Effect of bone marrow-derived stem cells on chondrocytes from patients with osteoarthritis

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Abstract. Increasing numbers of individuals are suffering from osteoarthritis every year, and the directed intra-articular injection of bone marrow stem cells has provided a promising treatment strategy for osteoarthritis. Although a number of studies have demonstrated that intra-articular injection of bone marrow stem cells produced desirable results, the mechanism underlying this effect has not been elucidated. In the current study, the effect of bone marrow stem cells on chondrocytes from patients with osteoarthritis was observed in a co-culture system. Human chondrocytes were obtained from patients with osteoarthritis who underwent surgical procedures and bone marrow stem cells were obtained from bone marrow aspirates, and then the chondrocytes were then cultured alone or cocultured with bone marrow stem cells in 0.4-μm Transwell inserts. The differentiation and biological activity of chondrocytes in the culture system were measured, and the inflammatory factors and OA-associated markers were also measured. The results indicated that coculture with human bone marrow stem cells increases cell proliferation of chondrocytes and inhibits inflammatory activity in osteoarthritis.

Introduction

Osteoarthritis (OA) is a chronic age-related disease, it affects the majority of the adult population, and is characterized by the slowly progressive destruction of articular cartilage, and degeneration of ligaments and menisci, as well as hypertrophy of the joint capsule (1,2). It is reported that changes in OA include decreased expression of chondrogenic markers [Aggrecan, collagen type II α1 (COL2A1) and SOX9], and enhanced expression of certain hypertrophic [matrix metallopeptidase (MMP13) and alkaline phosphatase] and fibroblastic [collagen I and collagen II (Col I, II and III, respectively)] markers (3,4).

Current therapies for OA include a number of noninvasive (drug treatment and physical therapy) and invasive therapies (drilling, debridement, osteochondral transplantation, autologous perichondral and periosteal grafts, and autologous chondrocyte implantation) to relieve the symptoms (5,6). Recently, stem cell based cell therapy was observed to provide a promising approach to OA treatment (7). Bone marrow-derived mesenchymal stem cells (BMSCs), which can be isolated from the bone marrow aspirate, and have multipotent differentiation potential (could differentiate into numerous tissues, such as bone, cartilage and fat), self-renewal capacity and immunomodulatory properties, has great potential for use in stem cell-based articular cartilage diseases (8).

Recent observations have shown that BMSCs also have shown desirable effects in the treatment of OA, probably via the secretion of bioactive trophic factors to exert potent anti-inflammatory, immunomodulatory, and antifibrotic effects (9,10). Emadedin *et al* (11) reported that intra-articular injection of autologous bone marrow mesenchymal stem cells in patients with OA of the knee did not result in local or systemic adverse events after a one-year follow-up period. In addition, all patients were partly satisfied with the results of the study, and magnetic resonance images (MRI) at baseline and six months post-stem cell injection displayed an increase in cartilage thickness, extension of the repair tissue over the subchondral bone and a considerable decrease in the size of edematous subchondral patches in three out of six patients. In another case, Buda *et al* (12) demonstrated that in a one-step arthroscopic technique for the treatment of osteochondral lesions of the knee with bone-marrow-derived cells, the result of clinical inspection and MRI demonstrated that the mean international knee documentation committee score prior to surgery was 29.9±13.2 and was 85.4±4.2 at 29±4.1 months ($P<0.0005$), while the knee injury and osteoarthritis outcome score before surgery was 35.1±11.9 and was 87.3±7.3 at 29±4.1 months ($P<0.0005$). Control MRI and biopsy samples showed osteochondral regeneration of the lesion site. Though the desired result of directed intra-articular injection of bone marrow stem cells in the treatment of OA diseases was observed, the mechanism underlying this effect has not been reported. Therefore, in the current study, the potent anti-inflammatory,

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immunomodulatory, and antifibrotic effects of BMSCs on chondrocytes in OA in a coculture system were explored, as well as the proliferation of chondrocytes following coculture with BMSCs, in order to evaluate the potential application of BMSCs in the treatment of OA.

Materials and methods

This study was approved by the ethics committee of the Southern Medical University (Nanjing, China) and informed consent was obtained from all patients.

Cell isolation and culture. BMSCs were harvested from patients who underwent bone marrow aspiration. BMSCs were isolated by density-gradient centrifugation at 500 x g for 5 min, resuspended and cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum (Hyclone, Logan, UT, USA) in 100-mm tissue culture flasks at 37˚C in a 5% CO_2 humidified incubator. After 24 h, the non-adherent cells were removed. After 10-14 days, adherent cells were trypsinized and sub-cultured.

Cartilage was harvested from patients with OA who underwent knee surgery, and chondrocytes were isolated and expanded as previously described (13). BMSCs and chondrocytes at passage 1 were used in this study.

Groupings. The chondrocytes were cultured in the six well plate, and BMSCs were seeded in the Transwell chamber (Corning Incorporated, Corning, NY, USA), with a 0.4-μm porous membrane at the bottom to prevent cell migration. Cells were divided into the following groups: Experimental group, chondrocytes cocultured with BMSCs; and control group, chondrocytes cultured alone. In each group $1x10^4$ BMSCs or chondrocytes at passage 1 were seeded. The culture medium was replaced every 2 days.

Cell proliferation. Chondrocyte proliferation was measured using cell counting kit-8 (CCK-8, Dojindo Molecular Technologies, Kumamoto, Japan) as previously described (14). NP cells (10^3 cells; 100 μl) from the experimental and control groups were seeded into every well of the 96-well plate. At different time points (1 day, 3 days, 5 days and 7 days), 10 μl CCK-8 solution was added into each well. After another 2 h, absorbance was measured spectrophotometrically at 450 nm using a Hitachi F-4500 fluorescence spectrophotometer (Hitachi, Ltd., Tokyo, Japan).

Protein release in the supernatant. Levels of major inflammatory proteins [interleukin (IL)-6, IL-8, CCL2/MCP-1, CCL3/MIP-1α, CCL5/RANTES, CXCL1/GROα], thrombospondin-1 (TSP-1) and tissue inhibitor of metalloproteinase-1 (TIMP-1) in the supernatant in each group at day 7 was quantified using ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The test was performed in triplicate.

Differentiation characteristics of chondrocytes. Relative expression of cyclooygenase 2 (COX-2), prostaglandin E2 (PGE2), tumor necrosis factor (TNF)-α, IL-1β, IL-6, and IL-8 was measured in the two groups at day 7 to evaluate the anti-inflammatory effects of BMSCs on OA chondrocytes. Relative expression of type I collagen, type III collagen, MMP13, SOX-9, Aggrecan and type II collagen was measured in two groups at day 7 to evaluate differentiation characteristics of chondrocytes. RNA was extracted from chondrocytes in the two groups according to previously described methods (15), and reverse transcribed into cDNA according to the manufacturer's instructions. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to evaluate the difference in gene expression in the two groups.

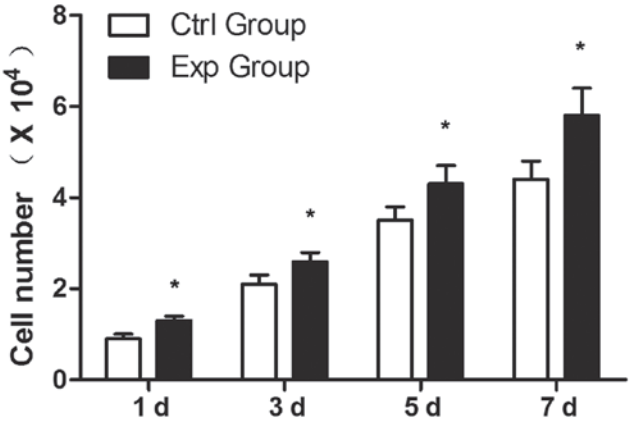

Figure 1. Proliferation of chondrocytes from patients with osteoarthritis. The chondrocytes proliferated gradually in the 2 groups with the increased culture time, and there was a significant difference between the 2 groups at different time points (*P<0.05).

RT-qPCR. RT-qPCR was performed in a final reaction volume of 20 μl containing 10 μl of 2X SYBR Green PCR Universal Master mix (Applied Biosystems, Warrington, UK), 300 nM of resuspended reference gene primer mix, 5 μl of diluted cDNA and 4 μl of RNase/DNase-free water. The primer sequences are shown in Table I. The thermal cycling conditions for RT-qPCR were as follows: 95˚C for 10 min followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. All reactions were performed in duplicate using an ABI PRISM® 7000 real-time PCR system (Applied Biosystems).

Statistical analysis. Differences in cell proliferation, gene expression and protein levels in each group were analyzed by one-way analysis of variance. Data were analyzed using SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Cell proliferation. The cell yields in the two groups showed an increase in cell number with the increase in culture time (Fig. 1). There was a significant difference between the two groups at different time points, and greater cell proliferation in the experimental group than that in the control group (P<0.05).

Release of inflammatory protein in the supernatant. It has been demonstrated that chondrocytes secrete various inflammatory proteins in OA (16); thus, the release of inflammatory protein in the supernatant in two groups was

Table I. Primer sequences for real-time polymerase chain reaction.

Gene symbol	Primer	Product (bp)
COX-2		382
Forward	5'-ATAACCCCGCCAAAAGGGG-3'	
Reverse	5'-AGGAACAGCATGCAGGTAGC-3'	
PGE-2		116
Forward	5'-GTCGTGTACCTGTCCAAGCA-3'	
Reverse	5'-GCGCTGGCGATGAACAAC-3'	
IL-1β		177
Forward	5'-CTGTCCTGCGTGTTGAAAG-3'	
Reverse	5'-TGCTTGAGAGGTGCTGATG-3'	
IL-6		184
Forward	5'-TAGTGAGGAACAAGCCAGAG-3'	
Reverse	5'-GCGCAGAATGAGATGAGTTG-3'	
IL-8		153
Forward	5'-CCAAACCTTTCCACCC-3'	
Reverse	5'-ACTTCTCCACAACCCT-3'	
Sox-9		281
Forward	5'-AGGTGCTCAAAGGCTACGAC-3'	
Reverse	5'-GGCATTCCCTGAAGACCTGG-3'	
COL2α1		373
Forward	5'-CGAAAGGTCAGACGGGTGAA-3'	
Reverse	5'-GGCATTCCCTGAAGACCTGG-3'	
Aggrecan		316
Forward	5'-ACCTCACCATGCCTTCACTG-3'	
Reverse	5'-GCTCTCACCTTTCACCACGA-3'	
COL1α2		70
Forward	5'-TTCTCTAGAACTTTGCTGCTCA-3'	
Reverse	5'-AAGCATATCATTGGTCCAGGG-3'	
COL3α1		354
Forward	5'-CGCCCTCCTAATGGTCAAGG-3'	
Reverse	5'-AGGGCCTGAAGGACCAGCTT-3'	
MMP13		198
Forward	5'-GACTTCCCAGGAATTGGTGA-3'	
Reverse	5'-TACCCCAAATGCTCTTCAGG-3'	
GAPDH		353
Forward	5'-CCACATCGCTGAGACACCAT-3'	
Reverse	5'-AAATGAGCCCCAGCCTTCTC-3'	

COX-2, cyclooxygenase-2; PGE-2, prostaglandin E2; IL, interleukin; COL, collagen; MMP, matrix metallopeptidase.

analyzed in the present study. The coculture system showed the inhibitory effect of inflammatory activity-related protein secretion. The results of ELISA demonstrated that the levels of inflammatory protein, such as IL-6, IL-8, CCL2/MCP-1, CCL3/MIP-1α, CCL5/RANTES and CXCL1/GROα, decreased in the coculture system, which indicated that BMSCs exerted anti-inflammatory effects.

The concentration of IL-6, IL-8, CCL2/MCP-1, CCL3/MIP-1α, CCL5/RANTES and CXCL1/GROα were measured (Fig. 2A-F). Production of IL-6 (9.00 ng/ml), IL-8 (4.10 ng/ml), CCL2/MCP-1 (4.50 ng/ml), CCL3/MIP-1α (3.6 ng/ml), CCL5/RANTES (25.60 ng/ml) and CXCL1/GROα (2.30 ng/ml) in the experimental group were significantly reduced compared with the production of IL-6 (3.50 ng/ml), IL-8 (1.90 ng/ml), CCL2/MCP-1 (1.10 ng/ml), CCL3/MIP-1α (1.2 ng/ml), CCL5/RANTES (75.30 ng/ml) and CXCL1/GROα (4.50 ng/ml) in the control group ($P<0.05$).

Expression of inflammatory genes in OA chondrocytes. A number of studies have reported that the expression of inflammatory genes in OA chondrocytes increased (16-18). The

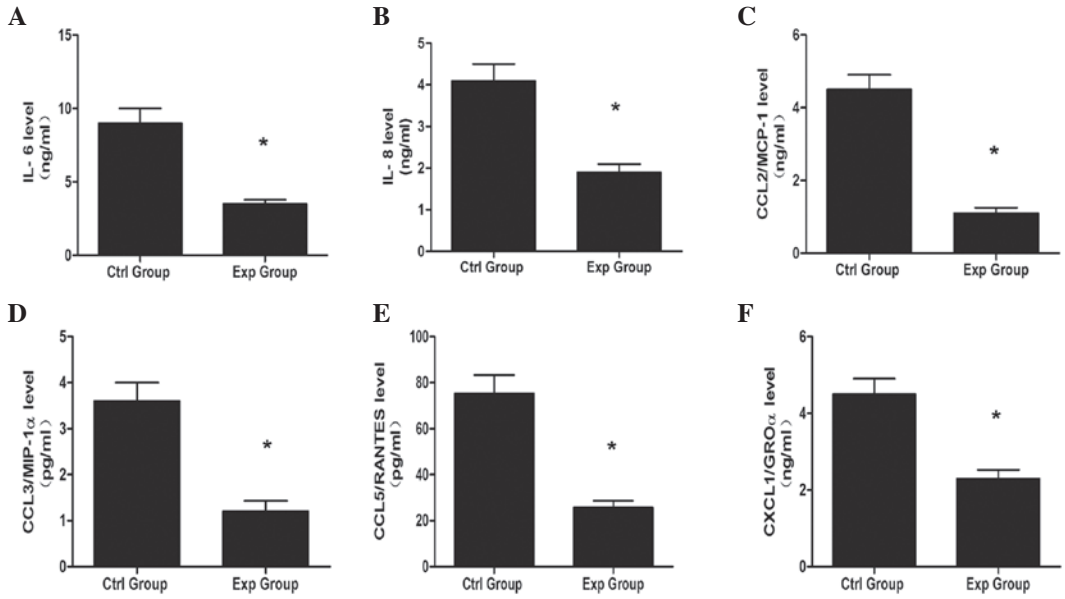

Figure 2. Level of inflammatory protein. These results showed the level of inflammatory proteins (A) IL-6, (B) IL-8, (C) CCL2/MCP-1, (D) CCL3/MIP-1α (E) CCL5/RANTES and (F) CXCL1/GROα in the experimental group is less than that in the control culture group, which indicated that BMSCs exerted the anti-inflammatory effect (*P<0.05). IL, interleukin.

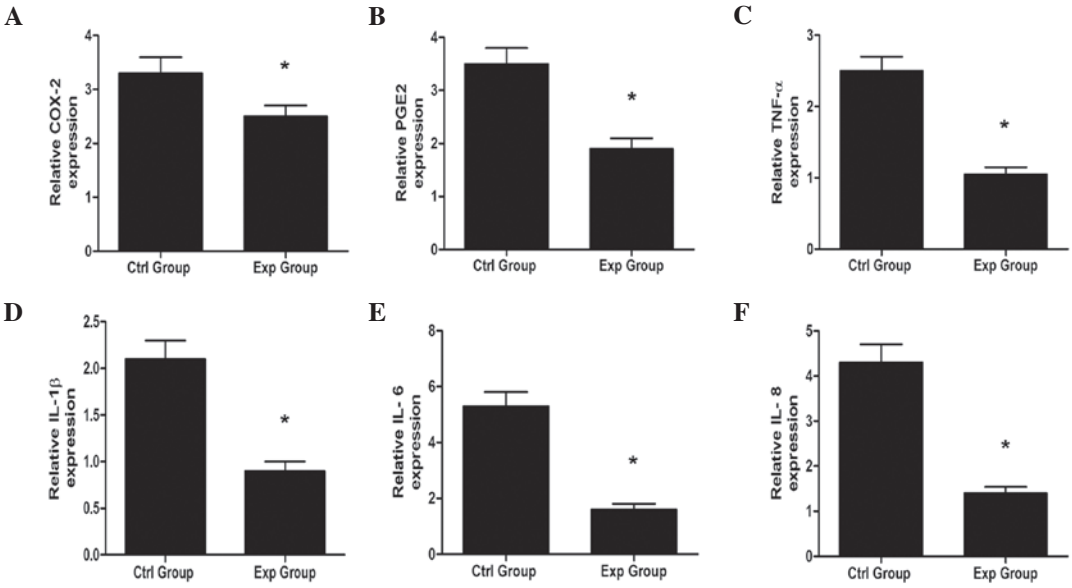

Figure 3. Expression of inflammatory genes. (A) COX-2, (B) PGE2, (C) TNF-α, (D) IL-1β, (E) IL-6 and (F) IL-8. There is a significant difference of the expression of inflammatory gene between 2 groups (*P<0.05), and the expression of inflammatory gene in the experimental group is less than that in the control group. COX-2, cyclooxygenase 2, PGE2, prostaglandin E2; TNF-α, tumor necrosis factor-α; IL, interleukin.

co-culture system notably resulted in a reduction of COX-2, PEG2, TNF-α, IL-6, IL-8 and IL-1β mRNA levels compared with the control group (Fig. 3; P<0.05).

OA chondrocyte differentiation. OA chondrocytes become fibrous and undergo hypertrophy, which was demonstrated by upregulation of fibrotic (collagen type I) and hypertrophic (osteopontin, type X collagen and matrix Gla) genes. However, the expression of fibrotic and hypertrophic genes in the experimental group decreased after coculture with BMSCs, and there were significant differences between the two groups. The chondrogenic gene (type II collagen and aggrecan) expression increased in the experimental group, while the SOX-9 expression decreased after coculture (Fig. 4). The results indicated that BMSCs showed chondroprotective and antifibrotic effects, as well as antihypertrophic effects.

Release of TIMP-1 and TSP-1 in the supernatant. TSP-1 has been reported to have the ability to upregulate TIMP-1. TIMP-1 could inhibit the vascularization of chondrocytes, which suggests that TIMP-1 has chondroprotective effects. The levels of TSP-1 and TIMP-1 were 63 and 735 pg/ml in the experimental group, while the level of TSP-1 and TIMP-1 was 42 and 598 pg/ml in the control, respectively (Fig. 5).

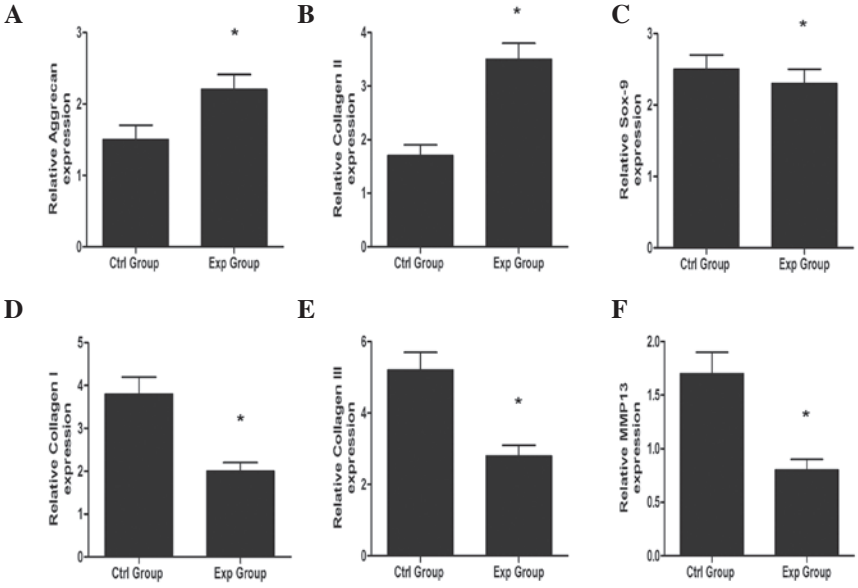

Figure 4. Differentiation of OA chondrocytes. (A) Aggrecan, (B) Collagen II, (C) SOX-9, (D) Collagen I, (E) Collagen III and (F) MMP-13. The expression of type II collagen and aggrecan the OA chondrocytes in co-culture groups is higher than that in the control group, while the gene expression of type I collagen, type III collagen and MMP13 in experimental group is less than that in the control group (*P<0.05). MP-13, matrix metallopeptidase 13; OA, osteoarthritis.

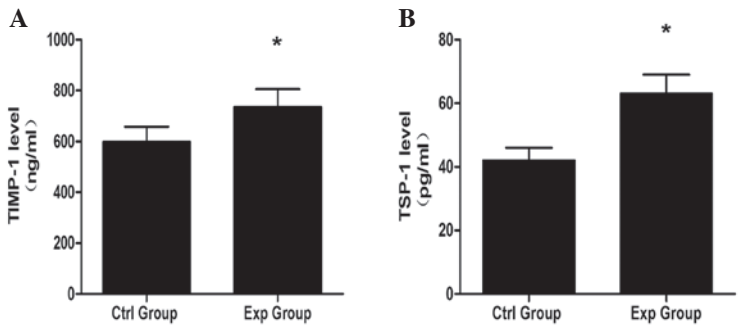

Figure 5. Level of growth factors. There is a significant difference in the concentration of protein between the 2 groups (P<0.05), and the level of (A) TIMP-1 and (B) TSP-1 in the supernatant in the experimental group is higher than that in the control group (*P<0.05). TIMP-1, tissue inhibitor of metalloproteinase-1; TSP-1, thrombospondin-1.

Discussion

Osteoarthritis (OA) is also termed chronic degenerative arthritis, degenerative joint disease or osteoarthrosis, and is characterized by progressive cartilage degeneration, subchondral bone impairment, the narrowing of the joint space, marginal osteophytosis, as well as loss of joint function (19). The common symptoms of osteoarthritis included joint pain, stiffness and a degree of loss of joint motion (20). It is reported that the inflammation is involved in the occurrence of OA, which promotes the ongoing joint degeneration (21). Recent studies have demonstrated that BMSCs have the potential for application in the treatment of OA as BMSCs have chondrogenic differentiation potential, and are also involved in the immunoregulation and tissue repair/regeneration by the secretion of various soluble factors. Firstly, BMSCs exhibit multilineage differentiation capacity and can develop into various cell types, including chondrocytes, osteoblasts and adipocytes (22). Secondly, BMSCs secrete a number of cytokines, such as hepatocyte growth factor, insulin-like growth factor-1, epidermal growth factor, keratinocyte growth factor, angiopoietin-1 and stromal derived factor-1, which possess a wide range of biological effects in the repair and regeneration of tissue (23). Therefore, in the present study the effect of BMSCs on chondrocytes from OA tissues was investigated, and the results indicated chondroprotective and antifibrotic effects, as well as antihypertrophic effects on the OA chondrocytes in a coculture system.

At first, the OA chondrocyte proliferation rate was analyzed in culture alone or coculture with BMSCs, and the results indicated that BMSCs significantly improved the cell proliferation rate, when compared with chondrocyte culture alone. It has been reported that BMSCs secrete different types of growth factors, including basic fibroblast growth factor (FGF-2), insulin-like growth factor 1 (IGF-1) and hepatocyte growth factor (HGF) (24). Among them, FGF-2 and IGF-1 have the capability to promote cell proliferation via the PI3-K pathway dependent signal pathway (25). Umeda *et al* (26) showed that BMSCs are more effective for increasing the proliferative capacity of nucleus pulposus cells via activation of rat nucleus pulposus cells by coculture with BMSCs. In the present study, the results of the CCK-8 assay confirmed the effects of BMSCs promoting chondrocyte cell proliferation.

The anti-inflammatory action of BMSCs on OA chondrocytes was investigated. It is well known that chondrocytes from patients with OA secrete various inflammatory cytokines and express inflammation activity-related genes (16). It has also been shown that certain OA inflammatory factors, such as IL-6, IL-8 and CXCL1/GROα, were involved in the progression of OA (27). The level of the inflammatory factors in the chondrocyte culture alone group and the coculture group were analyzed, a significant decrease in the release of inflammatory factors in the supernatant was observed after coculture with BMSCs, which indicated that BMSCs were anti-inflammatory in OA. BMSCs may inhibit macrophage activity and thereby suppress the production of catabolic mediators, such as IL-6, IL-8 and CXCL1/GROα (16,28). The mRNA expression of the main OA inflammatory factors, such as COX-2, TGF-α and PEG2, were also measured to evaluate the anti-inflammatory effect. An increase in the gene expression level of COX-2, TNF-α and PEG2 were observed during inflammatory and catabolic processes. The results showed that the mRNA expression of the predominant OA inflammatory factors in the coculture group was less than that in the chondrocyte culture alone group.

In addition, the differentiation of chondrocytes was also investigated in the coculture system. It is reported that chondrocytes lost their original phenotype during OA progression, whereby the chondrocytes become ossified and vascularized (29). The results of the present study showed that the expression of aggrecan and collagen II increased after coculture with BMSCs, while the expression of SOX-9 decreased. Furthermore, the expression of hypertrophic (MMP13) and fibroblastic (Collagen I and III) markers of chondrocytes co-cultured with ASCs decreased, which demonstrated that BMSCs exerted an antifibrotic and antihypertrophic effect on the OA chondrocytes. TSP-1 and TIMP-1 may be important in the antifibrotic and antihypertrophic process, and it was demonstrated that the secretion of TSP-1 and TIMP-1 increased in the coculture system.

In conclusion, co-culture with human BMSCs inhibits inflammatory activity and increases cell proliferation of OA chondrocytes, as well as exhibiting an antifibrotic and antihypertrophic effect, which may occur via the secretion of various growth factors and cytokines from BMSCs.

Acknowledgements

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