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The differentiation of mesenchymal stem cells by mechanical stress or/and co-culture system

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Abstract

Differentiation of mesenchymal stem cells (MSCs) into anterior cruciate ligament (ACL) cells is regulated by many factors. Mechanical stress affects the healing and remodeling process of ACL after surgery in important ways. Besides, co-culture system had also showed the promise to induce MSCs toward different kinds of cells on current research. The purpose of this study was to investigate the gene expression of ACL cells' major extracellular matrix (ECM) component molecules of MSCs under three induction groups. In addition, to follow our previous study, cell electrophoresis technique and mRNA level gene expression of MSC protein were also used to analyze the differentiation of MSCs. The results reveal that specific regulatory signals which released from ACL cells appear to be responsible for supporting the selective differentiation toward ligament cells in co-culture system and mechanical stress promotes the secretion of key ligament ECM components. Therefore, the combined regulation could assist the development of healing and remolding of ACL tissue engineering. Furthermore, this study also verifies that cell electrophoresis could be used in investigation of cell differentiation. Importantly, analysis of the data suggests the feasibility of utilizing MSCs in clinical applications for repairing or regenerating ACL tissue.

Keywords: Mesenchymal stem cells (MSCs); Anterior cruciate ligament cells (ACL cells); Mechanical stress; Co-culture system

The anterior cruciate ligament (ACL), an intraarticular ligament of the knee, is important for knee stabilization. Unfortunately, it is also the foremost vulnerable ligament [1]. Due to poor vascularization, the ACL has inferior healing capability and is usually replaced after significant damage has occurred [2]. Three options have been utilized for the repair or replacement of damaged ligaments using biological substitutes: autografts, allografts, and xenografts, but are not ideally suited for this purpose [3–6]. In addition, a variety of synthetic materials have been used for ligament replacement, but with limited success [7]. Since currently available replacements have a host of limitations, this has prompted the search for tissue-engineered solutions for ACL repair.

Mesenchymal stem cells (MSCs), precursors of mesenchymal tissues, can be derived from bone marrow stroma and differentiate into multiple non-hematopoietic cell lineages such as chondrocytes, osteoblasts, adipocytes, and myoblasts [8]. Even in older individuals, bone marrow stroma is relatively easily harvested. Growth factors, hormones, and other regulatory molecules are traditionally required in tissue engineering studies to direct the differentiation of stem cells along specific lineages. Previous studies have also shown that physical factors, including loading [9], electromagnetic fields [10], and ultrasound [11], play an important role in regulating the function of MSCs. Therefore, the regulatory mechanism of MSCs differentiation is an important issue in the development of ACL tissue engineering.

Mechanical stress is known to be one of the important factors in the regulation of ligament and tendon remodeling

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[12–14]. The effects of mechanical stress on cells are dependent on the magnitude [15,16], duration [17], and frequency [17] of mechanical stress. Breen reported that cyclic strain results in an increase in type I collagen expression in human pulmonary fibroblasts [12]. ACL cells also increased type I and type III collagen mRNA expression after cyclic stretching (10%, 0.16 Hz) [13]. Many papers have been published on the effects of mechanical stress affecting ACL cells [13,18]; however, little is known about the effects of mechanical stress on differentiation of MSCs.

In addition, Lange et al. revealed that when co-cultured with liver cells, MSCs have the potential to differentiate toward hepatocytic cells in vitro [19]. Ball et al. also revealed that direct co-culture of MSCs with fibroblasts leads to the differentiation of MSCs into myofibroblast-like cells [20]. Since different cells will release regulatory factors which could direct affect the differentiation of MSCs, the influence of the differentiation of MSCs co-culture with ACL cells was also estimated in this study.

The purpose of this study was to investigate the mRNA expression of ACL cells' major extracellular matrix (ECM) component molecules of MSCs under three induction groups. In addition, our previous paper reveals that most of the surface antigens and mRNA expression of ACL cells were similar with that of MSCs, besides the mRNA expression of MSC protein and the difference of cell electrophoretic mobility (EPM) [21]. Hence, the mRNA of MSC protein was also used in this study to assist the investigation of induction of MSCs in three groups. Furthermore, cell electrophoresis, a useful research tool which can assist cell identification was successfully used to discriminate MSCs and ACL cells in our previous study [21], this study also try to use cell electrophoresis to observe the differentiation of MSCs.

Materials and methods

Isolation and growth of MSCs. To isolate human MSCs, bone marrow aspirates of 10–20 ml were taken from the iliac crest of patients ranging in age from 40 to 60 years old. Institutional Review Board approval was obtained for the use of the human bone marrow. Nucleated cells were isolated with a density gradient [Ficoll/paque (Pharmacia)] and resuspended in complete culture medium. The culture medium used was Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Gibco-RBL Life Technologies, Paisley, UK) and antibiotic/antimycotic (penicillin G sodium 100 U/ml, streptomycin 100 μg/ml, amphotericin B 0.25 μg/ml, Gibco-BRL Life Technologies, Paisley, UK). All of the cells were plated in 15 ml of medium in a 75-cm^2 culture flask (Corning, USA) and maintained in a humidified atmosphere with 5% CO_2 at 37 °C. After 24 h, nonadherent cells were discarded, and adherent cells were thoroughly washed twice with PBS. The culture medium was changed twice a week, during which nonadherent cells were discarded. After the culture reached confluence, the cells were harvested with 0.2% trypsin and 1 mM EDTA for 5 min at 37 °C for subsequent characterization.

Isolation and growth of ACL cells. To isolate human ACL cells, tissue pieces were taken from the ACL reconstruction patients ranging in age from 40 to 60 years old. Patients enrolled in this research have signed an Informed Consent, and Institutional Review Board approval was obtained for the use of the ACL tissue pieces. Samples of ACL were obtained, cut into 1–2 mm pieces, and placed in polystyrene tissue-culture plates (Cony, USA). The culture medium used in the procedure was the same with the MSCs, as described previously. After approximately 21 days, the outgrowth cells were removed with 0.2% trypsin-EDTA for subculture. For this study, the fifth passage ACL cells were used for subsequent analysis.

Co-culture protocol. The ACL cells were seeded in the transwell insert membrane (with pores of 0.4 μm in diameter, Corning), and human MSCs were cultured in the lower chamber of the co-culture system for 7 days. The ratio of cell numbers of MSCs to ACL cells was controlled at 1:1.

Mechanical stretch application. After seeding on 6-welled tissue culture polystyrene plates (Costar, USA) for 7 days, the MSCs were detached by treatment with 10% trypsin-EDTA (Sigma), replaced on six-well, flexible-experiment, the bottomed plates (type I collagen coated, Flex I; Flexcell International, Mckeesport, PA, USA) at a density of 1×10^5 cells/well. In this mechanical loading was applied on MSCs by a Flexercell Tension Plus system 3000 (Flexcell International, Mckeesport, PA, USA). This system is a computer-driven instrument that simulates biological strain conditions using vacuum pressure to deform cells cultured on flexible, matrix-bonded growth surfaces of BioFlexRseries culture plates. MSCs were subjected to mechanical stress at a rate of 1 Hz with 10% elongation for 2 days [22]. Cell morphology before and after adding mechanical stress was viewed with a photomicroscope (Zeiss LAMBDA 10-2, Germany).

Real-time quantitative reverse transciptase polymerase chain reaction (RT-PCR). Total cellular RNA was extracted from MSCs using RNA extraction kit (Mix total RNA extraction kit, Bio-Rad, USA). cDNA synthesis was prepared from RNA isolation from these cells (Bio-Rad, USA). We performed and monitored real-time quantitative RT-PCRs by using an ABI Prism 7900 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA). The level of expression of each target gene was normalized to the reference gene glyceraldehyde phosphate dehydrogenase (GAPDH) and the resulting data were expressed as a ratio of the control. Data were collected with instrument spectral compensations by the Applied biosystems SDS 2.1 software, and analyzed using the threshold cycle number (C_t) relative quantification method. The type I collagen, type III collagen, tenascin C, and MSC protein genes were detected by the Quantitative *Taq*Man RT-PCR with the pre-designed assays (Assay ID: Hs00164004_m1, Hs00164103_m1, Hs00233648_m1, and Hs00414261_m1, Applied Biosystems, CA, USA).

Assessment of cell electrophoretic mobility. Electrophoresis was used to investigate the surface charge properties of MSCs under three induction groups by measuring their EPM. Prior to the mobility measurement, the cells were cultured in a fresh medium mentioned above for 4 h and redistributed in the buffer solution-containing 10 mM Tris–HCl and 291 mM glucose (pH 7.3–7.4) at a concentration of 1×10^5/ml. The electrophoretic behavior of MSCs and ACL cells were observed using an inverted microscope attached with a CCD camera, as described previously [23]. Briefly, cell suspension was introduced into a rectangular glass electrophoresis chamber similar to that used by Mironov and Dolgaya [24]. Agar bridges (2% agar) were placed in the ends of the chamber and connected to the DC supply (200 V) with two compartments containing Pt electrodes in saturated KCl solution. The hole for introducing cell suspension was located in the upper side of the chamber and was close to the agar bridge. Current passing through the chamber ranged from 1 to 4 mA. All measurements were conducted at 22 ± 2 °C and their duration did not exceed 30 min, to avoid the temperature increase of the chamber because of the heating effect of the microscope illumination. The magnitude of the velocity u was measured by recording the moving distance of cells in 10 s. The EPM μ was calculated by $\mu = ugS/I$, where S is the cross-sectional area of the observation chamber, I is the current, and g is the conductivity of medium measured separately. Each reported mobility was based on 10 readings and expressed as mean ± SEM.

Results

Cell morphology and orientation

When the MSCs were subjected to 10% elongation for 48 h, changes were induced in their morphology and partic-

ularly in their orientation (Fig. 1b). When cyclic strain was not applied, the cells show no particular orientation (Fig. 1a). After mechanical stress was applied, the cells orient perpendicularly to the strain axis and cause MSCs to change their morphology to spindle shape and also change their orientation. Hayakawa et al. also speculated that the stress fibers stretching and subsequently became reoriented obliquely to the direction of stretching [25]. However, the mechanism of stretch-dependent orientation was unclear.

Expression of ACL-related genes

The mRNA expression of type I collagen, type III collagen, and tenascin-C, typical markers of ligament cells in three groups [26], were assessed by real-time quantitative RT-PCR (Fig. 2). Group I was the MSCs exposure to mechanical stress. Group II was the MSCs co-culture with ACL cells. Group III was the MSCs exposure to mechanical stress after co-culture with ACL cells. The resulting data were expressed as a ratio of the control. As shown in Fig. 2a, no significant increase of type I collagen mRNA was observed in group I. In addition, the ratios of type I collagen for group II and group III were 3.35 ± 0.16 and 8.185 ± 0.52, respectively. The results reveal that the level of type I collagen mRNA was found to be significant up-regulated in both group II and group III ($p < 0.05$). The mRNA expression of type III collagen under three induction groups are shown in Fig. 2b and the results reveal

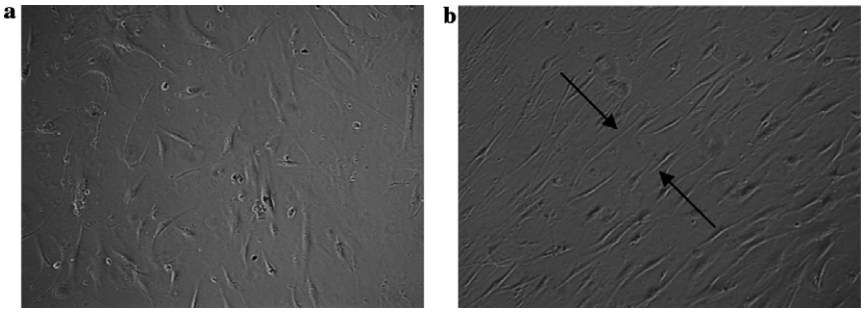

Fig. 1. Effects of mechanical stress on morphological change. Phase-contrast photomicrographs of MSCs before and after exposure to mechanical stress: (a) MSCs before the exposure to mechanical stress; (b) MSCs after the exposure to mechanical stress. The cells that were applied strain oriented perpendicularly to the strain axis. Arrow indicates direction of strain field. Original magnification, 100×.

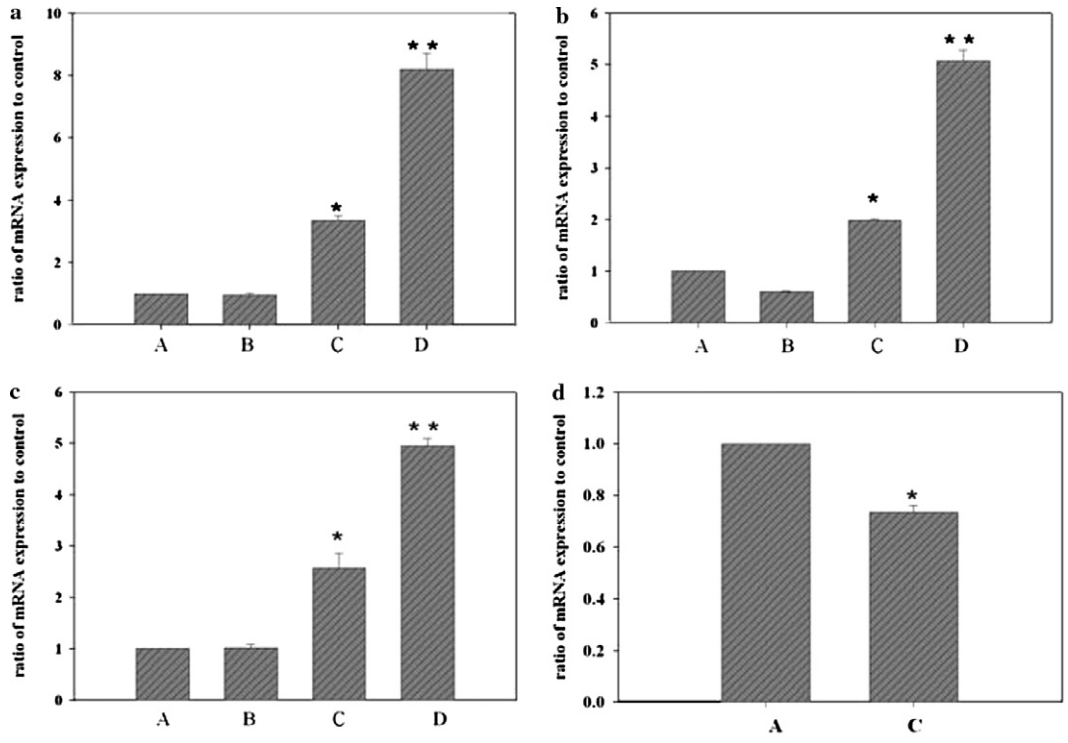

Fig. 2. mRNA expression of (a) collagen type I (b) collagen type III (c) tenascin-C (d) MSC protein under three induction groups. The resulting data were expressed as a ratio of the control. (A) Control; (B) MSCs after co-culture with ACL cells (group I); (C) MSCs exposure to mechanical stress (group II); (D) MSCs exposure to mechanical stress after co-culture with ACL cells (group III). Asterisks denotes significant differences ($^{*}p < 0.05$, $^{**}p < 0.01$) of formazan absorbance compared to the control, which was calculated by Student's *t*-test.

the level of type III collagen mRNA was also found to be up-regulated in group II and group III. Besides, significant increases were also observed for the mRNA expression of tenascin-C in group II and group III ($p < 0.05$), and without significant increase could be observed in group I (Fig. 2c).

In addition, MSC protein is suggested a specific gene of MSCs in our previous paper. To our knowledge, it is the first time to use the mRNA expression of MSC protein to discriminate MSCs and ACL cells and the result show that mRNA expression of MSC protein indeed can assist the discrimination of MSCs and ACL cells [21]. Therefore, MSC protein was also used for the application of observation the variation of MSCs during the three induction groups. The mRNA expression of MSC protein in group II is shown in Fig. 2d and significant downregulation of MSC protein expression in group II was observed ($p < 0.05$). In contrast, the MSC protein expression cannot be detected after 40 cycles in group I and group III.

Electrophoretic analysis

Since mRNA expression of MSC protein can assist the discrimination of MSCs and ACL cells, our previous study also reveal that it is not suggested to use the gene of MSC protein only to differentiate MSCs from other cells because MSCs themselves do not express a high level of this gene [21]. Therefore, a simple and cheap method, cell electrophoresis, was developed to assist in identification of MSCs differentiating into ACL cells and the value of the electrophoretic mobility (EPM) for human MSCs (-3.05 ± 0.16 μm cm/V s) is significantly more negative than that for human ACL cells (-2.05 ± 0.08 μm cm/V s) in the same electric field ($p < 0.001$) [21]. Electrophoresis, the movement of a charged entity in a response to an applied electric field, is proposed to investigate the differentiation of MSCs into ACL cells. Fig. 3 shows the EPM of MSCs under three induction groups and the EPM value of MSCs for group I is about

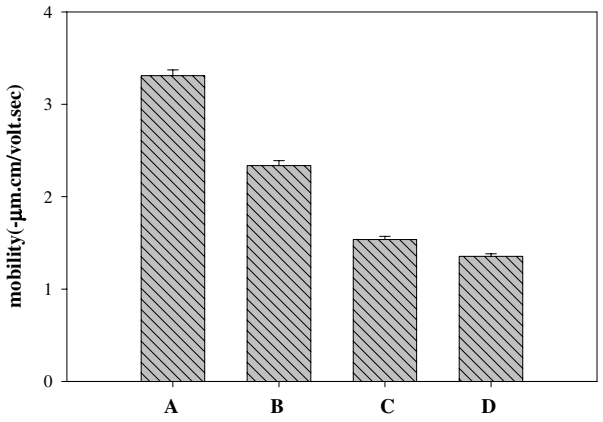

Fig. 3. The EPM of MSCs. (A) Control; (B) MSCs after co-culture with ACL cells (group I); (C) MSCs exposure to mechanical stress (group II); (D) MSCs exposure to mechanical stress after co-culture with ACL cells (group III).

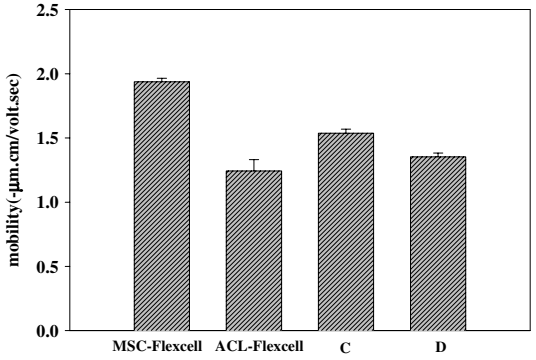

Fig. 4. The EPM of MSCs and ACL cells on Flexcell well. (C) MSCs exposure to mechanical stress (group II); (D) MSCs exposure to mechanical stress after co-culture with ACL cells (group III).

-2.34 ± 0.053 μm cm/V s, which is approach to the EPM value of ACL cells (-2.05 ± 0.08 μm cm/V s) [21].

However, the EPM values of MSCs under induction group II and group III are 1.54 ± 0.03 and 1.35 ± 0.03, respectively, which are lower than that of ACL cells (-2.05 ± 0.08 μm cm/V s). It is suggested that the reason is the cells under induction group II and group III have been changed from the normal tissue culture polystyrene plates to the flexible Flexcell under the exposure to mechanical stress. Our previous study reveals that although the precise mechanism by which the variation of electrophoretic mobility of cultured cells was unknown, the biomaterials could influence the cell mobility [23]. Therefore, for the sake of excluding the materials effect, the EPM of MSCs and ACL cells seeding on the Flexcell well are shown in Fig. 4 and the result reveals that both of the EPM of cells on group II and group III decreased in comparison with MSCs. In addition, the EPM of cells on group III is 1.35 ± 0.03, which is also approach to the EPM value of ACL cells on the Flexcell (1.24 ± 0.09 μm cm/V s).

Discussion

Our study shows mRNA expression of ACL cells' typical markers of MSCs with significant increase in group II and group III, especially in group III. Both of the cells under group II and group III were exposure to mechanical stress, therefore, it is suggested that mechanical stress appeared to promote the increase of mRNA expression of ECM gene of cells. It is reasonable to speculate that mechanical signals may trigger cell-surface stretch receptors and adhesion sites, resulting in cascades that involve activation of gene responsible for the synthesis and secretion of key ligament ECM components [26].

In addition, the mRNA of MSC protein cannot be detected after 40 cycles in group I and group III, besides, both of the EPM values of cells under induction group I and group III are approach to the EPM value of ACL cells. Since cell electrophoresis analysis reveals the net surface charge and overall surface membrane protein property, it

is suggested that the surface charge density was changed in cell differentiation and displayed the similar surface charge with the cells that induction wanted (ACL cells). An enormous amount of information is expressed on the cell membrane and the charged conditions on cell surface are of complicated nature. However, the cell surface proteins in the periphery depend on the gene products of intracellular metabolism [21]. Since both group I and group III undergo the process of co-culture with ACL cells, it is suggested that co-culture effect would induce the differentiation of MSCs. Because the culture medium was supplemented with serum capable of supporting the culture of any cell type, but not ligament-specific regulatory molecules, it is presumed that the specific regulatory signals released from ACL cells appear to be responsible for supporting the selective differentiation toward ligament cells in group I and group III.

In group II, although mechanical stress applied to MSCs result in significant increase in typical markers of ligament cells, including type I collagen, type III collagen, and tenascin-C, the mRNA of MSC protein also could be detected after the procedure of mechanical stress in comparison with co-culture effect. In addition, to combine with the EPM data, it is suggested that presumably the influence of mechanical stress may be via cell binding sites, leading to cell membrane protein change and result in change of cell mobility, however, the EPM of cells under induction group II is not approach the EPM of ACL cells. Therefore, it is suggested that these cells were promoted to secrete key ligament ECM components under the exposure of mechanical stress; however, they were not induced to differentiate into ACL cells.

In group III, mRNA of MSC protein cannot be detected and the EPM of cells is approach to the ACL cells. Besides, it is suggested that the MSCs have the tendency to differentiate cells with the characteristic of ACL-like after co-culture with ACL cells. Hence, after mechanical stress was applied, the secretion of key ligament extracellular matrix (ECM) components is enhanced and the increase level was higher than group II. The complex anatomy of the human ligaments is dependent on the interaction, orientation, and relationships between molecules, such as matrix proteins, and cells [27]. Therefore, ECM plays an important role in ligament tissue engineering. Since combined regulation (group III) display high secretion of ECM component, it is suggested that combined regulation could be used to induce MSCs differentiation and promote matrix remolding.

Although secretion of key ECM components and cell differentiation could be control in this study, mechanical stimulation enhance secretion of ECM components and co-culture effect induce cell differentiation for reasons that are not entirely clear. More detailed studies of cell receptors involved in transducing the mechanical signals and the regulatory factors released from ACL cells will be needed to refine discuss in the future for the ACL reconstruction and tissue engineering. Importantly, analysis of the data suggests the feasibility of utilizing MSCs in clinical applications for repairing or regenerating ACL tissue.

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