J Orthopaed Traumatol (2007) 8:21–24
DOI 10.1007/s10195-007-0152-2

ORIGINAL

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The effects of transforming growth factor β1, insulin-like growth factor 1 and leptin on the proliferation of fetal chondrocytes

Received: 27 December 2006
Accepted: 22 January 2007
Published online: 5 March 2007

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Abstract One method to exogenously enhance the repair of articular cartilage is the local application of growth factors. This method is based on the chondrogenic effects of some agents and their potential ability to enhance cell migration. Human fetal chondrocytes were isolated and cultured. Their proliferation under the influence of different agents was microscopically evaluated. Fetal calf serum at 5% and 10% concentrations induced microscopically visible cell proliferation. Transforming growth factor beta one (5 and 10 ng/ml), insulin-like growth factor 1 (5 and 10 nmol/l) and leptin (1 and 2 ng/ml) accelerated proliferation of the cells towards the increasing gradient of the agents. Fibroblast growth factor beta (5 and 10 ng/ml), bone morphogenic protein two (10 ng/ml) and laminin (1 mg/ml) did not affect cell proliferation. This study suggests that different agents can play a role in the proliferation of fetal chondrocytes.

Key words Fetal chondrocytes • Proliferation

Introduction

Articular cartilage possesses neither blood supply nor lymphatic drainage, and articular chondrocytes are known to be ineffective in adult life to respond to injuries. After injury, there are two ways to restore the joint surface: the first is to enhance or initiate a process of fibrocartilage healing, for example by drilling the subchondral bone; the second way is to transplant tissue that can either increase chondrocyte proliferation or act as new cartilage, for example by the implantation of autologous chondrocytes or stem cells.

One method to exogenously enhance the proliferation of articular cartilage is the intra-articular injection or local application of different growth factors, with or without a scaffold on which healing can be structured [1–5]. This method is based on the chondrogenic effects of these agents and their potential ability to enhance cell migration.

The optimal agents and their combination, concentration, timing and way of delivery have to be defined in further studies. Transforming growth factor (TGF)-β1, insulin-like growth factor (IGF)-1 and fibroblast growth factor (FGF)-β can enhance chondrogenesis and synthesis of cartilage-specific matrix, allowing the formation of tissue which is hyaline cartilage-like [1–4]. Bone morphogenic proteins (BMPs) promote many aspects of chondrogenesis and leptin, a cytokine-type hormone, plays a major role during the early phase of proliferation and differentiation of chondrocytes in the epiphyseal growth plate [6]. Laminin, an extracellular matrix component, is involved in the definition of differentiating areas in the bone, i.e. the periphery, which eventually give rise to the perichondrium [7].

In the present study, we created a system to easily assess in vitro the effects of different agents, in different concentrations, on the proliferation of fetal chondrocytes.

Materials and methods

Cell isolation and expansion

We used human embryonal cartilage cells retrieved from epiphyses of the femurs and tibias of human fetuses between 18 and 22 weeks gestational age, which were all aborted for social reasons without known chromosomal anomalies. The use of human embryonic cells for this purpose was in accordance with the ethical standards of the 1964 Declaration of Helsinki. Cell culturing, preparation of the dishes and proliferation tests were done in a dedicated tissue laboratory under sterile conditions.

Chondrocytes were isolated by digestion with 0.15% type 2 collagenase for 22 hours and resuspended in Dulbecco's modified Eagle's medium containing 4.5 mg/ml D-glucose, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 mM HEPES buffer, 100 U/ml penicillin, 100 mg/ml streptomycin and 0.29 mg/ml L-glutamine (basic medium).

The chondrocytes were plated in tissue culture flasks and cultured in basic medium supplemented with 10% fetal bovine serum in a humidified 37° C, 5% CO_2 incubator. After four days, the cells were removed to 35-mm tissue culture dishes, using 0.25% trypin-EDTA solution. To the tissue culture dishes, we added a DCCM-1 medium without serum, 1% L-glutamine and antibiotics (100 µg/ml streptomycin, 100 U/ml penicillin and 1.25 µ/ml nystatin) and the dishes were incubated at 37° C. The cells reached confluence in one week. Microscopic examinations revealed that the cells had a typical morphology of chondrocyte cultures.

Preparation of the dish for cell migration tests

The 35-mm tissue culture dish was prepared in the following way. One half of each dish was left untouched. The cells in the other half were scraped mechanically using a sterile disposable cell scraper (Greiner Bio-One) and then the whole plate was washed with 2 ml medium. Accurate removal of cells from half of the dish was microscopically examined and assured.

On the half sphere of the cell-free zone, a cylindrical glass ring of 8 mm diameter and 6 mm height was adhered to the margin of the dish using biologic glue (Histoacryl sterile, Aesculap). In this cylinder, we placed the 10 µl of the examined agent mixed with 150 µl 1% hyaluronic acid gel (Biotechnology General, Israel), which allows a slow release of the agent. Only a minimal amount of glue was used to fix the glass cylinder to the margin of the dish, in order to prevent instability in the medium. Then we added to each dish 2 ml DCCM-1 medium, a defined medium lacking serum. We used methylene blue as a control marker to demonstrate a slow release from the cylinder, which lasted around 36 hours.

The tested agents

The agents tested were TGF-β1, IGF-1, FGF-β, BMP-2, laminin and human leptin. We also examined the effects of different concentrations of fetal calf serum (1%, 2.5%, 5% and 10%) on the proliferation of the cells. The dishes were incubated at 37° C in a humidified atmosphere equilibrated with 5% CO_2.

Evaluation of the proliferation response to agents

Evaluation of cell proliferation in response to the agents was determined microscopically every two days. As control we used dishes that contained hyaluronic acid only or those containing 10% fetal calf serum which was able to promote cell proliferation.

Cell proliferation was assessed by the bell shape formation towards the chemotactic gradient from glass cylinder. In this way a cone of cells with its edge points to the glass cylinder was created. Sporadic proliferations with no specific direction were ignored. Proliferation was scored as + (equivalent to that of 5% fetal calf serum), ++ (equivalent to that of 10% fetal calf serum) and +++ (superior to that of 10% fetal calf serum).

Results

Fetal calf serum in 5% and 10% concentrations induced microscopically visible cell proliferation. The 10% concentration promoted proliferation faster and more prominently than the 5% concentration. Under these concentrations, cell proliferation was clearly noticed on day 4 of incubation. It became even more prominent on days 8 and 10 (Fig. 1a). Lower concentrations of 1% and 2.5% fetal calf serum failed to affect the proliferation in comparison to a control dish which contained only hyaluronic acid (Fig. 1b, c).

FGF-β (5 and 10 ng/ml), BMP-2 (10 ng/ml), and laminin (1 mg/ml) did not affect cell proliferation (Table 1). TGF-β1 (5 and 10 ng/ml), IGF-1 (5 and 10 nmol/l), and leptin (1 and 2 ng/ml) caused intensive proliferation, which was on day 4 already more prominent than the control dishes that contained hyaluronic acid only or 10% fetal calf serum. This became even more prominent at days 8 and 10. The cells showed a clear orientation towards the cylinder of glass containing these agents (Fig. 2). The higher concentrations of the tested agents caused a more extensive and a quicker proliferation of the cells following the agent gradient.

Discussion

Damage to articular cartilage is a frequent clinical problem and is commonly considered irreversible. Full-thickness defects may lead to the formation of fibrocartilage of low

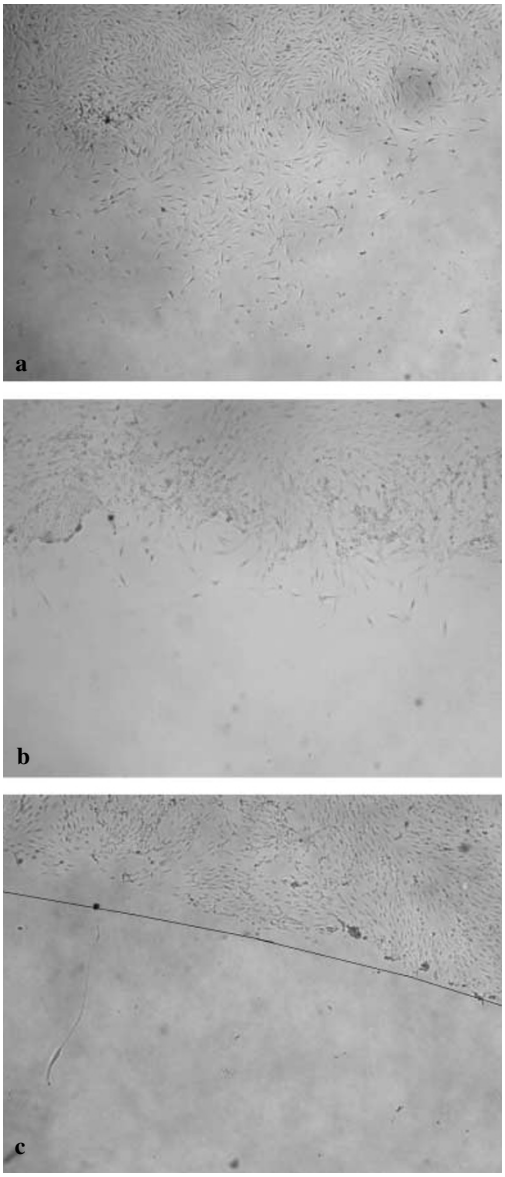

Fig. 1a-c *Chondrocyte proliferation in control situations*. **a** Response to fetal calf serum 10% after 8 days. **b** Negative control with only hyaluronic acid (no proliferation). **c** Response to 2.5% fetal calf serum

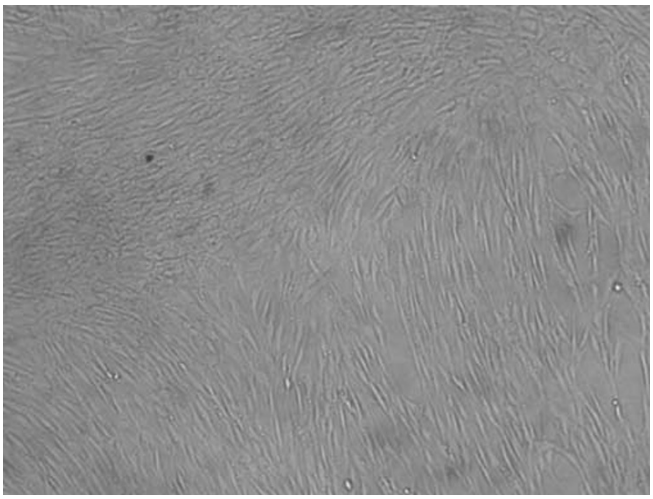

Fig. 2 Chondrocyte migratory response to TGF-β1

mechanical quality, while partial-thickness lesions hardly show any repair response. Advances in molecular biology have revealed the potential of growth factors, differentiation factors, and cytokines in affecting chondrocyte proliferation. The chondrogenic effects of these agents and their potential ability to enhance cell migration could be used for cartilage repair.

TGF-β1 is a well-known potent growth factor implicated in chondrogenesis both in vitro and in vivo [1, 4]. This study showed that TGF-β1 can promotes chondrocyte proliferation. The 5 and 10 ng/ml concentrations of TGF-β caused intensive chondrogenesis which was on day 4 already more prominent than the control, and became more prominent on days 8 and 10.

Chondrocytes exposed to a combination of FGF and TGF-β1 cease the production of collagen type 2 and undergo a massive increase in cell number [3]. A one-day exposure to TGF-β was sufficient for induction of the chondrogenic repair response in full-thickness defects of the articular cartilage in rabbits [8]. Despite this, in our study FGF-β at a concentration of 5 and 10 ng/ml had no effect on chondrocyte proliferation.

Table 1 Migration response of primary human chondrocytes to various growth factors

Agent	Concentration	Migration response
FGF-β	5 and 10 ng/ml	None
TGF-β1	5 and 10 ng/ml	+++
IGF-1	5 and 10 nmol/l	+++
BMP-2	10 ng/ml	None
Laminin	1 mg/ml	None
Leptin	1 and 2 ng/ml	+++

+++, Proliferation superior to that of 10% fetal calf serum

BMPs promote almost every aspect of chondrogenesis [9]. They also act as regulators in chondrocyte differentiation during embryonic development [10]. BMP-2 can drive embryonic stem cells to cartilage or osteoblasts, depending on supplementary co-factors [11]. Like FGF-β, BMP-2 at a concentration of 10 ng/ml had no effect on chondrocyte proliferation in this study.

Many metabolic processes of systems and organs are affected by leptin, which affects skeletal growth via acceleration of IGF-1 and affects the chondrogenic axis-lineage all along. Especially at the early phase, the chondrogenic proliferation and differentiation of chondrocytes in the epiphyseal growth plate are subjected to this factor [12, 13]. One and two ng/ml concentrations of leptin had positive effects on the proliferation of fetal chondrocytes in this study.

The positive effect of fetal calf serum on the proliferation of chondrocytes can be related to the fact that fetal calf serum influences the proteoglycan synthesis of cartilage cells. Van Susante et al. [14] investigated the responsiveness of bovine chondrocytes to growth factors (IGF-1, TGF-β2 and BMP-2) in medium with different serum concentrations, and showed that proteoglycan synthesis dramatically decreased, as the concentration of fetal calf serum in the medium decreased.

Despite these results, there is still a lack of knowledge regarding the interactions among the external environment, the cells and the different agents. Of the substances that we examined, BMP-2, FGF-β, and laminin did not affect cell proliferation, in spite of the fact that in previous studies these substances had an effect on the proliferation of different kinds of cells. TGF-β1, IGF-1 and leptin exerted a noticeable influence on proliferation of the chondrocytes. This result suggests that leptin, as a regulator of energy balance, as much as TGF-β1 and IGF-1, have the ability to influence the proliferation of fetal chondrocytes. The study suggests that different agents can play a major role in the proliferation of fetal chondrocytes. In vivo studies that examine cartilage repair in animals are planned, based on the findings from this in vitro study.

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