

Development of Serum-Free Media for Primary Culture of Human Articular Chondrocytes

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Abstract Human articular chondrocytes (HAC) were cultivated as a monolayer in a serum-free medium for primary culture (SFM-P). An optimized SFM-P provides 95% proliferation rate of that obtainable from primary and secondary chondrocyte cultures grown in a control medium with serum. The gradual decrease in the amounts of synthesized glycosaminoglycan and type II collagen was improved by coating the culture dishes with type IV collagen and fibronectin. A significant improvement in the expression of type II collagen and aggrecan mRNA could be achieved. In addition, the monolayer cultures showed better synthesis of the extracellular matrices than alginate-bead cultures in SFM-P.

Key words: Chondrocytes, primary culture, serum-free medium

Several unsuccessful attempts have previously been made to treat injured joints; however, systematic evaluation of the cartilage repair techniques has not yet been reported. Current therapies for treating cartilage defects include shaving the articular surface, perforating the underlying subchondral bone, periosteal and perichondral grafts, osteotomy, and artificial joint replacement. Unfortunately, these rigorous techniques do not restore the native structure of cartilage [16]. Under such a scenario, the recently developed autologous chondrocyte transplantation (ACT), in combination with a periosteal graft, could be a potential relief measure. In the ACT cartilage repair technique, a serum-containing medium is generally used for the *in vitro* cultivation of the patient's chondrocytes [4]. However, the use of a serum-free medium for primary culture (SFM-P) is recommended to avoid the problems associated with viral infection and

for economical efficiency. Moreover, articular cartilage *in vivo* is an avascular, aneural connective tissue composed of chondrocytes and an abundant extracellular matrix (ECM) and does not contact directly with blood. Therefore, in order to overcome the unidentified dilemma caused by cartilage growth and transplants, it is highly preferable for the cells to be grown without serum *ex vivo*.

Articular chondrocytes grown in a monolayer culture are profoundly affected by the type and concentration of serum used [5]. For this reason, attempts to develop serum-free media for cartilage date back to the 1960s [3]. Although there have been a lot of research on defined medium, the use of serum-containing medium was inevitable for the isolation and primary culture of chondrocytes to support cell adhesion. For the stimulation of cell adhesion, cell culture dishes can be coated with collagen, fibronectin, poly-L-lysine, and so on, if serum is not used.

Recently, another approach for the treatment of cartilage injuries exploits the possibility of using constructs consisting of chondrocytes cultured on suitable scaffolds made of biocompatible and biodegradable materials. In this case, it is also necessary to cultivate the chondrocytes with SFM-P. Subsequently, three dimensional cultures of chondrocytes in SFM-P may be the new articular cartilage repair technique [11].

In this study, the possibility of using a serum-free medium for the large-scale proliferation of human articular chondrocytes was examined. A serum-free medium was developed by supplementing the basal medium with epidermal growth factor (EGF) in a monolayer culture and transforming growth factor- β 1 (TGF- β 1) in three dimensional cultures. The serum-free medium was used from the beginning of chondrocyte isolation, and the cells were subcultured under serum-free conditions. The growth of chondrocytes and synthesis of ECM were compared with those of the cells grown in the serum-containing medium.

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MATERIALS AND METHODS

Isolation of Chondrocytes

Primary cultures of human chondrocytes were prepared from articular cartilage obtained from joint replacement surgery. Briefly, the cartilage was cut into small slices, incubated, and washed in medium before slices were digested with 0.2% collagenase (Worthington Biochemical Co., Lakewood, NJ, U.S.A.) for 12–24 h at 37°C. After incubation, the slices of cartilage were almost completely digested. Undigested fragments were removed by passing the solution through a nylon mesh. Isolated cells were washed 3 times by centrifugation and were resuspended in phosphate buffered saline (PBS). The cell number and viability were measured in a hemocytometer after staining with trypan blue.

Primary Cell Culture Condition

The SFM-P contained ITSE (10 µg/ml insulin, 5.5 µg/ml transferrin, 5 µg/ml selenium, and 2 µg/ml ethanolamine), 50 µg/ml ascorbic acid-2-phosphate, 100 µg/ml kanamycin, 200 µg/ml insulin, and 10 ng/ml EGF. The cells were incubated at 37°C with 5% CO₂ in air. The media were replenished twice a week by 50% (v/v). To enhance the adhesiveness during primary culture of chondrocytes, the culture dishes were coated with a mixture of 5 µg/cm² fibronectin (BD Bioscience, Los Angeles, CA, U.S.A.), 2.5 µg/cm² poly-L-lysine (Sigma, St. Louis, MO, U.S.A.), and 25 mg/cm² type IV collagen (BD Bioscience) for 1 h at 37°C or overnight at 4°C, and then washed with culture medium before use. Type I collagen-coated dishes (BD Bioscience) were used.

Alginate Bead Culture

Low viscosity alginate (2%, Sigma) was dissolved in PBS and autoclaved at 115°C for 20 min [19]. Pelleted chondrocytes were suspended in alginate solution (2×10⁶ cells/ml), and the alginate-chondrocyte suspension was allowed to gel by carefully adding a small amount of 102 mM CaCl₂. Gelation occurred instantly. After removal of the remaining CaCl₂, the beads were washed 4 times with 0.15 M NaCl and then twice with culture medium.

Growth Rate and Viability

Growth was analyzed by counting the number of cells with a hemocytometer during the primary and secondary cultures, following trypsinization in monolayer cultures. Alginate was dissolved by adding 1.0 ml of 55 mM sodium citrate in 0.15 M NaCl. The recovered cells were counted using a hemocytometer, and their viability was estimated with the trypan blue exclusion method [14].

Assays of Glycosaminoglycans (GAG)

Samples were tested for the presence of proteoglycan using the Blyscan glycosaminoglycan assay (Biocolor, Belfast,

Ireland). This assay is based on the specific binding of the cationic dye, 1,9-dimethylmethylene blue, to the sulfated glycosaminoglycan chains of proteoglycans. The procedure was carried out according to the manufacturer's instructions. Samples (500 µl) were added to 500 µl of Blyscan dye reagent and mixed for 30 min at room temperature. The glycosaminoglycan-dye complex was recovered by centrifugation and the pellets were washed and resuspended in 1 ml of dissociation buffer. Absorbance at 656 nm was measured.

RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.) [13]. Cells were lysed by the addition of 1 ml of TRIzol reagent. The total RNA was subsequently isolated according to the manufacturer's instructions. Complementary DNA was synthesized from 1 µg of total RNA per sample using AMV reverse transcriptase (1st Strand cDNA Synthesis Kit, Roche Molecular System, Inc., Mannheim, Germany). The reaction was carried out in a final volume of 20 µl, with 5 mM MgCl₂, 1 mM each of deoxynucleotides, 1.6 µg of oligo-(dT), 50 U/µl RNase inhibitor, and 20 U/µl AMV reverse transcriptase in 50 mM KCl and 10 mM Tris-HCl at pH 8.3. The mixture was incubated at 25°C for 10 min, 42°C for 60 min, heated to 99°C for 5 min, and flash-cooled to 4°C. PCR amplifications for types I and II collagens, aggrecan, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were performed for 30 cycles of 30 sec/95°C denaturation, 30 sec/58°C annealing, and 2 min/72°C extension, using recombinant Taq DNA polymerase (Takara, Tokyo, Japan).

The specific primers used: for type I collagen, forward primer (CCCAAGGACAAGAGGCAT), reverse primer (GCAGTGGTAGGTGATGTTCTG); for type II collagen, forward primer (CTGGCTCCCAACACTGCCAACGTC), reverse primer (TCCTTTGGGTTTGCAACGGATTGT); for aggrecan, forward primer (TGAGGAGGGCTGGAA-CAAGTACC), reverse primer (GGAGGTGGTAATTGC-AGGG AACA); and for GAPDH, forward primer (GCTCTCCAGAACATCATCCCTGCC), reverse primer (CGTTGTCATACCAGGAAATGAGCTT). PCR products were visualized on a 2% agarose gel by ethidium bromide stain and compared against a 1 kb DNA ladder (Promega, Madison, WI, U.S.A.) used as a molecular weight marker.

Western Blot Analysis

Whole cell lysates were prepared by extracting proteins in lysis buffer [50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% (v/v) Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium pyrophosphate, 100 mM sodium fluoride, 0.01% (v/v) aprotinin, 4 µg/ml pepstatin A, 10 µg/ml leupeptin, and 1 mM phenylmethyl-sulfonyl fluoride (PMSF)]. Proteins were separated by SDS-PAGE and transferred onto PVDF membranes (Invitrogen). Membranes were blocked with 5%

skim milk in Tris-buffered saline containing 0.1% Tween 20 [10], and incubated sequentially with an antibody to type II collagen (Chemicon International, Inc., Temecula, CA, U.S.A.) and peroxidase-conjugated secondary antibody (KPL, Gaithersburg, MD, U.S.A.), and the proteins visualized with TMB membrane peroxidase substrate (KPL).

Chemical Staining of Cells

After monolayer cultivation, the cells were fixed with 10% buffered formalin for 30 min at room temperature, and then washed in distilled water [12]. After fixation, the cells were stained with 1% Alcian Blue 8GX (Sigma) in 3% glacial acetic acid (pH 2.5) for 30 min at room temperature, briefly washed in distilled water, and then allowed to 'blue' for 20 min in running tap water. Finally, the cells were dehydrated in sequential concentrations of 70%, 80%, 90%, 95%, and 100% ethanol, and then cleared in xylene and mounted in Permount (Fisher Scientific, Pittsburgh, PA, U.S.A.) [6].

RESULTS AND DISCUSSION

Articular chondrocytes in monolayer cultures are usually affected by the types and concentration of serum used. Since the serum from animal origin may cause several problems such as viral contamination, many attempts have been made to develop serum-free media for the cultivation of cartilage-derived cells. Recently, research on various growth factors and additives that affect proliferation of chondrocytes and ECM synthesis have been reported. In most cases, they basically describe the observation of the effects of growth factors on growth or change in ECM in defined serum-free medium. Therefore, chondrocyte isolation and its primary culture in optimal serum-free medium that can provide proper cell growth and ECM synthesis should be investigated.

Various kinds of serum-free media have been used for different cell lines in cultures [15]. ITSE and ascorbic acid-2-phosphate were added for the optimization of a serum-free medium. In this study, insulin is known for its effects as a growth factor and its synergetic effects on stimulating cell growth [1]. Hence, insulin was chosen as an additive, and 200 $\mu\text{g}/\text{ml}$ was found to be optimal. EGF was also used to stimulate the growth of chondrocytes. Utilization of such a serum-free medium yielded 95% growth, compared with that with serum. In addition, in order to verify the effect of EGF, cells were cultivated without EGF and the result showed a drastic decrease in cell growth (Fig. 1). EGF is known to enhance DNA synthesis and growth in various cell lines [9], with several lines of evidence suggesting that this factor may influence cartilage metabolism under normal and presumably pathological conditions: First, human articular chondrocytes grown at high density in primary

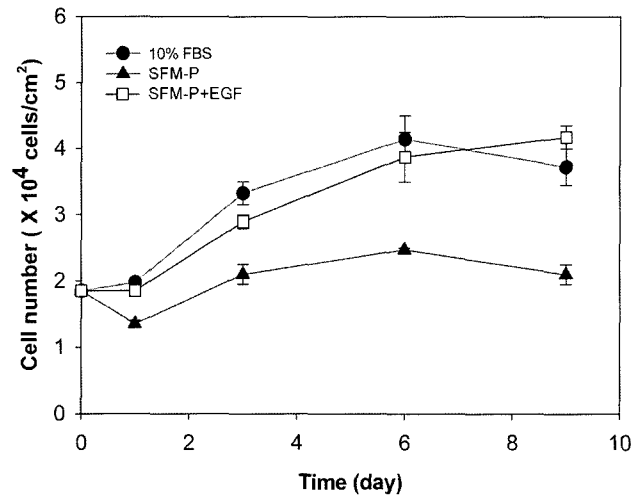


Fig. 1. Effect of various media on chondrocyte growth. ●, DMEM with 10% FBS; ▲, SFM-P; and □, SFM-P with EGF.

culture produce EGF; Second, high concentrations of EGF have been found in the synovium and synovial fluids of patients suffering from rheumatoid arthritis and to a lesser extent in those suffering from osteoarthritis; Third, some of the large cartilage-type proteoglycan-agrecan molecules have an EGF-like motif in their carboxy-terminal third globular domain [18].

The amount of GAG secreted during cell growth is usually proportional to the number of chondrocytes present in the culture. Therefore, the amount of GAG secreted into the medium was measured in the present study and the result showed that the cells in serum-free conditions produced up to 73% of GAG, compared with that in the serum-containing culture (Fig. 2). In early studies on rabbit chondrocytes, it was reported that EGF blocked ECM synthesis; however, in the study on rats, EGF was shown

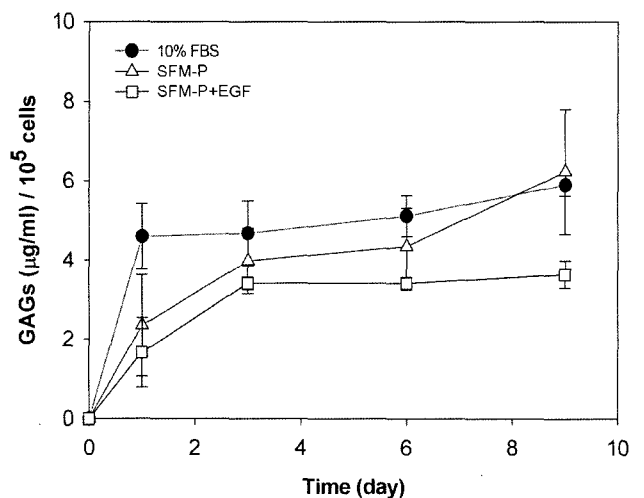


Fig. 2. Total sulfated GAG synthesis in various media. ●, DMEM with 10% FBS; ▲, SFM-P; and □, SFM-P with EGF.

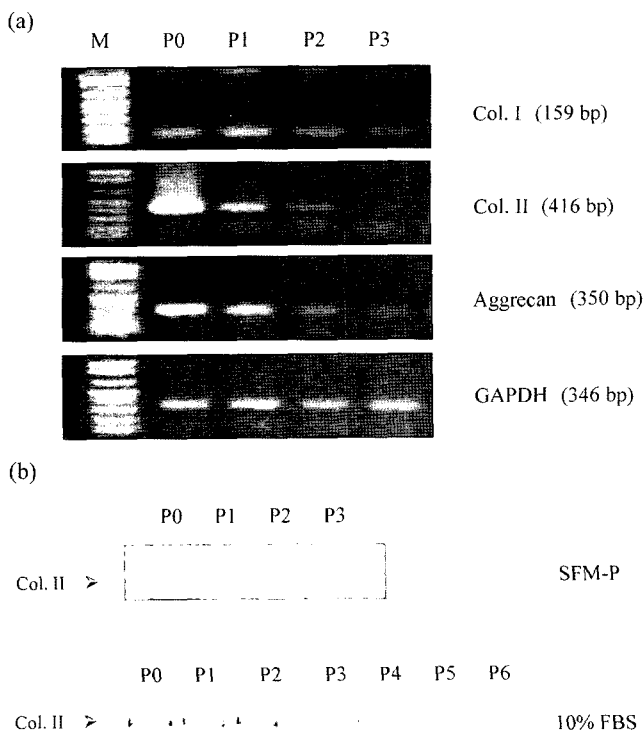


Fig. 3. Synthesis of ECM during different passages (P) of chondrocytes subcultured in SFM-P. (a) RT-PCR and (b) Western blot.

to promote proteoglycan synthesis [8, 17]. It has been reported that the effects of EGF are different with monolayer and three-dimensional cultures, and also by the age of the donor [18].

In general, if monolayer subcultures in a serum-containing medium were performed, de-differentiation of chondrocyte occurs after 4–5 passages [2]. Figure 3 shows the results from RT-PCR and Western blot, indicating that the ability to synthesize Type II collagen was lost after three passages of monolayer culture in a serum-free medium. Considering both transcription and translation at mRNA and protein levels, respectively, it was clear that the Type II collagen synthesis did not occur after three passages. By coating the culture dish with fibronectin, poly-L-lysine, and type IV collagen, de-differentiation was delayed and ECM synthesis lasted longer. The same result was obtained from alginate bead cultures, in which TGF- β 1 was more effective than EGF for Type II collagen synthesis (Fig. 4). Alcian blue-stained monolayer chondrocytes in SFM-P showed that proteoglycan synthesis similar to that of a serum-containing medium was achieved.

The patients' age should not be overlooked when considering ACT. The older the patient is, the slower the growth of chondrocytes and the greater the decreases of chondrocytic characters. Therefore, this aspect should also be considered, when developing a serum-free medium. In

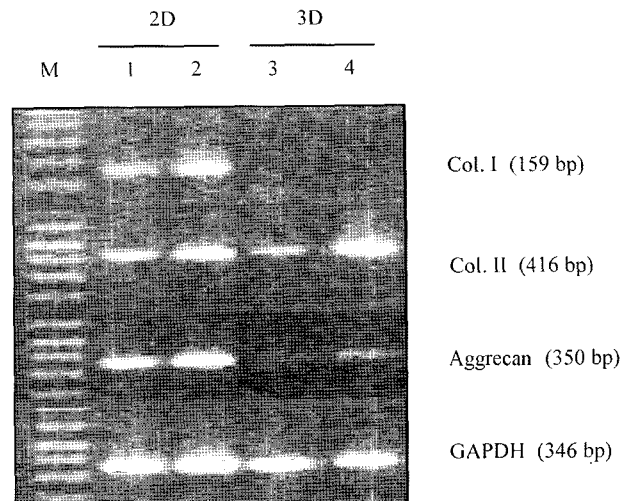


Fig. 4. Comparison of 2D and 3D culture conditions in SFM-P for ECM synthesis with growth factors. M, 1 kb DNA ladder; Lane 1, SFM-P (P2); lane 2, SFM-P+ECM coating (P3); lane 3, SFM-P(P3); and lane 4, SFM-P+TGF- β 1 (P3).

this research, GAG synthesis in a serum-free medium was compared with patients' ages from 10 to 70 years. As expected, the GAG synthesis was less in older cells (data not shown). This corresponded with the results by Huckle *et al.* [7], who showed that chondrocytes from young people synthesized more GAG and lasted at least for five passages. Therefore, in serum-free ACT, the age of the patient must be heavily considered.

In conclusion, a serum-free medium for the isolation and primary culturing of chondrocytes was successfully developed. This study demonstrated that chondrocytes can be grown to large numbers *in vivo* in a serum-free medium from primary cultures. The cell growth and ECM synthesis in SFM-P were similar to those of chondrocytes cultured in serum-containing medium, indicating that use of this serum-free medium for the isolation and primary culture could alleviate the problems arising from the use of human serum or FBS in developing a cell therapy in ACT.

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