Effects of Silkworm Hemolymph and Cartilage-specific Extracellular Matrices on Chondrocytes and Periosteum-derived Progenitor Cells

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Abstract In order to enhance the repair of defects in articular cartilage via cell therapy with autologous chondrocytes, as well as with periosteum-derived progenitor cells (PDPCs), silkworm hemolymph (SH) and a variety of cartilage-specific extracellular matrices (ECMs) including type II collagen, proline, chondroitin 4-sulfate, and chondroitin 6-sulfate were assessed with regard to their efficacy as media supplements. SH, a known anti-apoptotic agent, was found to enhance cell growth, as was shown by the results of a 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay. According to the results of reverse transcriptase polymerase chain reaction (RT-PCR) analyses, the cartilage-specific ECMs were found to stimulate the expression of hyaline cartilage-specific genes, most notably type II collagen and Sox9, in monolayer cultures of PDPCs.

Keywords: chondrocytes, periosteum-derived progenitor, silkworm hemolymph, extracellular matrix, cartilage

INTRODUCTION

Articular cartilage has a fairly limited self-renewal proliferation property, as a result of its avascular structure. Therefore, numerous researchers have become interested in establishing methods by which cartilage can be repaired via cell therapy or *in vitro* tissue engineering [1]. However, large quantities of cells are generally required in order to make an artificial construct as similar as possible to actual tissue. Yet, it is rather difficult to cultivate sufficient autologous chondrocytes *in vitro*, as chondrocytes are dedifferentiated fibroblast-like cells, with relatively low proliferative potential during serial passages in monolayer cultures [2-6]. In order to acquire engineered cartilage and to secure sufficient amounts of cells for the treatment of defects in the articular cartilage, a great many studies have been conducted to enhance the isolation and cultivation of chondrogenic progenitor cells, such as mesenchymal stem cells and periosteum-derived progenitor cells (PDPCs), which are progenitor cells with chondrogenic potential [7,8]. In a recent study, we reported the isolation of human PDPCs, using immunophenotypes for chondrogenesis [9]. The redifferentiation of dedifferentiated cells by a variety of *in vitro* culture systems has also been the focus of recent research. In this case, the culture systems have classically been designed to resemble actual *in vivo* conditions, either by virtue of the construction of an actual dynamic three-dimensional environment [10-12] or by the induction of cartilage-specific combinations of environmental factors [2,13-16]. However, the requirements for redifferentiation have yet to be investigated for human chondrocytes expanded in the presence of different factors.

Silkworm hemolymph (SH) has been demonstrated to exert an inhibitory effect on apoptosis of human and mammalian cell cultures, as well as in insect cell cultures [18]. It has been determined that the 30 K protein group in the SH, which consists of five proteins, constitutes a primary factor with regard to this inhibitory effect on apoptosis. The SH-mediated regulation can augment cell longevity by minimizing the incidence of cell death [19,20]. SH was also known to enhance the production of recombinant protein [19].

Cells on their own basement ECMs tend to differentiate to a more significant degree than others, as the result of the activation of cell type-specific genes. Complex ECMs have been shown to promote tissue-specific differentiation of pluripotent embryonic stem cells depending on the type of matrix used. In particular, a cartilage extract harboring a variety of cartilage matrix components has been shown to promote chondrogenesis *in vivo* [17]. Other early studies have addressed the roles played by cartilage-specific ECMs secreted by chondrocytes, includeing type II collagen and glucosamine sulfates. However, these results have been generated either in three-dimensional culture systems or in *in vivo* systems. [2,14-16].

In this study, in order to obtain the desired engineered cartilage and to secure a sufficient quantity of host cells

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for the repair of defective cartilage, the effects of SH on the proliferation of chondrocytes and PDPCs were assessed. In addition, the effects of a variety of cartilage-specific ECMs, including proline, type II collagen, chondroitin 4-sulfate (C_4S), and chondroitin 6-sulfate (C_6S), on the expression of hyaline cartilage-specific genes, most notably type II collagen and Sox9 of dedifferentiated chondrocytes and PDPCs in a monolayer culture, were studied in order to create an environment similar to that of actual *in vivo* conditions.

MATERIALS AND METHODS

Primary Culture of Human Articular Chondrocytes

Human articular cartilage was minced and digested with 0.2% human type II collagenase (Washington Biochemical Co., NJ, USA) in Dulbecco's Modified Eagle's Medium-low glucose (DMEM) (Gibco BRL, MD, USA) for 16 to 20 h at 37°C. The digested solution was then centrifuged for 3 min at 1,200 rpm. The cell pellets were resuspended in DMEM containing 10% (v/v) fetal bovine serum (FBS) (Gibco BRL, MD, USA) and antibiotic-antimycotic solution and incubated at 37°C in an atmosphere containing 95% humidified air with 5% CO_2 on seeded culture plates.

Primary Culture and Isolation of PDPCs

The minced periosteum explants were placed on a 100-mm culture dish (Becton Dickinson Labware, NJ, USA) with the cambium side (bone surface) of the periosteum placed against the dish. The explants were cultured in DMEM containing 10% (v/v) FBS and antibiotic-antimycotic solution at 37°C in 95% humidified air and 5% CO_2 for one week. At the end of passage number 5, the PDPCs were isolated as described in our earlier study [9].

Cultivation with SH and Various ECMs

Passage number 5 of the chondrocyte culture and passage number 7 of the PDPC culture were treated with three concentrations of SH, 1, 3, and 10% (v/v), and compared via the MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide) assay method on days 1, 3, 5, 7, and 9. In addition, a variety of ECMs, such as proline (Sigma, 115 g/mL), type II collagen (Sigma, 30 g/mL), chondroitin 4-sulfate (C_4S) (Sigma, 80 g/mL), chondroitin 6-sulfate (C_6S) (Sigma, 80 g/mL), and fibronectin (Sigma, 12.5 g/mL) were tested and analyzed by the same method. The fibronectin was compared to another ECM supplement as a cartilage-non-specific ECM and culture with 10% FBS was used as a negative control.

RT-PCR

Total RNA was extracted with TRIzol reagent (Invitrogen, CA, USA). Total RNA was subsequently isolated in accordance with the manufacturer's instructions [21].

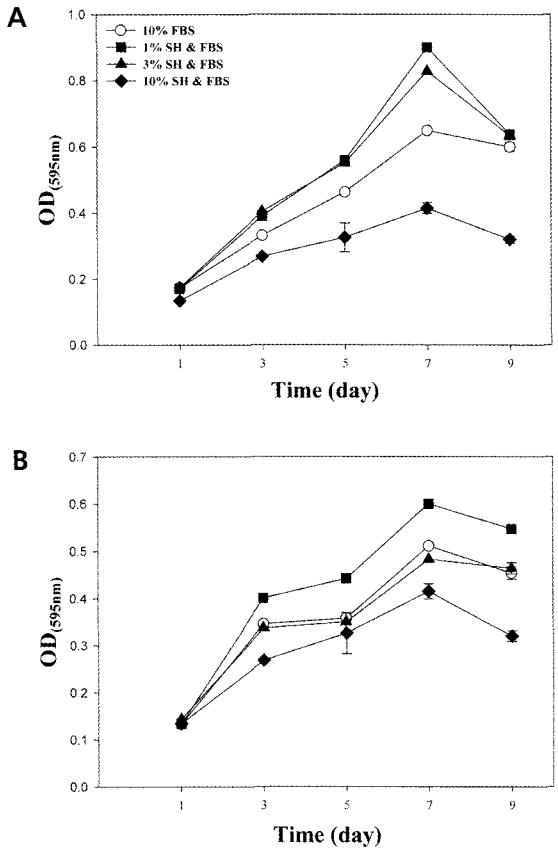

Fig. 1. Effects of SH on the growth rates of (A) chondrocytes and (B) PDPCs.

Complementary DNA was synthesized from a 1 μg total RNA per sample using AMV reverse transcriptase. The reaction was conducted in a final volume of 20 L, with 5 mM $MgCl_2$, 1 mM each deoxynucleotide, 1.6 g oligo (dT), 50 U RNase inhibitor, and 20 U AMV reverse-transcriptase in 50 mM KCl and 10 mM Tris/HCl at a pH of 8.3. The mixture was incubated for 10 min at 25°C, 60 min at 42°C, then heated to 99°C for 5 min and flash-cooled to 4°C. PCR amplifications for type II collagen, Sox9, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were conducted for 30 cycles of 30 sec/95°C denaturation, 30 sec/61°C annealing, and 30 sec/72°C extension, using recombinant Taq DNA polymerase (Takara Korea Biomedical, Seoul, Korea).

The specific primers used were as follows: for type II collagen, forward primer (CTGGCTCCCAACACTGCCAACGTC), reverse primer (TCCTTTGGGTTTGCAACGGATTGT); for human Sox9, forward primer (GGTTGTTGGAGCTTTCCTCA), reverse primer (TAGCC TCCCTCACTCCAAGA); and for GAPDH, forward primer (GCTCTCCAGAACATCATCCCTGCC), reverse primer (CGTTGTCATACCAGGAAATGAGCTT) [22].

RESULTS AND DISCUSSION

Silkworm hemolymph (SH) was administered at three

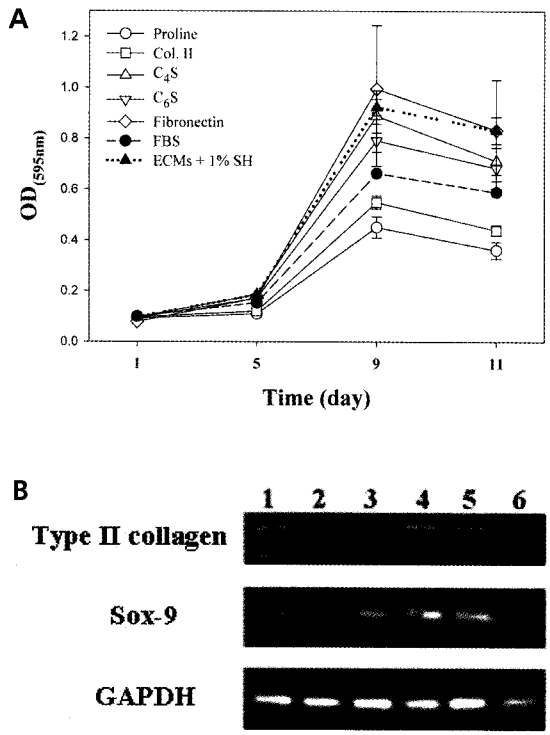

Fig. 2. Effects of various ECMs on (A) growth rate and (B) chondrocyte-specific gene expression in chondrocytes. 1, type II collagen; 2, fibronectin; 3, C_4S; 4, C_6S; 5, proline; 6, 10% FBS.

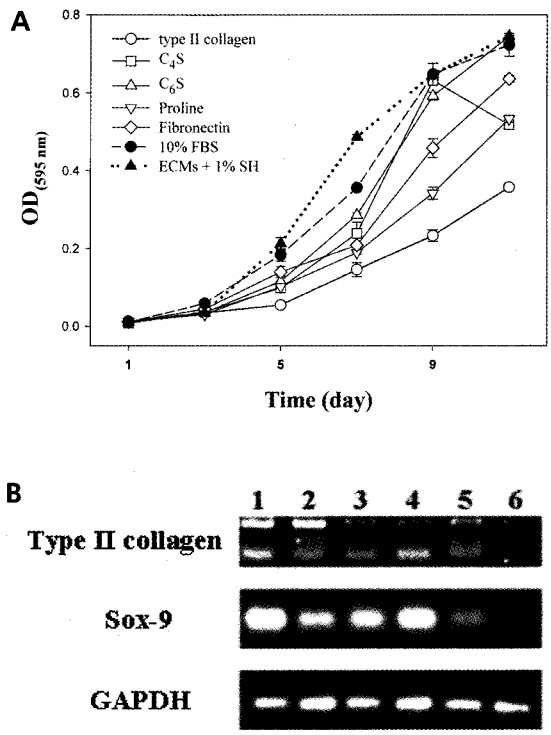

Fig. 3. Effects of various ECMs on (A) growth rate and (B) chondrocyte-specific gene expression in PDPCs. 1, type II collagen; 2, proline; 3, C_4S; 4, C_6S; 5, fibronectin; 6, 10% FBS.

concentrations, 1, 3, and 10% (v/v) to the cultures of chondrocytes and PDPCs (Fig. 1). The 1% SH concentration resulted in the most profound cell growth, and the largest cell mass was achieved at day 7 in each of the cell lines. Compared to the negative control culture in the chondrocytes, a 1.55-fold growth promotion was observed with SH. The shape of the cells did not change significantly in the presence of SH. The addition of various ECMs, including proline, type II collagen, C_4S, C_6S, and fibronectin to the chondrocyte cultures resulted in diverse growth results, as shown in Fig. 2A. Although the growth rates of the chondrocyte cultures supplied with type II collagen and proline were lower than that of the control, a faster growth rate and higher cell mass than those seen in the negative control were observed in the culture treated with 1% SH, and all cartilage-specific ECMs, which included proline, type II collagen, C_4S, and C_6S. Furthermore, the expression of the differentiated chondrocyte-specific genes, Sox9, and type II collagen, was clearly detected in the monolayer cultures containing proline, type II collagen, C_4S, and C_6S. In the culture to which fibronectin, a cartilage-nonspecific ECM, had been added, Sox9 and type II collagen genes were not detected (Fig. 2B).

The PDPCs were isolated from the periosteum and characterized by flow-cytometric analysis with several antibodies for known surface markers for chondrogenic progenitor cells and human mesenchymal progenitor cells, namely, CD9, CD90, CD105, SH2, SH3, and SH4 [9]. The growth rate of the PDPC cultures supplied with each of the individual ECMs were all lower than those of the control. However, the addition of 1% SH and various ECMs, including proline, type II collagen, C_4S, and C_6S to the PDPC cultures caused growth in excess of that seen in the negative control, as is shown in Fig. 3A. In addition, the expression of Sox9 and type II collagen genes was verified in the monolayer cultures of PDPCs to which proline, type II collagen, C_4S, and C_6S had been added, a result comparable to that seen with the redifferentiated chondrocytes (Fig. 3B).

Ample amounts of both chondrocytes and PDPCs were acquired by the addition of SH to the culture medium. In addition, cartilage-specific ECMs caused PDPCs, the chondrogenic progenitor cells, as well as the dedifferentiated chondrocytes, to express the cartilage-specific genes, Sox9, and type II collagen, in the monolayer culture. These results indicated that the expansion and chondrogenesis of the PDPCs and the dedifferentiated chondrocytes can occur simultaneously. As a result, this should reduce the time required for the construction of tissue engineered cartilage using a three-dimensional culture with scaffold *in vitro*, or via the direct injection of the cells into the human body.

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