Osteoblast responses to thin nanohydroxyapatite coated on roughened titanium surfaces deposited by an electrochemical process

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Purpose. The purpose of this study was to investigate in vitro osteoblast responses to the thin nano-hydroxyapatite (HA) coating on porous implant surfaces.
Materials and Methods. Surface characteristics of nano-HA coating were evaluated by x-ray diffractometer (XRD) and Fourier transform infrared spectroscopy (FTIR). Murine preosteoblast cell (MC3T3-E1) proliferation, alkaline phosphatase (ALP) activity, and osteocalcin release on nano-HA coated surfaces were compared with HA-coated surfaces.
Results. The XRD pattern demonstrated that the peak of nano-HA coating matched well with the standard HA patterns. FTIR spectra also showed that the coating consisted of pure HA crystals. Significant increases in cell proliferation, total protein on day 7, ALP activity on day 14 and day 21, and osteocalcin production on day 21 ($P < .05$) were observed for nano-HA coated surfaces.
Conclusions. It was concluded that thin nano-HA coating, deposited by the electrochemical process, improved proliferation and differentiation of osteoblasts. (Oral Surg Oral Med Oral Pathol Oral Radiol 2013;116:e311-e316)

The use of dental implants is a popular alternative to rehabilitate function and esthetics of patients who are missing teeth. The success becomes more predictable when adequate local and systemic conditions are present to provide bone healing during the osseointegration process.[1,2] Implant success is questionable when local bone condition is inferior, however. For example, osteoporosis is directly related to the decrease of matrix bone volume. Because of osteoporosis, women during menopause have a reduced osteoblastic activity to synthesize bone matrix, rather than increasing the number of osteoclasts, which may cause less bone-implant contact with lower torque resistance.[3] Interests have been concentrated on how to improve implant osseointegration in those with osteoporosis.

In the 1980s, Bonfield and colleagues developed hydroxyapatite (HA)-reinforced polyethylene (PE), which showed bioactive ability because of the presence of HA.[4–6] HA has been a crucial focus of research in the biomaterials field for many years[4]; however, its brittleness and low tensile strength limit its application.[7,8] Therefore, HA was designed to coat implant surfaces, which demonstrated that HA coatings improved osteoblast function and bone integration with implants.[9,10]

Studies showed that nanomaterials (1-100 nm) were better suited for clinical applications because the molecules on a nanoscale have different properties, which are determined by the size, pattern, and folding.[11,12] Xiong et al.[13] found that nano-HA–coated titanium-niobium alloy enhanced in vitro proliferation of the osteoblastlike cells significantly more than the uncoated alloy. The in vivo test done by Luiz et al.[14] and the in vitro study done by Wang et al.[15] indicated that nano-HA could enhance new bone formation at the initial healing stage.

To date, there are many techniques to deposit nano-HA coating on metallic surfaces, such as the hydrothermal process,[13] the electrohydrodynamic atomization spray method,[16] plasma spray method,[17] biomimetic method,[18,19] electrophoresis method,[20] radiofrequent magnetron sputter deposition,[21] and the electrochemical deposition method. Many methods have their own limitations, however; for instance, coating thickness is not thin enough,[12,22–26] processing tem-

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perature is too high,[13,20] there is poor interfacial strength between coatings and the substrate,[20] and it is difficult to deposit uniform coatings on irregular surfaces.[12,26]

Electrochemical deposition is 3-dimensional in coverage, more preservative of implant roughness, and less thick than plasma spraying.[27,28] Through changing the concentration of the electrolytes, the morphology and thickness of HA crystals can be controlled.[29] This method can also coat irregularly shaped substrates. The temperature required is not very high. Studies demonstrated that the needlelike apatite, deposited by the electrochemical method, benefited the bonding between implant and bone during the early stages.[30] Our laboratory produced nano-HA coated on porous titanium surfaces by the electrochemical method. This coating was a very thin and rodlike HA nanocrystal in nature, which improved bone integration with implant surfaces.[31,32]

Therefore, the purpose of this study was to investigate in vitro osteoblast responses to the thin nano-HA coating on porous titanium surface.

MATERIAL AND METHODS

Surface treatments of titanium samples

Plates of 10 × 10 × 1 mm were roughened as previously described by Yang et al.[33] In brief, samples were polished, sandblasted, and washed with acetone, 75% alcohol, and distilled water in an ultrasonic cleaner. Subsequently, samples were treated by a solution containing HF and HNO_3 and then treated by a solution containing HCl and H_2SO_4.

Preparation of HA coatings

The preparation of HA coatings was similar to previous studies.[32,34,35] In brief, the titanium samples were used as the working electrode (cathode), while a platinum (Pt) plate functioned as the counter electrode. The electrolytes were prepared by dissolving analytical grade Ca(NO3)2 (0.6 mmol/L), and NH4H2PO4 (0.36 mmol/L) into distilled water to a Ca/P ratio of 1.67. NaNO3 (0.1 mol/L) was added to improve the conductivity of the electrolytes. The deposition process was conducted with a DC power source at 3.0 V at 85°C for 30 minutes.

Surface analysis

The crystal structure of HA coatings was examined using an x-ray diffractometer (XRD; Philips XD-98 The Netherlands) with Cu Kα radiation. Spectroscopic analysis of the grown CaP microcrystals was carried out by Fourier transform infrared spectroscopy (FTIR) using K Br pellet technique.

Cell culture and seeding

Murine preosteoblast cells (MC3T3-E1) were cultured in α-minimum essential medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and 1% penicillin and streptomycin and maintained at 37°C in a 5% CO_2 humidified atmosphere (standard culture conditions). When cells were seeded at a density of 1×10^5 on each Ti disk, basic medium was supplemented with 10 mmol/L β-glycerolphosphate (Sigma-Aldrich, St. Louis, MO, USA) and 50 mg/L ascorbin-2-phosphate (Sigma-Aldrich, USA). Medium was changed every 3 days.

Cytoplasmic total protein assay

Total protein is an indication of the proliferative and biosynthetic capacities of bone cell cultures. Cells were lysed by CelLytic Buffer (Sigma-Aldrich). The cellular protein concentration was determined by bicinchoninic acid (BCA) protein assay kit (Pierce, Holmdel, NJ, USA). In brief, a 5-μL volume of cell lysate mixed with 15 μL phosphate-buffered saline (PBS) and 200 μL working assay solution was incubated at 37°C for 30 minutes. The resulting optical densities were measured at 562 nm with a spectrophotometer. Bovine serum albumin was used to generate a standard curve.

Alkaline phosphatase activity assay

Alkaline phosphatase (ALP) activity was determined by a commercial phosphatase substrate kit (Wako, Osaka, Japan), which is a colorimetric endpoint assay measuring the enzymatic conversion of p-nitrophenyl phosphate (pNPP) to the yellowish product p-nitrophenol (pNP) in the presence of ALP. Following gentle removal of culture medium and washing with PBS, the cells were lysed using CelLytic Buffer (Sigma, USA). A 20-μL volume of cell lysate mixed with 100 μL working assay solution was shaken for 60 seconds with a plate mixer, and then incubated at 37°C for 15 minutes. Then, the addition of 80 μL stop solution to each well terminated the reaction. After that, the 96-well plate was shaken for another 60 seconds and read at 405 nm with a spectrophotometer. ALP activity was calibrated by per-unit total cellular protein. ALP activity was expressed as nanomoles of p-nitrophenol liberated per microgram of total cellular protein per hour.

Osteocalcin release assay

The production of osteocalcin was measured as the release of extracellular matrix protein into the culture medium by a mouse osteocalcin enzyme immunoassay (EIA) kit (Biomedical Technologies, Stoughton, MA,