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Surface functionalization of nanoporous alumina with bone morphogenetic protein 2 for inducing osteogenic differentiation of mesenchymal stem cells

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ABSTRACT

Many studies have demonstrated the possibility to regulate cellular behavior by manipulating the specific characteristics of biomaterials including the physical features and chemical properties. To investigate the synergistic effect of chemical factors and surface topography on the growth behavior of mesenchymal stem cells (MSCs), bone morphorgenic protein 2 (BMP2) was immobilized onto porous alumina substrates with different pore sizes. The BMP2-immobilized alumina substrates were characterized with scanning electron microscopy (SEM) and X-ray photoelectron spectroscopy (XPS). Growth behavior and osteogenic differentiation of MSCs cultured on the different substrates were investigated. Cell adhesion and morphological changes were observed with SEM, and the results showed that the BMP2-immobilized alumina substrate was able to promote adhesion and spreading of MSCs. MTT assay and immunofluorescence staining of integrin β1 revealed that the BMP2-immobilized alumina substrates were favorable for cell growth. To evaluate the differentiation of MSCs, osteoblastic differentiation markers, such as alkaline phosphatase (ALP) activity and mineralization, were investigated. Compared with those of untreated alumina substrates, significantly higher ALP activities and mineralization were detected in cells cultured on BMP2-immobilized alumina substrates. The results suggested that surface functionalization of nanoporous alumina substrates with BMP2 was beneficial for cell growth and osteogenic differentiation. With the approach of immobilizing growth factors onto material substrates, it provided a new insight to exploit novel biofunctional materials for tissue engineering.

1. Introduction

Tissue engineering is a radical new approach to repair and replace damaged or diseased tissues. It involves the use of a combination of cells, engineered scaffolds and suitable biochemical and physiochemical factors to improve or replace biological functions [1]. Scientific advances in biomaterials, stem cells, growth and differentiation factors, and biomimetic environments have created unique opportunities to fabricate tissues by assembling engineered 3-dimensional (3D) biocompatible scaffolds, cells, and biologically active molecules into functional structures resembling native tissues [2–11]. The major challenge now facing tissue engineering is the need of scaffold for more complex functionality, as well as both functional and biomechanical stability destined for transplantation. The ideal scaffolds provide a framework and initial support for cell attachment, proliferation, differentiation and formation of extracellular matrix (ECM) [12].

Material surface properties have significant effects on cellular behavior. Several features of the implant surface such as chemical composition, topography, roughness, and stiffness play important roles in implant osteointegration [13,14]. Due to the biocompatibility and excellent mechanical properties, porous alumina substrates have received a great deal of attention in bone tissue engineering, since bone cells can penetrate throughout the interconnected pores and grow on their biocompatible surfaces, which would promote bone ingrowth by providing a 3D environment [15,16]. We have demonstrated that it was possible to influence cellular attachment, differentiation and mineralization of osteoblasts by changing the nanopore size of alumina [17]. Swan et al. demonstrated that nanoporous alumina with the pore size of 72 nm was favorable for osteoblast adhesion [18]. Our previous study proved that nanoporous alumina was able to promote the adhesion and proliferation of MSCs [19]. These reports indicated that nanoporous alumina could be used as an ideal cell culture scaffold in tissue engineering.

Besides surface topography, local chemistry property of the substrate is considered as another important factor that affects cellular behaviors, which regulates cell–material interaction. Surface coating with growth factors is a feasible approach to change the surface chemical property of substrates. However, quick diffusion of the coated growth factors from the substrates reduced the effective time, and the frequent high-dose growth factors may result in minus effect on cell behavior. To avoid deleterious effects, surface modification by immobilizing adhesive peptides, growth factors, or hormones onto

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biomaterial surfaces via either chemicals or a physical strategy has been introduced and been proven as a valuable method to promote desirable cell–substrate interactions and to enhance cell functions at the cell-tissue interface[20–26].

Bone morphogenetic proteins (BMPs) play important roles in bone and cartilage formation. Among the members of BMP family, bone morphorgenic protein 2 (BMP2) has been demonstrated to be able to stimulate osteogenic differentiation and promote bone formation [27,28]. Kim et al. proved that BMP2-immobilized polycaprolactone scaffolds were able to stimulate the osteogenic differentiation of mesenchymal stem cells (MSCs) [29].

Lack of quickly self-renewable cell source is another bottleneck in the current development of tissue engineering. MSCs are a promising cell source in therapeutic and regenerative medicine. They can be easily harvested and cultured from a wide range of tissues. Furthermore, as undifferentiated cell types, MSCs are able to differentiate into a variety of cell types, such as osteoblasts, chondroblasts, myoblasts, adipocytes and tenocytes [30–34], under certain stimulation. Due to these advantages, MSCs have been becoming an attractive cell source for the repair of damaged or defective tissues/organs in tissue engineering.

In this study, the synergistic effect of substrate topography and chemical cue (BMP2), which was immobilized onto alumina substrates with different pore sizes to fabricate surface functionalized materials, on the proliferation and osteogenic differentiation of MSCs was investigated.

2. Materials and methods

2.1. Nanoporous alumina

Nanoporous alumina substrates with the pore size of 20 and 100 nm were purchased from Whatman International Ltd, England. The substrates were ultrasonically cleaned twice in ethanol for 30 min each, and then dried at room temperature. They were stored in a vacuum oven until use. Smooth alumina purchased from Alfa Aesar was used as control.

2.2. BMP2 immobilization

The alumina substrates were submersed in a 2 mg/ml solution of dopamine (10 mM Tris buffer, pH 8.5) overnight in the dark [35,36]. The substrates were then rinsed with distilled water to remove unattached dopamine and dried under nitrogen flow. The polydopamine-grafted substrates were then submersed in BMP2 solution (100 ng/mL) (BioVision Incorporated, USA) with 10 mM Tris buffer (pH 8.5) and incubated overnight in a humid atmosphere at room temperature [36,37]. The substrates were then washed three times with sterile PBS to remove unattached BMP2 and air-dried in a sterile environment for the following cell experiments. The polydopamine-grafted alumina substrates were denoted as PDOP-alumina; the BMP2-immobilized PDOP-alumina substrates were denoted as BMP2-PDOP-alumina.

2.3. Surface characterization

The surface topography of the alumina substrates was imaged by scanning electron microscope (SEM; JEOL 7000FK, Japan). The chemical composition of the substrates was determined by X-ray photoelectron spectroscopy (XPS) using a Model PHI 5600 system (Perkin elmer, USA) with an Al Kα source (1486.6 eV).

2.4. MSC cell culture

MSCs were purchased from the Health Science Research Resources Bank, Japan. The cells were cultured in 25 cm^2 flasks at 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 of penicillin and 100 μg/ml of streptomycin. Upon reaching confluence, cells were detached 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 substrate. After cells have adhered to the material, the alumina membrane was transferred to a new cell culture plate. The medium was changed every 3 days.

2.5. Cell viability

MTT assay was employed to estimate viabilities of the cells on the substrates. Briefly, after MSCs were cultured on the different substrates for 7 days, the medium was changed and 200 μl of MTT (5 mg/ml) (Wako, Japan) was added and incubated at 37 °C for another 4 h. The medium containing MTT was removed and 1.5 ml dimethyl sulfoxide (DMSO) was added to each well of the plate to dissolve the formazan crystals. The optical density of the solution was measured at the wavelength of 490 nm with a microplate reader (Bio-Rad 680, USA).

2.6. Immunofluorescence staining

After 2 days of culture, MSCs were fixed with 4% paraformaldehyde at room temperature for 20 min. Samples were then washed three times with PBS and permeabilized with 0.25% Triton X-100 at room temperature for 30 min. The samples were then incubated with 1% bovine serum albumin (BSA)/PBS at 37 °C for 1 h. Subsequently, goat monoclonal antibody against integrin β1 (1:200) (Santa Cruz Biotechnology) was added and kept at 4 °C for overnight. Mouse-antigoat Rhodamine-conjugated secondary antibody (1:100) (Santa Cruz Biotechnology) was added at room temperature for 10 min. Samples were rinsed three times with PBS and stained with 0.5 μM FITC-conjugated phalloidin at room temperature for 2 h. The nuclei of MSCs were stained with 10 μg/ml DAPI at room temperature for 5 min, after which mounting medium (10 μl) was dispensed on the samples. The stained samples were finally observed by a confocal laser scanning microscope (Nikon, Japan).

2.7. Cell morphological observation and analysis

To observe adhesion and morphologies of MSCs on the different substrates, the cells were rinsed with PBS after incubation for 4 days, then fixed with 2.5% glutaraldehyde (Wako, Japan) in PBS for 1 h at room temperature. After thorough washing with PBS, the cells were dehydrated in a graded series of ethanol (70%, 80%, 90% and 99.5%) (Wako, Japan) for 15 min each and air-dried at room temperature. The fixed samples were sputter-coated with gold (Canon E-200S, Japan) and imaged by SEM.

To quantify the differences in cell morphology imaged by SEM, the length and width of MSCs were measured using ImageJ software. The ratio of obtained cell length to cell width was denoted as cell elongation ratio. At least 30 cells were measured in each group.

2.8. Alkaline phosphatase activity

The quantitative detection of alkaline phosphatase (ALP) activity was determined by an assay based on the hydrolysis of p-nitrophenylphosphate to p-nitrophenol to evaluate the osteogenic differentiation of MSCs. In brief, after MSCs were cultured on the different substrates for 1, 2 and 3 weeks, 20 μl of the cell lysate was added to 100 μl working reagent. The samples were then incubated at 37 °C for 15 min and the reactions were then stopped with 80 μl sodium hydroxide. The absorbance at the wavelength of 405 nm was measured with a spectrophotometric microplate reader (Bio-Rad 680). ALP activity was normalized to the total intracellular protein production and expressed as micromoles per milligram protein per minute.