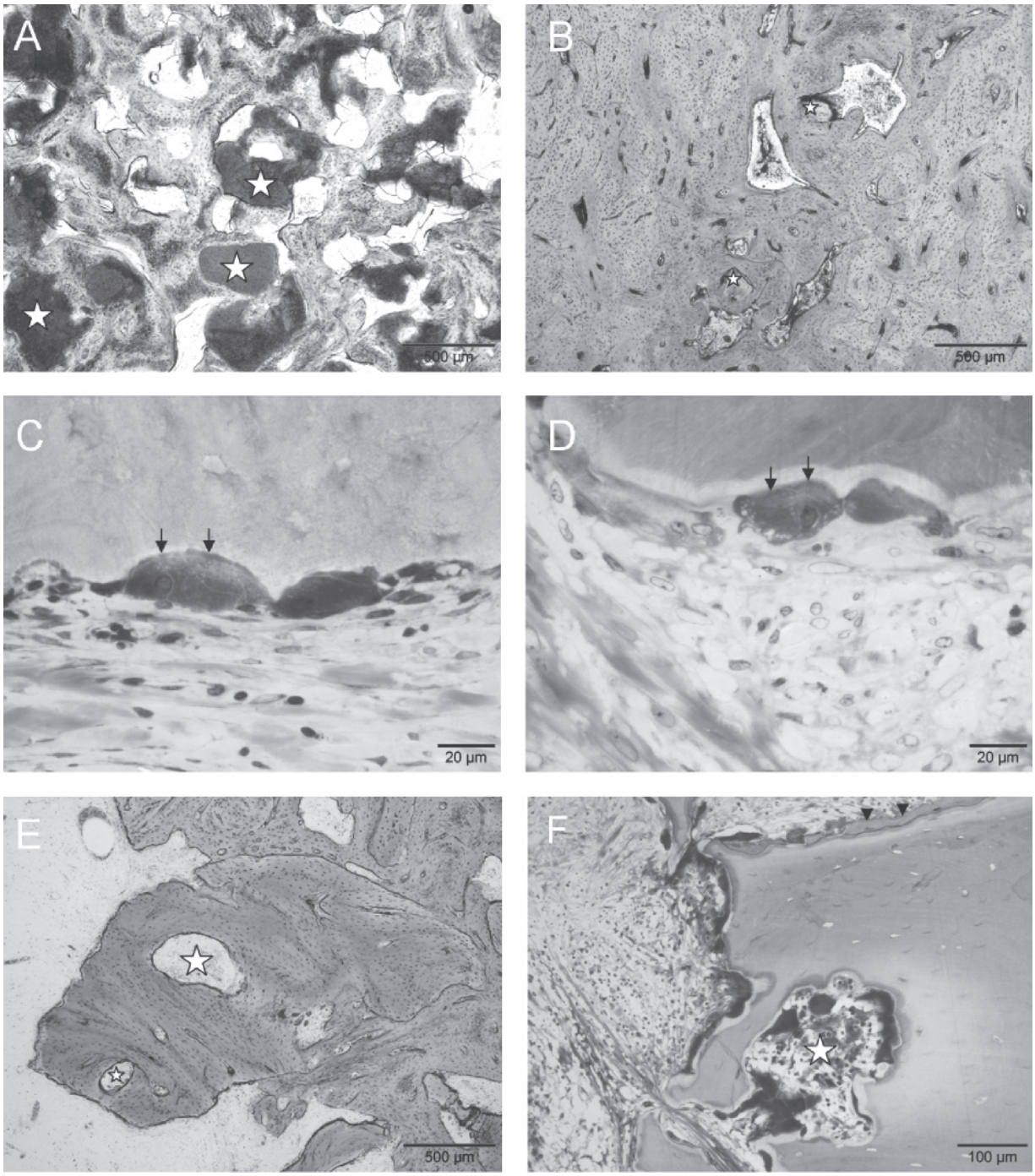

Figure 1. Light microscopy of bone defects in the mandibula of mini-pigs five weeks after implantation of bone substitute materials. **A.** Lingual the centre of a defect filled with NanoBone® is bridged with bone surrounding NanoBone® granules (stars). Bone and granules are in the process of remodelling. **B.** Margin of a former defect displaying small remnants of Bio-Oss® (stars). **C., D.** Osteoclasts with visible ruffled borders (arrows) are situated in resorption lacunae of bone (**C**) and Bio-Oss® (**D**). **E., F.** High numbers of osteoclasts attach to surface and to formerly vascular channels (stars in **E**) of Bio-Oss® granules located near bone. They may also form resorptive trails (star in **F**). Thick plastic sections, toluidine blue. Scale bars are given in the lower right corner of the micrographs.

The vestibular wall of the defects was bridged by thin trabeculae of woven bone contacting granules of biomaterial surrounded mainly by connective tissue. In the centre of the defects these walls contained only connective tissue and biomaterial. The region of the former canalis mandibularis consisted partly of osseous trabeculae but mainly of connective tissue and granules.

Degradation of NanoBone® and Bio-Oss® implants

In all the histological samples analysed multinuclear cells attached to the surface of biomaterial granules were observed. These had the same shape and size as osteoclasts adhering to bone tissue (Fig. 1C, D). Most of these cells demonstrated the features of osteoclasts such as polarisation, ruffled border and TRAP activity (Fig. 1D; 2A, C). Signs of osteoclastic resorption of the biomaterials were evident as resorption lacunae on the granule surface (Fig. 1C, F; 2A, C) and, in the case of Bio-Oss®, in the formerly vascular osteon channels of this bovine biomaterial (Fig. 1E). The dimensions of the lacunae corresponded to those on bone surfaces in our samples. However, the proportion of osteoclasts lying in erosion lacunae was smaller than in bone tissue. There was also evidence of resorptive trail formation in NanoBone® (Fig. 2C) as well as in Bio-Oss® (Fig. 1F). In Bio-Oss® this was identified by osteoclasts situated in channels not surrounded by bone lamellae (Fig. 1F).

The cytoplasm of osteoclasts located on biomaterial often contained differently sized vacuoles and particles stained with toluidine blue, Goldner's trichrome and von Kossa (Fig. 2A, C, D). Such particles were also found near the free surfaces of these cells.

Osteoclasts attached to biomaterial were regularly found in areas with new formation of bone and remodelling respectively, though not on all granules. Frequently bone formation and resorption of the biomaterial proceeded in direct continuity on the granules (Fig. 1F; 2A). Osteoclasts attached to granules also occurred in defect regions without visible bone formation. This was true especially for NanoBone® implants, where granules in the connective tissue were often completely surrounded

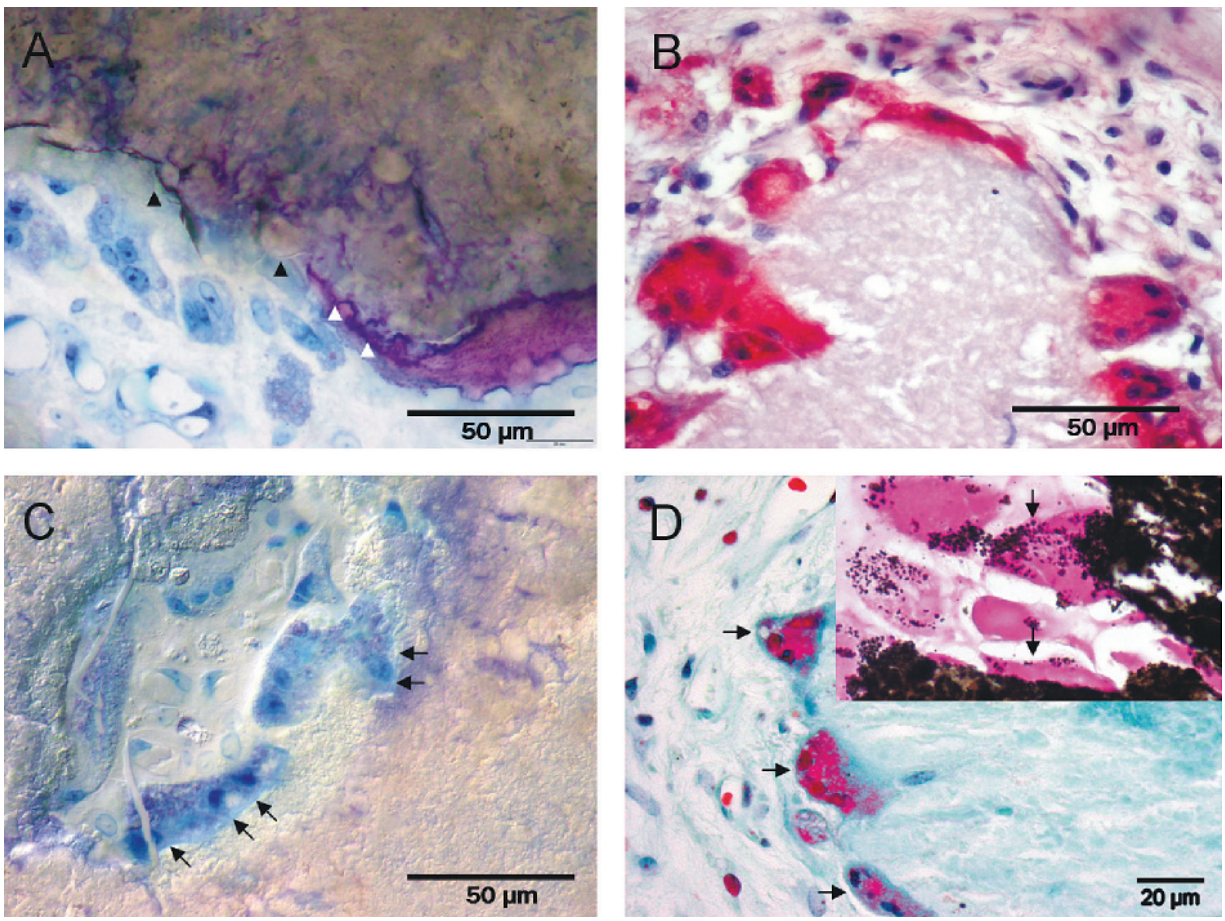

Figure 2. Light microscopy of bone defects in the mandibula of mini-pigs five weeks after implantation of NanoBone®. **A.** Bone deposition (white arrowheads) and osteoclastic resorption (black arrowheads) proceed in direct continuity on a granule. **B.** Granules surrounded only by connective tissue may be covered with TRAP-active osteoclasts but rarely show resorption lacunae. **C.** Osteoclasts containing particles and demonstrating ruffled borders (arrows) are attached to a granule. **D.** The particles in the cytoplasm of osteoclasts (arrows) are stainable with Goldner's trichrome and von Kossa staining (inset). **A., C.** Thick plastic sections, toluidine blue. **B.** Paraffin section, TRAP activity. **D.** Thin plastic sections, Goldner's trichrome and von Kossa (inset) respectively. Scale bars are given in the lower right corner of the micrographs.

by multinuclear cells. These cells were TRAP-reactive, but rarely situated in resorption lacunae. Large multinuclear cells without the features of osteoclasts only occasionally contacted granules of either biomaterial.

DISCUSSION

In the experimental design used for this study we were able to demonstrate that two bone replacement materials based on hydroxyapatite are degraded by osteoclasts. The periphery of the former defects consisted of newly formed bone without biomaterial residues, demonstrating that complete biodegradation of the tested materials is possible. Concerning NanoBone®, this confirms the results of other studies carried out with the same animal model [3, 15, 27], which have reported only small amounts of the material eight months after implantation. The fast, extensive degradation of NanoBone® is explained by a high degree of nano- and microporosity and a loose arrangement of small HA crystals [12], physical properties that promote degradation [11, 13, 18, 19, 24].

Concerning the biodegradation of Bio-Oss®, opinions in the literature vary but the prevalent view is of resorbability [10]. However, Duda and Pajak [10], amongst others, have observed Bio-Oss® remnants in patients even years after implantation. Experiments performed in the cranial bones of dogs [22, 31] could not demonstrate signs of biodegradation after several months, while in goats degradation by osteoclast-like cells was found [21]. A recent human study [35] also reports degradation by osteoclasts. The reasons for the conflicting results may lie in factors such as animal model, implantation site and the local mechanical environment, which influence the remodelling of bone substitute materials [2, 20]. An impressive example of the influence of mechanical forces is described for orthodontic movements in the dog, where 12 months after implantation Bio-Oss® remained an inactive filler in non-utilised areas of the mandibula but was extensively degraded in zones with mechanical strain [1]. Such zones must be constantly remodelled and we found osteoclasts resorbing biomaterial predominantly in defect areas with extensive bone remodelling. During the remodelling processes osteoclasts form channels in the first, rapidly formed woven bone, which are filled by vascularised connective tissue. To the extent that these channels come into contact with biomaterial, osteoclast progenitor cells may attach to the granules and differentiate to active cells. In our study degradation was characterised by resorption pits and trails in HA granules, but also by small mineral particles within and near the attached osteoclasts. Thus the removal of biomaterial resorption products by osteoclasts seems to follow the mechanisms demonstrated for bone [29]. That osteoclasts simultaneously resorb and phagocytise calcium phosphate ceramics was demonstrated by transmission electron microscopy *in vitro* [7, 17, 39] and *in vivo* [34]. We found evidence, as did Wenisch et al. [34], that material released into the extracellular space can then be phagocytised by mononuclear macrophages.

Osteoclasts were also attached to granules situated in vascularised connective tissue in areas without bone formation (a defect centre on the vestibular side). We observed this especially after implantation of NanoBone®. This reflects the situation in natural bone, where the arrival of osteoclasts is triggered by bare mineralised bone surfaces created by the activity of collagenases [5]. However, on NanoBone® embedded in connective tissue osteoclasts were rarely situated in resorption lacunae. This suggests that activating signals [4] are lacking in these regions. For the maintenance of the scaffold function of bone substitutes the inactivity of osteoclasts on biomaterial in areas without bone formation is imperative.

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