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Fabrication of hydrogels with elasticity changed by alkaline phosphatase for stem cell culture

Hiroyuki Toda [a], Masaya Yamamoto [a], Hiroshi Uyama [b], Yasuhiko Tabata [a,*]

[a] Department of Biomaterials, Institute for Frontier Medical Sciences, Kyoto University, 53 Kawara-cho Shogoin, Sakyo-ku Kyoto 606-8507, Japan
[b] Division of Applied Chemistry, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan

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ABSTRACT

The objective of this study is to design hydrogels whose elasticity can be changed by alkaline phosphatase (ALP) in cell culture and evaluate the effect of hydrogel elasticity on an osteogenic gene expression of cells. Hydrogels were prepared by the radical polymerization of acrylamide (AAm), *N,N′*-methylenebisacrylamide (BIS), and Phosmer™M containing phosphate groups (PE-PAAm hydrogels). The storage modulus of PE-PAAm hydrogels prepared was changed by the preparation conditions. When human mesenchymal stem cells (hMSC) were cultured on the ALP-responsive PE-PAAm hydrogels in the presence or absence of ALP, the morphology of hMSC was observed and one of the osteogenic differentiation markers, Runx2, was evaluated. By ALP addition into the culture medium, the morphology of hMSC was changed into an elongated shape without cell damage. ALP addition modified the level of Runx2 gene expression, which was influenced by the modulus of PE-PAAm hydrogels. It is concluded that the elasticity change of hydrogel substrates in cell culture had an influence on the Runx2 gene expression of hMSC.

Statement of Significance

Stem cells sense the surface elasticity of culture substrates, and their differentiation fate is biologically modified by substrate properties. Most of experiments have been performed in static conditions during cell culture, while the in vivo microenvironment is dynamically changed. In this study, we established to design an enzyme-responsive hydrogel whose elasticity can be changed by alkaline phosphatase (ALP) in cell culture to mimic in vivo conditions. As a result, the cells were deformed and the gene expression level of an osteogenic maker, Runx2, was modified by ALP treatment. This is the novel report describing to demonstrate that the dynamic alteration of hydrogel substrate elasticity could modulate the osteoblastic gene expression of human MSC in vitro.

1. Introduction

It is well recognized that stem cells sense the surface and bulk properties of culture substrates, and their differentiation fate is biologically modified by substrate properties, such as elasticity, ligand presentation, and surface morphology [1–4]. To investigate the effect of substrate elasticity on the cell response, various natural and synthetic polymer hydrogels have been designed as cell culture substrates [5–9]. In the case of natural polymers, such as collagen, matrigel, hyaluronic acid, and alginate, both the polymer concentration and crosslinking conditions are changed to prepare the hydrogel with varied elasticity. However, the hydrogel elasticity could not be changed independent of the biochemical property of the natural polymers due to the difference in preparation conditions. The inconsistency in biochemical properties often makes it difficult to evaluate the effect of elasticity alone on cellular behavior. On the contrary, the hydrogels of synthetic polymers, such as poly(acrylamide) (PAAm), poly(ethylene glycol) (PEG), and poly (dimethyl siloxane) (PDMS), do not have any specific biochemical effects, and can be designed to have a wide range of substrate elasticity. For example, PAAm hydrogels with elasticity ranging from 0.01 to 100 kPa were used as culture substrates for different types of cells, such as MSC, neural stem cells (NSC), fibroblasts, and endothelial cells [10–13]. PEG-based hydrogels with elasticity ranging from 0.1 to 1000 kPa were also studied as culture sub-

* Corresponding author.
E-mail address: yasuhiko@frontier.kyoto-u.ac.jp (Y. Tabata).

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strates for different cell types, such as MSC, NSC, fibroblasts, and endothelial cells [6]. PDMS with elasticity ranging from 10 to 1000 kPa was used for NSC to evaluate neuronal maturity [14]. Taken together, the synthetic hydrogel is a good material to investigate the influence of the substrate elasticity on the behavior of stem cells. The elasticity of the local microenvironment around cells in the body depends on tissue type and condition and ranges over more than two orders of magnitude. For example, the elasticity of the normal liver is around 1 kPa, while that of the cirrhotic liver is around 10 kPa [15]. Previous reports showed that the elasticity of the artery and vein ranges from 10 kPa to 10 MPa [16]. Although the elasticity range of bone tissue is from 10 MPa to 100 GPa, that of pre-osteoblast is from 10 to 100 kPa [6]. Generally, cells constantly sense the mechanical properties of their microenvironment by adhering and actively pulling, and then the resulting resistance induces a change in cell shape. Irrespective of cell type, the modulation of traction forces by changing the elasticity of the synthetic hydrogels is essential to the mechanosensitivity [17], spreading and migration [18], stiffness [19], and differentiation of cells [11,20]. Researches have been performed in static conditions, while the elasticity of substrates is not changed during the cell culture. Static conditions do not always reflect the in vivo condition because the body microenvironment is dynamically changed. For example, with the maturation of mesodermal cardiomyocytes to adult myocardium, the elasticity of their microenvironment increases 9 times by changing the collagen expression and localization [21]. The elasticity of the liver depends on the fibrosis stage while the elasticity of the fibrotic liver tissue is higher than that of the normal one [22]. During the development and progression of tissue fibrosis, myofibroblasts enhance their harmful contractile activity responding to the level of stress in the surrounding tissue [23]. Wang et al. reported that the mechanical integrity of collagen networks in the human bone deteriorates with age, and such adverse changes correlate well with the decreased toughness of aged bone [24]. As one trial to mimic in vivo conditions changed dynamically, stimuli-responsive hydrogels are designed and prepared. Polymer hydrogels either physically or chemically crosslinked show controlled and reversible shape changes in response to various external stimuli, such as thermal, electrical, magnetic, pH, ultra violet (UV)/visible light, enzymic reaction, ionic or metallic interactions and combinations [25].

In this study, PAAm-based hydrogels are designed as an enzyme-responsive hydrogel, whose elasticity can be changed by alkaline phosphatase (ALP) as an extrinsic cue in cell culture. PAAm hydrogels containing the Phosmer™M unit with phosphate groups (PE-PAAm) were prepared as culture substrates, and the hydrogel elasticity was changed by the amount of crosslinking agent and Phosmer™M added in hydrogel preparation. Human mesenchymal stem cells (hMSC) were cultured on PE-PAAm hydrogels. Change in the hydrogel elasticity or the cell morphology and the gene expression of Runx2, which is one of the important osteogenic differentiation factors, was evaluated in the presence or absence of ALP. We examine the effect of hydrogel preparation conditions on the ALP-induced change in elasticity.

2. Materials and methods

2.1. Materials

Acid phosphoxy ethyl methacrylate (Phosmer™M) was obtained from Uni-Chemical Inc., Nara, Japan. ALPs derived from calf intestine (CIP) and bacteria (BAP) were purchased from Wako Pure Chemical Inc., Osaka, Japan. $Na^{125}I$ (740 MBq/ml in 0.1 N NaOH aqueous solution) was purchased from NEN Research Products, Perkin Elmer, Inc., Waltham, MA. Other chemicals were obtained from Nacalai tesque Inc., Kyoto, Japan, and used without further purification.

Native collagen type I was isolated from Wistar rats' tails [26]. Briefly, 600 mg of tendons dissected from rat tails was placed on a culture dish and exposed to UV light at 365 nm overnight. The fiber-like materials were suspended in 200 ml of sterile 0.1% acetic acid and stirred at 4 °C for 48–96 h. The resulting solution was left without agitation for 24 h to allow the undissolved materials to naturally sediment, and the supernatant was obtained as the collagen solution.

2.2. Radioiodination of collagen type I

Collagen type I were radioiodinated by the conventional chloramine T method [27]. Briefly, 5 μl of $Na^{125}I$ (740 MBq/ml in 0.1 N NaOH aqueous solution) was added to 30 μl of an aqueous solution of collagen type I (1 mg/ml). Then, 0.2 mg/ml chloramine T in 0.5 M potassium phosphate-buffered solution (pH 7.5) containing 0.5 M NaCl (100 μl) was added to the solution mixture. After agitation at room temperature for 2 min, 100 μl of phosphate-buffered saline solution (PBS, pH 7.4) containing 0.4 mg of sodium metabisulfate was added to the reaction mixture to stop radioiodination. The reaction mixture was passed through PD-10 column (GE Healthcare UK Ltd., UK) to remove the uncoupled ^{125}I molecules from the ^{125}I-labeled protein. The protein amount in the ^{125}I-labeled protein solution was quantified by using Micro BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). The radioactivity of ^{125}I-labeled protein was measured on the gamma counter (ARC-360, Aloka Co., Ltd., Japan) to calculate the specific radioactivity of the ^{125}I-labeled protein.

2.3. Preparation of PE-PAAm hydrogels containing phosphate groups

Acrylamide (AAm), *N,N′*-methylenebisacrylamide (BIS), and Phosmer™M were co-polymerized to prepare hydrogels containing phosphate groups, in different conditions (Table 1). Briefly, AAm and Phosmer™M (10 and 20 mol%) were dissolved in 25 v/v% acetic acid to give the total monomer concentration 250 mmol. BIS at concentrations ranging from 0.0065 to 0.65 mmol and potassium peroxodisulfate (KPS, 10 mg) were added to the mixed monomer solution, and then the solution weight was adjusted to 10 g by 25 v/v% acetic acid. The solution was poured into a space between 2 glass plates with a gap of 300 μm (8.5 × 9.5 cm), followed by copolymerization at 70 °C for 1 h. The resulting PE-PAAm hydrogels were washed with PBS to remove unreacted reagents. After an equilibrium swelling with PBS, the PE-PAAm hydrogels were punched out to prepare disks with a diameter of 14 mm. The PE-PAAm hydrogels were named as $Gel_{Px,By}$, where x is the molar concentration of Phosmer™M and y is the weight concentration of BIS.

2.4. Evaluation of swelling ratio of PE-PAAm hydrogels

As a control, PAAm hydrogels were prepared by the polymerization of AAm monomer (250 mmol), BIS (0.0065, 0.065, 0.325, and 0.65 mmol), and KPS (10 mg) solution without Phosmer™M in the mold of glass plates described above. Similarly, the PE-PAAm and PAAm hydrogels were punched out to prepare the 14 mm-diameter disks. After swelling in double-distilled water (DDW), HCl, and NaOH aqueous solution at different pH values, or PBS for 1, 3, 6, 12, 24, 48, and 72 h, the diameter of disks was measured. The swelling ratio was calculated by D_t/D_0, where D_t and D_0 (=14 mm) are the diameter (mm) of PE-PAAm and PAAm hydrogels at time t and time 0, respectively. Experiments were performed for 3 specimens independently for each sample.