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Mesoporous surface topography promotes bone cell differentiation on low elastic modulus Ti–25Nb–25Zr alloys for bone implant applications

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ABSTRACT

Reducing the elastic modulus of metallic bone implants can decrease the stress shielding effect between the implants and bone tissue. Ti alloys containing elements without biological side effects have shown considerable potential as alternatives of biomedical Ti–Al–V alloys in bone implant applications. Ti alloys with porous surface topography have been shown to enhance cell response. This paper developed a rapid electrochemical anodization process for the creation of a low elastic modulus Ti–25Nb–25Zr (Ti25Nb25Zr) alloy with mesoporous topography for bone implant applications. We also investigated the effects of the mesoporous surface topography on bone cell differentiation. The surface mesotopography presented a pore size of <20 nm. Compared to untreated Ti25Nb25Zr alloys, the proposed electrochemical anodization treatment increased the formation of focal adhesion complex protein (vinculin) and the subsequent integrin-mediated pathway (i.e., the expression of focal adhesion kinase). This in turn enhanced the expression of various osteogenic markers, including the gene and protein expression of osteopontin, bone sialoprotein, and type I collagen, from human bone marrow mesenchymal stem cells. An increase in osteogenic markers is expected to promote osseointegration on Ti25Nb25Zr alloys used in bone implant applications. To the best of our knowledge, this is the first study to enhance osteogenic gene and protein expression by producing a low elastic modulus Ti25Nb25Zr alloy with a mesoporous surface structure.

1. Introduction

Alpha/beta (α/β)-type Ti6Al4V alloy is commonly used in the manufacturing of orthopedic implants, due to its good biocompatibility and mechanical properties. However, some previous studies have reported potential biological side effects associated with Al and/or V elements [1–3]. This has led to the development of Ti alloys that use Zr and/or Nb in place of Al and/or V [4–6]. More recently, researchers have focused on developing potential nontoxic β-type TiNbZr alloys which possess lower elastic modulus than that (more than 100 GPa) of α/β-type Ti alloys [7,8]. Reducing the elastic modulus to a level close to that of human bone (1–40 GPa) [9] has been shown to prevent implant loosening and bone resorption by reducing the stress shielding effect between

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bone and the orthopedic implant [10,11]. However, up to now, little information is available regarding the bone formation ability of β-type TiNbZr alloy implants with a lower elastic modulus (≈70 GPa).

Some researchers have sought to promote cell response by giving Ti-based implants a porous surface topography [12–15]. However, most of these surface treatment methods are exceedingly complex. In a previous report, the authors of this study used electrochemical anodization to create a mesoporous surface structure on β-type Ti-25Nb-25Zr (Ti25Nb25Zr) alloys, which have an elastic modulus of only 70 GPa. We then investigated the corrosion resistance, protein adsorption, and cell adhesion/proliferation/migration associated with these alloys [16]. However, the effects of mesoporous surface topography of low elastic modulus Ti25Nb25Zr alloys on bone cell differentiation have yet to be fully elucidated.

Our objective in fabricating mesotopographic surface structures on Ti25Nb25Zr alloys was to enhance the differentiation of bone cells. In this study, we investigated the expression of various osteogenic markers from human bone marrow mesenchymal stem cells (hBMSCs) on mesotopographic Ti25Nb25Zr alloys. We also investigated the formation of focal adhesion complex protein and integrin-mediated pathway as well as their correlation to bone cell differentiation.

2. Materials and methods

2.1. Material preparation

For the preparation of specimens, we employed the same methods outlined in our previous report [16]. Biomedical β-type Ti25Nb25Zr discs, with a diameter of 15 mm, were ground using silicon carbide papers from # 80 to #1200, followed by applying anodic current (0.2 A) to the ground Ti25Nb25Zr specimens using an electrochemical galvanostat in alkaline solution (5 M NaOH) at room temperature for about 10 min. Electrochemically anodized specimens were designated as Ti25Nb25Zr-A, whereas un-anodized (or un-treated) ground specimens were designated as Ti25Nb25Zr-G. Ground Ti specimens were used as reference group in subsequent cell differentiation tests.

2.2. Surface characters

The surface characters of the Ti25Nb25Zr specimens were investigated using methods similar to those employed in our previous reports [16]. In brief, surface topography was observed using field emission-scanning electron microscopy (FE-SEM). Focused ion beam milling was used to make the Ti25Nb25Zr-A specimens for cross-sectional analysis by transmission electron microscopy (TEM). The surface structure dimension was measured using Image-Pro® Plus image analysis software based on FE-SEM micrographs. Various surface roughness parameters, including the arithmetic average roughness (R_a) and the mean average roughness (R_z), were measured using an atomic force microscope (AFM) with a 5 μm × 5 μm scanning area. Surface energy, or wettability, was analysed using a contact-angle goniometer, according to the measurement of contact angle of a droplet of polar water and nonpolar diiodo-methane on the specimens. Nanoindenter, with Berkovich (three-sided pyramid) diamond as indenter head, was used to measure the surface elastic modulus of the test specimens. The continuous contact stiffness measurement technique was utilized to acquire a series of elastic modulus. The maximum indentation depth was approximately 1000 nm. For ion release analysis, the test specimens were immersed in neutral phosphate-buffered saline (PBS) for 7 d. Afterwards, the amounts of released ions (Ti, Nb, and Zr ions) were analysed using inductively coupled plasma-atomic emission spectrometer (ICP-AES). The amounts of metallic ions released from each test specimen (exposure area: 1.5 cm^2) were expressed in parts per billion (ppb). At least, three specimens from each test group were examined using FE-SEM, AFM, contact-angle goniometer, nanoindenter, and ICP-AES analyses.

2.3. Cell response

The hBMSCs used in this study are isolated and incubated, and show the significant differentiation potential to mesenchymal cells. To study the osteogenic differentiation, the cell culture medium, containing low glucose Dulbecco's modified Eagle medium (DMEM) supplemented with 50 g/ml ascorbic-2 phosphate, 10^{-8} M dexamethasone, and 10 mM β-glycerophosphate, was changed per two days. The experimental processes for evaluating the cell response were similar to those employed in our previous reports and more detailed information was described elsewhere [17].

2.3.1. Focal adhesion complex and integrin-mediated pathway

After culturing hBMSCs on Ti25Nb25Zr specimens for 3 h, the effect of surface mesoporosity on cell adhesion was examined using immunofluorescent staining in order to characterize the expression of focal adhesion complex protein (vinculin). The culture medium comprised low glucose DMEM supplemented with 10% fetal bovine serum. Adherent cells were subsequently fixed using 4% paraformaldehyde and permeabilized using 0.2% Triton X-100 in phosphate buffered salin. The cells were incubated with mouse monoclonal anti-vinculin overnight at 4 °C, and secondary antibodies were then conjugated using fluorescein isothiocyanate. Nuclei were stained using 4 0,6-diamidino-2-phenylindole. Cell images were obtained using confocal laser scanning microscopy (CLSM): blue color for nuclei and bright green points for focal adhesion complex protein, vinculin.

We also investigated the expression of proteins involved in the integrin-mediated pathway; i.e., expression associated with focal adhesion kinas (FAK). Specific protein expression was detected using the Pierce Enhanced Chemiluminescence Detection Kit with appropriate antibodies, including anti-phospho-FAK (Tyr 397) and anti-GAPDH. Relative photographic density was quantified using Multi Gauge software. Following incubation of hBMSCs on Ti25Nb25Zr alloys for a period of 3 h, signaling via integrins was confirmed by phospho-FAK (p-FAK) analysis in conjunction with Western blot analysis.

2.3.2. Cell differentiation

2.3.2.1. Gene expression. After seeding hBMSCs (10^5 cells/disc) on specimens, semiquantitative reverse transcription polymerase chain reaction was utilized to evaluate the expression levels of transcription factors and osteogenesis-related genes. After culturing the hBMSCs specimens for 3, 5, or 7 d, total RNA was isolated using the RNeasy Mini Kit. The quantity of isolated RNA was assessed using a microplate photometer at 260 nm. The gene expression levels for two major transcription factors of hBMSCs, Runx2 and Osterix, were evaluated after osteogenic culturing for 3 or 5 d. After osteogenic culturing for 7 d, we evaluated the expression levels of various osteogenic genes, including osteopontin (OPN), bone sialoprotein (BSP), and type I collagen (Col I). For this analysis, expression levels were normalized using a housekeeping gene (GAPDH).

2.3.2.2. Protein expression. Western blot analysis was used to analyze osteogenesis-related protein expression. After hBMSCs (10^5 cells/disc) had been incubated on specimens for 7 or 14 d, total cell lysates were collected using RIPA buffer and separated using 10% polyacrylamide gel before being transferred onto a nitrocellulose membrane. Specific protein expression was determined

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