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Effect of the nanostructure of porous alumina on growth behavior of MG63 osteoblast-like cells

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It is well known that cellular responses to materials, in terms of adhesion, migration and proliferation, are highly affected by the surface characteristics of the materials. The investigation of the effect of material surface topography on cell behaviors is of great importance for the development of implanted biomaterials in tissue engineering. Alumina is one of the most popular implant materials used in orthopedics, but few data are available concerning the potential cellular responses of MG63 to nanoporous alumina. The present study investigated the size effect of nanoporous alumina substrates on MG63 cell behaviors in terms of cell viability, expression of integrin β1, alkaline phosphatase (ALP) activity and changes of cell morphology, respectively. Cell viability was measured by means of MTT assay and integrin β1 expression was detected by immunofluorescence staining and real-time PCR. Scanning electron microscopy (SEM) was used to observe cell morphology. Cell function was evaluated by detecting the ALP activity and mineralization. Results showed that cell viability and expression of integrin β1 were decreased with the increasing pore size, however, the increasing pore size of the alumina resulted in elongated cell morphology, enhanced ALP activity and mineralization. This study showed that the surface topography of nanoporous alumina plays an important role in regulating the behaviors of MG63 osteoblast-like cells and porous alumina can be regarded as useful substrate in tissue engineering.

[**Key words:** MG63 osteoblast-like cells; Nanoporous alumina; Surface topography; Proliferation; Alkaline phosphatase activity]

Tissue engineering is an emerging interdisciplinary filed that applies the principles of biology and engineering to the development of various substitutes that restore and maintain the function of defected tissues. Over the last decade, developments have enhanced the field of tissue engineering from the simple cell cultures to precisely engineered materials that closely mimic native tissues in both construction and function. Biologically inspired materials are being designed with the aim of improving the integration of medical implants and improve their clinical performance (1). However, disadvantages of the implanted materials are still existed, such as lack of direct chemical bonding and interaction with the surrounding environments. In order to solve those complicated problems, great efforts have been paid to promote biocompatibility and enhance the cell–material interaction. Among the various approaches, a promising strategy is the design of topographically patterned surfaces that is similar with the extracellular matrix (ECM) of the tissues to mimic the biological and mechanical properties of the original tissue (2–4).

The cell adhesion, migration and proliferation behaviors are significantly affected by the biochemistry of the local microenvironment and the surface topography of the substrates on which cells are cultured. Previous studies have shown that deliberately patterned topographical and chemical features can provide controllable cell–material interaction and influence cellular responses ranging from morphology and alignment through adhesion, growth and differentiation (5–7). In general, structures with surface topography on mesoscale and microscale sizes have been widely studied to investigate the effect of microstructure on cell behavior. Recently, how to control the cell behavior within microenvironments on the nanoscale has attracted more and more attention. Cells were verified to respond to surface protrusion topography that has nanoscale features in a controllable manner. Microfabricated pillar arrays of different geometries were proved to control cell orientation and cell function (8,9). Vertical nanowires arrays with high aspect ratios were also reported to affect the adhesion, morphology and growth of living cells (10–12).

Nanophase alumina has been widely used as an implant biomaterial in bone tissue engineering for its biocompatibility, and has been demonstrated significant biointegration and cell ingrowth (13,14). Recently, there has been increasing research attentions focused on porous alumina. Nanoporous alumina was recognized as an important orthopedic material and as a template in the fabrication of nanostructures such as nanowires and nanotubes (15). Favorable results have been verified by researchers using microporous and nanophase alumina with cells. Swan et al demonstrated that nanoporous alumina membranes with the average pore size of 72 nm showed favorable osteoblast adhesion (16). Hepatoma cell

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line HepG2 was observed homogeneously grown over the nanostructured alumina surface; also, membrane protrusions/filopodia was detected distributing around the edge of cells and interacting with the nanopores (17). Furthermore, marrow stromal cells on nanoporous alumina substrate were proved to have better osteogenic differentiation ability as compared with amorphous alumina surface (18). Based on these reports, it suggests that surface roughness plays an important role in the cell attachment and subsequent proliferation and differentiation.

On nanoporous alumina, the main surface feature which can be sensed by cells is the pore size. In order to study the effect of different nanopore size on cell behaviors, human MG63 osteoblast-like cells were employed to investigate cellular behaviors and morphology with nanoporous alumina membranes having different pore sizes of 20, 100 and 200 nm. Our results demonstrated that different pore size has different effects on cell behaviors, and it is possible to control the cellular behaviors with pore sizes. It provides a new insight to exploit this novel biocompatible material for tissue engineering.

MATERIALS AND METHODS

Nanoporous alumina substrate characterization Nanoporous alumina membranes (Anodisc Whatman International Ltd., UK) with pore diameters of 20, 100 and 200 nm were used in this study. The membranes were ultrasonically cleaned twice in ethanol for 30 min each, and then dried at room temperature in a clean bench. The surface topography of the membranes was examined with scanning electron microscope (JEOL 7000FK, JEOL, Japan).

MG63 cell culture The osteoblast-like MG63, a human osteosarcoma cell line, was obtained from Riken BioResource Center (Japan). MG63 cells share a lot of characteristic features of osteoblasts and are commonly used in evaluating material properties. The cells were cultured in 25 cm^2 flasks at the 37°C in a humidified incubator containing 5% CO_2. The culture medium was Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/ml streptomycin. When reaching confluence, cells were treated with 0.05% trypsin/0.02% EDTA, collected by centrifugation and resuspended in DMEM. The cells were counted using a hemacytometer and approximately 1×10^5 cells were seeded onto the alumina membranes. MG63 cells cultured on cover glass was used as control. The membranes were then incubated at 37°C for 6 h to allow the cells to attach to the material, and then the alumina membranes were transferred to a new cell culture plate.

Cell viability Cell viability was determined by MTT assay. Briefly, after MG63 cells were cultured on nanostructured alumina surface for 1, 2 and 4 days. 100 μl of MTT (5 mg/ml) (Wako, Japan) was added to each well and incubated at 37°C for another 4 h. Then, 0.5 ml dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals. The absorbance of each solution was measured at the wavelength of 490 nm with a microplate reader (Bio-Rad 680, Bio-Rad, USA).

Immunofluorescence staining of cytoskeleton and integrin β1 After 2 days of culture, MG63 cells adhered to different substrates were washed with PBS and fixed with 4% paraformaldehyde at room temperature for 20 min. Samples were then washed with PBS for three times to remove excess paraformaldehyde. Fixed cells were permeabilized with 0.25% Triton X-100 at room temperature for 30 min. Next, the treated samples were blocked with 1% bovine serum albumin (BSA)/phosphate buffered saline (PBS) at 37°C for 1 h. Subsequently, goat monoclonal antibody against integrin β1 (1:200) (Santa Cruz Biotechnology) was added at 4°C overnight. After that, mouse–antigoat Rhodamine-conjugated secondary antibody (1:100) (Santa Cruz Biotechnology) was added at room temperature for 10 min in the dark. Then, samples were rinsed with PBS for three times and stained with 0.5 μM Alexa Fluor 488 conjugated phalloidin at room temperature for 2 h. The nuclei of MG63 cells were stained with 10 μg/ml DAPI and counterstained with mounting medium at room temperature for 5 min. The stained samples were finally observed with confocal laser scanning microscopy (CLSM, Nikon, Japan). In order to distinguish the stained cells, the fluorescent images (1.2×1.2 mm) were taken at the position where the cells having low density on the substrates and at least 3 pictures were taken for each group from different glass and membranes.

Real-time PCR Total RNA of the cells on different substrates was extracted using RNeasy Mini Kit (Qiagen, USA) according to manufacturer's instructions. Reverse transcript were performed using High Capacity RNA-to-cDNA Kit (ABI, USA) with 1.5 μg total RNA in a final 20 μl reaction volume. Real-time PCR were performed with Taqman Gene Expression Master Mix (ABI) on an ABI 7300 real-time PCR system. Predesigned MGB probes of glyceraldehydes 3-phosphate dehydrogenase (GAPDH, Hs99999905_m1), and integrin β1 (ITGB1, Hs00559595_m1) (ABI) were used to detect relative gene expressions. Expression levels of GAPDH was used as an endogenous control. Expression level was normalized to GADPH and calculated using standard curve method.

Cell morphology by scanning electron microscope (SEM) To observe cell morphologies on nanoporous alumina substrates, the cells were cultured for 4 days and rinsed with PBS, and then fixed with 2.5% glutaraldehyde (Wako) in PBS for 1 h at room temperature. After thorough washing with PBS, the cells were dehydrated in a graded ethanol series (50%, 60%, 70%, 80%, 90%, 95% and 99.5%) (Wako) for 15 min each and air-dried at room temperature. The fixed samples were sputter-coated with gold (E-200S, Canon, Japan) and cell morphology was observed using SEM.

Cell elongation analysis using the morphology of MG63 To quantify the differences in cell morphology observed in the SEM results, the length and width of MG63 cells were measured using Image J software. The ratio of obtained cell length to cell width was denoted as the MG63 cell elongation ratio. At least 30 cells were measured in each group.

Alkaline phosphatase (ALP) activity Alkaline phosphatase (ALP) activity was determined by an assay based on the hydrolysis of *p*-nitrophenylphosphate (*p*-NPP) to *p*-nitrophenol (*p*-NP). In brief, MG63 cells were cultured on nanostructured alumina substrates or cover glass for 1, 2 and 4 days, respectively. Then, 20 μl of the triton lysate was added to 100 μl working reagent. The samples were then incubated at 37°C for 15 min. After incubation, the reaction was stopped with 80 μl sodium hydroxide. The absorbance at the wavelength of 410 nm was measured with a spectrophotometric microplate reader (Bio-Rad 680). The ALP activity was normalized by the total intracellular protein production and expressed as micromoles per milligram protein per minute.

Total intracellular protein content was measured by bicinchoninic acid (BCA) protein assay. The cell based scaffolds were assayed after culture for 1, 2 and 4 days. After medium was removed from the cell culture, MG63 cells were washed with PBS and lysed by 1% Triton X-100 with three freeze–thaw cycles. Total intracellular protein content in the cell lysates were measured using BCA assay kit (Pierce Chemical Co., USA). In brief, 25 μl of triton lysate was added to 200 μl of BCA working solution and the mixture was incubated for 30 min at 37°C. The protein concentration was determined from the absorbance at 570 nm wavelength by spectrophotometric microreader (Bio-Rad 680).

Mineralization assay For detection of mineralization, alizarin red staining was performed after incubation for 4 weeks. Briefly, cultured cells were fixed in 4% paraformaldehyde (PFA) and treated with 40 mM alizarin red S (pH 4.1, Sigma) for 20 min at room temperature with gentle shaking. After aspiration of the unincorporated dye, the samples were washed four times with distilled water while shaking for 5 min. For quantification of staining, 10% v/v acetic acid was added to each sample and incubated for 30 min with shaking. The surface layer on the substrate was collected with acetic acid (10%, v/v) and transferred to a microcentrifuge tube. The microcentrifuge tubes were heated to 85°C for 10 min and then were centrifuged at 20,000 *g*/min for 15 min. The supernatant was transferred to a new microcentrifuge tube and neutralized with 10% v/v ammonium hydroxide (19). The absorbance of supernatant was measured at wavelength of 405 nm with a spectrophotometric microplate reader (Bio-Rad 680).

Statistical analysis Data were represented as mean ± standard deviation. Statistical analysis to compare results between two groups was carried out by unpaired Student's *t* test and a value of $p < 0.05$ was considered statistically significant.

RESULTS

Characterization of the alumina surfaces The representative SEM images of nanoporous alumina with different pore sizes were shown in Fig. 1. The diameter and thickness of the alumina membranes are 13 mm and 60 μm, respectively. Although the manufacturer proclaimed that the pore size of the alumina were 20, 100 and 200 nm, the actual average pore sizes of the alumina are around 31 nm, 94.7 nm and 210 nm, respectively. The alumina surfaces were smooth and had randomly distributed circular pores.

Cell viability Cell viability of MG63 on different alumina substrates was investigated with MTT assay after incubation for 1, 2 and 4 days, respectively. The cells cultured on cover glass were used as control. Our result showed that there was no significant statistical difference between the glass and aluminum after culturing for 2 and 4 days (data not shown). The effect of nanoporous alumina substrates on viability of MG63 cells was shown in Fig. 2. MG63 cells cultured on alumina substrate showed higher cell viability than that on cover glass. Cell viability decreased gradually with the increasing pore sizes of alumina substrate. Smaller pore sizes as 20 nm had more cell numbers of MG63 on the surface than lager pore size such as 100 and 200 nm at day 2 and day 4, respectively. The results indicated that smaller pore sized alumina were advantageous for cell growth.