Chondrogenic differentiation of canine myoblasts induced by cartilage-derived morphogenetic protein-2 and transforming growth factor-β1 in vitro

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Abstract. The meniscus has limited ability to repair itself following injury. However, tissue engineering provides new means of meniscus repair. Myoblasts, which possess the potential of multi-directional differentiation, may be ideal seed cells in meniscus tissue engineering. Myoblasts from different animals showed slight differences in morphology and in the potential to differentiate. In the present study, we isolated myoblasts from canines and induced chondrogenesis in order to establish a new experimental model of seed cells. Myoblasts were isolated and harvested from Beagle canines. To induce chondrogenesis, cartilage-derived morphogenetic protein-2 (CDMP-2) at different concentrations (10, 20, 50 and 100 ng/ml), transforming growth factor-β1 (TGF-β1; 10, 20, 30 and 50 ng/ml), and different concentrations of CDMP-2 (10, 20, 50 and 100 ng/ml) together with TGF-β1 (20 ng/ml) were added to the cultured pellets. After 21 days of in vitro culture, chondrogenic differentiation was evaluated by histological and immunohistochemical techniques. The degree of gene expression was measured by quantitative RT-PCR. Based on the histological staining of glycosaminoglycan, using the toluidine blue dye-binding method, we found that CDMP-2 initiated chondrogenic differentiation of myoblasts, as did TGF-β1. Furthermore, CDMP-2 conferred a stronger stimulatory effect than TGF-β1. The combination of CDMP-2 and TGF-β1 synergistically induced chondrogenesis of myoblasts. This synergistic chondrogenic effect of CDMP-2 together with TGF-β1 was further confirmed by quantification of glycosaminoglycan using dimethylmethylene blue dye-binding assay and immunohistochemical analysis of the expression of cartilage-specific proteins collagen I and II. Canine myoblasts can be induced into chondrocytes by CDMP-2 and TGF-β1 in vitro, suggesting that myoblasts are suitable as seed cells for meniscus tissue engineering.

Introduction

Meniscus is a complex fibrocartilaginous tissue, which is essential to the knee joint for shock absorption, load distribution, maintenance of stability and protection of articular cartilage (1). For symptomatic meniscus injury, a partial or total meniscectomy is often performed as the intrinsic healing capacity of the meniscus is limited (2). This, however, often leads to osteoarthritis (3). Other therapeutic methods of preserving the function of the meniscus are limited, and the result is not always satisfactory (4).

In recent years, meniscal regeneration using tissue engineering techniques have been carried out based on the loading and culture of suitable cells into appropriate scaffolds (5). To date, several cell sources have been used for meniscus repair, including meniscus fibrochondrocytes, chondrocytes or bone marrow-derived mesenchymal progenitor cells (6). However, the use of these cell sources have prompted issues concerning cell selection, acquisition of seed cells and the quality of repaired tissues (7). Myoblasts are regarded as ideal donor cells due to their abundant source, obtainability, stem cell-like potential, extended survival ability and rapid proliferative activity in vitro (8). Myoblasts are considered superior in biopsy, manipulation and clinical application than other stem cells, such as chondrocytes or bone mesenchymal stem cells (9). Therefore, myoblasts may be suitable for providing autogenous cells for the treatment of meniscal injuries.

Chondrogenesis proceeds through the aggregation of cells into prechondrogenic condensation and then differentiation of cells into chondrocytes. Numerous growth factors in the transforming growth factor-β1 (TGF-β1) superfamily have been found to enhance or induce certain stem cells to become chondrocytes in vitro (10,11). Since cartilage-derived morphogenetic protein-2 (CDMP-2) belongs to the TGF-β superfamily, it is a key factor in the regulation of the development of the appendicular skeleton, particularly at the early stages of chondrogenesis of the limb (12). Expression of CDMP-2 is mainly

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localized at the condensing mesenchyme of the limb as well as in future joint spaces, and is directly correlated with the location, number and timing of developing joints. Nevertheless, little is known about the effects of CDMP-2 on the differentiation of myoblasts in vitro. TGF-β1 was initially reported to be essential for the chondrogenesis of certain stem cells (13). However, TGF-β1 alone may not be sufficient for the induction of the differentiation of myoblasts into chondrocytes. We, therefore, tested the chondrogenic effect of CDMP-2 alone and together with TGF-β1 on the differentiation of myoblasts into chondrocytes using a high-density pellet culture system.

Materials and methods

Myoblast culture. Beagle canines from the Agricultural Institute of Shanghai Jiaotong University were used as the source of myoblasts. Myoblasts from the hind limb of adult Beagle canines were isolated and harvested by mechanical decomposition and two-step enzyme digestion (14). The cells were then purified by a method involving differential adherent velocity and flow cytometry. Myoblasts were resuspended in media containing Dulbecco’s modified Eagle’s medium/F12 (DMEM/F12; Gibco BRL) supplemented with 10% fetal bovine serum (FBS; Gibco BRL), 100 U/ml penicillin G, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B. The myoblast suspension was seeded on 100-mm dishes and cultured at 37°C in a humidified atmosphere of 95% air and 5% CO₂. To remove the non-adherent cells, the medium was changed on the fifth day after seeding, and it was changed again every four days thereafter. Myoblasts were passaged four times prior to being collected for use.

Pellet culture. For chondrogenic differentiation, 3.0x10^5 myoblasts in a 15-ml conical polypropylene tube were centrifuged at 500 g for 5 min at 20°C (15) and cultured at 37°C with 5% CO₂ in 0.5 ml DMED-high glucose (Sigma) medium containing 1% FBS, insulin (6.25 μg/ml; Sigma) and ascorbate 2-phosphate (50 μg/ml; Sigma). Human CDMP-2 at different concentrations (10, 20, 50 and 100 ng/ml; PeproTech EC), TGF-β1 (10, 20, 30 and 50 ng/ml; Sigma) and different concentrations of CDMP-2 (10, 20, 50 and 100 ng/ml) together with TGF-β1 (20 ng/ml) were added to the cultured pellets. The medium was changed every 3 days for 21 days. The concentration of CDMP-2 and TGF-β1 selected was based on a previous study (16).

Histology. Pellets were harvested following 21 days of culturing, fixed in 10% buffered formalin for 2 h, embedded in 2% agarose for easy manipulation and kept in 70% ethanol overnight. Samples were embedded in paraffin and 5-μm sections were cut. Sections were stained with toluidine blue and 0.01 M PBS (pH 7.4) were applied as a primary antibody at 4°C, overnight. Sections were then incubated with the secondary antibody, rabbit anti-mouse immunoglobulin (Dako) for 60 min followed by mouse PAP kit (Dako). Collagen type I and II were visualized by the reactions with 0.05% dianinobenzidine containing 0.1% H₂O₂.

RT-PCR. Pellets (n=20) cultured for 21 days were digested with collagenase I and II (3 mg/ml; LSL Co.), hyaluronidase (1 mg/ml; Sigma) and 0.25% trypsin (Sigma) for 3 h at 37°C. RNA was extracted from the digested pellets using TRIzol (Invitrogen), then converted to cDNA and amplified by PCR in a PCR machine with 1 cycle at 94°C (5 min), 35 cycles at 94°C (30 sec), 57°C (30 sec) and 72°C (30 sec), followed by 1 cycle at 72°C (5 min). The mRNAs analyzed were collagen I (681 bp) and collagen II (447 bp) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (211 bp). Prime sequences for GAPDH, collagen I and collagen II as follows: GAPDH, sense 5’CTCTTATGCAACACAGTGCC3’, antisense 5’GTACTCTGCTTGTGGATCC3’; collagen I, sense 5’ATGCCCAGAGTCTACGAGTG3’, antisense 5’TCTTGGAAGCTTCTTCTGATC3’; collagen II, sense 5’TTCGCCAGTTCAAGATGTC3’, antisense 5’CTTCAGCACCTGTCTCACC3’.

GAG measurement. Pellets were harvested following 21 days of induction for GAG quantitation, washed with PBS and digested with 200 μl papain solution (Sigma; 280 μg/ml in 50 mM sodium phosphate, pH 6.5, containing 5 mM N-acetyl cysteine and 50 mM EDTA) for 24 h at 65°C. GAG content was measured by the reaction with dimethylmethylene blue (DMMB; Sigma). Optical density was determined at 525 nm using shark chondroitin sulfate (Sigma) as the standard.

Statistical analysis. The data are expressed as the means ± SD. Statistical significance for total GAG content (μg/pellet) [H] thymidine incorporation value between groups was determined using one-way analysis of variance (ANOVA).

Results

CDMP-2 promotes chondrogenesis of myoblasts. All pellets of the myoblasts showed a spherical shape after 21 days under different culture conditions. In the absence of factors or in the presence of TGF-β1 alone, the expression of collagen II in the pellets was not detected by immunohistochemistry (Fig. 1A). Expression of collagen II in the pellets was upregulated by either CDMP-2 (Fig. 1C) alone, or in combination with TGF-β1 (Fig. 1D). Higher cell density in the collagen II-positive region in the pellets indicated that more cartilage matrix was produced after myoblasts differentiated into chondrocytes. Only some cells incubated with TGF-β1 alone differentiated into chondrocytes (Fig. 1B). The expression of collagen II was observed all over the pellets when treated with CDMP-2 together with TGF-β1 (Fig. 1D). The results suggest a synergistic effect of CDMP-2 and TGF-β1 on chondrogenesis related to myoblasts.
Collagen I, expressed by chondrocytes, was expressed under both induced situations of cell culture but was more strongly expressed in the sections that expressed collagen II (Fig. 1E-H). CDMP-2 in the presence of TGF-β1 strongly upregulated the expression of collagen I in the pellets. The results indicated that most cells treated with CDMP-2 and TGF-β1 had differentiated into chondrocytes. Chondrogenesis of the pellets was further confirmed by RT-PCR (Fig. 2). Products of all PCR amplifications were sequenced to exclude the possibility of false-positive results. Results of the RT-PCR were in accordance with the immunohistochemical analysis. In all pellets, collagen I mRNA was uniformly expressed. Collagen II mRNA was expressed in both pellets cultured with CDMP-2 alone or in combination with TGF-β1 (Fig. 2, lanes 3 and 4), but it was not detected in pellets cultured without either growth factor or in the presence of TGF-β1 alone (Fig. 2, lanes 1 and 2).

**Dose effect of CDMP-2 and TGF-β1 on myoblast chondrogenesis.** To evaluate the dose effect of CDMP-2 and TGF-β1 on myoblast chondrogenesis, the pellets were cultured in medium containing different doses of CDMP-2 (10, 20, 50 and 100 ng/ml), TGF-β1 (10, 20, 30 and 50 ng/ml) and CDMP-2 (10, 20, 50 and 100 ng/ml) together with TGF-β1 (20 ng/ml), respectively, for 21 days. We assessed the GAG (a main matrix element of cartilage) expression in the pellets by histological staining using the toluidine blue dye-binding method. Metachromatic-staining of pellets represented GAG expression by differentiated chondrocytes. The metachromatic-staining region of the pellets grown in the presence of CDMP-2 combined with TGF-β1 (20 ng/ml) was correlated to the CDMP-2 concentration. An apparent effect was observed at 50 ng/ml (Fig. 3C); the maximal chondrogenic effect was noted at 100 ng/ml (Fig. 3D).

To further evaluate the dose effect of CDMP-2 and TGF-β1 on myoblast chondrogenesis, we quantified GAG deposition of pellets using the DMMB-binding method (Fig. 4). Pellets cultured in the presence of growth factors showed a significant stimulation of GAG expression with CDMP-2 exhibiting a stronger stimulatory effect than TGF-β1. An increase in GAG expression was induced by TGF-β1 at 30 ng/ml (P<0.01) and by CDMP-2 at 50 ng/ml (P<0.01), but a significant stimulatory effect was detected at a lower dose of CDMP-2 (10 ng/ml) combined with TGF-β1 (20 ng/ml) (P<0.01).
Meniscal injury is one of the most common injuries to the knee. The meniscus is essential for normal knee function, and loss of the meniscus increases the risk of subsequent development of degenerative alterations in the knee. Tissue engineering may offer new methods for the regeneration of meniscus lesions or for the complete replacement of a degenerated (part of total) meniscus by the production of newly synthesizing meniscal tissue. Tissue engineering is based on a smart and unique combination of exogenous cells, specific stimuli and matrix scaffold in an in vitro or in vivo environment (17). In terms of cell sources, several cell sources have been used for meniscus repair, including meniscus fibrochondrocytes, chondrocytes and bone marrow-derived mesenchymal progenitor cells (18).

Myoblasts are satellite cells that proliferate near the muscle fibers. Myoblasts have been considered a candidate cell source for tissue engineering and regenerative medicine by virtue of their potential to differentiate into chondrocytes, fibroblasts, osteoblasts and other tissues of mesenchymal origin (19,20). Compared to the above-mentioned cell sources, myoblasts represent a more promising source for cartilage engineering, as they are relatively abundant and easily accessible with minimal donor site morbidity (21). At the same time, myoblasts further promote the development of tissue engineering, as they have higher cell yield and a more rapid proliferative ability during in vitro expansion (22).
In the present study, we demonstrated that expanded canine myoblasts seeded within conical polypropylene tubes can be efficiently induced to undergo chondrogenic differentiation when treated with CDMP-2. The in vitro chondrogenic differentiation of myoblasts requires the complex involvement of growth factors and cell-cell and cell-matrix interactions, similar to developmental chondrogenesis in vivo. Expression of members of the TGF-β superfamily of growth factors has been localized to sites of bone repair as well as to sites of embryonic bone and cartilage formation in vivo (23). Furthermore, the chondroinductive effects of the TGF-β superfamily members, particularly the bone morphogenetic proteins (BMPs) and the TGF-βs, have been well established in embryonic and adult mesenchymal cells (24).

Recent reports have demonstrated the critical role of intracellular signaling cascades activated by the TGF-β family members in promoting cartilage-specific gene expression, including the mitogen-activated protein (MAP) kinases, whose major subtypes include p38, extracellular signal-regulated kinase-1 (ERK-1) and c-Jun N-terminal kinase (JNK or stress-activated protein kinase). To date, several growth factors have been demonstrated to enhance or induce various stem cells to become chondrocytes in vitro. However, BMPs play an important role in the development of embryonic skeleton and growth and retention of adult skeleton (25). CDMP-2 is a member of the BMP family. CDMP-2 is essential for chondrogenesis. CDMP-2 initiates limb mesenchymal cells, periosteum-derived cells and ATDC5 (a mouse clonal teratocarcinoma chondrogenic cell line) to differentiate into chondrocytes in vitro and even cartilage-like structures in vivo. Moreover it enhances GAG production of chondrocytes (26).

In the present study, we evaluated the effects of CDMP-2 alone and in combination with TGF-β1 on the chondrogenesis of myoblasts using a high-density pellet culture system in vitro. The results demonstrated that high concentrations of CDMP-2 (50 ng/ml) alone stimulated the chondrogenesis of myoblasts, as did CDMP-2 together with TGF-β1. The chondrogenic effect on myoblasts was the strongest in the pellets cultured with CDMP-2 (50 ng/ml) and TGF-β1 (20 ng/ml).

TGF-β1 is a key factor in the regulation of cellular differentiation in cartilage formation. TGF-β1 was found to be highly expressed in embryonic cartilage. It has been reported that TGF-β1 stimulated collagen II and GAG expression of periosteum-derived cells in a dose-dependent manner (27). Repairing of partial thickness articular defects in vivo was enhanced by TGF-β1 treatment that induced mesenchymal cell recruitment from the synovium (28). Our results showed that low doses of TGF-β1 alone were not sufficient to promote myoblast chondrogenesis. When tested at doses of TGF-β1 (10, 20, 30 and 50 ng/ml), we observed that higher doses (30 and 50 ng/ml) sufficiently initiated myoblast differentiation into chondrocytes. However, the chondrogenic effects of 50 ng/ml TGF-β1 showed a significant difference when compared to combined CDMP-2 (50 ng/ml) and TGF-β1 (20 ng/ml). These data indicate that TGF-β1 has weaker chondrogenic effects on myoblasts than CDMP-2. We provide evidence that CDMP-2 in combination with TGF-β1 synergistically promotes chondrogenesis of myoblasts.

In conclusion, in the present study we demonstrated that higher doses of CDMP-2 or TGF-β1 stimulated myoblast differentiation into chondrocytes. Low doses of CDMP-2 with TGF-β1 showed a marked chondrogenic effect on myoblasts. Therefore, combination of CDMP-2 and TGF-β1 may be beneficial for the treatment of cartilage lesions using myoblasts.

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