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The synergetic effect of hydrogel stiffness and growth factor on osteogenic differentiation

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ABSTRACT

Cells respond to various chemical signals as well as environmental aspects of the extracellular matrix (ECM) that may alter cellular structures and functions. Hence, better understanding of the mechanical stimuli of the matrix is essential for creating an adjuvant material that mimics the physiological environment to support cell growth and differentiation, and control the release of the growth factor. In this study, we utilized the property of transglutaminase cross-linked gelatin (TG-Gel), where modification of the mechanical properties of TG-Gel can be easily achieved by tuning the concentration of gelatin. Modifying one or more of the material parameters will result in changes of the cellular responses, including different phenotype-specific gene expressions and functional differentiations. In this study, stiffer TG-Gels itself facilitated focal contact formation and osteogenic differentiation while soft TG-Gel promoted cell proliferation. We also evaluated the interactions between a stimulating factor (i.e. BMP-2) and matrix rigidity on osteogenesis both *in vitro* and *in vivo*. The results presented in this study suggest that the interactions of chemical and physical factors in ECM scaffolds may work synergistically to enhance bone regeneration.

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1. Introduction

Natural bone development reflects the significant role of physical stimuli in bone regeneration, which involves continuous turnover of bone extracellular matrix (ECM) and mineralization through bone resorption and formation. Mechanical loading during normal activities such as climbing and walking helps to enhance bone formation and directs the newly formed cells along the local loading direction. Yet, reduced loading during long-term immobilization or microgravity results in bone loss [1]. The healing process of bone fracture due to aging or accidental incidence also demonstrates the importance of mechanical factors, as the bone is subjected to mechanical regulation during regeneration from soft tissues. A stiff fixation minimizes interfragmentary movement, which results in limited callus formation. On the other hand, interfragmentary micromotion during flexible fixation stimulates callus formation and improves the repair process, whereas unstable

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http://dx.doi.org/10.1016/j.biomaterials.2014.02.040 0142-9612/© 2014 Elsevier Ltd. All rights reserved. fixation prevents the fracture from healing [2–4]. The mechanical properties of fixation influence the healing progression in a direct (intramembraneous) and/or an enchondral pattern that aids the process of ossification [5]. Thus, the mechanical signals are as important as chemical signals in the regulation of bone development, remodeling, and regeneration.

Two dimensional (2-D) in vitro studies have shown that the regulation of important cellular processes, such as proliferation, differentiation, and apoptosis, is controlled by the mechanical properties and geometry of the cells and their surrounding environment [6–11]. Apart from cellular processes, the microenvironment may control the cell shape and cytoskeletal tension, and may influence cell-matrix and cell-cell interactions [7,12]. Although 2-D in vitro studies have identified several important mechanical cues that can manipulate cell fate [12,13], cells present on a coated 2-D surface might not truly reflect their characteristics in a 3-D environment. At the cellular scale, the surrounding fluid and the ECM exert stresses on cells, and the cells may respond to the applied forces through integrin bindings in an omnidirectional manner [14–19]. The number of integrin-ligand bonds formed by a cell with its surroundings, dictates the extent of cell-matrix interactions [20], the ease with which they migrate through it, and the degree of generated intracellular tension [21–24], which are crucial factors







during tissue remodeling, morphogenesis, and differentiation, and normal physiological functioning. Since 2-D cell-matrix interactions are bidirectional, and 3-D are omnidirectional, it might not be suitable to extrapolate the effects of biological signaling due to cell-matrix interactions from 2-D experiments [25]. In addition, the variation of mechanical properties of ECM may lead to concentration gradients existing within the 3-D physiological structure, and this phenomenon cannot be achieved in 2-D *in vitro* studies. The differences in rigidity profiles can potentially affect the behaviors of cells in the center of the matrix different from the behaviors of those close to the periphery, because of the dissimilar local concentrations [26]. Therefore, cell responses to the substrate rigidity should be studied or re-examined in a physiologically relevant 3-D microenvironment.

One of the major challenges in the 3-D study of cell responses to substrate rigidity is the lack of suitable substrates. Even though some researchers [27,28] may have identified correlations between the matrix rigidity and cell phenotype in 3-D, many available materials are not suitable for 3-D studies because of the rigidity. A soft material cannot be assembled into a 3-D structure that can support cell survival throughout a long-term study, and a rigid material limits the cell survival and restrains the variation of substrate rigidity. For instance, by using thixotropic polyethylene glycol-silica gel, Pek et al. [27] showed that cell differentiation can be controlled by modulating the matrix stiffness through variations in the concentrations of the nanoparticles. Though physically crosslinked silicate polymer hydrogels have shown attractive biological properties, their applications as scaffold structures for bone regeneration are limited because of their insufficient mechanical properties [29,30]. On the contrary, silk fibroin protein is rather stable and mechanically robust, but the utilization of silk is restrained from elastomeric biomaterial applications because of its inherent tendency to form stiff materials (>1 MPa) as a result of the beta sheet crystal formation [13]. Hence, the development of a 3-D material that can mimic the physiological ECM allows the investigation of cell responses to substrate mechanics, which are independent of both biochemical and transport properties would be a major breakthrough in tissue engineering.

Injectable transglutaminase cross-linked gelatin (TG-Gel) may be one of the ideal alternative materials owing to its mechanical strength required for supporting cell adhesion, survival, and organization during the regeneration process without compromising on the bioactive substances [31]. The matrix stiffness of TG-Gel can be conveniently tuned by controlling the concentration of gelatin. Hence, TG-Gel was adopted in our 3-D study to examine the effects of rigidity on precursor cells. Moreover, chemical milieus are important to the mimic, as the bone formation, maintenance, and regeneration constitute a complex process involving the interactions of many cellular elements in vivo, including growth factors, hormones, cytokines, and ECM components [32]. However, the effects of mechanical changes and their interactions with the growth factors have yet to be adequately explored, and the understanding of their influences may provide new insights into the processes of development, disease, and material-cell-based regeneration. Among the osteoinductive growth factors, BMP-2 has been found to be essential for the initialization of bone regeneration [33]. Therefore, we investigated the effects of chemical stimulating factor (i.e. BMP-2) and matrix rigidity on osteogensis in both in vitro and in vivo models.

2. Methods and materials

2.1. Cell culture

Mouse C2C12 myoblast cells (ATCC, American Type Culture Collection, Manassas, VA) were initially cultured in a growth medium (GM) consisting of high glucose Dulbecco's modified Eagle medium (DMEM, Mediatech, VA) supplemented with 10% (v/v) fetal bovine serum (FBS, Lonza, MD) and 1% (v/v) penicillin-streptomycin (PS, Mediatech, VA) in a humidified atmosphere of 5% CO₂ at 37 °C. The medium was changed every 2–3 days. When the cells reached 70% confluence, they were subcultured using 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) solution.

2.2. Materials and reagents

Unless otherwise stated, all chemicals and reagents were purchased from Sigma–Aldrich (St. Louis, MO). The preparation of microbial transglutaminase (TG) and gelatin gel (Gel) were reported elsewhere [34,35]. Briefly, gelatin Type A 300 Bloom (Sigma Aldrich, MO) was dissolved and autoclaved in distilled water to generate 10% gelatin stock. Then, 10% gelatin stock solution was diluted with phosphate-buffered saline (PBS, 137 mm NaCl, 2.7 mm KCl, 10 mm Na₂HPO₄, and 1.8 mm KH₂PO₄) to produce gels with final concentrations of 3%, 6%, and 9%. TG from *Streptomyces mobaraense* was obtained from Ajinomoto (Tokyo, Japan), and was further purified with SP Sepharose Fast Flow beads (Sigma–Aldrich, MO). The activity of TG was titrated by the o-phthaldialdehyde (OPA) assay using casein as a substrate [36], and the protein concentration was tested by the Bradford method (Bio-Rad, Hercules, CA) [37] utilizing Bovine Serum Albumin (BSA) as a standard. Gel and TG were stored at 4 °C and -80 °C, respectively.

2.3. Mechanical testing

To quantify the rigidity and porosity of the substrate, transglutaminase crosslinked gelatin (TG-Gel) was created by mixing each 100 μ L of pre-heated gel solution with 7 μ L of TG. The TG-Gel was shaped into cylinders with the height to diameter ratio (L/D) of 1.25 to avoid mechanical failure modes, such as buckling. Furthermore, the TG-Gel containing trapped bubbles was discarded to avoid obtaining irregular mechanical properties.

2.3.1. Yield strength measurement

The Young's modulus or yield strength ($E_{modulus}$ [kPa]) of TG-Gel was measured using the unconfined compression test, where only lateral deformation of the TG-Gel was induced through compression between two plates. The force (F [N]) required to compress the TG-Gel was recorded at every 1 [cm] of deformation to deduce the stress (σ [kPa]) versus strain relationship. The strain (L^*) is defined by equation (1):

$$L^* = \frac{\Delta L}{L}$$
(1)

where ΔL denotes the amount of deformation and *L* represents the initial height of the TG-Gel. The Young's modulus of the TG-Gel was determined from the slope of the stress versus strain graph and by equation (2):

$$E = \frac{\sigma}{L^*}$$
(2)

2.3.2. Porosity measurement

The porosity of the fabricated 3-D scaffold was measured using liquid displacement method as described [38]. Briefly, the TG-Gel was immersed in ethanol of a known volume (V_1) contained in a graduated cylinder for 5 min. Ethanol was selected because it could readily penetrate the open pores of the TG-Gel without inducing swelling or shrinkage. The porosity of the scaffold (ε) is calculated by equation (3):

$$\varepsilon(\%) = \frac{V_1 - V_3}{V_2 - V_3} \times 100\%$$
(3)

where V_2 corresponds to the total volume of the ethanol-impregnated scaffold along with ethanol, and V_3 denotes the amount of residual ethanol in the cylinder after the removal of ethanol-impregnated TG-Gel.

2.4. In vitro 3-dimensional TG-Gel model

In vitro 3-D TG-Gel-Cell construct was created to evaluate the effects of matrix rigidity on cellular responses. A pre-myoblast C2C12 cell line was selected to study osteogenic differentiation because C2C12 is a pluripotent mesenchymal precursor cell that provides a model system to study the early stage of osteoblast differentiation during bone formation in muscular tissues and its ALP expression is close to the baseline level when the cells are cultured in 2-D environment [31]. The prepared gel was first heated and liquefied at 60 °C in water bath. Then, every 100 μ L of the gel solution was evenly mixed with C2C12s at a density of 2×10^6 cells/ml and $5 \,\mu$ L of TG to create the TG-Gel-Cell mixture. Afterwards, 20 μ L of the TG-Gel-Cell mixture was seeded into each well of 48-well suspension plates. After incubation for 1 h at 37 °C, 1 mL of growth medium (GM) was added into each well containing the solidified gel through TG chemical cross-linking. The GM was supplemented with 30 ng/ml BMP-2 (R&D Systems, Minneapolis, MN) to determine the effects of BMP-2 on osteo-genesis with the aid of TG-Gel with various rigidities.

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