Acta Biomaterialia 35 (2016) 185–193

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Full length article

Matrices secreted during simultaneous osteogenesis and adipogenesis of mesenchymal stem cells affect stem cells differentiation

Rong Cai [a,b], Tomoko Nakamoto [a], Takashi Hoshiba [a,c], Naoki Kawazoe [a], Guoping Chen [a,b,*]

[a] Tissue Regeneration Materials Unit, International Center for Materials Nanoarchitectonics, National Institute for Materials Science, 1-1 Namiki, Tsukuba, Ibaraki 305-0044, Japan
[b] Department of Materials Science and Engineering, Graduate School of Pure and Applied Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8577, Japan
[c] Frontier Center for Organic Materials, Yamagata University, 4-3-16 Jonan, Yonezawa, Yamagata 992-8510, Japan

ARTICLE INFO

Article history:
Received 20 August 2015
Received in revised form 3 February 2016
Accepted 8 February 2016
Available online 9 February 2016

Keywords:
Stepwise mimic
Osteogenesis
Adipogenesis
Mesenchymal stem cells
Extracellular matrix

ABSTRACT

The extracellular matrix (ECM) plays a pivotal role in regulating stem cell functions. The ECM dynamically changes during tissue development. It remains a great challenge to mimic the dynamically changing ECM. In this study, we prepared novel types of extracellular matrices that could mimic the dynamic variation of extracellular matrices, which were derived from simultaneous osteogenesis and adipogenesis of human bone marrow-derived mesenchymal stem cells (MSCs). Four ECMs simultaneously mimicking early osteogenesis and early adipogenesis (EOEA), early osteogenesis and late adipogenesis (EOLA), late osteogenesis and early adipogenesis (LOEA), late osteogenesis and late adipogenesis (LOLA) were prepared. The stepwise osteogenesis–co-adipogenesis-mimicking matrices had different compositions and different effects on the osteogenic and adipogenic differentiation of MSCs. The matrices could provide very useful tools to investigate the interaction between ECM and stem cells and the role of ECM on stem cell differentiation.

Statement of Significance

Extracellular matrices (ECMs) are dynamically remodeled to regulate stem cell functions during tissue development. Until now, mimicking the ECM variation during stem cell differentiation to single cell type has been reported. However, there is no report on simultaneous mimicking of stem cell differentiation to two types of cells. In this study, we prepared the mixture ECMs derived from simultaneous osteogenesis and adipogenesis of MSCs at different stages and found that they could regulate stem cell differentiation. The concept is new and the ECMs are novel. No such ECMs have been reported previously. The matrices will provide very useful tools to investigate the interaction between ECM and stem cells and the role of ECM on stem cell differentiation.

1. Introduction

The extracellular matrix (ECM) interacts with cells and provides various signals for manipulating cell functions and maintaining the homeostasis of living organisms [1–3]. The composition of the ECM is dependent on the type of tissues and organs [4]. For example, the articular cartilage is characterized by the abundance of type II collagen and aggrecan, while bone tissues are mainly made up of type I collagen [5,6]. Adipose tissues also produce ECM with specific biochemical composition [7]. The different types of ECM have specific effects on the regulation of cellular response and function [8].

The ECMs are not only tissue-specific but are also dynamically remodeled and balanced during the development and aging process to maintain the activity of each tissue and organ. When the balance is broken, disorders and diseases may occur. It has been reported that imbalance of the ECM in bone may result in disorders such as osteoporosis and osteoarthritis [9,10].

During the tissue development progress, the ECMs surrounding bone marrow-derived mesenchymal stem cells (MSCs) are remodeled according to the different stages of differentiation [11,12]. Abnormal ECM dynamics are documented in clinical studies of many diseases [2,13]. For example, the ECMs in pathological cases such as osteoporosis have different characteristics compared with the normal tissues [14]. The ECMs in osteoporosis cases have a

* Corresponding author at: Tissue Regeneration Materials Unit, International Center for Materials Nanoarchitectonics, National Institute for Materials Science, 1-1 Namiki, Tsukuba, Ibaraki 305-0044, Japan.
E-mail address: Guoping.Chen@nims.go.jp (G. Chen).

http://dx.doi.org/10.1016/j.actbio.2016.02.009
1742-7061/

mixture composition derived from osteogenesis and adipogenesis of MSCs. So far, the ECMs during either osteogenesis or adipogenesis have been examined to understand their roles in pathological cases which disrupt the balance of osteogenesis and adipogenesis [15,16]. Although decellularized osteoporotic bone tissues can been used, the stages of osteogenesis and adipogenesis are different in each patient. Decellularized osteoporotic bone tissues are not suitable for the investigation of ECM roles in the diseases caused by disruption of bone homeostasis. To solve this problem, ECM models which possess the composition of both osteogenic and adipogenic states and the different stages of osteogenesis and adipogenesis are strongly desirable.

Previously, we reported "stepwise osteogenesis-mimicking matrices" and "stepwise adipogenesis-mimicking matrices" that respectively replicated the ECM secreted at different stages of osteogenesis or adipogenesis [11,12]. These matrices had different effects on proliferation and differentiation of MSCs. Although these ECM models can mimic ECM changes during osteogenesis and adipogenesis separately, they cannot simultaneously mimic osteogenesis and adipogenesis to make their complex ECM. Therefore, in this study, four novel stepwise development-mimicking matrices were developed to mimic the dynamically changing ECM derived from MSCs undergoing simultaneous osteogenesis and adipogenesis. The composition of the ECMs and their effects on the proliferation and osteogenic and adipogenic differentiation of MSCs were compared.

2. Materials and methods

2.1. MSCs expansion

Human bone marrow-derived mesenchymal stem cells (MSCs) of passage 2 were purchased from Lonza (Walkersville, MD). MSCs were expanded and characterized as described previously [17]. Cells were cultured in MSCGM™ (Lonza, Walkersville, MD) in a humidified atmosphere of 5% CO_2 at 37 °C. After reaching 80% confluence, the cells were subcultured. The subcultured cells (passage 4) were collected by treatment with trypsin/EDTA solution and suspended in the medium for further use.

2.2. Simultaneous osteogenesis and adipogenesis of MSCs

The MSCs (passage 4) were seeded on tissue culture polystyrene (TCPS) plates at a density of 5000 cells/cm^2. To induce cells undergoing simultaneous osteogenesis and adipogenesis, MSCs were cultured in mixture media composed of osteogenic medium (OM) and adipogenic medium (AM) at different ratios. Osteogenic medium was prepared from Dulbecco's modified Eagle's medium (DMEM, Sigma, Louis, MO) supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY), 1000 mg/l glucose, 584 mg/l glutamine, 100 units/ml penicillin, 100 mg/l streptomycin, 0.1 mM nonessential amino acid, 1 mM sodium pyruvate, 0.4 mM proline, 50 mg/l ascorbic acid, 10 nM dexamethasone and 10 mM β-glycerolphosphate. The adipogenic medium was prepared by adding 10% FBS, 4500 mg/l glucose, 584 mg/l glutamine, 100 units/ml penicillin, 100 mg/l streptomycin, 0.1 mM nonessential amino acid, 1 mM sodium pyruvate, 0.4 mM proline, 50 mg/l ascorbic acid, 1 μM dexamethasone, 0.5 mM methyl-isobutylxanthine, 10 μg/mL insulin, and 100 mM indomethacin in DMEM. Based on our previous study, mixture media at OM/AM ratio of 95/5, 85/15, 70/30 and 50/50 were used [18]. The cells were cultured in the mixture media for 7, 14 and 21 days and media were replaced every 3–4 days. The different stepwise stages of simultaneous osteogenesis and adipogenesis of MSCs were obtained by controlling the OM/AM ratio and culture time. Four types of cells at different stepwise osteogenesis–co-adipogenesis stages were obtained. They are early osteogenesis and early adipogenesis stage (EOEA) cells, early osteogenesis and late adipogenesis stage (EOLA) cells, late osteogenesis and early adipogenesis stage (LOEA) cells, and late osteogenesis and late adipogenesis stage (LOLA) cells. As controls, cells at osteogenesis early stage and adipogenesis early stage as well as undifferentiated stage of MSCs were also obtained as in our previous reports [11,12]. Detailed culture conditions are shown in Table 1.

2.3. Investigation of simultaneous osteogenesis and adipogenesis of MSCs

To confirm the stepwise differentiation of MSCs as above described, alkaline phosphatase (ALP) staining, Alizarin red S staining and gene expression analysis were carried out. After MSCs were cultured at the conditions as shown in Table 1, the cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS for 10 min. For ALP staining, the cells were incubated with 0.1% naphthol AS-MX phosphate (Sigma, St. Louis, MO) and 0.1% fast blue RR salt (Sigma, St. Louis, MO) in 56 mM 2-amino-2-methyl-1,3-propanediol (pH 9.9, Sigma, St. Louis, MO) working solution at room temperature for 10 min, washed with PBS twice and then observed under an optical microscope. For Alizarin red S staining, the fixed cells were incubated with 0.5% Alizarin red S (Sigma, St. Louis, MO) solution at room temperature for 10 min, extensively washed twice with PBS, and then observed under an optical microscope.

Expression of the genes encoding alkaline phosphatase (*ALP*, an early stage marker of osteogenesis), bone sialoprotein 2 (*IBSP*, a late stage marker of osteogenesis) [19], and lipoprotein lipase (*LPL*, an early stage maker of adipogenesis) [20] was investigated by real-time PCR analysis. Total RNA was extracted from the cells cultured at conditions listed in Supplementary Table 1 by using RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instruction. Total RNA (1.0 μg) was used as a first strand reaction that contained random hexamer primers and murine leukemia virus reverse transcriptase (Applied Biosystems, Foster City, CA). Real-time PCR was used to quantify 18 S rRNA, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), *ALP*, *IBSP*, and *LPL*. The reaction was performed with 10 ng of cDNA, 90 nM PCR primers, 25 nM PCR probe and Fast Start TaqMan Probe Master (Roche Diagnostics, Tokyo, Japan). The expression level of 18 S rRNA was used as an endogenous control and gene expression level relative to *GAPDH* was calculated using the comparative Ct method [21]. The sequences of primers and probes are based on our previous studies listed in Supplemental Table 1 [11,12]. The primers and probes were obtained from Applied Biosystems and Hokkaido System Science (Sapporo, Japan).

2.4. Preparation of stepwise osteogenesis–co-adipogenesis-mimicking matrices

MSCs that were cultured at conditions as shown in Table 1 were used to prepare the stepwise osteogenesis–co-adipogenesis-mimicking matrices. The cultured cells were washed twice with PBS and decellularized by incubation with PBS containing 0.5% Triton X-100 and 20 mM NH_4OH for 5 min at 37 °C, followed with treatment with DNase I (100 μg/ml, Roche, Basel, Switzerland) and RNase A (100 μg/ml, Nacalai Tesque, Kyoto, Japan) for 1 h at 37 °C. Removal of cellular components by decellularization was confirmed by staining of cytoskeleton and cell nuclei. The cells before and after decellularization were fixed with 4% paraformaldehyde for 10 min and treated with 0.2% Triton X-100 for 2 min. Actin filaments were visualized by incubation with phalloidin-Alexa 488 (Invitrogen, Carlsbad, CA) at room temperature for 20 min. Cell nuclei were visualized by staining with 4′-6-