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Enhanced in vivo responses of osteoblasts in electrostatically activated zones by hydroxyapatite electrets

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Abstract Matured osteoblasts were proved to be located in the bone formation accelerated by induced large surface charges on the electrically polarized hydroxyapatite (HA) ceramics regardless of the charge polarities, whereas the spatial cell distribution patterns were different. Polarized HA ceramic plates with an average electric charge of 3.9 μCcm^{-2} were implanted in widely spaced defects of canine femora for 3 and 7 days. The osteoblasts were identified by immunochemical detections of osteocalcin and osteopontin. Expressions of osteocalcin and osteopontin were detected throughout the gaps between the implanted HA plates and the cut cortical bone surfaces, especially in the vicinities of the cut cortical bone surfaces and the osteoids regardless of the polarity of the induced charges. Additionally, the newly formed bone tissue that directly bonded to the negatively charged HA surfaces was lined by an osteoblast layer. As soon as 7 days after the implantation, the presence of well-developed osteoblasts suggested that the electrostatic force of the HA ceramics had conditioned the field in the biointerface zone of the polarized HA surfaces.

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1 Introduction

Electrostatic force has continually been recognized as one of the important factors for bone reconstruction but, compared to growth factors, it has been the subject of fewer studied. Electrostatic conditions have primarily been discussed from the perspectives of piezoelectric properties of bones and the electrical current stimulation of cells. Yasuda and Fukada [1] first proved that bone had piezoelectric properties and that the electrical energy converted from mechanical stress affected the proliferation and functions of cells in bones. Bassett [2, 3] demonstrated that the mechanically stressed bones induced negative charges on the compressed parts and positive charges on the tensile parts due to the piezoelectricity of bone collagen. Based on these findings, the current stimulations were extensively examined with various methods. However, the electric current frequently provoked necrosis and insufficient bone repair. Another approach to electrical stimulation was implantation of polymer beads with dissociated groups [4–6]. Although the osteogenic effects of charges on polymer surfaces are still controversial, the electrical charges of the dissociated groups were not recognized as being effective for osteoconduction. Moreover, multinucleated giant cells were found on the positively charged surfaces. Thus

pioneering studies resulted in proof of the non-osteoconductive phenomena partly ascribed to the use of insufficiently biocompatible materials. The responses of the surrounding tissues were much more strongly governed by the interfacial reactions of the dissociated groups and the migration of electrons from or to the surrounding substances than the surface charges of the dissociated groups. Although an effect of the electrostatic charges was estimated in vivo using an electret polymer of corona-charged fluoroethylene propylene without the reaction effect of the dissociated groups and the surrounding substances with electron migrations [7], no remarkable enhancement was observed in the proliferation, adhesion and differentiation of neurocytes.

We have more recently disclosed that exclusively large surface charges were inducible on biocompatible ceramics of hydroxyapatite (HA) by electrical polarization [8, 9] due to proton transport [10, 11]. The negatively charged surfaces of the HA electrets enhanced their osteobonding abilitity in canine and rabbit femora and tibiae [12–15]. The formation of direct bonding bone was observed at 1 week after implantation, even in a wide clearance of 0.5 mm between the HA and bone. In the vicinity of the positively charged surfaces, the osteoid tissues surrounded by osteoblastic cells occupied the clearance between the ceramic electret and the bone. Although the monolayer cells attached to the calcified tissues in the vicinities of the polarized HA were recognized to be osteoblasts from their morphology, the cells were insufficiently identified and the spatial distributions of the cell activities were still unclear. In the present study, the expressions of the genetic markers of the osteoblasts in the electrostatically conditioned zone generated by the HA ceramic were confirmed by immunochemical staining.

2 Materials and methods

HA ceramics sintered in a saturated water vapor atmosphere at 1,250°C were obtained from the pure synthetic HA powder described in detail in previous publications [16]. After being crystallographically identified by conventional X-ray diffractometry (XRD) and FT-IR spectrometry, the HA ceramic specimens with a size of 5.0 mm × 8.0 mm × 1.0 mm were electrically polarized in a DC field of 1.0 $kVcm^{-1}$ with a pair of Pt electrodes in air at 300°C for 1 h. Confirmation of the polarization charges of samples chosen at random was examined by the thermally stimulated depolarization current (TSDC) method. The measurements were performed from room temperature (RT) to 500°C in air at a heating rate of 5.0°Cmin^{-1}.

The polarized samples were implanted in the femoral and femoral diaphyses fully described in previous reports [12, 13]. The experiments were carefully completed by veterinarians in accordance with the Guidelines for Animal Experimentation (Tokyo Medical & Dental University) as well as the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Pub. No. 85-23, Rev. 1985). Rectangular holes of 1.4 mm × 5.0 mm at intervals of more than 15 mm were obtained in the femoral bones of six male beagle dogs with a 0.7-mm dental fisher bur. One each of the 36 polarized and the 12 unpolarized samples was placed in each hole so that the 5.0 mm × 8.0 mm faces of the sample would confront the cut cortical bone faces. The clearances between the observational faces of the samples and the cut cortical bone faces were fixed at 0.2–0.5 mm. The samples were rigidly held by the friction between the lateral faces of the samples and the bone faces. The bones containing the samples were harvested at 3 and 7 days after implantation. The extracted tissues were decalcified with Plank-Rychlo solution for 1–2 weeks at 4°C. The morphological evaluations of the tissue reaction in the gaps between the ceramics and cortical bones were examined using transversal sections stained with hematoxylin eosin (H–E). Osteocalcin and osteopontin were detected by immunochemical staining methods using rabbit antihuman osteocalcin antibody (Biogenesis Ltd.) [17] and rabbit antimouse bone sialoprotein antibody (Cosmo Bio Co., Ltd.) [18], respectively.

3 Results

A typical TSDC spectrum of the polarized HA samples is shown in Fig. 1. The TSDC curve with a maximum current density of 3.2 $nAcm^{-2}$ increased at ca. 180°C, reached a maximum at 370°C, and then gradually decreased. The average stored charge was as large as 3.9 μCcm^{-2} and corresponded well with the published results [9–12]. The calculated half-life of the charge was ca. 200 years. Therefore, the electrically polarized HA ceramic samples were recognized as electrets. We abbreviated the negative charge-induced surface, the positive charge-induced surface, and the surface of the unpolarized HA as the N-surface, P-surface and 0-surface, respectively.

Three days after implantation, no new bone formation was observed in the gap spaces, regardless of the magnitude and polarity of the surface induced charges of the ceramics (Fig. 2). The gaps between the HA samples and the cut cortical bones were occupied by fibrin of fibrinogen layers containing erythrocytes of nucleated cells, while the extracellular matrix and cells were not strictly identified. Therefore, we discontinued immunochemical examination of the 3-day sections.

Figure 3a shows that the newly formed bone layers (NB) of ca. 0.02 mm in thickness contacted the N-surfaces

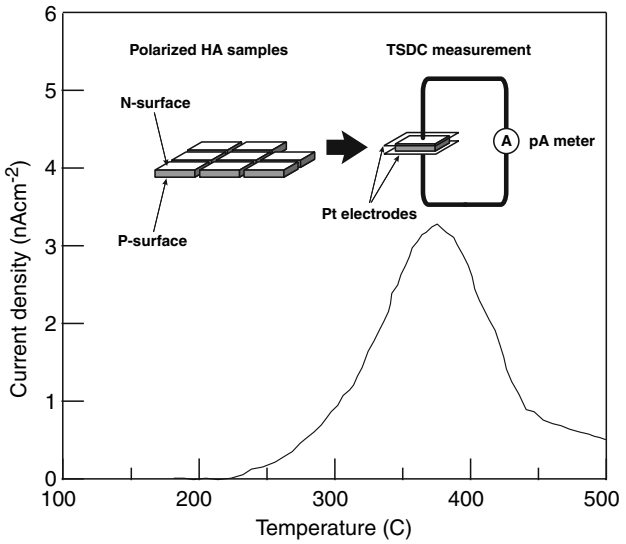

Fig. 1 Schematic illustrations of electrical polarization treatment (inset) and TSDC spectra of HA ceramic electrets. HA ceramics were tightly clamped with a pair of Pt electrodes. Electrical field of DC 1.0 $kVcm^{-1}$ was applied for 1 h to HA samples heated at 300°C. Surfaces in contact with anode and cathode are denoted as N-surfaces and P-surfaces, respectively. Stored charges of samples chosen at random were destructively investigated by TSDC method. TSDC spectrum of HA ceramic sample polarized at 300°C for 1 h in DC field of 1 $kVcm^{-1}$

without any inclusion 7 days after implantation. The bone layers were accompanied by monolayered osteoblast-like cells (OL) on the bone surfaces opposite the electret surface and maturing osseous cells (MO) in the newly formed bones. New bone formation was also observed on the cut cortical bones with a cement line (CL) indicating the boundary between the newly formed and accomplished bones. The spaces between the newly formed bones were filled with unidentified fibrous tissues and blood capillaries (BC) with erythrocytes.

The gaps between the P-surfaces and the cortical bones were occupied by osteoid tissues surrounded by osteoblastic cells 7 days postoperatively. Almost all of the osteoids (Os) were isolated from the ceramic electret surface by fibrin granulation-like tissues while a small part of the osteoids were in direct contact with the P-surface. The layer-structured osteoblast-like cells (OL) were ubiquitous at the margins of the osteoids and significantly larger than those in the N-surfaces. The unpolarized HA ceramic surfaces (0-surfaces) were isolated from the osteoid tissues by dominant fibrin multilayers (Fb) with osteoprogenitor cells (Fig. 3b). Therefore, we discontinued immunochemical examination of the 0-surface sections. In all groups, the phagocyte reaction with multinucleated giant cells reported in the histological investigations concerning the charged surfaces of polymer beads was not found in the gap areas 7 days after implantation.

Expressions of osteocalcin and osteopontin are shown in Figs. 4 and 5, respectively. Both expressions were detected in the entire gap spaces between the ceramic electrets and the cut cortical bone surfaces. In particular, the expressions were considerably dominant in the vicinities of the cut cortical bone surfaces and the osteoids regardless of the polarity of the electret charges. Although relatively strong and sporadic detections of osteocalcin were found in the newly formed bones in contact with the N-surfaces, no other remarkable differences in distribution were recognized between osteocalcin and osteopontin expressions.

4 Discussion

At 3 days after implantation, the histological responses had no considerable differences, while we reported that formation of the fibrin network was accelerated by the induced surface charges up to day 1 in rat tibiae [19]. The drastic bone formation for the N-surface after 7 days requires further immunochemical investigations for cell identification.

Osteocalcin is recognized to be a noncollagenous protein marker of osteoblasts and odontoblasts and is known to be mostly incorporated into the bone matrix bound to HA [20]. Osteopontin is also recognized to be one of the major

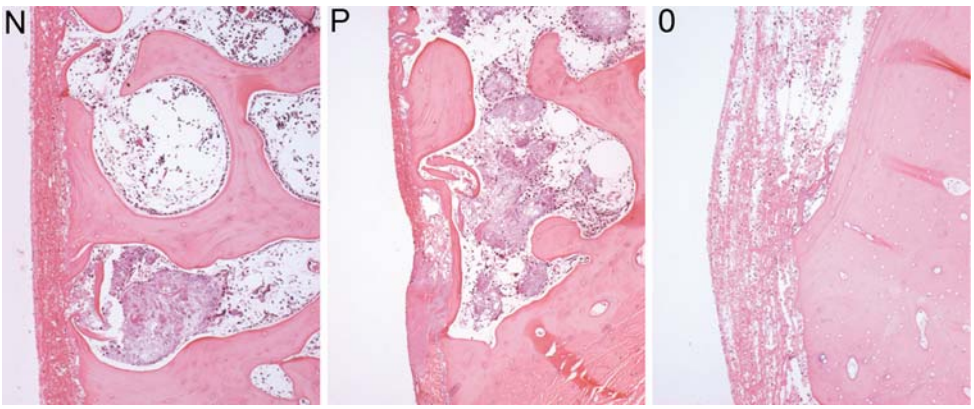

Fig. 2 H&E stained sections of tissue in gaps of 0.2–0.5 mm wide between HA ceramics and cortical bones at 3 days after implantation in the vicinity of N-surface and P-surface in comparison to that of 0-surface

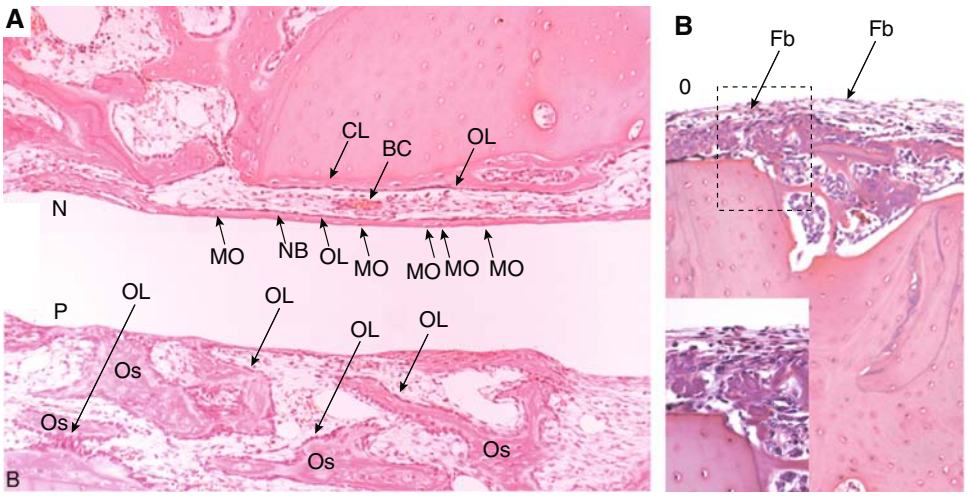

Fig. 3 H&E stained sections of formed bone in gaps of 0.2–0.5 mm wide between HA ceramics (HA) and cortical bones at 7 days after implantation in the vicinities of N-surface (N) and P-surface (P) (left: **a**) in comparison to that of 0-surface (right: **b**). Inset shows magnified image of area enclosed in broken line frame. BC: blood capillaries, CL: cement line, Fb: Fibrin layers, MO: maturing osseous cells, NB: newly formed bone, OL: osteoblast-like cells, and Os: Osteoids

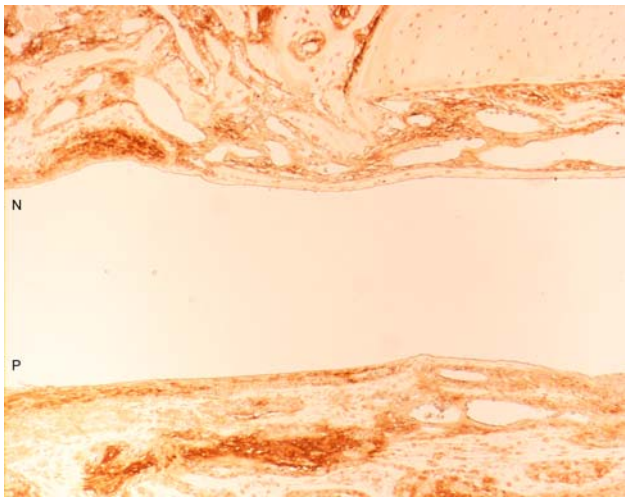

Fig. 4 Immunochemical detection of osteocalcin of electrically polarized HA ceramics

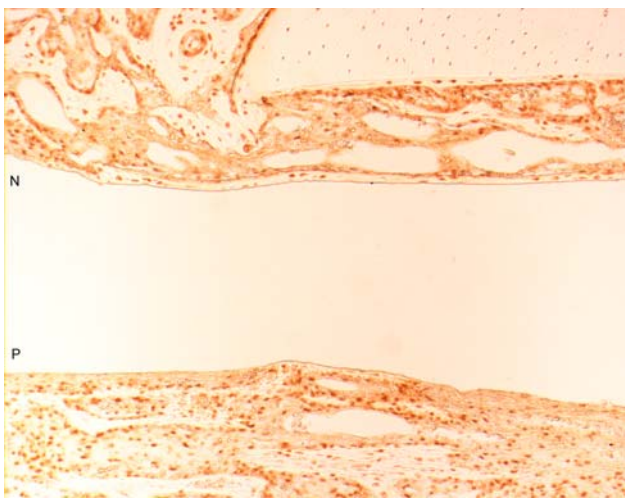

Fig. 5 Immunochemical detection of osteopontin of electrically polarized HA ceramics

noncollagenous proteins in bones [21] and is shown to be an early marker of cells of the osteoblast lineage [22].

The detections of osteocalcin and osteopontin expressions in the vicinities of both the N- and P-surfaces implies that the matrix in the gaps between the ceramic electret surfaces and the cut cortical bones were recognized as secretions derived from cells and that the calcified tissues were assignable as bone. Although the newly formed boned directly bonding to the N-surfaces showed the multilayer structure of the osteopontin positive stripes, the intensities of the staining were very weak. This weakness could be attributed to the incompatibility between the antigens and antibody because of the different species.

Even in the relatively earlier stage of the first 7 days after implantation, the existence of well-developed osteoblasts suggests that the electrostatic forces of the ceramic electrets conditioned the fields in the vicinities of the electret surfaces. The range of force was estimated to be more than 0.3 mm from the surface. These phenomena should serve as evidence that the HA ceramic electrets could provided the electrostatic effects in living organisms [23].

5 Conclusion

Huge surface charges of electrically polarized HA ceramics were demonstrated to enhance their abilities to form bone, although the bone formation processes varied according to charge polarity. Based on the results of immunochemical staining, the electrostatically conditioned zones generated by HA ceramic electrets were shown to activate osteoprogenitor cells in the vicinities of the electrets.

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