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Role of S100A12 in the pathogenesis of osteoarthritis

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ABSTRACT

S100A12 is a member of the S100 protein family, which are intracellular calcium-binding proteins. Although there are many reports on the involvement of S100A12 in inflammatory diseases, its presence in osteoarthritic cartilage has not been reported. The purpose of this study was to investigate the expression of S100A12 in human articular cartilage in osteoarthritis (OA) and to evaluate the role of S100A12 in human OA chondrocytes. We analyzed S100A12 expression by immunohistochemical staining of cartilage samples obtained from OA and non-OA patients. In addition, chondrocytes were isolated from knee cartilage of OA patients and treated with recombinant human S100A12. Real-time RT-PCR was performed to analyze mRNA expression. Protein production of matrix metalloproteinase 13 (MMP-13) and vascular endothelial growth factor (VEGF) in the culture medium were measured by ELISA. Immunohistochemical analyses revealed that S100A12 expression was markedly increased in OA cartilages. Protein production and mRNA expression of MMP-13 and VEGF in cultured OA chondrocytes were significantly increased by treatment with exogenous S100A12. These increases in mRNA expression and protein production were suppressed by administration of soluble receptor for advanced glycation end products (RAGE). Both p38 mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) inhibitors also suppressed the increases in mRNA expression and protein production of MMP-13 and VEGF. We demonstrated marked up-regulation of S100A12 expression in human OA cartilages. Exogenous S100A12 increased the production of MMP-13 and VEGF in human OA chondrocytes. Our data indicate the possible involvement of S100A12 in the development of OA by up-regulating MMP-13 and VEGF via p38 MAPK and NF-κB pathways.

1. Introduction

Osteoarthritis (OA) is a degenerative joint disease and is the most prevalent type of arthritis. It is characterized by degradation of the existing cartilage matrix and production of new connective tissue in the form of osteophytes on the joint surface and at the joint margins [1,2]. Cartilage is composed of highly differentiated chondrocytes and extracellular matrix (ECM) and is primarily an avascular tissue. In early OA, uncontrolled production of matrix-degrading enzymes, including matrix metalloproteinases (MMPs) and aggrecanases, occurs, which results in the progressive degradation of articular cartilage [3,4]. Among MMPs, MMP-13 (collagenase 3) plays a major role in the degradation of type II collagen (COL2), which is the major type of collagen found in cartilage, and has been shown to be overexpressed in OA [4,5]. Aggrecanases belong to a disintegrin and metalloproteinase with thrombospondin type 1 motifs (ADAMTS) family, and the degradation of aggrecan by ADAMTS-4 (aggrecanase 1) and ADAMTS-5 (aggrecanase 2) is an early event in OA cartilage damage [6]. Recently, it has been postulated that endochondral ossification is essential for the development of osteoarthritis [7]. The process of endochondral ossification requires the hypertrophic differentiation of chondrocytes, which involves the secretion of type X collagen (COL10) and the conversion of avascular cartilage tissue into highly vascularized bone tissue via degradation of cartilage matrix and vascular invasion [4,8]. During endochondral ossification in a developing bone, neovascularization from the subchondral bone takes place in the cartilage growth plate, resulting in the resorption of cartilage ECM and the replacement by bone matrix [9]. The vascular invasion depends on an angiogenic switch by vascular endothelial growth factor (VEGF) [8,10]. VEGF is expressed in the chondrocytes of OA cartilage, and a linear correlation was observed between VEGF immunoreactivity and OA development [11]. Activation of runt-related transcription factor-2 (RUNX2) contributes to chondrocyte hypertrophy and matrix breakdown [12]. In recent studies,

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it was reported that hypoxia-inducible factor-2α (HIF2α) enhanced the promoter activities of RUNX2, COL10, MMP-13, and VEGF and was important for the endochondral ossification of cultured chondrocytes [7,10].

The S100 protein family is made up of more than 20 known low molecular weight (9–14 kDa) intracellular calcium-binding proteins that control the key cellular pathways, including the regulation of cytoskeleton, cell migration and adhesion, and host oxidative defense. S100 proteins have also been demonstrated to have important extracellular pro-inflammatory effects and cytokine-like activities in addition to their intracellular functions [13]. A subgroup of S100 proteins called "calgranulins," which include S100A8, S100A9, and S100A12, are constitutively expressed at high levels in neutrophils and are associated with various inflammatory conditions [14,15]. In inflammatory compartments, S100A12 is markedly overexpressed, and elevated serum levels of S100A12 have been reported in patients suffering from various inflammatory disorders, such as rheumatoid arthritis, psoriatic arthritis, Crohn's disease, ulcerative colitis, and Kawasaki disease [13]. Moreover, S100A12 has been shown to interact with the receptor for advanced glycation end products (RAGE) [16]. RAGE is a multi-ligand cell surface receptor that belongs to the immunoglobulin superfamily and plays an important role in inflammation, diabetes, Alzheimer's disease, and cancer [17]. It was reported that S1000A12 contributed to the pathogenesis of inflammatory lesions via interaction with RAGE after release to the extracellular compartment [16]. Although many reports state the involvement of S100A12 in inflammatory diseases, the presence of S100A12 in OA cartilage has not been reported. The purpose of this study was to investigate the expression of S100A12 protein in human OA articular cartilage and to evaluate the role of S100A12 in human OA chondrocytes.

2. Materials and methods

2.1. Reagents

Goat anti-human S100A12 antibody and recombinant human interleukin-1β (IL-1β) were purchased from R&D Systems (Minneapolis, MN). Recombinant human S100A12 protein was purchased from MBL (Aichi, Japan). PD98059, SP600125, and BAY11-7082 were purchased from Sigma (St. Louis, MO). SB203580 was purchased from Calbiochem (La Jolla, CA). Recombinant soluble RAGE (sRAGE) was purchased from Biovendor (Heidelberg, Germany).

2.2. Immunohistochemistry for S100A12

Human cartilage was obtained from patients who fulfilled the American College of Rheumatology criteria for OA and underwent total hip or knee arthroplasty ($n = 8$) or from age-matched non-OA patients who suffered a femoral neck fracture of the hip ($n = 6$). These experiments were performed in accordance with a protocol approved by the Ethics Committee of Nagoya University, and all patients gave written consent for the use of their tissues for this research. Non-OA and OA human articular cartilage specimens were fixed in 10% formalin, embedded in paraffin, and cross-sectioned. For immunohistological analyses of S100A12, sections were deparaffinized in xylene and rehydrated in graded alcohol. After washing with phosphate buffered saline (PBS), the sections were first incubated with 1% Triton X-100 for 15 min and then with 3% hydrogen peroxide/methanol for 10 min. Next, they were blocked with 10% rabbit serum for 10 min and incubated for 1 h at room temperature with goat anti-S100A12 antibody (1:100 dilution) as the primary antibody. For the negative controls, tissue sections were incubated with non-immunized rabbit IgG as a substitute of the primary antibody. The primary antibody was detected by the avidin–biotin conjugate method, which was applied according to manufacturer's instructions using Histofine SAB-PO kit (Nichirei, Tokyo, Japan). Peroxidase activity was detected using Histofine DAB kit (Nichirei). After the sections were rinsed with water, the nuclei were counterstained with hematoxylin. The number of S100A12 positive stainable cells was counted in the articular cartilage. Each section was observed under a light microscope (×100). The mean number of positive cells (/300 cells) was calculated for each group. All slides were evaluated independently by two blinded observers.

2.3. Cell culture

Cartilage samples were obtained at the time of joint replacement surgery from the knees of OA patients. Articular cartilage from the femoral condyles, tibial plateaus, and articular surface of the patella was cut into small pieces with a scalpel. Cartilage samples were minced and digested at 37 °C with trypsin–EDTA solution for 30 min, incubated with 3 mg/ml type II collagenase (Worthington Biochemical Co, Lakewood, NJ) in Dulbecco's Modified Eagle's Medium (DMEM; Gibco-BRL, Belgium) for 18 h, filtered through a nylon mesh, and washed extensively. Next, the isolated chondrocytes were seeded in 75 cm^2 culture flasks and incubated in DMEM containing 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 mg/ml) at 37 °C in an atmosphere of 5% CO_2. At confluence, the cells were detached and seeded in 12-well plates at a density of 2×10^5/well. Chondrocyte cultures were made serum-free overnight before each experiment.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted with an RNeasy kit (Qiagen, Hilden, Germany), and reverse transcription was performed using High

Table 1
Sequences of primers used for RT-PCR.

Gene	Oligonucleotide sequence		Product size (bp)
	Forward	Reverse	
MMP13	5′-GGTGGTGATGAAGATGATT-3′	5′-TCAGTCATGGAGCTTGCT-3′	107
MMP3	5′-GGCACAATATGGGCACTTTA-3′	5′-AATGACCGGCAAGATACAGAT-3′	144
MMP1	5′-CTGGGAGCAAACACATCTGA-3′	5′-CTGGTTGAAAAGCATGAGCA-3′	239
ADAMTS4	5′-GTCCCATGTGCAACGTCA-3′	5′-GCCATCTTGTCATCTGCCA-3′	102
ADAMTS5	5′-TGACAAGTGCGGAGTATG-3′	5′-AGGCAGTGAATCTAGTCTGG-3′	147
COL10A1	5′-ATGCATATGGAGGTAGGC-3′	5′-AGAGAGGCTTCACATACG-3′	128
RUNX	5′-CCTCCCTGAACTCTGCACCA-3′	5′-TGGTAGAGTGGATGGACGGG-3′	153
HIF2A	5′-CCTCCCTGAACTCTGCACCA-3′	5′-TGGTAGAGTGGATGGACGGG-3′	172
COL2A1	5′-AGAGGGGATCGTGGTGACAA-3′	5′-GGCAGCAAAGTTTCCACCAA-3′	144
Aggrecan	5′-CCAGGAGGTATGTGAGGA-3′	5′-CGATCCACTGGTAGTCTTG-3′	185
VEGFA	5′-AAGGAGGAGGGCAGAATCAT-3′	5′-ATCTGCATGGTGATGTTGGA-3′	226
S100A12	5′-CTGTGCATTGAGGGGTTAACATTAG-3′	5′-CCGAACTGAGTATTGGTGGAAGAT-3′	97
GAPDH	5′-TGCACCACCAACTGCTTAGC-3′	5′-GGCATGGACTGTGGTCATGAG 3′	87

Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to manufacturer's protocols. Relative quantification of gene expression was performed using the LightCycler480 SYBR Green I Master (Roche Diagnostics, Mannheim, Germany). Primers for MMP13, MMP3, MMP1, VEGF, COL2A1, aggrecan, COL10A1, RUNX2, HIF2A, ADAMTS4, ADAMTS5, A100A12, and GAPDH were used for the analysis. Expression levels were measured in triplicate and normalized to the expression of GAPDH. The sequences of the primers are listed in Table 1.

2.5. Enzyme-Linked Immunosorbent Assay (ELISA)

Supernatants of the solution containing the cultured cells after treatment with S100A12 from various experiments were collected and stored at −20 °C before analysis. Production of MMP-13 was analyzed using an ELISA kit obtained from GE Healthcare (Buckinghamshire, UK) according to manufacturer's protocol using duplicate wells for each sample. VEGF was analyzed using an ELISA kit obtained from R&D Systems (Minneapolis, MN).

2.6. Statistical analysis

All data are presented as mean ± standard deviation (SD). Statistics were analyzed using the Student's *t* test. *P* values <0.05 were considered statistically significant.

3. Results

3.1. S100A12 expression in non-OA and OA human cartilage

Immunohistochemical analyses revealed that S100A12 expression was markedly increased in the superficial and deep zones of OA cartilage (Fig. 1A). Densely stained nuclei and membranes were observed in the superficial zone of OA cartilage, whereas only faint or no staining was observed in non-OA cartilage. Fig. 1B shows that S100A12 expression in OA cartilage was significantly higher (88.2 ± 9.3%) than that observed in non-OA cartilage (14.9 ± 4.1%; $P < 0.01$).

3.2. IL-1β induces expression of S100A12 in human OA chondrocytes

We examined mRNA expression of S100A12 in cultured human OA chondrocytes treated with IL-1β (10 ng/ml) for 24 h using real-time RT-PCR. The expression of S100A12 in OA chondrocytes was significantly upregulated by IL-1β stimulation (Fig. 2A).

3.3. Effect of exogenous S100A12 on human OA chondrocytes

To investigate the effect of exogenous S100A12, human OA chondrocytes were treated with recombinant human S100A12 (100 nM) for 24 h. We examined the expression patterns of MMP-1, MMP-3, MMP-13, ADAMTS4, and ADAMTS5 as proteinases, which degrade the cartilage matrix, HIF2α as a catabolic regulator of osteoarthritic changes, COL2 and aggrecan as cartilage matrix proteins, RUNX2 and COL10 as markers for hypertrophy of chondrocytes, and VEGF as a marker of vascular proliferation. The mRNA expressions of MMP-1, 3, and 13 and VEGF were significantly increased by treatment with S100A12 (Fig. 2B). There was no significant difference in mRNA expressions of ADAMTS4, ADAMTS5, and HIF2α. Expression of hypertrophic markers RUNX2 and COL10 also showed no significant changes. By contrast, the expression of matrix proteins COL2 and aggrecan were significantly decreased. In addition, we examined protein production of MMP-13 and VEGF in the supernatants of cultured cells using ELISA. The amount of MMP-13 protein was significantly increased at

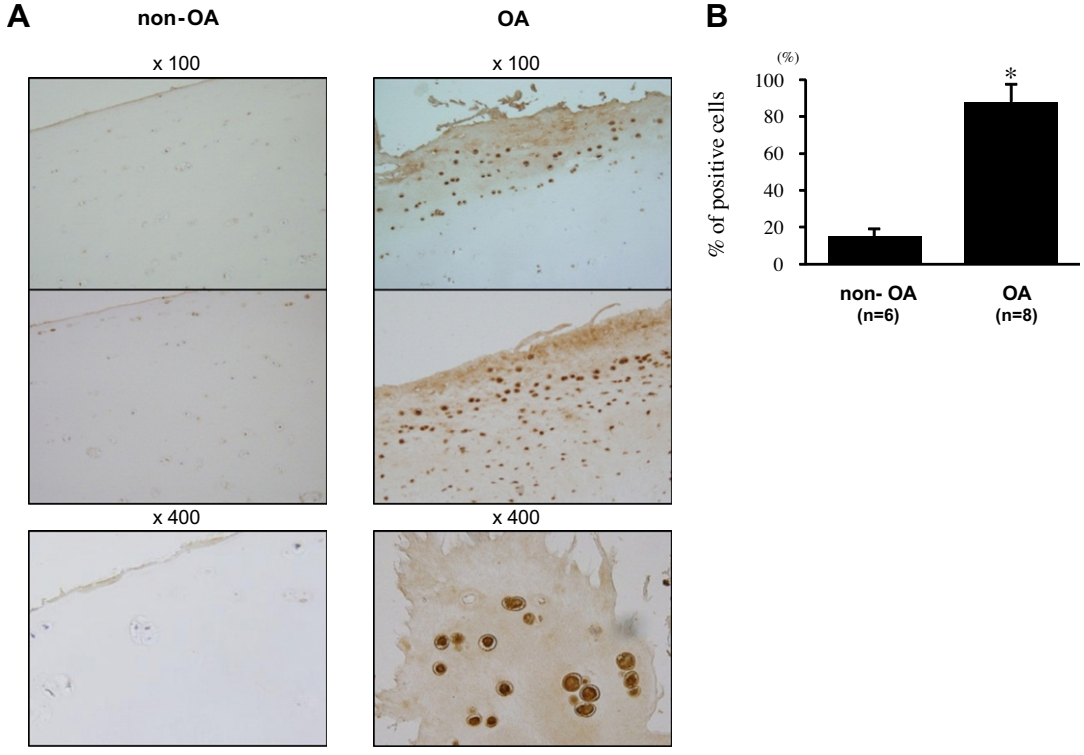

Fig. 1. Expression of S100A12 in human articular cartilage. (A) Immunohistochemical analyses of human non-OA and OA cartilage for S100A12. Data are represented for non-OA (left panels) and OA (right panels) donors, and upregulation of S100A12 was demonstrated in OA cartilage. Original magnification 100× and 400× are shown. (B) Histograms of S100A12 positive cells (%) in non-OA ($n = 6$) and OA ($n = 8$) cartilage. Data are expressed as mean ± SD. *$P < 0.01$ compared with non-OA cartilage.

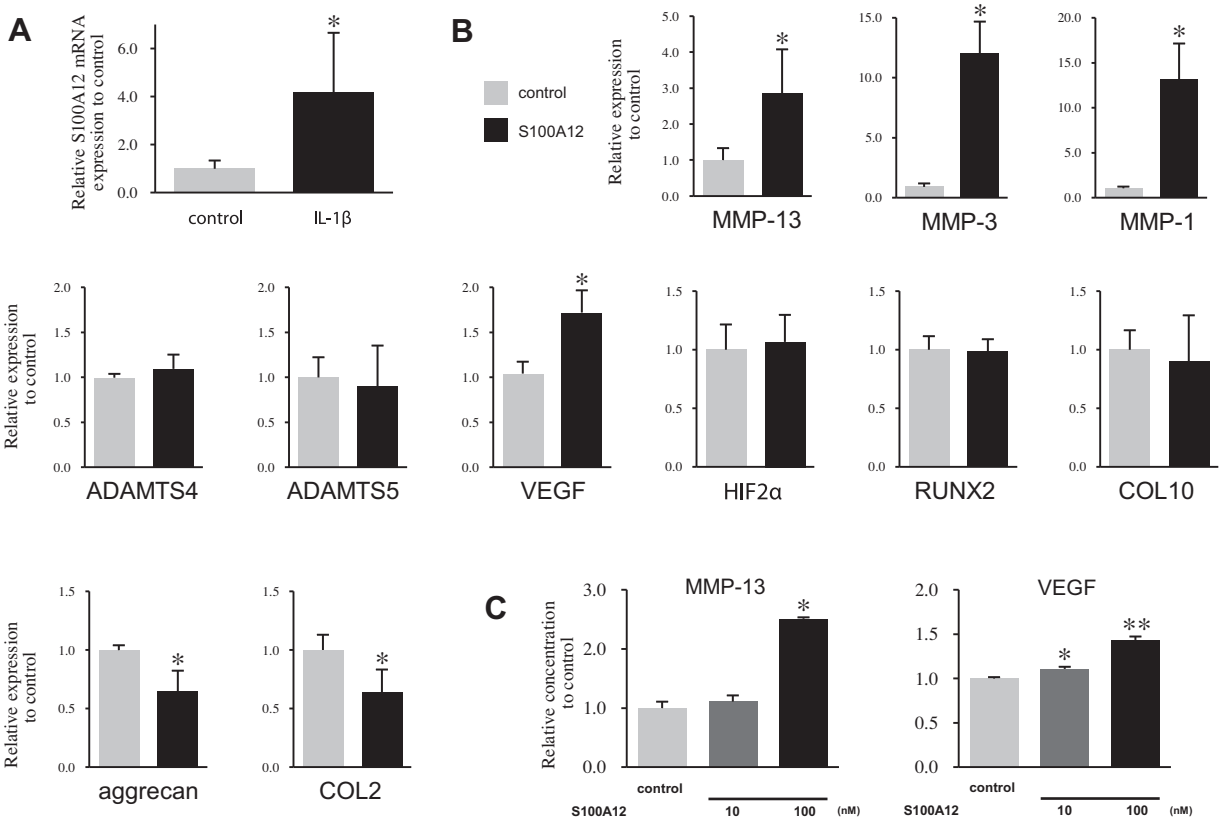

Fig. 2. Expression of mRNA and protein production in human OA chondrocytes stimulated with IL-1β and S100A12. (A) S100A12 mRNA expression in OA chondrocytes stimulated with IL-1β (10 ng/ml) for 24 h was measured by real-time RT-PCR ($n = 5$) and normalized to GAPDH. (B) Expression of mRNA in OA chondrocytes stimulated with S100A12 (100 nM) for 24 h was measured by real-time RT-PCR ($n = 5$) and normalized to GAPDH. (C) Production of MMP-13 and VEGF in the culture medium of OA chondrocytes stimulated with S100A12 (10 nM and 100 nM) for 24 h was measured by ELISA ($n = 3$). Data are expressed as mean ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with each control sample.

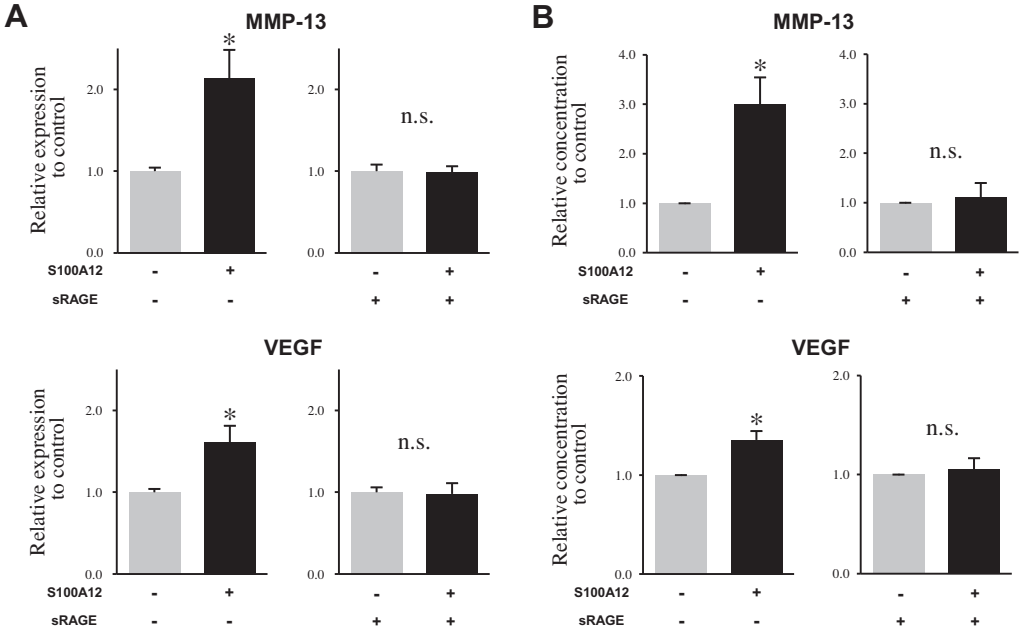

Fig. 3. Expression of mRNA and protein production of MMP-13 and VEGF in human OA chondrocytes pretreated with sRAGE. OA chondrocytes were pretreated without sRAGE or with sRAGE at 100 μg/ml for 30 min and then stimulated with S100A12 (100 nM) for 24 h. (A) Expression of MMP-13 and VEGF mRNA was measured by real-time RT-PCR ($n = 6$) and normalized to GAPDH. (B) Protein production of MMP-13 and VEGF in the culture medium was measured by ELISA ($n = 5$). Data are expressed as mean ± SD. $^{*}P < 0.01$ compared with each control sample, n.s. = not significant.

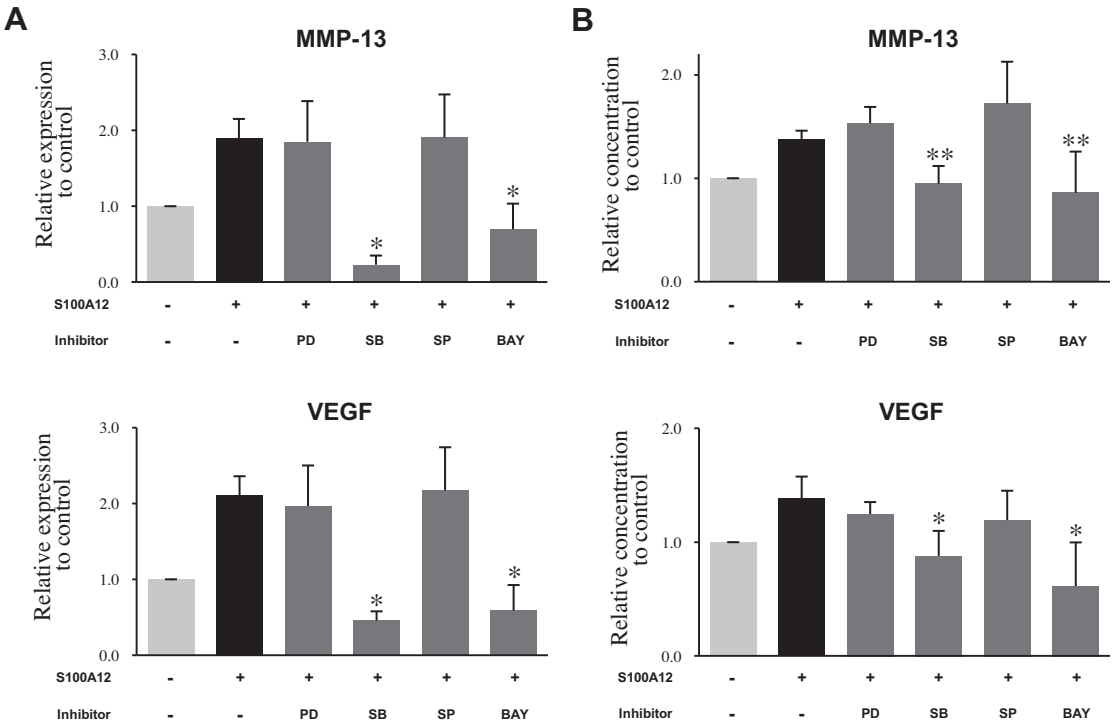

Fig. 4. Expression of mRNA and protein production of MMP-13 and VEGF in human OA chondrocytes pretreated with MAPK and NF-κB inhibitors. OA chondrocytes were pretreated with PD98059 (PD; 10 μM), SB203580 (SB; 5 μM), SP600125 (SP; 5 μM), or BAY11-7082 (BAY; 10 μM) for 30 min and stimulated with S100A12 (100 nM) for 24 h. (A) Expression of MMP-13 and VEGF mRNA was measured by real-time RT-PCR ($n = 5$) and normalized to GAPDH. (B) Protein production of MMP-13 and VEGF in the culture medium was measured by ELISA ($n = 4$). Data are expressed as mean ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with control cells treated with S100A12 alone.

an S100A12 density of 100 nM. The amount of VEGF protein was also significantly increased in a dose-dependent manner (Fig. 2C).

3.4. Pretreatment with soluble RAGE (sRAGE) inhibits the effect of S100A12 in human OA chondrocytes

To investigate whether S100A12-induced expression of MMP-13 and VEGF in human OA chondrocytes was mediated by binding to RAGE, we used excess amount of sRAGE to block the binding of S100A12 to RAGE. sRAGE, which comprises the extracellular domains of RAGE, is thought to competitively inhibit binding between the cell surface receptor and its ligands. Addition of sRAGE to the ligands is expected to cause impairment of RAGE-mediated cellular responses and consequently reveals the involvement of RAGE in signaling events. Cells were pretreated with 100 μg/ml of sRAGE for 30 min, followed by incubation with S100A12 (100 nM) for 24 h. Treatment with sRAGE significantly inhibited S100A12-mediated upregulation of MMP-13 and VEGF mRNA and thus, inhibited increased protein production of MMP-13 and VEGF in the culture medium (Fig. 3).

3.5. p38 mitogen-activated protein kinase (MAPK) and nuclear factor-B (NF-B) inhibitors suppress the effects of S100A12 in human OA chondrocytes

We investigated cell signals in chondrocytes stimulated with S100A12 using MAPK and NF-κB inhibitors. Cells were pretreated with PD98059 (10 μM), SB203580 (5 μM), SP600125 (5 μM), or BAY11-7082 (10 μM) for 30 min, followed by incubation with S100A12 (100 nM) for 24 h. The expression of MMP-13 and VEGF mRNA was significantly suppressed by pretreatment with p38 (SB203580) and NF-κB (BAY11-7082) inhibitors compared with that in controls treated with S100A12 alone (Fig. 4A). Protein production of MMP-13 and VEGF was also significantly attenuated by pretreatment with p38 and NF-κB inhibitors (Fig. 4B). Pretreatment with ERK (PD98059) and JNK (SP600125) inhibitors had no significant effect on S100A12-induced mRNA expression or protein production of MMP-13 and VEGF.

4. Discussion

In this study, we demonstrated for the first time marked upregulation of S100A12 expression in human OA cartilage. Among other S100 proteins, expression of S100A8 and S100A9 was markedly enhanced in arthritic cartilage, and *in vitro* S100A8 exhibited direct potency in the stimulation of human and murine chondrocytes to produce MMPs and to cause aggrecanase-mediated pericellular matrix degradation [18,19]. S100A4 was shown to be highly expressed in OA cartilage compared with normal cartilage, and it stimulated RAGE-dependent signaling cascades leading to activation of MMP-13 in OA cartilage [20]. S100A11 was secreted by chondrocytes. Its expression was upregulated in OA cartilages, and S100A11/RAGE activation resulted in hypertrophy [21,22]. Similar to these S100 proteins, S100A12 was expressed in human OA cartilage in this study. Furthermore, a statistically significant increase in the number of S100A12 positive cells was observed in OA cartilage compared with non-OA cartilage. These results suggest that S100A12 is associated with the progression of OA.

Although S100A12 has been detected in the synovial fluids of OA patients, the origin of S100A12 remains unknown [23]. It has been reported that S100A12 is expressed and secreted by neutrophils, monocytes, and activated macrophages [24–26]. Although the involvement of inflammatory cells in OA remains disputable, based on the report that increased mononuclear cell infiltration and overexpression of mediators of inflammation were observed in OA synovial tissue, it can be said that S100A12 could be released

from these inflammatory cells in synovial tissue [27,28]. On the other hand, it is speculated that another candidate which could release S100A12 is a chondrocyte itself. In this study, an increase in S100A12 expression was observed in human OA cartilage and mRNA expression of S100A12 was significantly upregulated by IL-1β in human OA chondrocytes. Furthermore, in our preliminary experiments, mRNA expression of S100A12 in OA chondrocytes was significantly upregulated by S100A12 (data not shown). Taken together, these findings suggest that S100A12 may act in an autocrine manner in OA chondrocytes.

We also examined the effect of exogenous S100A12 at a concentration of 100 nM (approximately 1.2 μg/ml) on OA chondrocytes. In chronic inflammatory arthritis, such as rheumatoid arthritis and psoriatic arthritis, concentrations of S100A12 are higher in the synovial fluid than in serum [29,30]. A recent study using mass spectrometry reported that the expression of S100A12 in synovial fluid of OA patients was about one-quarter of that in rheumatoid arthritis patients [23]. Considering that the concentration of S100A12 in synovial fluid of patients with rheumatoid arthritis has been reported to be 3–70 μg/ml, an S100A12 concentration of 100 nM could be possible in synovial fluid of OA patients [29,31].

We demonstrated that the addition of S100A12 to cultured human OA chondrocytes resulted in the upregulation of MMP-13 and VEGF mRNA and the subsequent release of MMP-13 and VEGF proteins into the culture medium. We speculate that S100A12 could be involved in the development of OA by upregulating the synthesis of MMP-13 and VEGF, which could lead to cartilage degradation and angiogenesis. On the other hand, expression of ADAMTS4/5, HIF2α, COL10, and RUNX2 mRNA showed no significant change. We speculate that the degradation of proteoglycans and the hypertrophy of chondrocytes and osteogenesis may be modulated primarily by other factors such as IL-1, IL-8, tumor necrosis factor (TNF), syndecan, and S100 family proteins other than S100A12 [7,18,21,32,33]. Interaction of S100A12 with RAGE plays a key role in inflammatory reactions in various diseases [13]. S100A12 binds to RAGE on endothelial cells, mononuclear phagocytes, and lymphocytes, and triggers cellular activation through the NF-κB pathway in a RAGE-mediated manner [16]. In this study, upregulation of expression of MMP-13 and VEGF mRNA was significantly attenuated when S100A12 was quenched by sRAGE, suggesting that RAGE plays an important role in the pathway of this effect.

Finally, we investigated the signaling pathways of MAPK and NF-κB using specific inhibitors. Pretreatment with inhibitors of p38 and NF-κB, but not ERK or JNK, significantly attenuated the S100A12-induced increase in mRNA expression and protein production of MMP-13 and VEGF in OA chondrocytes. In RAGE-mediated pathways of other S100 proteins in chondrocytes, exogenous S100A4 increased the production of MMP-13 via MAPK (ERK1/2, p38, and JNK) and NF-κB, S100B increased the production of MMP-13 via MAPK (ERK1/2, p38, and JNK) and NF-κB, and exogenous S100A11 induced hypertrophy of chondrocytes via MAPK (p38) [20,21,33]. Thus, p38 MAPK and NF-κB pathways could also be involved in the S100A12-induced upregulation of MMP-13 and VEGF in OA chondrocytes.

In conclusion, this study demonstrated for the first time that S100A12 was expressed in human OA articular cartilage. Exogenous S100A12 upregulated the expression of MMP-13 and VEGF mRNA, and increased the production of MMP-13 and VEGF proteins in human OA chondrocytes. These increases were suppressed by treatment with soluble RAGE. Inhibitors of p38 MAPK and NF-κB were found to abrogate S100A12-induced production of MMP-13 and VEGF. Thus, these pathways could be involved in S100A12-induced production of MMP-13 and VEGF in human OA chondrocytes. S100A12 may contribute to OA progression by increasing the production of MMP-13 and VEGF.

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