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Co-culture with human synovium-derived mesenchymal stem cells inhibits inflammatory activity and increases cell proliferation of sodium nitroprusside-stimulated chondrocytes

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ABSTRACT

Rheumatoid arthritis (RA) and osteoarthritis (OA) are primarily chronic inflammatory diseases. Mesenchymal stem cells (MSCs) have the ability to differentiate into cells of the mesodermal lineage, and to regulate immunomodulatory activity. Specifically, MSCs have been shown to secrete insulin-like growth factor 1 (IGF-1). The purpose of the present study was to examine the inhibitory effects on inflammatory activity from a co-culture of human synovium-derived mesenchymal stem cells (hSDMSCs) and sodium nitroprusside (SNP)-stimulated chondrocytes. First, chondrocytes were treated with SNP to generate an *in vitro* model of RA or OA. Next, the co-culture of hSDMSCs with SNP-stimulated chondrocytes reduced inflammatory cytokine secretion, inhibited expression of inflammation activity-related genes, generated IGF-1 secretion, and increased the chondrocyte proliferation rate. To evaluate the effect of IGF-1 on inhibition of inflammation, chondrocytes pre-treated with IGF-1 were treated with SNP, and then the production of inflammatory cytokines was analyzed. Treatment with IGF-1 was shown to significantly reduce inflammatory cytokine secretion in SNP-stimulated chondrocytes. Our results suggest that hSDMSCs offer a new strategy to promote cell-based cartilage regeneration in RA or OA.

1. Introduction

Excessive inflammatory activation can induce the production of several types of pro-inflammatory enzymes, cytokines, and chemokines that may lead to chronic inflammatory diseases such as rheumatoid arthritis (RA) and osteoarthritis (OA) [1,2]. Pro-inflammatory enzymes include inducible forms of nitric oxide synthase (iNOS) that catalyze the production of nitric oxide (NO) and mediate inflammation and autoimmune diseases [3,4]. NO activates cyclooxygenase-2 (COX-2), resulting in the increased release of pro-inflammatory prostaglandins associated with inflammation and several joint diseases [5,6]. Pro-inflammatory cytokines, including interleukin (IL)-1β, IL-6, tumor necrosis factor α (TNF-α), chemokine (C-C motif) ligand 2 (CCL2)/monocyte chemotactic protein-1 (MCP-1), and CCL3/macrophage inflammatory protein-1α (MIP-1α), are primarily introduced during inflammatory activation [7].

Mesenchymal stem cells (MSCs), also referred to as multipotent stromal progenitor cells, have been differentiated to cells of the mesodermal linage such as, chondrocytes, osteoblasts, and adipocytes [8,9]. Moreover, MSCs are known to regulate immunomodulatory activity such as, suppression of T and B cell proliferation and differentiation, dendritic cell maturation, and natural killer cell activity *in vitro* [10–13]. Specifically, MSCs have been shown to secrete various growth factors, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (FGF2), hepatocyte growth factor (HGF), and insulin-like growth factor 1 (IGF-1) upon stimulation [14].

In particular, IGF-1 has been reported to promote the cell proliferation and survival rate in many cell types via PI3-K pathway-dependent signaling and to influence anti-apoptotic effects through regulation of the mitochondrial cytochrome-c/caspase pathway [15–18]. In addition, it has been shown to prevent oxidative stress and reduce inflammatory responses [19–21].

In this study, we hypothesized that co-culture of human synovium-derived MSCs (hSDMSCs) and sodium nitroprusside (SNP)-stimulated chondrocytes would inhibit inflammatory activity and recover chondrocyte proliferation. Based on our results, it was determined that co-culture of hSDMSCs and SNP-stimulated chondrocytes inhibited inflammatory cytokine secretion and inflammation activity-related gene expression, while increasing the IGF-1 concentration and chondrocyte proliferation rate.

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2. Materials and methods

2.1. Isolation and culture of chondrocyte and SDMSCs

Human chondrocytes and hSDMSCs were obtained from the synovium of patients in accordance with the regulations of the Ethics Committee of Yonsei Sarang Hospital. Chondrocytes were isolated following a standardized procedure [22]. Briefly, minced articular cartilage was digested at 37 °C with 0.5% pronase (Sigma) for 1 h and 0.2% collagenase (Sigma) for 45 min in Dulbecco's modified Eagle's medium (DMEM; Hyclone) with 25 mM HEPES (Sigma) and 1× penicillin/streptomycin (Hyclone). The cells were cultured at 37 °C with 5% CO2 in complete DMEM containing 10% fetal bovine serum (FBS) (Gibco) and 1× penicillin/streptomycin (Hyclone) (Supplementary Fig. 1A and 1B).

Synovial membrane (SMs) specimens were rinsed twice with Hanks' balanced salt solution (HBSS; Gibco) supplemented with 1× penicillin/streptomycin (Hyclone). The specimens were then finely minced, and digested with 0.2% collagenase (Sigma) in high-glucose DMEM (Hyclone) containing 1× penicillin/streptomycin (Hyclone). Following 2 h incubation at 37 °C, cells were collected by centrifugation, washed twice, resuspended in high-glucose DMEM supplemented with 10% FBS (Hyclone) and 1× penicillin/streptomycin (Hyclone), and plated in a T-25 culture flask. Cells were allowed to attach for 4 days, and non-adherent cells were removed by changing the culture medium (Supplementary Fig. 1C and 1D).

2.2. MTT assay

Cells were seeded at a density of 1.5×10^5 cells/well in 24-well plates. After 16 h, cells were then treated with various concentrations of SNP (0.25–2 mM) (Sigma). Cell survival rate was determined using the established 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based assay. Each well was incubated with the MTT solution (Sigma) for 4 h, and the absorbance of each well was measured at 590 nm using a spectrophotometer.

2.3. NO measurement

Chondrocytes seeded a density of 1.5×10^5 cells/well were cultured in 24-well plates under CO_2 in a humidified incubator for 16 h at 37 °C and then incubated with 1 mM SNP. After 24 h, the amount of nitrite converted from NO was measured by mixing the culture media (100 μl) with an equal volume of Griess reagent (0.1% naphthylethylene diamine, 1% sulfanylamide, and 2.5% H_3PO_4). The optical density was measured at 540 nm.

2.4. IL-6, CCL2/MCP-1, MIP-1a and IGF-1 ELISA

Production of IL-6, CCL2/MCP-1, CCL3/MIP-1a, and IGF-1 from SNP-treated cells or cells co-cultured with hSDMSCs and SNP-stimulated chondrocytes were determined using enzyme-linked immunosorbent assay (ELISA) using commercially available ELISA kits (R&D Systems) according to the manufacturer's instructions, respectively.

2.5. Co-culture with hSDMSCs and SNP-stimulated chondrocyte in transwell systems

Chondrocytes were plated on the bottom of wells of 6-well transwell cell culture systems (Pore size 0.4 μm; Corning) using complete medium and appropriate culture environment. After 16 h, cells were then treated with 1 mM SNP for 24 h. The hSDMSCs were cultured onto the membrane of transwell cell-culture inserts and allowed to grow overnight. The next day the hSDMSCs were washed with PBS and the transwell inserts were placed into the 6-well plates containing the chondrocytes seeded at the onset of the experiment. The cells were incubated for 3 weeks. An illustration of the co-culture system is provided in Supplementary Fig. 2.

2.6. Analysis of mRNA expression

Total RNA was prepared from samples using a total RNA Extraction kit (iNtRON, Korea) according to the manufacturer's instructions. cDNA synthesis and PCR were conducted using an RT premix (Bioneer) and Takara Ex Taq DNA polymerase (Takara), respectively, according to the manufacturer's protocols. PCR analysis was performed with gene-specific primers (Supplementary Table 1). The PCR conditions were as follows: 32 cycles at 94 °C for 30 s, 58.2–59.8 °C for 30 s, and 72 °C for 30 s. The RT-PCR products were analyzed by electrophoresis in 1.5% agarose gels.

2.7. Statistical analysis

All data are presented as the mean ± standard error of the mean (SEM). Comparisons of multiple groups were performed by one-way analysis of variance (ANOVA), followed by pair-wise comparisons with a Bonferroni post hoc test. Differences were considered statistically significant at $p < 0.05$. All data were analyzed using the GraphPad Prism software, version 5.00 (GraphPad software).

3. Results

3.1. SNP-stimulated chondrocytes produce NO, IL-6, CCL2/MCP-1, and CCL3/MIP-1α

Cytotoxicity experiments were performed using 0.25, 0.5, 1, and 2 mM concentrations of SNP to treat chondrocytes, and the cell survival rate was determined using the MTT assay. Treatment with SNP for 24 h caused significant chondrocyte death in a dose-dependent manner (Fig. 1A). Specifically, we determined that 1 mM SNP should be used for chondrocyte treatment, because cells treated with 1 mM SNP exhibited an approximately 50% cell survival rate (54.3 ± 1.5%) (Fig. 1A).

Chondrocytes were used to assess the potential inflammation activity of SNP, since chondrocytes can produce NO, inflammatory cytokines, IL-6, CCL2/MCP-1, and CCL3/MIP-1α, upon stimulation with 1 mM SNP (Fig. 1B–E). These results showed that production of NO (Con., 0 ± 0.6 μM; 1 mM SNP, 21.5 ± 1.3 μM), IL-6 (Con., 0 ± 0.5 pg/ml; 1 mM SNP, 244 ± 19.5 pg/ml), CCL2/MCP-1 (Con., 0 ± 0.2 pg/ml; 1 mM SNP, 145.4 ± 3.8 pg/ml), and CCL3/MIP-1α (Con., 0 ± 0.3 pg/ml; 1 mM SNP, 27.6 ± 0 pg/ml) were significantly increased when compared to the control.

3.2. Co-culture of hSDMSCs with SNP-stimulated chondrocytes decreases cytokine production, but increases IGF-1 concentration

Several previous studies have demonstrated that MSCs have important immunomodulatory properties *in vitro* via co-culture with mononuclear cell [23–25] as well as *in vivo* through various clinical trials [26,27]. Moreover, co-cultured with MSCs in RA was inhibited inflammation activity [28–30]. Thus, we hypothesized that in a co-culture of hSDMSCs and SNP-stimulated chondrocytes, inflammatory activity may be inhibited and chondrocyte proliferation may be recovered. FACS analysis performed to confirm this showed that the established cells had hSDMSC characteristics (Supplementary Fig. 3). hSDMSCs were

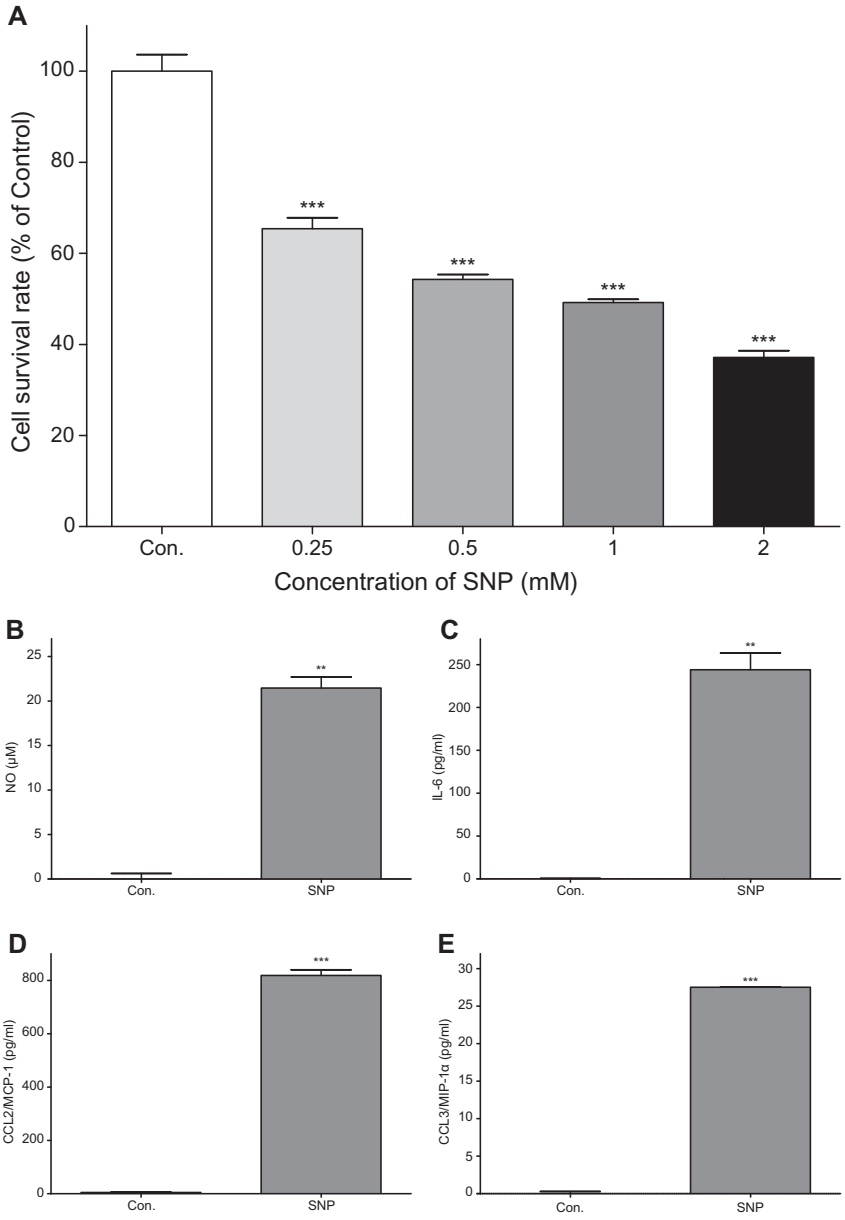

Fig. 1. The cytotoxic effects of sodium nitroprusside (SNP) on chondrocytes. (A) Dose-dependent effect of SNP on cell viability of chondrocytes. Approximately 1.5×10^5 chondrocytes/well were seeded in 24-well plates. Cells were incubated for 16 h with various concentrations of SNP (0.25, 0.5, 1.0, and 2.0 mM). Cytotoxicity was assessed after 24 h using MTT assay. (B–E) Stimulation with SNP resulted in NO production, and release of the inflammatory cytokines, IL-6, CCL2/MCP-1, and CCL3/MIP-1α by chondrocytes. Approximately 1.5×10^5 chondrocytes/well were seeded in 24-well plates. Cells were incubated for 24 h with 1 mM SNP, and then production of NO, IL-6, CCL2/MCP-1, and CCL3/MIP-1α was analyzed. The values shown are the means ± SEM from 6 independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to the control alone.

co-cultured with SNP-stimulated chondrocytes according to methods described in the Section 2, and the production of inflammation activity-related cytokines was analyzed (Fig. 2A–C).

In the co-culture system, IL-6 and CCL3/MIP-1α were significantly reduced at 1 week compared to the SNP-stimulated chondrocytes, but CCL2/MCP-1 was not. After 2 weeks, all inflammatory cytokines were reduced in the co-culture system when compared to the SNP-stimulated chondrocytes (Fig. 2A–C).

Several studies have demonstrated the ability of MSCs to secrete various growth factors [14]. Specifically, IGF-1 has been shown to prevent oxidative stress and reduce inflammatory responses [19–21]. Therefore, we analyzed the IGF-1 concentration in the co-culture system, and found that IGF-1 was significantly increased during cultivation as compared to the SNP-stimulated chondrocytes (Fig. 2D). Thus, these findings indicated that the hSDMSCs were able to secrete IGF-1 and that IGF-1 could prevent inflammatory activity in SNP-stimulated chondrocytes.

3.3. Inhibition of inflammatory activity-related gene expression and increase of SNP-stimulated chondrocyte proliferation in a co-culture system

Evaluation of the inhibition of inflammatory activity-related gene expression in a co-culture system showed that iNOS, COX-2, and MMP-3 mRNA levels were decreased (Fig. 3A). The co-culture system notably resulted in a time-dependent reduction of iNOS, COX-2, and MMP-3 mRNA levels compared to the SNP-stimulated chondrocytes. In contrast, GAPDH levels were not affected by the co-culture system or in SNP-stimulated chondrocytes. In addition, the proliferation rate was increased about 5-fold, 3.2-fold, and

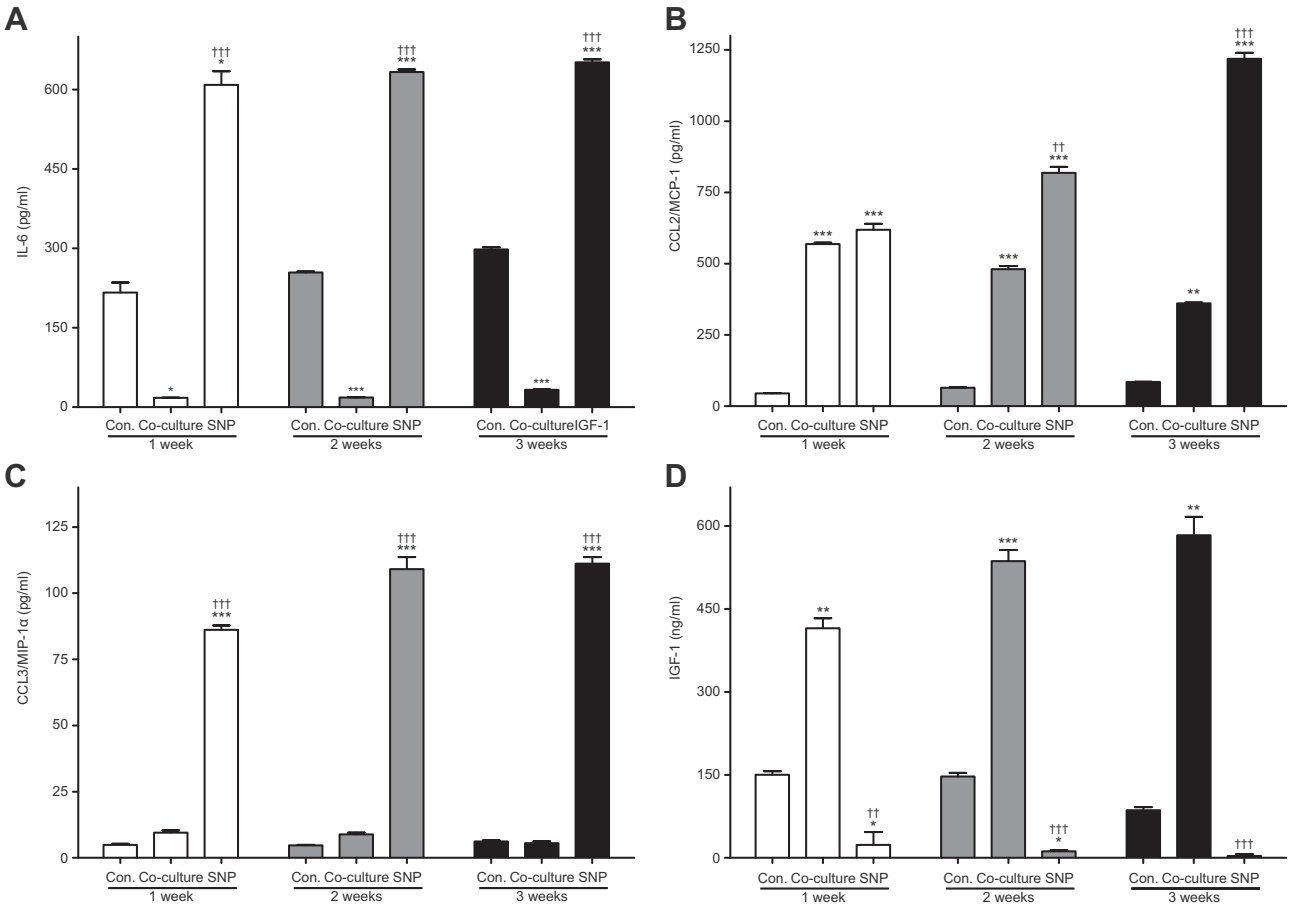

Fig. 2. Inhibitory effect on inflammatory activity by co-culture of hSDMSCs with SNP-stimulated chondrocytes for 3 weeks. (A–C) Secretion of the inflammatory cytokines, IL-6, CCL2/MCP-1, and CCL3/MIP-1α from the co-culture of hSDMSCs with SNP-stimulated chondrocytes. (D) Concentration of IGF-1 from the co-culture of hSDMSCs with SNP-stimulated chondrocytes. The values shown are the means ± SEM from 6 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to the control alone. $^{\dagger\dagger}P < 0.01$ and $^{\dagger\dagger\dagger}P < 0.001$ compared to the co-culture of hSDMSCs with SNP-stimulated chondrocytes.

4.8-fold at 1, 2, and 3 weeks, respectively, when compared to the SNP-stimulated chondrocytes (Fig. 3B).

3.4. Exogenous IGF-1 inhibits cytokine production in SNP-stimulated chondrocytes

Our results showed that co-culture of the hSDMSCs and SNP-stimulated chondrocytes inhibited inflammatory cytokine secretion and inflammation activity-related gene expression, and increased cell proliferation and IGF-1 secretion (Figs. 2 and 3). Several previous studies demonstrated that IGF-1 reduced inflammatory responses and oxidative stress *in vitro* and *in vivo* [19–21]. To confirm this, chondrocytes were treated with 1 mM SNP for 24 h after pre-treatment with IGF-1 (20 ng/ml) for 2 h, and the production of IL-6, CCL2/MCP-1, and CCL3/MIP-1α were analyzed (Fig. 4A–C). Production of IL-6 (Con., 210.4 ± 4.2 pg/ml; 20 ng/ml IGF-1 + 1 mM SNP, 294.5 ± 0.4 pg/ml; 1 mM SNP, 600.6 ± 3.5 pg/ml), CCL2/MCP-1 (Con., 4.8 ± 1 pg/ml; 20 ng/ml IGF-1 + 1 mM SNP, 265.8 ± 32 pg/ml; 1 mM SNP, 46.6 ± 0.4 pg/ml), and CCL3/MIP-1α (Con., 1 ± 0.6 pg/ml; 20 ng/ml IGF-1 + 1 mM SNP, 3.34 ± 0 pg/ml; 1 mM SNP, 27.6 ± 0 pg/ml) were significantly reduced compared to chondrocytes treated with 1 mM SNP.

4. Discussion

In the present study, we determined that SNP-stimulated chondrocytes secreted inflammatory factors, thus generating an *in vitro* model of RA or OA. We then demonstrated that co-culture of hSDMSCs with SNP-stimulated chondrocytes inhibited the secretion of inflammatory cytokines and expression of inflammatory activity-related genes, while increasing the concentration of IGF-1 in culture media and the chondrocyte proliferation rate. Moreover, pre-treatment with IGF-1 inhibited inflammatory cytokine secretion by SNP-stimulated chondrocytes.

RA and OA are chronic inflammatory diseases of the knee joint [1,2]. *In vitro* and *in vivo* studies have demonstrated that pro-inflammatory cytokines and chemokines produced by synoviocytes and chondrocytes, as well as cells from other joint tissues, can be measured in the synovial fluids of RA and OA patients and contribute to the disruption of the balance between anabolism and catabolism [31–33]. Existing drug therapies for RA and OA provide, at best, symptomatic relief from pain, but they fail to prevent cartilage damage and subsequent destruction of other joint tissues [34].

MSCs have been isolated and identified from various tissues, such as bone marrow, adipose tissue, and the umbilical cord vein [35–37]. MSCs provide a feasible cell source for cartilage repair because they can differentiate into multiple lineages, including adipocytes, osteoblasts, and chondrocytes. In addition, MSCs have demonstrated important immunomodulatory properties *in vitro* when co-cultured with mononuclear cells [23–25], as well as *in vivo* in various clinical trials [26,27]. Interestingly, MSCs secreted distinctively different cytokines such as VEGF-α, IGF-1, EGF, keratinocyte growth factor, angiopoietin-1, and stromal derived factor-1 when compared to dermal fibroblasts [14,38]. Specifically,

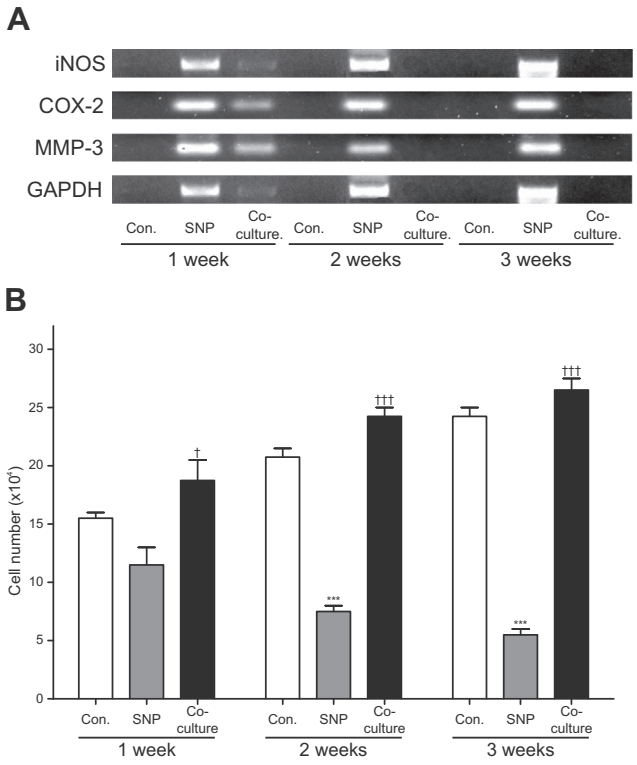

Fig. 3. Inhibitory effect on the expression of inflammation activity-related genes and the increase of cell proliferation by co-culture of hSDMSCs with SNP-stimulated chondrocytes for 3 weeks. (A) RT-PCR analysis of iNOS, COX-2, and MMP-3 mRNA expression from the co-culture of hSDMSCs with SNP-stimulated chondrocytes for 3 weeks. Lane 1, 4, and 7; control chondrocytes, Lane 2, 5, and 8; co-culture of hSDMSCs and SNP-stimulated chondrocytes, Lane 3, 6, and 9; SNP-stimulated chondrocytes. Lane 1, 2, and 3; co-culture for 1 week, Lane 4, 5, and 6; co-culture for 2 weeks, and Lane 7, 8, and 9; co-culture for 3 weeks. (B) Analysis of chondrocyte cell number from co-culture with hSDMSCs and SNP-stimulated chondrocytes. The values shown are the means ± SEM from 6 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the control alone. $^{\dagger\dagger}P < 0.01$ and $^{\dagger\dagger\dagger}P < 0.001$ compared to the co-culture of hSDMSCs and SNP-stimulated chondrocytes.

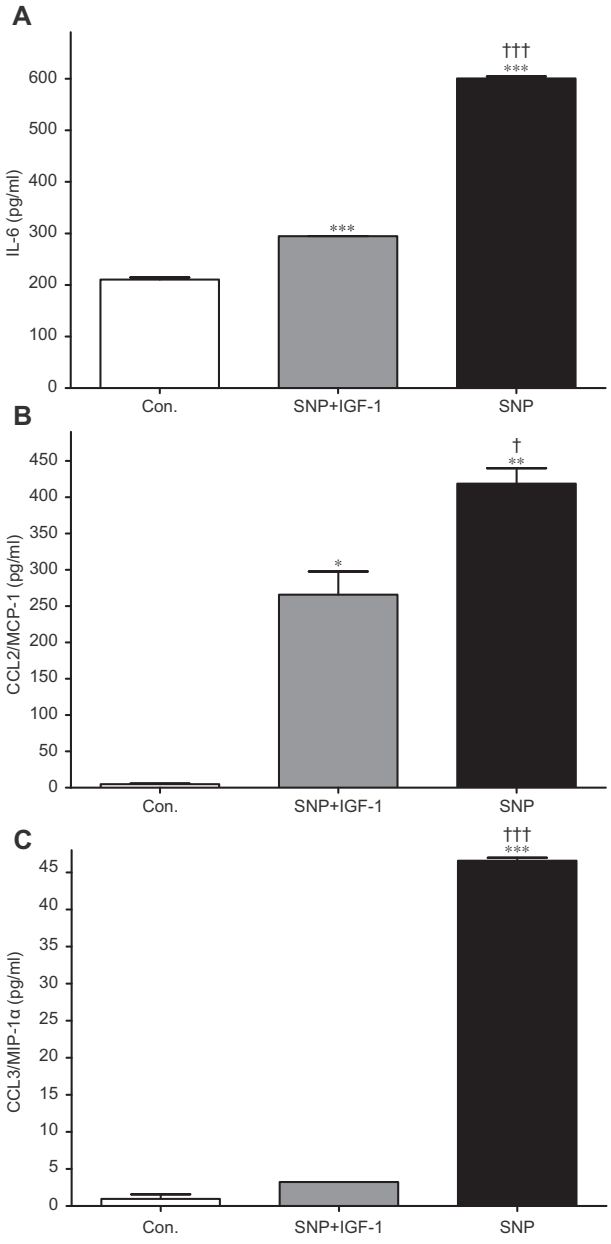

Fig. 4. Inhibitory effect of insulin-like growth factor-1 (IGF-1) on inflammatory activity of SNP-stimulated chondrocytes. (A–C) Inflammatory cytokine secretion by SNP-stimulated chondrocytes treated with IGF-1 (20 ng/ml). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to the control alone. $^{\dagger}P < 0.05$ and $^{\dagger\dagger\dagger}P < 0.001$ compared with SNP-stimulated chondrocytes treated with IGF-1.

hSDMSCs have been shown to have robust proliferative capacity and superior chondrogenic potential, thus attracting increased interest in cartilage tissue engineering and clinical applications [39]. Importantly, the phenotype profile and chondrocyte differentiation capacity of hSDMSCs are not affected by age or OA etiology. Our results showed that co-culture with hSDMSCs and SNP-stimulated chondrocytes increased IGF-1 secretion. SNP-stimulated chondrocytes reduced secretion of inflammatory cytokines, inhibited expression of inflammation activity-related genes, such as iNOS, COX-2, and MMP-3, and increased chondrocyte proliferation rate.

We demonstrated that co-culture of hSDMSCs with SNP-stimulated chondrocytes resulted in IGF-1 secretion. To confirm the anti-inflammatory effect of IGF-1, we pre-treated chondrocytes with IGF-1 for 2 h, and then stimulated with SNP for 24 h. Our results showed that IGF-1 was significantly inhibited secretion of inflammatory cytokines by SNP-stimulated chondrocytes. In particular, IGF-1 is known to be an endocrine and autocrine/paracrine growth factor that is a primary mediator of growth hormone effects on developmental growth [18]. However, IGF-1 can potentiate TNF-α-induced c-Jun N-terminal kinase (JNK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation in endothelial cells [40]. Moreover, IGF-1 protects oxidative stress-induced apoptosis in intestinal epithelial cells and induced pluripotent stem cells (iPSCs) [21,41]. Several studies have demonstrated that IGF-1 has potent survival effects on vascular cells and prevents oxidized LDL-induced apoptosis of vascular smooth muscle cells, suggesting that IGF-1 activity could contribute to the atherosclerotic process [17,42–44]. We indicated that our co-culture system showed improved anti-inflammation activity with SNP-stimulated chondrocytes. Therefore, the presented data suggests a new strategy for promoting cell-based cartilage regeneration in RA and OA.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.077.

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