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The alignment of MC3T3-E1 osteoblasts on steps of slip traces introduced by dislocation motion

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ABSTRACT

Bone tissue shows a highly anisotropic microstructure comprising biological apatite and collagen fibrils produced by the mutual activities of bone cells, which dominates its mechanical function. Accordingly, directional control of osteoblasts is crucial for forming anisotropic bone tissue. A new approach was proposed for controlling cell directionality by using crystallographic slip traces caused by dislocation glide. Dislocations were introduced into α-titanium single crystals by plastic deformation of $(01\bar{1}0)[2\bar{1}\,\bar{1}0]$ slip system, inducing a step-like structure with acute angles between the surface normal and the slip plane. Topographical properties of step patterning, including step interval and step height, could be controlled by varying the compressive plastic strain. The step geometry introduced by plastic deformation strongly influenced osteoblast elongation, and it aligned preferentially along slip traces. Ti substrates under 10% plastic strain with step height of approximately 300 nm and step interval of 10 μm induced osteoblast alignment most successfully. Actin stress fibers elongated parallel to slip traces, with polarized vinculin accumulation between steps.

1. Introduction

The anisotropic structure of biological tissues is crucial for living organisms to be able to adapt to various types of physical or chemical environments, and tissue-specific directional morphology is important for their proper anisotropic function. Biological development starts as a single cell that differentiates into highly organized tissues, and various cues such as chemical molecules and the physical and mechanical environments govern tissue genesis. Thus, controlling cell behavior *in vitro* by regulating the environment surrounding the constituent cells could be used to achieve tissue morphogenesis with appropriate directionality. Bone tissue, in particular, exhibits a highly anisotropic microstructure that is characterized by the preferential orientation of biological apatite (BAp) *c*-axis and collagen fibrils [1]. This anisotropic feature of bone tissue, especially the degree of preferential alignment of BAp crystals, has been shown to dominate the bone mechanical performance depending on the portion of bone affected by the *in vivo* stress distribution [2]. For example, in the mandible of a monkey, the preferential alignment of *c*-axis in BAp crystallites locally depends on the loading direction caused by biting and chewing stresses. Accordingly, there is a pressing need to fabricate biomimetic materials designed from the viewpoint of anisotropic bone microstructure. The *in vitro* establishment of the bone-mimetic orientational structure can lead to designing of implantable materials that are effective for the regeneration of bone anisotropy. Among bone cells, osteoblasts are responsible for bone formation, and osteoblast cultivation *in vitro* has been well established both in cell lines and in primary cells [3–5]. Because aligned or migrated osteoblasts in a preferential direction are considered to provide oriented extracellular matrix [6–8], controlling the behavior of osteoblastic cells, especially the anisotropic cell alignment and migration, may enable the realization of anisotropic morphogenesis of bone tissue. In a natural bone matrix environment, osteoblasts are known to adhere to the nano- and microscale topography of matrix proteins and inorganic components [9]. With an emphasis on such cell–extracellular matrix interactions via nano- and microscale topographic features, cultured cell responses to geometrical cues have been widely studied in various types of cells. Contact guidance by topographic cues was first reported by Harrison in 1912 [10], following which many studies have examined the relationship between surface patterning and cell orientation. Coupled with progress in the field of nanotechnology, microfabrication techniques have been applied to biomedical materials [6,7,11–28]. However, such technologies have limitations because of their complicated processes and the requirement of specialized equipments.

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In this article, a crystallographic approach to control cell direction and arrangement is proposed. α-Titanium is considered one of the most attractive materials for biomedical applications. From an atomistic viewpoint, it has a hexagonal close packed (hcp) crystal lattice, and single crystals of hcp metals are known to deform extensively by slip deformation or twinning. The plastic deformation of α-Ti single crystals, in which atoms are arranged in an orderly manner in the same direction of three dimensional space, induced the appearance of nanoscale step patterns with acute angles introduced by slip traces after dislocation glide. This surface topography results from atom movements produced by operative dislocation, and thus, such a geometrical structure cannot be achieved by other processing technologies. The aim of the present study is to clarify the effects of this characteristic topographical structure on the arrangement of osteoblasts behavior.

2. Materials and methods

2.1. Fabrication of stepped titanium substrates

Titanium single crystals were grown from α-titanium rods (purity: 99.8%) using an optical floating-zone apparatus (SCI-MDH-20020, NEC Machinery Corp.) at a crystal growth rate of 2.5 mm h^{-1} under high-purity argon gas flow. The single crystals were slowly cooled to room temperature (40 h). The crystallographic orientations of the crystals were analyzed and then indexed by using Laue back-scattered X-ray diffraction. Parallelepipeds oriented for compression (dimensions: $2 \times 2 \times 5$ mm^3) were cut from the single crystals by electric discharge machining. These specimens were grinded using #2000 emery papers, following which they were sonicated in acetone and ethyl alcohol. Prior to loading, the surfaces of all specimens were electrochemically polished to eliminate the effect of surface roughness or surface damage and to obtain reasonably smooth surfaces. Compression tests were carried out by using an Instron-type testing machine (AG-100k NX, Shimadzu, Kyoto, Japan) at a nominal strain rate of 1.67×10^{-4} s^{-1}. Fig. 1(a) shows the compressive axis of the specimen ina stereographic triangle. The loading axis was set to operate a prismatic slip system of $(01\bar{1}0)[2\bar{1}\,\bar{1}0]$ with a Schmid factor of 0.5, indicating the most efficient orientation for the dislocation glide. The first loading axis was described as an open circle, which moves toward $(11\bar{2}0)$ as the compressive strain increases. A compressive strain of 1–10% was applied to avoid the activation of the double slip that can be caused by excess loading.

2.2. Surface characterization

The slip traces on the specimen surface were observed by using an optical microscope equipped with Normarski interference contrast (BX60M, OLYMPUS, Tokyo, Japan). The step intervals were measured through 12 images (1320 μm × 1760 μm) per specimen, followed by an analysis using Image J software (NIH, MD, USA). It should be noted that because of the hierarchical geometry of the surface structure introduced by slip deformation, the nanoscale distribution of slip traces cannot be observed only with light microscopy.

Three-dimensional imaging and quantification of the surface topographic features were performed by using an atomic force microscope (SPM-9500J3, Shimadzu, Kyoto, Japan). Topographical images of 30×30 μm^2 sections of the substrate surfaces were obtained in contact mode using silicon nitride cantilevers (OMCL-TR400PSA, Olympus, Tokyo, Japan). Height images were captured and analyzed by scanning at a scan rate of 2 Hz, following which each step length was calculated by using the obtained heights. As mentioned before, limited slip traces are observable under an optical microscope, and therefore the measurable step heights corresponding to the step intervals on the specimen surface were estimated to be >100 nm.

2.3. Cell culture

MC3T3-E1 cells (RIKEN Cell Bank, Tokyo, Japan) were seeded at a density of 1×10^5 cells/well in 96-well polystyrene plates (Iwaki, Tokyo, Japan) containing the titanium single- crystal substrates. The cells were maintained in α-minimum essential medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Gibco, Invitrogen), 100U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) at 37 °C in a humidified 5% CO_2 incubator.

2.4. Cell orientation

After culturing for 24 h, the cells on the substrates were washed in PBS and fixed with methanol for 10 min. After drying in air, the cells were stained with 5% Giemsa staining solution (Wako, Osaka, Japan) for 10 min. The cells were then washed in distilled water and dried in air.

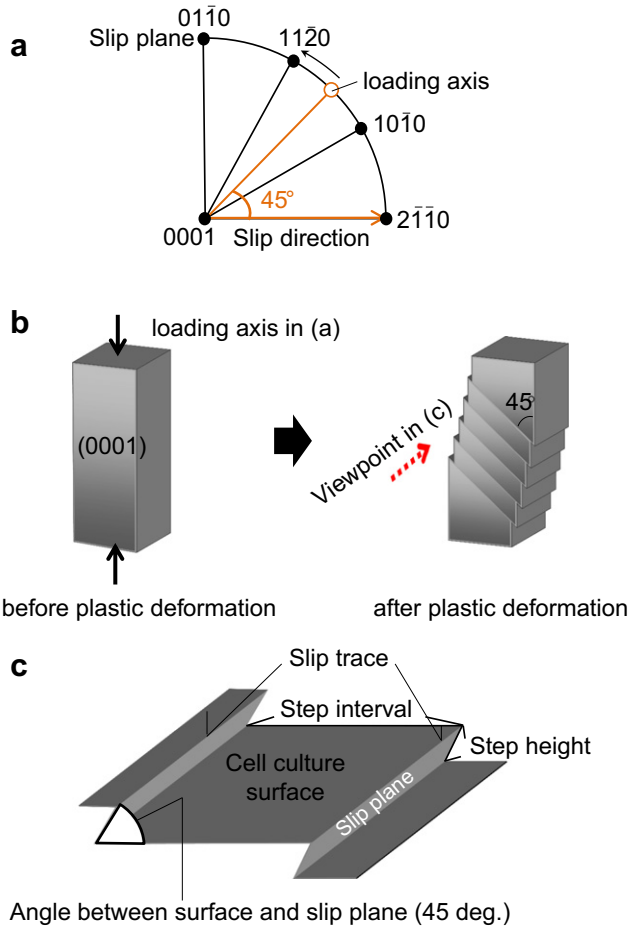

Fig. 1. (a) The orientation of the specimen in the stereographic triangle. The ° mark indicates the first loading axis, which moves toward $(11\bar{2}0)$ as the compressive plastic strain increases. The loading condition was adjusted as the prismatic slip system of $(01\bar{1}0)[2\bar{1}\,\bar{1}0]$ is activated. (b) A diagram representing the plastic deformation process. After plastic deformation, the characteristic stepped geometry by the operative slip system of $(01\bar{1}0)[2\bar{1}\,\bar{1}0]$ appeared on the surface of the specimen. (c) A schematic illustration of the stepped geometry on the surface introduced by the slip trace. From left to right, the steps go downhill. The step interval is the distance between the slip planes, and the step height corresponds to the strain amount. The flat space between two steps is applied for cell culture. The angle between the culture surface and the slip plane is 45°, which is produced by the prismatic slip system in the Ti single crystal with hcp.

The orientation of the cells on the Ti substrates was examined relative to the slip trace by taking photographs of the cells using a brightfield microscope (BX60M, OLYMPUS, Tokyo, Japan). The images were analyzed with Image J software (NIH, MD, USA). The criteria for the inclusion of cells for quantification are that the cells should not be in contact with other cells and they should not be in contact with the image perimeter.

The degree of cell orientation was characterized by the angular standard deviation, S.D. (σ), for a wrapped normal distribution [29,30]. Here, the probability distribution function was adapted from Fisher [31] for a periodicity of π radians, where μ is the mean angle and ρ, the mean resultant length. These parameters were determined from a set of n measured cell orientations, θ_i, by the following equations:

$$f(\theta) = \frac{1}{\pi}\left(1 + 2\sum_{p=1}^{\infty}\rho^{p^2}\cos(2p(\theta-\pi))\right), \tag{1}$$

$$\rho = \frac{1}{n}\sqrt{\left(\sum_{i=1}^{n}\cos 2\theta_i\right)^2 + \left(\sum_{i=1}^{n}\sin 2\theta_i\right)^2}, \tag{2}$$

$$\mu = \tan^{-1}\left(\sum_{i=1}^{n}\sin 2\theta_i / \sum_{i=1}^{n}\cos 2\theta_i\right). \tag{3}$$

The angular standard deviation (σ) was determined by the following equation:

$$\sigma = \frac{1}{2}\sqrt{-2\ln\rho}. \tag{4}$$