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ORIGINAL ARTICLE

EFFECT OF EXOGENOUS PROTEOGLYCANS ON CHONDROGENESIS OF BONE MARROW MESENCHYMAL STEM CELLS

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Abstract Proteoglycans are one of the most important components of the extracellular matrix in the cartilage and the levels of proteoglycans, such as versican and aggrecan, increase during chondrogenesis. The purpose of this study was to investigate the effect of exogenous proteoglycans from salmon nasal cartilage on chondrogenesis of mesenchymal stem cells. Mesenchymal stem cells derived from bone marrow aspiration of rabbit femurs were induced to chondrogenic lineage using a pellet culture technique. Pellets were cultured in the medium with or without cell growth factors, with or without proteoglycans, or a combination of these agents. Pellets treated with cell growth factors became hypertrophic and showed lacuna formation, and synthesis of cartilage matrix was recognized histologically. The expression of type II collagen and aggrecan mRNA were decreased in pellets incubated with a combination of cell growth factors and proteoglycans, compared to those incubated with only cell growth factors. Exogenous proteoglycans may down-regulate the expression of cartilage-specific mRNA directly, or may interact with growth factors in the culture medium. As the increase of glycoprotein during chondrogenesis is important for determining the direction and degree of differentiation, exogenous proteoglycans may have a similar effect.

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Key words: mesenchymal stem cells; proteoglycan; chondrogenesis.

原 著

外因性プロテオグリカンが骨髄由来間葉系幹細胞の軟骨分化に与える影響

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抄録 間葉系幹細胞の軟骨分化過程においてバーシカン，アグリカンといったプロテオグリカン（PG）が発現することは知られている．本研究では，外因性サケ鼻軟骨由来PGを間葉系幹細胞のペレットカルチャーに添加し，組織学的およびmRNA発現について検討を行った．その結果，成長因子（GF）単独およびGFとPGの両方を添加した2群において，細胞の形態が軟骨様に変化し，組織学的に細胞外マトリックスの産生を認めた．軟骨特異的遺伝子である2型コラーゲンおよびアグリカンのmRNA発現は，コントロール群およびPG群ではほとんど発現を認めないが，GF群では高度な発現を認めた．しかしGFとPGを併用するとGF単独群に比してその発現は有意に減弱した．PGの直接的な作用により遺伝子発現が抑制されたか，PGとGFの相互作用による影響と考えられた．軟骨分化の過程で発現する糖タンパク質は，分化の方向性や程度を決定する重要な因子であると考えられ，外因性のPGにも同様の効果があるものと考えられる．

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Introduction

Articular cartilage comprises an extracellular matrix consisting of proteoglycans (PGs), collagens, and water. Interaction between PGs and collagen provides unique structural and physiological properties for cartilage to function in weight bearing and joint motion. PGs are major and important components in articular cartilage.

It is also considered that PGs play an important role in chondrogenesis. PGs such as versican and aggrecan, which contain chondroitin sulfate chains, are known to increase during chondrogenesis.[1] A previous study has shown that chondrogenesis can be regulated by interactions between matrix substrate molecules.[2] It has also been demonstrated that heparan sulfate PG enhances the ability of growth factors to induce mesenchymal stem cells (MSCs) to undergo chondrogenesis in pellet culture.[3]

MSCs have the ability of differentiating into cells of numerous tissue lineages, such as bone, fat, cartilage, muscle tissue, and neurons.[4-6] In addition to their multipotency, MSCs can be acquired by bone marrow aspiration without permanently damaging tissues, efficiently expanded in monolayers by serial passages without altering their differentiation potential, and may be a suitable autogenous cell source for regenerative medicine.

Chondrogenesis of MSCs begins with the migration of MSCs during skeletal tissue development to sites destined to become the long bones. The MSCs then undergo condensation, proliferation, and chondrogenic differentiation and hypertrophy.[7] Pellet culture of MSCs, which simulates condensation, is a key technique for inducing the differentiation of multipotent MSCs into the chondrogenic lineage.[8] Although this approach has been shown to induce chondrogenic differentiation of MSCs to some extent, its effects are limited, and stimulation with cell growth factors is necessary for sufficient differentiation into the chondrogenic lineage.[9-16]

The purpose of this study was to investigate the effect of exogenous PGs on chondrogenic differentiation of MSCs, and to clarify that PGs augment chondrogenesis acceleration instead of cell growth factors. If PGs have ability to augment chondrogenesis, they will be a useful material for regenerative medicine of articular cartilage.

Methods

All animal experiments in this study were performed in accordance with the Guidelines for Animal Experimentation of Hirosaki University.

Isolation and culture of MSCs

Skeletally mature female Japanese White rabbits were used for this study. Under general anesthesia, bone marrow aspirates were taken from the femurs to isolate MSCs. Nucleated cells were isolated using a density gradient (Histopaque®, Sigma-Aldrich) and resuspended in complete culture medium consisting of αMEM (Invitrogen), 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen), 250 ng/ml amphotericin B (ICN Biomedicals), and 2 mM L-glutamine, and incubated at 37℃ in a humidified atmosphere containing 95% air and 5% CO_2. After 24 h, non-adherent cells were discarded, and adherent cells were thoroughly washed twice with phosphate-buffered saline. The confluent cells were transferred to the next passage using 0.25% trypsin and 1 mM EDTA (Invitrogen) for up to four passages.

Preparation of PGs

Extraction and purification of PGs from salmon nasal cartilage were performed by the method as described previously.[17]

Table 1 Description of the primers and probes

Gene	Access no.	Primer 5' → 3'	Probe 5' → 3'	PCR Product (bp)
GAPDH	L23961	F: CGACCACTTCGGCATTGTG R: CCCGTCCACGGTCTTCTG	CCACGGTGCACGCCAT	76
Aggrecan	L38480	F: CTGCAGGCTGCCTACGA R: CAGTCTGATCAGCCAGCCA	CCACCAGTGCGACGCC	64
Collagen type I	D49399	F: CCGCGACCTGAGACTCA R: GCATCCATAGTGCATCCTTGGTT	CCACTGCTCCACTCTGG	81
Collagen type II	S83370	F: CCCCACGCCCACTCG R: CCCAGCTTTTGTTTTGCAGTCT	CCCAGTTCAGGTCTCTTG	67
Collagen type X	AF247705	F: CCAGGACCTCCAGGACTATCAG R: TCCTCTCTCCCCTTGTTTTCCT	CTGGCAAACCTGGCTTC	75

Pellet culture

A pellet culture system was used for chondrogenic differentiation.[8] Approximately 2.5 $\times 10^6$ MSCs were placed in a 15-ml polypropylene tube and centrifuged at 450 x g for 10 min. The pellets were cultured in 500 μl chondrogenic medium that contained 10^{-7} M dexamethasone in high-glucose DMEM supplemented with 6.25 μg/ml insulin, 6.25 μg/ml transferrin, and 6.25 ng/ml selenious acid (ITS supplement; Invitrogen), then 50 μg/ml ascorbate-2-phosphate, 40 μg/ml proline (Wako), and 100 μg/ml pyruvate were also added. The pellets were cultured with or without 500 ng/ml recombinant human bone morphogenetic protein-2 (BMP-2; R&D Systems) and 10 ng/ml recombinant human transforming growth factor-β3 (TGF-β3; R&D Systems), with or without 100 μg/ml PGs from salmon nasal cartilage, or a combination of these agents. The pelleted cells were incubated at 37℃ with 5% CO_2, and the medium was replaced every 3-4 days for 21 days.

Histology

After 21 days of culture, the pellets were harvested and fixed in 10% buffered formalin overnight at room temperature. The fixed pellets were embedded in paraffin, cut into 5-μm-thick sections, and stained with hematoxylin-eosin (H-E) and alcian blue.

RNA isolation and real-time polymerase chain reaction (real-time PCR)

Ten pellets were mixed together and digested with 3 mg/ml collagenase, and 1 mg/ml hyaluronidase (Sigma) for about 3 h at 37℃ to remove matrix proteins. Total RNA was extracted by using an RNeasy Micro Kit (Qiagen) and cDNA was synthesized by reverse transcription. Gene expression levels of GAPDH, type I/II/X collagen and aggrecan in cultured MSCs were quantified by real-time PCR (Applied Biosystems). Primer and probe sequences (Applied Biosystems) used in the PCR amplifications are listed in Table 1. The reactions were heated at 50℃ for 2 min and 95℃ for 10 min followed by 40 cycles of 95℃ for 15 s and 60 ℃ for 1 min using an ABI Prism 7000 Sequence Detection System. For each primer/probe set, a C_t value was determined as the cycle number at which the fluorescence intensity reached a specific value in the middle of the exponential region of amplification. Relative gene expression levels were determined by employing the comparative C_t method (ABI User Bulletin 2), and standardizing the levels to that of *Gapdh*.

Statistical analysis

Analyses were performed using SPSS software version 12.0. Scheffe's test was used for statistical analysis, and differences at $p < 0.05$ were taken as significant.

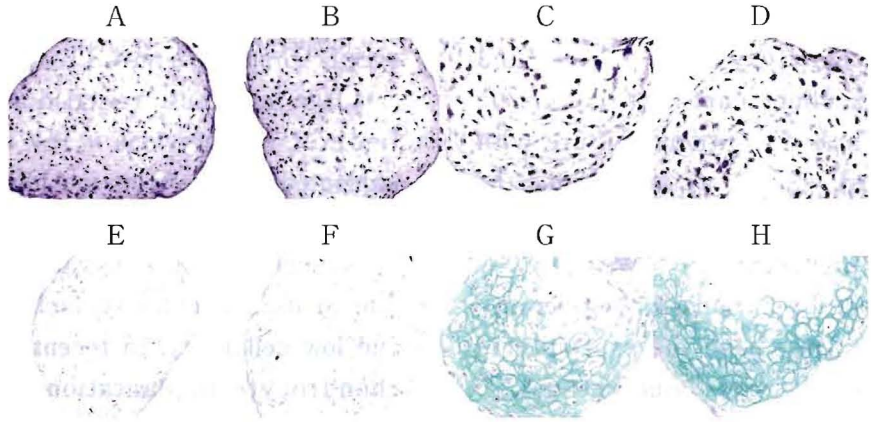

Fig. 1 Histological examination. Cells were cultured under the following conditions and stained with H-E (A-D) or alcian blue (E-H). Control (0 ng/ml BMP-2 and TGF-β3, without PG; A, E), only PG (0 ng/ml BMP-2 and TGF-β3, with 100 μg/ml PG; B, F), only growth factors (500 ng/ml BMP-2 and 10 ng/ml TGF-β3, without PG; C, G), PG and growth factors (500 ng/ml BMP-2 and 10 ng/ml TGF-β3, with PG; D, H). ×400.

Results

MSCs made small, spherical form 24 h after pellets were centrifuged. The histological sections (Fig. 1) showed a chondrocyte-like cell morphology only in cells that had been induced with BMP-2 and TGF-β3. Pellets treated with only PGs showed small and low cell counts, as in the non-treated controls. In the histological sections of MSCs treated with cell growth factors, hypertrophic cells and lacuna formation were observed, and a cartilage matrix was synthesized, as demonstrated by the appearance of PGs stained with alcian blue. There was no difference in staining between pellets that had been incubated with and without PG treatment.

The expression of type II collagen and aggrecan mRNA were decreased in pellets incubated with growth factors and PGs compared to those incubated with growth factors in the absence of PGs (Fig. 2). There was no difference in the expression of type I and type X collagen mRNA between pellets incubated with or without PGs.

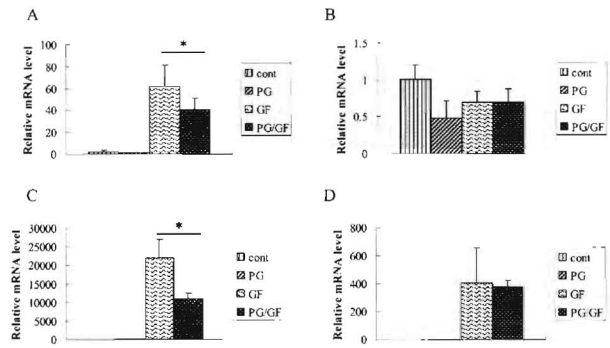

Fig. 2 Quantification of gene expression in MSCs pellet-cultured for 21 days in medium with or without cell growth factors, with or without proteoglycans, or a combination of these agents. Aggrecan (A), type I collagen (B), type II collagen (C), and type X collagen (D). *$p < 0.05$.

Discussion

PGs are one of the most important components of the extracellular matrix. They are composed of glycosaminoglycan chains bonded to a backbone protein molecule known as a core protein. The predominant PG in articular cartilage is chondroitin sulfate, which serves a direct, primary role providing the osmotic resistance necessary for cartilage to withstand compressive loads. In addition, increased PG synthesis is associated with matrix assembly and cartilage formation. It has been reported that chondrogenesis can be regulated by interactions between matrix substrate molecules, such as PGs, and receptors on the cell surface.[3] Our previous study has

also demonstrated that exogenous PGs increased the number of chondrocytes in culture, and histologically excellent cartilage-like tissue was formed in three-dimensional culture with PGs.[18] In the current study, we investigated the effect of PGs from salmon nasal cartilage on chondrogenic differentiation.

MSCs are an ideal cell source for regenerative medicine because they have the capability to differentiate into numerous tissue lineages.[4-6] They can be also derived from bone marrow without permanently damaging tissues, and have high proliferative ability. Therefore, we used MSCs obtained by bone marrow aspiration, and these were induced to differentiate into chondrogenic lineage using a pellet culture system.[8]

In this study, we investigated whether exogenous PGs from salmon nasal cartilage accelerated the chondrogenesis of MSCs. Growth factors such as BMP-2 and TGF-β3 are known to induce MSCs to undergo chondrogenesis, but it is unclear whether this process is accelerated by PGs. From the results of this study, it was clearly evident that chondrogenic differentiation of MSCs depended upon cell growth factors and did not require PGs. Addition of exogenous cartilage PGs to cell growth factors inhibited the expression of type II collagen and aggrecan mRNA in MSC culture, but had no effect on differentiation by the histological examination. One reason may be that PGs work to downregulate cartilage-specific mRNAs in the presence of exogenous PGs. Another reason may be that PGs interact with growth factors in the culture medium.

There were several limitations to this study. First, only a single time period was evaluated. This choice was based on previous studies investigating the time course of the pellet culture of MSCs using other treatment protocols.[8,9,13] Second, protein expression levels were not assessed. Thus, future studies should re-evaluate the expression levels of cartilage-specific proteins. Third, PGs from salmon nasal cartilage may cause rejection as foreign protein. However, our previous *in vivo* study where we implanted the scaffolds including PGs to rabbit knees showed no rejection.

Articular cartilage has poor healing potential due to its avascularity, lack of nerve supply, and low cellularity. In recent years, autologous chondrocyte implantation (ACI) has been shown to be feasible for patients with localized articular cartilage defects.[19] However, several problems remain. The regenerative tissue from ACI is a fibrous cartilage which is mechanically and biologically inferior in quality compared to hyaline cartilage.[20] Also, such cells are limited in terms of both their source and proliferation capacity. The best approach for repair of injured cartilage is a topic of current interest, and further studies are needed to investigate the application of PGs for this purpose, and how PGs induce MSCs to undergo chondrogenesis.

In conclusion, while PGs had no effect on chondrogenesis of MSCs by histological examination, PGs inhibited the expression of cartilage-specific mRNA in MSC culture. We found that PGs affect MSCs at some level, and further studies are needed to clarify this mechanism of function.

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