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Mouse embryonic stem cells cultured under serum- and feeder-free conditions maintain their self-renewal capacity on hydroxyapatite

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ABSTRACT

New tissue engineering techniques based on embryonic stem (ES) cells and artificial scaffolds are required for regenerative medicine. Because artificial scaffolds can regulate the differentiation states of ES cells, compatibility between the cells and artificial scaffolds is important. To our knowledge, this study is the first report showing that mouse ES (mES) cells can be maintained in undifferentiated state on hydroxyapatite coated with gelatin. In contrast to previous studies, our culture medium was serum-free and included a GSK-3 inhibitor. Under these conditions, mES colony morphology was similar to that of an undifferentiated state; mES cells expressed the pluripotent-specific factors Oct-3/4 and Nanog, and they maintained the ability to differentiate into the three germ layers. Moreover, a GSK-3 inhibitor blocked the expression of integrin subunits that bind to laminin which are known to induce the differentiation of mES cells. These findings indicate that mES cells can be cultured under serum- and feeder-free conditions and maintained in an undifferentiated state on a composite with hydroxyapatite and that this composite can be used to control the differentiation of stem cells.

1. Introduction

Based on the widespread availability of pluripotent stem cells, such as embryonic stem (ES) cells and induced pluripotent stem (iPS) cells, and the ability to propagate and expand these cells in vitro, a major area of research in regenerative medicine has been the improvement of stem-cell therapy technique [1,2]. However, techniques that allow three-dimensional culturing of large tissue architectures are still under investigation, and they are unlikely to be applied in a clinical setting in the immediate future. Another therapeutic option is, tissue engineering of the composites of ES cells and artificial scaffolds; thus this option can be developed in parallel with techniques that drive in vitro organogenesis of stem cells.

To fabricate new composites, it is essential that the nature of the interaction between scaffold materials and cultured cells is understood. It is known that tissue cells sense and respond to the chemical components and mechanotransduction of their substrate [3,4]. The differentiation state of stem cells is also regulated by extracellular matrix (ECM), which is a natural scaffold [5,6]. For example, ES cells cultured on type I and type IV collagen remain in an undifferentiated state, whereas laminin and fibronectin have altered their characteristics. Understanding the compatibility of scaffolds and ES cells is a priority in the design of composites [5].

Although mouse ES (mES) cells are generally cultured on feeder cells with serum and leukemia inhibitory factor (LIF) in the culture medium, the use of cellular and protein components is inconvenient for clinical applications because these components can cause immune reactions. Under natural condition, mES cells are innately programed for self-replication, which does not require extrinsic instruction [7]. Recently, techniques for stable ES generation in the absence of feeder cells using serum-free culture media supplemented with chemical compounds and/or ECM components have been reported [8,9]. Sato et al. showed that the propagation of serum- and feeder-free ES cells on a gelatin-substrate with LIF in the medium is enhanced by an indirubin compound, 6-bromoindirubin-3′-oxime (BIO), which inhibits glycogen synthase kinase-3 (GSK-3) [10]. Treatment of mES cells with BIO results in increased β-catenin activity, indicating that activation of a canonical Wnt signaling promotes maintenance of stem cell properties [10]. In the case of ECM, Laminin-511 allows the self-renewal of mES cell in the absence of feeder cells and LIF [11]. The integrin family of cell surface adhesion receptors transduces signals from the ECM to the cells; thus, integrins are also essential for controlling the differentiation state of ES cells [5].

Depending on the intended use, various biomaterials have been widely developed as scaffolds. For example, owing to its good biocompatibility, hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, HAp), a major component of tissues such as bone, is used as an artificial scaffolds for bone regeneration. Although prosthetic bone materials, such as HAp, are less invasive for patients than autogenous grafts which are obtained by surgical procedures, they have a limited role in the treatment. Therefore,

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Table 1
Sequence of primers and probe numbers used for qRT-PCR.

Genes	Forward primer	Reverse primer	Probe number
Oct-3/4	gaggctacagggacacctttc	gtgccaaagtggggacct	6
Nanog	ttcttgcttacaagggtctgc	cagggctgccttgaagag	6
Integrin α1	ccaatgagactgttcctgagc	ttgggaaatgccctctttt	31
Integrin α2	aggaaaagaacccactcctgt	ttctgctgtgcagctgatgt	64
Integrin α3	aggatatgtggcttggagtga	gaccacagcaccttggtgta	81
Integrin α5	tcatcctagccattctttttgg	gggagggaacgtttgaaga	15
Integrin αv	accaggatggcttcaatgat	agcctgtggaccttccatt	32
Integrin α6	gctgttggagctccctatga	atgtcccctcgagaacctg	60
Integrin β1	tgacaacaatgaagctatcg	atgtcgggaccagtaggaca	109
Integrin β3	gtgggagggcagtcctcta	caggatatcaggaccttgg	31
GAPDH	gggttcctataaatacggactgc	ccattttgtctacgggacga	52

composites of HAp, with organic materials and/or cultured cells may be more effective for bone regeneration [12,13]. It was previously reported that mES cells can be cultured on apatites, but these conditions do not permit an undifferentiated state to be maintained [14,15]. We think that different culture conditions from the previous studies will be needed for the development of composites that allow mES cells to remain in an undifferentiated state and to proliferate in sufficient quantities.

The purpose of this study was to assess the compatibility of mES cells grown under serum- and feeder-free conditions with HAp, with the aim of developing new bone prosthetic materials. In combination of LIF, we added BIO or its kinase-inactive analog, 1-methyl-6-bromoindirubin-3′-oxime (MeBIO) to the culture medium. Then to study whether mES cells could be maintained in an undifferentiated state in the presence of HAp, we evaluated colony morphology, Oct-3/4 and Nanog expression, and the ability of mES cells to differentiate. Finally, the expression of integrins, which are cytoadherence proteins, was examined to understand the interaction between the HAp scaffold and the differentiation state of mES cells.

2. Materials and methods

2.1. HAp preparation and characterization

HAp powders were synthesized by a wet method and uniaxially pressed into pellets at 120 MPa. They were sintered at 1250 °C for 2 h under a water vapor stream [16,17]. Then, sintered HAp pellets were ultrasonically cleaned in acetone, ethanol, and distilled water. About 80% of the prepared HAp pellets had a density of more than 95% of the theoretical density value (3.16 $g \cdot cm^{-3}$), and they were provided in the following experiments.

The crystal phases of the product were determined with a powder X-ray diffractometer (XRD) in the 2θ range of 20° to 70° using Cu Kα radiation at 40 kV and 40 mA (PW1700, PANalytical, Tokyo, Japan). Chemical bonding in HAp was measured using a Fourier transform infrared spectrometry (FT-IR, JASCO FT/IR-500 spectrometer) between 4000 and 400 cm^{-1} using KBr pellets [18]. The surface roughness of the specimens was quantified using a laser profile micrometer with a resolution of 0.01 μm (VF-7500, Keyence, Japan). Six specimens were scanned at three different positions. The Ra values are presented as means ± SD.

2.2. Cell culture

The feeder-free ES cell line used in this study was generated from a C57BL/6 N ES cell line, B6-6 (AES0172), provided by RIKEN BioResource Center (http://www.brc.riken.jp/lab/cell/english/). The original B6-6 ES cells were cultured under a serum- and feeder-free condition in the presence of LIF and BIO according to the method reported previously [10]. The resultant colonies having a typical round, compact shape were passaged several times and were stored in liquid nitrogen until use. This feeder-free B6-6 cell line (AES0187) will also be available from RIKEN BioResource Center soon. The cells were cultured on glass or HAp coated with 0.1% gelatin in ES maintenance medium which consisted of Knock-out DMEM®, supplemented with 15% knockout serum replacement (KSR), L-glutamine, nonessential amino acids (nAA), 10 ng/ml LIF (all from Invitrogen), 2-mercaptoethanol (2-ME, Wako), and BIO or MeBIO, which is N-methylated analog of BIO that serves as a relevant kinase inactive control, depending on the experiments [10]. For neural differentiation, dissociated mES cells were cultured in a low cell adhesion 96-well plate with G-MEM supplemented with 10% KSR, L-glutamine, nAA, 2-ME, pyruvate, hDkk1, and hLefty-1 to form aggregations. On day 7, the cell aggregates were transferred to a Matrigel-coated (BD Biosciences) dish in DMEM/F12 supplemented with N2 [9]. For differentiation into mesodermal progenitors, ES cells were plated on a plastic dish coated with collagen IV (BD Biosciences), and cultured in DMEM/F12 supplemented with 10% FCS, 2 mM glutamine, 0.1 mM nAA, and 0.1 mM 2-ME for 6 days. For endodermal differentiation, ES cells were cultured with 1% FCS, 100 ng/ml activin, glutamine, nAA, and 2-ME on a collagen IV-coated dish for 6 days [19].

2.3. Morphology of mES cells

The morphology of mES cells on the different scaffold substrates was observed by a scanning electron microscopy (SEM; Hitachi S-3400NX, Tokyo, Japan) at 10 kV. The cells on each substrate were prepared for SEM by fixation in 2% glutaraldehyde for 2 h, dehydration in a graded series of ethanol, critical point drying (Hitachi, ECP-2), and coating with platinum (Hitachi, E102).

2.4. Alkaline phosphatase staining

Alkaline phosphatase staining was performed after fixation with 4% paraformaldehyde, using a kit and following the manufacturer's protocol (Wako).

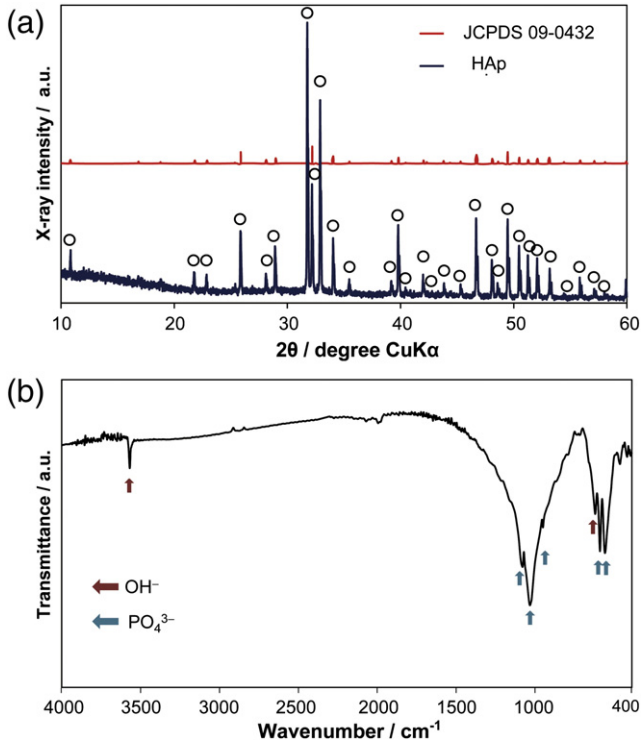

Fig. 1. X-ray diffraction patterns (a) and FT-IR spectra (b) of hydroxyapatite.