***In vitro* culture of human chondrocytes (1): A novel enhancement action of ferrous sulfate on the differentiation of human chondrocytes**

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Abstract

Chondrogenic differentiation of mesenchymal cells is generally thought to be initiated by the inductive action of specific growth factors and depends on intimate cell-cell interactions. The aim of our investigation was to characterize the influences of basic fibroblast growth factor (bFGF) and ferrous sulfate (FeSO_4) on proliferation and differentiation of human articular chondrocytes (HAC). This is the first report of the effects of FeSO_4 on chondrogenesis of HAC. Multiplied chondrocytes of hip and shoulder joints were cultured in chondrocyte growth medium supplemented with bFGF, FeSO_4 , or both bFGF + FeSO_4 for 4 weeks. A 20 μl aliquot of a cell suspension containing 2×10^7 cells ml^{-1} was delivered onto each well of 24-well tissue culture plates. Cells cultured with the growth medium only was used as a control. Alamar blue and alcian blue staining were done to determine the chondrocyte proliferation and differentiation, respectively, after 4 weeks. The samples exposed to bFGF, FeSO_4 , and combination of both indicated sufficient cell proliferation similar to the control level. Differentiations of the HAC exposed to bFGF, FeSO_4 , and bFGF + FeSO_4 were 1.2-, 2.0-, and 2.2-fold of the control, respectively. Therefore, chondrocyte differentiation was significantly enhanced by the addition of FeSO_4 and bFGF + FeSO_4 . The combined effects of bFGF and FeSO_4 were additive, rather than synergistic. These results suggest that treatment with ferrous sulfate alone or in combination with basic fibroblast growth factor *etc.*, is a powerful tool to promote the differentiation of HAC for the clinical application.

Introduction

The availability of autologous chondrocytes needed for cartilage repair is limited. In 1977, it was first attempted to use the cultured chondrocytes for an articular cartilage repair (Green 1977). In a previous study, chondrocytes and tissue engineered product of poly-lactic acid failed to produce mature cartilage tissue one month after transplantation (Ito et al. 1992). Therefore, there is an essential need for supplying nutrients to the seeding cells in the early stage of transplantation to promote the chondrocyte viability, attachment, proliferation, extracellular matrix (ECM) production and maintaining of the cellular morphology. To date extensive studies have been undertaken to investigate the effects of many growth

factors such as insulin (Stevens and Hascall 1981; Harper and Harper 1987) and insulin like growth factor-1 (Bohme et al. 1992), transforming growth factor- α (TGF- α) (Panagakos 1994), - β (TGF- β) (Redini et al. 1991), thyroid hormone (Quarto et al. 1992), bone morphogenic proteins (Pizette and Niswander 2000), and other agents such as heparin (Brown and Balian 1987), vanadate (Kato et al. 1987), and retinoic acid (De Luca et al. 2000). *In vitro* chondrocytes culture of hyaluronic acid has been reported to show stimulatory effect (Fukuda et al. 1996; Kawasaki et al. 1999), inhibitory effect (Wiebkin and Muir 1975; Shimazu et al. 1993), or no effect (Larsen et al. 1992) on cell proliferation and differentiation.

Cyr61, a product of a growth factor-inducible im-

mediate-early gene, promoted cell proliferation, migration, and adhesion (Kireeva et al. 1996). Pleiotrophin (the heparin-binding growth-associated molecule) enhanced chondrogenesis in micromass culture (Dreyfus et al. 1998).

Although the basic fibroblast growth factor (bFGF) has been studied extensively for many years, its roles in cell attachment, proliferation and differentiation remains largely disputed. Basic FGF promoted articular cartilage repair (Toolan et al. 1996), and parenchymal cell proliferation and differentiation (Slack et al. 1987; Kimelman et al. 1988). However, another study reported that bFGF suppressed chondroitin sulfate synthesis (Matsusaki et al. 1998). In an *in vivo* study, bFGF acted as an angiogenic factor and induced capillary formation around the implanted composite of collagen sponge scaffold (Fujisato et al. 1996).

Chondroitin sulfate proteoglycans are found in various tissues and thought to play important roles in various cellular interactions (Yamagata et al. 1989; Sugiura et al. 1993). In a great number of mammalian and avian chondroitin sulfate proteoglycans, the glycosaminoglycan chains bear sulfate at position 4 or 6 of *N-acetylgalactosamine* residues. Sulfate groups in the form of heparan sulfate and keratan sulfate are also present in proteoglycans at the cell surface and in the extracellular matrix. We therefore, speculated that supplementation with exogenous sulfate ion in the medium during chondrocyte cultures may beneficially affect the chondrocyte differentiation.

In the present study, the effects of FeSO_4 alone or in combination with bFGF on human articular chondrocyte (HAC) proliferation and differentiation were investigated using a micromass culture system.

Materials and methods

Chondrocytes and medium

Human articular chondrocytes from the first passage of articular cartilage of hip (about 85%) and shoulder (about 15%) joints, and chondrocyte growth medium were commercially obtained from Cell Application, INC (San Diego, CA, USA). Chondrocyte growth medium contains basal medium (No. 410–450) and growth supplement (No. 411-GS). Sterile bFGF was purchased from Promega (Madison, WI, USA) and FeSO_4 was from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Both bFGF and FeSO_4 were dis-

solved in distilled Milli-Q water, and solution of FeSO_4 was sterilized by filtration through a 0.22 μM Millipore filter.

Cell culture methods

The acquired HAC had a 95.8% cell viability and were seeded in monolayer at a density of 2×10^4 cells cm^{-2} in Corning® 75 cm^2 cell culture flasks (Corning® Type 430720, Corning Co. Ltd., NY, USA). When HAC reached sub-confluent, the cells were trypsinized (trypsin-EDTA (Gibco) in PBS) and sub-cultured in monolayer. After adequate cell growth, chondrocytes at the third passage were trypsinized and high-density micromass cultures were initiated by spotting 4×10^5 cells in 20 μl medium onto each well of Costar 24-well microplates for tissue culture (Costar® Type 3513, Corning Co. Ltd.).

To allow the cells to settle and adhere to the plate, culture plates were incubated in a CO_2 incubator for 2h at 37 °C. The wells were then added with the growth medium (1 ml/well) supplemented with bFGF (10 ng ml^{-1}), FeSO_4 (50 $\mu\text{g ml}^{-1}$), or both bFGF + FeSO_4 . HAC cultured onto 24-well tissue culture microplates with the growth medium only was used as a control. The cells were further cultured in 5% CO_2 and 95% air at 37 °C for 4 weeks. The control and sample-containing media were changed every 2–3 days. At least three cultures were run for each case. All experiments were repeated at least twice, and similar results were obtained.

Cell morphology assay

Cell morphology was determined by inverted light microscopy and photographs were taken with Fuji films.

Proliferation assay

Cell proliferation was quantitatively measured by alamar blue (Biosource, International, Inc, Camarillo, CA, USA) assay after 4 weeks of culture. The assay demonstrates the metabolic activity of the cells by detection of mitochondrial activity. The indicator dye of alamar blue was incorporated into the cells and was reduced and excreted as a fluorescent product. At the end of culture period, the media from all wells were discarded, and the wells were filled with 1 ml/well of 20-fold diluted alamar blue solution with the fresh

medium. The culture plates were incubated for 4h at 37 °C. After the incubation period, two aliquots of 100 μ l of solution from each well were transferred into new wells of Costar tissue culture 96-well microplates (Costar® Type 3595, Corning Co. Ltd.). Equal volume of fresh medium per well (total three wells) served as blank. The extent of cell proliferation was quantitated by Cytofluor II fluorescence multiwell cell reader (PerSeptive Biosystems, Framingham, MA, USA) at 535 nm excitation and 590 nm emission. Blank values were subtracted from experimental values to eliminate background readings.

Differentiation assay

After alamar blue assay, medium from all wells was discarded and the micromass cultures were washed once with 0.5 ml/well of PBS (–) at a room temperature. Proteoglycans are typical contents of the cartilage matrix. The extent of chondrogenesis was determined by staining the cartilage specific proteoglycans with alcian blue (Wako Pure Chemical Industries, Ltd., Osaka, Japan) as detailed earlier (Kistler et al. 1990). Briefly, the cultures were stained in 0.5 ml/well with 1% alcian blue, pH 1.0 overnight at 4 °C. The alcian blue solution was then removed and the micromasses were rinsed with 3% acetic acid and distilled water. The cartilage proteoglycans was extracted by 4-M guanidine hydrochloride and the bound dye was measured at a wavelength of 600 nm using an ELISA reader (Bio-Tek Instruments, Inc., Winooski, VT). A 100 μ l of fresh 4-M guanidine hydrochloride per well, total three wells, served as blank. Blank values were deducted from experimental values to eliminate background readings.

Statistical analysis

Comparing control with samples exposed to various factors assessed the statistical significance of the cell proliferation and differentiation. Student's *t*-test was used to compare the samples. Statistical significance was represented by $p < 0.05$. Data were indicated as the mean $\pm$ s.d.

Results

Cell morphology

Following two hours of cell spotting in high-density

micromass culture, cells were aggregated (Figure 1a). After 4 weeks of culture, the chondrocytes predominantly formed a uniform sheet of chondrogenic cells. The control cells showed few poorly defined cluster formations (Figure 1b). The visualization of the cartilage nodules was usually achieved within one week following the spotting of the micromass culture. These nodules were better visualized by staining the proteoglycans with the alcian blue. The culture exposed to bFGF had slightly higher ECM components under light microscopy. Cultures exposed to FeSO₄ (Figure 1c), and both bFGF + FeSO₄ (Figure 1d) showed a denser ECM components. Cultures exposed to these factors also exhibited poorly defined nodules formation. There was no effect of these factors on cell morphology, which maintained a rounded shape. In all cultures, chondrocytes spread from the edge of micromass culture and the spreading cells were spindle in shape.

Cell proliferation assay

Chondrocyte proliferation was quantitatively determined by alamar blue assay after 4 weeks of culture and is shown in Figure 2. All values of the samples exposed to the factors were expressed as percentage of the control average value. Basic FGF or FeSO₄ alone, and/or the combination with both factors slightly decreased the cell proliferation in comparison to the control, but the inhibition was not significant. However, all samples showed sufficient proliferation close to the control level. The values of cell proliferation for the samples exposed to bFGF, FeSO₄, and bFGF + FeSO₄, were 83.7%, 93.7%, and 90.8%, respectively.

Alcian blue staining

The bound form of proteoglycans with the alcian blue was extracted with 4-M guanidine hydrochloride. Their amounts were expressed as percentage of the control average value (Figure 3). Intensities of alcian blue staining in all cultures exposed to the factors were found to be higher than that in the control culture. The cells exposed to bFGF revealed a slightly higher cell differentiation (115.9%) but this was not significant when compared to control ($p > 0.05$). While, chondrogenic differentiation of the cells exposed to FeSO₄, and bFGF + FeSO₄ were 195.5% and 218.1%, respectively, both cultures showed a significantly higher ($p < 0.05$) proteoglycon product-

Figure 1. Appearance of micromass cultures of human articular chondrocytes at day 0 and after 4 weeks. a) Day 0 (magnification, $\times 100$); b) control, 4 weeks, ($\times 100$); c) FeSO_4 , 4 weeks, ($\times 100$); d) bFGF + FeSO_4 , 4 weeks, ($\times 100$).

ion when compared to the control. When multiple comparisons among the samples exposed to the factors were done with respect of bFGF to FeSO_4 or bFGF + FeSO_4 , they were found to be statistically different at a significance level of 0.05. But when the cultures exposed to FeSO_4 were compared with the cultures exposed to bFGF + FeSO_4 , it was found not to be significantly different ($p > 0.05$).

Differentiation index

Cell differentiation index (cell differentiation/cell proliferation) for control, bFGF, FeSO_4 , and bFGF + FeSO_4 cultures were 1, 1.38, 2.09, and 2.40, respectively (Figure 4). Cultures exposed to FeSO_4 , and bFGF + FeSO_4 elicited significantly higher cell differentiation than the control culture ($p < 0.05$).

Discussion

In reconstructive surgery, autologous cartilage grafting is commonly performed to reconstruct cartilage defects. Because of the limited supply of fresh autologous cartilage, many investigators concentrate on the *in vitro* production of cartilage tissue. Several growth factors regulate the metabolism and differentiation of cartilage cells. Measurement of proteoglycans synthesis and cell proliferation assessed the biological effects of bFGF and FeSO_4 . In this study, FeSO_4 alone or in combination with bFGF significantly enhanced chondrocyte differentiation and both factors showed cell proliferation satisfactorily near to the control level. Cells were grown as high-density micromass, which resembles to 3-dimensional tissues. On day 0, following 2h of settlement period, the cells

CELL PROLIFERATION

Figure 2. Cell proliferation of human articular chondrocytes in control, bFGF, FeSO₄, and bFGF + FeSO₄ after 4 weeks.

in all cultures aggregated leaving spaces among the aggregates (Figure 1a). The morphology under light microscopy showed that the cells exposed to FeSO₄, and especially the combination of bFGF + FeSO₄ appeared to elicit a denser ECM production than the control (Figure 1b–d). This morphological phenomena was confirmed biochemically by alcian blue assay (Figure 3). The best stimulation for cell differentiation was obtained with the culture exposed to the combination of bFGF + FeSO₄. To evaluate the rela-

CELL DIFFERENTIATION

Figure 3. Cell differentiation of human articular chondrocytes in control, bFGF, FeSO₄, and bFGF + FeSO₄ after 4 weeks.* $p < 0.05$.

DIFFERENTIATION INDEX

Figure 4. Differentiation index of human articular chondrocytes in control, bFGF, FeSO₄, and bFGF + FeSO₄ after 4 weeks. Differentiation indices were expressed as the ratios of cell differentiation/cell proliferation.* $p < 0.05$.

tive value of each component on cell differentiation, the pattern of expression strongly suggested that the effects of bFGF were purely additive to the effects of FeSO₄ rather than synergistic. Although we have shown that FeSO₄ alone or in combination with bFGF stimulated *in vitro* chondrocyte differentiation, we do not have direct proof whether it has the same effects *in vivo*. The effects of bFGF on cell culture remain largely controversial. No stimulatory effect of bFGF on the matrix synthesis of human chondrocytes was reported (Bujia et al. 1996). In another study, human nasal septal cartilage did not increase cell proliferation in response to bFGF (Quatela et al. 1993). In our study, bFGF slightly inhibited proliferation but slightly increased differentiation of HAC. Our findings were in contrast to previous studies where bFGF stimulated chondrocyte proliferation (Bujia et al. 1994; Seko et al. 1995). The increased cell differentiation in our study, however, was in agreement with a recent report that an administration of bFGF into cartilaginous defects promoted the differentiation of chondrocytes and the matrix synthesis (Fujimoto et al. 1999). These variable effects of bFGF on the cell proliferation and differentiation may be partially due to chondrocytes derived from different organs and tissues. It was previously reported that in response to bFGF, the proliferation of human auricular chondrocyte was increased but not that of human nasal septal chondrocytes (Quatela et al. 1993). Yet another study using rib and ear chondrocytes from the same

rabbit reported that the origin and cellular differentiation states of chondrocytes caused different cell proliferation to bFGF (Lee et al. 1977). The different doses of bFGF also affected the outcome of chondrocyte proliferation and differentiation. One recent report demonstrated that low concentrations [10 ng ml^{-1}] of bFGF stimulated cartilage matrix production, but high concentrations (1000 ng ml^{-1}) inhibited matrix production (Mancilla et al. 1998). Different level of chondrocyte proliferation with different doses of bFGF was also reported in another study (Matsusaki et al. 1998). As the values of cell proliferation and differentiation with FeSO_4 are firstly reported here, we could not compare these results with other studies. The localization of chondroitin sulfate in developing and adult cartilage taken with the facts that no study of chondrocytes with FeSO_4 was done led us to analyze the effects of FeSO_4 on chondrogenesis. In a more recent study, we have evaluated chondrocytes of knee joints from different human origin treated with FeSO_4 (unpublished data). We have found similar stimulation in cell differentiation as we noted in this study. But cell proliferation, contrary of this study was increased. Therefore, we can state, as for bFGF that different findings of chondrocyte proliferation by FeSO_4 may be contributed to the different origin sites of chondrocytes. The mechanism of chondrogenic enhancement by FeSO_4 is considered as follows: Sulfate ion molecule interacts with adenosine triphosphate to form adenosine 5'-phosphosulfate (APS), which further interacts with a phosphate ion molecule to accumulate 3'-phosphoadenosine 5'-phosphosulfate (PAPS). PAPS is the activated form of sulfate, and chondroitin sulphotransferase transfers sulfate of PAPS to *N-acetylgalactosamine* in chondroitin and forms chondroitin sulfate. High amounts of chondroitin sulfate is present in proteoglycans. We speculate that FeSO_4 was the supplier of sulfate for accumulation of APS and PAPS, which ultimately formed the proteoglycans.

Although we did not attempt to see the effects of other growth supplements, we assume that other growth supplements when used in multiple combinations with FeSO_4 would result in enhanced chondrogenesis. In this study, we used sulfate in the form of ferrous sulfate only. We did not try to study the effects of sulfate from other metal salts such as magnesium sulfate, copper sulfate, etc. Iron in the form of ferrous sulfate is routinely used for treating anemia. Human body can consume large amounts of ferrous sulfate without many side effects. On the

contrary, ions like silver, copper, mercury and nickel released from metallic biomaterials have adverse biological effects even when they were used in low doses (Wataha et al. 2000).

This study suggests that cultured chondrocytes in the presence of FeSO_4 potentially enhanced chondrogenesis for cartilage tissue engineering. The present work firstly shows the usefulness of FeSO_4 in the human cartilage tissue engineering because of its biological safeties as well as its stimulatory action. Further studies with this molecule to see its time-dependent and dose-dependent effects *in vivo* will be needed, and, the effects on primary undifferentiated cells and in combination with other molecules are subjected for further studies. Some of these are under consideration for future investigation in our laboratory.

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