Simvastatin inhibits CD44 fragmentation in chondrocytes

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Abstract

In human osteoarthritic chondrocytes, the hyaluronan receptor CD44 undergoes proteolytic cleavage at the cell surface. CD44 cleavage is thought to require transit of CD44 into cholesterol-rich lipid rafts. The purpose of this study was to investigate whether statins exert a protective effect on articular chondrocytes due to diminution of cholesterol. Three model systems of chondrocytes were examined including human HCS-2/8 chondrosarcoma cells, human osteoarthritic chondrocytes and normal bovine articular chondrocytes. Treatment with IL-1β + Oncostatin M resulted in a substantial increase in CD44 fragmentation in each of the three chondrocyte models. Pre-incubation with simvastatin prior to treatment with IL-1β + Oncostatin M decreased the level of CD44 fragmentation, decreased the proportion of CD44 that transits into the lipid raft fractions, decreased ADAM10 activity and diminished the interaction between CD44 and ADAM10. In HCS-2/8 cells and bovine articular chondrocytes, fragmentation of CD44 was blocked by the knockdown of ADAM10. Inhibition of CD44 fragmentation by simvastatin also resulted in improved retention of pericellular matrix. Addition of cholesterol and farnesyl-pyrophosphate reversed the protective effects of simvastatin. Thus, the addition of simvastatin exerts positive effects on chondrocytes including reduced CD44 fragmentation and enhanced the retention of pericellular matrix.

Highlights

- Simvastatin treatment inhibited the IL-1β + Oncostatin M induced CD44 fragmentation in chondrocytes
- Inhibition of CD44 fragmentation improved pericellular matrix retention
- Fragmentation of CD44 was blocked by the knockdown of ADAM10
- Simvastatin diminished co-localization of ADAM10 and CD44 due to lipid raft disruption

1. Introduction

CD44 is a single-pass transmembrane glycoprotein receptor and in many cell types, serves as a primary receptor for the glycosaminoglycan hyaluronan (HA)[1]. In articular chondrocytes, HA and proteoglycan-rich cell-associated matrices are anchored to the plasma membrane via the binding of HA to CD44 [1-3]. Previous reports have demonstrated proteolytic cleavage of CD44 from the cell surface and shedding in several tumor cell systems [4]. Our previous study demonstrated similar proteolytic cleavage of CD44 from the cell surface of articular chondrocytes, and moreover, that the shedding of CD44 is higher in chondrocytes derived from patients undergoing total knee replacement as well as *in vitro* models used to mimic osteoarthritis (OA) [5]. For example, in human OA chondrocytes, a substantial proportion of the CD44 undergoes degradation as compared to normal chondrocytes derived from human ankle cartilage [5]. CD44 cleavage also can be induced in normal articular chondrocytes by treatment with IL-1β, phorbol myristate acetate or HA oligosaccharides [5,6]. The signature pattern of CD44 degradation, present in both malignant cells and OA chondrocytes, includes the cleavage of the extracellular domain of CD44 by a metalloproteinase such as membrane type I (MT1-MMP, aka

[1] **Abbreviations:** HA, hyaluronan; OA, osteoarthritis; OSM, Oncostatin M; BAC, bovine articular chondrocytes; HAC, human articular chondrocytes; MT1-MMP, membrane type I metalloproteinase; ICD, intracellular domain; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; FPP, farnesyl-pyrophosphate; GGPP, geranylgeranylpyrophosphate; Mev, mevalonic acid; GGTI, geranylgeranyltransferase 1 inhibitor; FTI, farnesyltransferase inhibitor; MβCD, methyl-β-cyclodextrin

MMP14), ADAM17 or ADAM10 (Fig. 1A) [7]. The metalloproteinase action releases a 70 kD CD44 ecto-domain into the extracellular matrix, leaving a 18-20 kD C-terminal truncation fragment within the plasma membrane (termed CD44-EXT) [7]. The CD44-EXT fragment is then cleaved within the intramembranous domain by γ-secretase, releasing a 15 kD intracellular domain (CD44-ICD) into the cytoplasm [5]. The release of these CD44 domains can exert negative influences on chondrocyte function. A previous study reported that release of the CD44-ICD into the cytoplasm of chondrocytes competitively blocks interactions between full-length CD44 and cytoskeletal adaptor proteins—interactions that are required to stabilize retention of a pericellular matrix [6]. In other cell systems, release of the shed CD44 ecto-domain acts as a decoy receptor for HA, preventing HA binding to the cell surface [8]. Thus, proteolytic cleavage of CD44 not only results in a loss of full-length CD44 but also the generation of two potential dominant negative domains, leading to continued, prolonged failure of chondrocytes to assembly and to retain a HA / proteoglycan-rich pericellular matrix.

All of the relevant metalloproteases and γ-secretases that participate in CD44 cleavage are localized in cholesterol-rich lipid rafts [5]. In articular chondrocytes, full-length CD44 also transits into this lipid raft environment [5,9]. It is for these reasons that the shedding of CD44 can be blocked by chemical inhibitors of matrix metalloproteinases and by cholesterol chelators such as methyl-β-dextran—agents which disrupt lipid rafts [5,6,9]. However, complete and prolonged removal of cholesterol from plasma membranes is not sustainable or suitable for clinical uses. In this study, we examined the use of statins as an approach to provide a more limited and sustainable diminution of cholesterol.

Statins, a family of inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, are principal therapeutic agents for the treatment of hypercholesterolemia due to results in a dramatic reduction in circulating low-density lipoprotein (LDL)-cholesterol [10]. In addition, by inhibiting the conversion of HMG-CoA to L-mevalonic acid, statins prevent the synthesis of important isoprenoids, such as farnesyl-pyrophosphate (FPP) an important precursor of cholesterol biosynthesis, as well as side reactions that generate geranylgeranylpyrophosphate (GGPP), all of which are shown in Figure 3A [11]. Prenylation also occurs in many cellular and systemic regulatory pathways and there is mounting experimental evidence that these non-cholesterol related properties induce the pleiotropic effects of statins involved in immunomodulation [12], neuroprotection [13], and cellular senescence [14]. In chondrocytes, studies have shown that statins have protective, anti-inflammatory effects where they may function in immunoregulation of inflammatory cytokines or more directly as inhibitors of MMP expression [15-17]. Another study demonstrated that statins increased the mRNA levels of bone morphogenetic protein-2, aggrecan and collagen type II [18]. In an *in vivo* animal model, statins were shown to reduce cartilage degradation in OA rabbits [19]. Based on the above observations, this study addressed whether statins exert a protective effect that included the inhibition of CD44 cleavage in chondrocytes and improved retention of pericellular matrix.

2. Material and Methods

2.1 Cell Culture

Bovine articular chondrocytes (BAC) were isolated from full-thickness slices of cartilage from metacarpophalangeal joints of 18–24-month-old steers as described previously [5]. Human articular chondrocytes (HAC) were isolated from knee

cartilage obtained following replacement surgery with institutional approval. These cartilage samples were from patients ranging in age from 63 to 73 years. Chondrocytes were isolated from full-thickness slices of normal-looking articular cartilage regions by sequential digestion with Pronase (EMD Biosciences) and collagenase P (Roche) [20]. The primary chondrocytes were cultured in DMEM:Ham's F12 nutrient media mixture (Sigma-Aldrich) containing 10% fetal bovine serum (FBS). The chondrocytes were plated as high-density monolayers (5.0 x 10^5 cells/cm^2) for analysis of primary cells or as low-density monolayers (5,000 cells/cm^2) for particle exclusion assay analysis. HCS-2/8 cells are a continuous long-term culture line derived from a human chondrosarcoma [21]. HCS-2/8 cells were grown in DMEM supplemented with 10% FBS. The HCS-2/8 cells were passaged at confluence using 0.5% trypsin, 0.2% EDTA.

2.2 Treatment of Cells

HCS-2/8 cells, BAC and HAC were pretreated with the indicated dose of simvastatin (Sigma-Aldrich) for 48h prior to stimulation with 0.5 ng/ml IL-1β (R&D Systems) and 10 ng/ml Oncostatin M (OSM; Cell Signaling Technology) for 24h in fresh culture medium. In some experiments, BAC were treated with simvastatin in the presence of mevalonic acid (Mev; Sigma-Aldrich), fernesyl pyrophosphate (FPP; Sigma-Aldrich) or geranylgeranyl pyrophosphate (GGPP; Sigma-Aldrich), water soluble cholesterol (Chol; Sigma-Aldrich) then stimulated with IL-1β + OSM. For experiments designed to inhibit prenyltransferases, medium was supplemented with 10μM geranylgeranyltransferase 1 inhibitor; GGTI-286 (Calbiochem) and 10μM farnesyltransferase inhibitor; FTI-277 (Calbiochem) prior to stimulation. HCS-2/8 cells and BAC were treated with GI254023X as ADAM10 inhibitor. In some

experiments HCS-2/8 cells, BAC and HAC were stimulated with IL-1β+OSM in the presence of the γ-secretase inhibitor DAPT (Calbiochem). The final concentration of DMSO was set to 0.1% in all culture conditions.

2.3 siRNA Treatment

MT1-MMP, ADAM10 and ADAM17 siRNA were purchased from Santa Cruz Biotechnology. Negative-control siRNA was purchased from Sigma Aldrich. HCS-2/8 cells and BAC were transfected with 90 pmol of the indicated siRNAs for 48h using Lipofectamine RNAiMax transfection reagent (Invitrogen) according to the manufacturer's protocol. Knockdown of target proteins was confirmed by western blot with indicated antibodies.

2.4 Western Blotting

Total protein was extracted using Cell Lysis Buffer (Cell Signaling Technology) containing protease inhibitor cocktail (Thermo Fisher Scientific). Equivalent protein concentrations were loaded into NuPAGE® Novex® 4–12% gradient sodium electrophoresis mini gels (Invitrogen). After electrophoresis, proteins within the acrylamide gel were transferred to a nitrocellulose membrane using a Criterion blotter apparatus (Bio-Rad), and the nitrocellulose membrane was then blocked in 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T). An antibody specific for the CD44 cytoplasmic tail (anti-cytotail) was used [5]. CD44, ADAM10 (Abcam), ADAM17 (Abcam), MT1-MMP (Abcam), flotillin-1 (Abcam) and β-actin (Cell Signaling Technology) were detected with primary antibodies followed by appropriate secondary antibody. After incubation with corresponding HRP-conjugated secondary antibody, detection was performed using enhanced chemiluminescence reagents (Novex ECL; Invitrogen, West Pico; Thermo Fisher

Science). In some experiments, X-ray films were then digitized using a Chemi-Doc Imager (Bio Rad). Pixel intensity of bands were quantified using ImageJ software.

2.5 Co-immunoprecipitation

Cell monolayers were extracted using 10 mM Tris, pH 7.5, with 2 mM EDTA, 1% Triton X-100, and protease and phosphatase inhibitors. Magnetic protein G Dynabeads® (Invitrogen) were used after the product protocol that for most experiments included the incubation of a preformed antibody-Dynabead complex (10 µg of a primary antibody diluted in 200 µl of PBS-Tween 20) with cell lysates for 60 min. The recovered protein fractions were mixed with 10 µl of NuPAGE® SDS sample buffer, 4 µl of reducing agent, and 6 µl of deionized water and incubated 10 min at 70 °C and loaded into a 4 –12% NuPAGE® Novex® Tris acetate gradient mini gel and analyzed by Western blot analysis.

2.6 Particle Exclusion Assay

Bovine chondrocytes were cultured overnight in 6 well plates (5,000 cells/cm^2). The medium was replaced with a suspension of formaldehyde-fixed erythrocytes in PBS containing 0.1% BSA [5]. Cells were photographed using a microscope with a CCD camera (BZ-8000, Keyence). The presence of cell-bound pericellular matrix was seen as a particle-excluded zone surrounding the chondrocytes. Cells were treated with calcein-AM to provide contrast and delineate the extent of the plasma membrane. Total excluded pericellular matrix areas and cell areas of fifty cells from randomly selected microscopic fields were measured using analysis software (BZ-8000, Keyence) and mean percentage of the pericellular matrix areas were calculated.

2.7 Sucrose density-gradient centrifugation

HCS-2/8 cells were lysed in HEPES buffer containing 1% Triton X-100, followed by homogenization with 10 strokes with a dounce homogenizer. The lysate was then mixed with an equal volume of 80% sucrose in HEPES buffer, followed by overlaying with 1.5 ml of 30% sucrose and 1.5 ml of 5% sucrose solutions. The sucrose gradient was centrifuged for 21h at 32000 rpm in an SW41 rotor (Beckman-Coulter) and ten 1ml fractions were harvested separately. Thirty microliter aliquots from each fraction, number 1–10, were used for subsequent western blot analysis.

2.8 Statistical analysis

Most experiments were repeated at least twice. Experiments performed using human OA chondrocytes were repeated using cells derived from the cartilage of three different osteoarthritic patients. Experiments using bovine articular chondrocytes represent at least two independent preparations of cells per experiment. Results are displayed as representative experiments and are presented as mean ± SD. Statistical analysis were performed using Stata version 13 software and statistical differences were assessed using the Student's t test and one-way ANOVA/Bonferroni post hoc test analysis. $P < 0.05$ was considered significant.

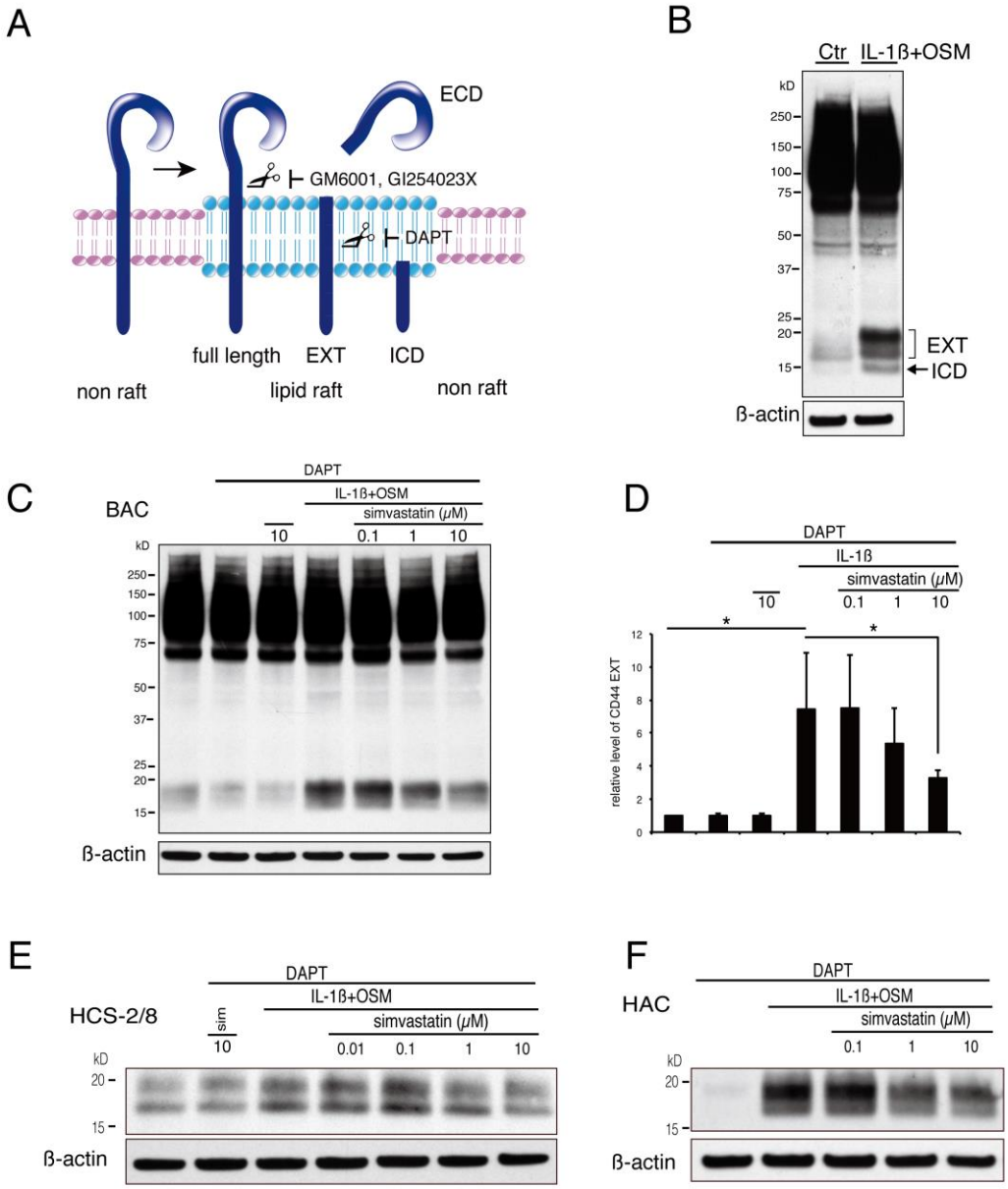
3. Results

3.1 Simvastatin inhibits CD44 fragmentation induced by IL-1β + Oncostatin M stimulation

As previously reported [5], treatment of human or bovine articular chondrocytes with inflammatory cytokines such as IL-1β induces a state of enhanced catabolism that

includes CD44 fragmentation. Upon treatment of primary bovine articular chondrocytes (BAC) with 0.5 ng/ml IL-1β and 10 ng/ml OSM for 48h, the enhanced fragmentation was revealed by the generation of 18-20 kD doublet CD44-EXT bands and the 15 kD CD44-ICD bands on Western blots (Fig. 1B). For analysis of the effect of simvastatin we used the γ-secretase inhibitor DAPT (*N*-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester) during IL-1β+OSM stimulation to block the generation of the 15 kD CD44-ICD (Fig. 1A). As expected, blocking the second cleavage step with DAPT blocked and resulted in an accumulation of 17-20 kd CD44-EXT, products of the first cleavage step, and therefore a suitable condition for the analysis of the inhibition of CD44 fragmentation by simvastatin [5]. The addition of simvastatin, most effective at 10 µM, affected a significant reduction in the formation of CD44-EXT peptides in bovine articular chondrocytes (BAC, Fig. 1C). Similar reductions in IL-1β+OSM-induced generation of CD44-EXT peptides was observed in HCS-2/8 cells (Fig. 1E) and human OA articular chondrocytes (HAC, Fig. 1F) due to 48 h pre-incubation with simvastatin; again most effective at 10 µM.

Figure 1

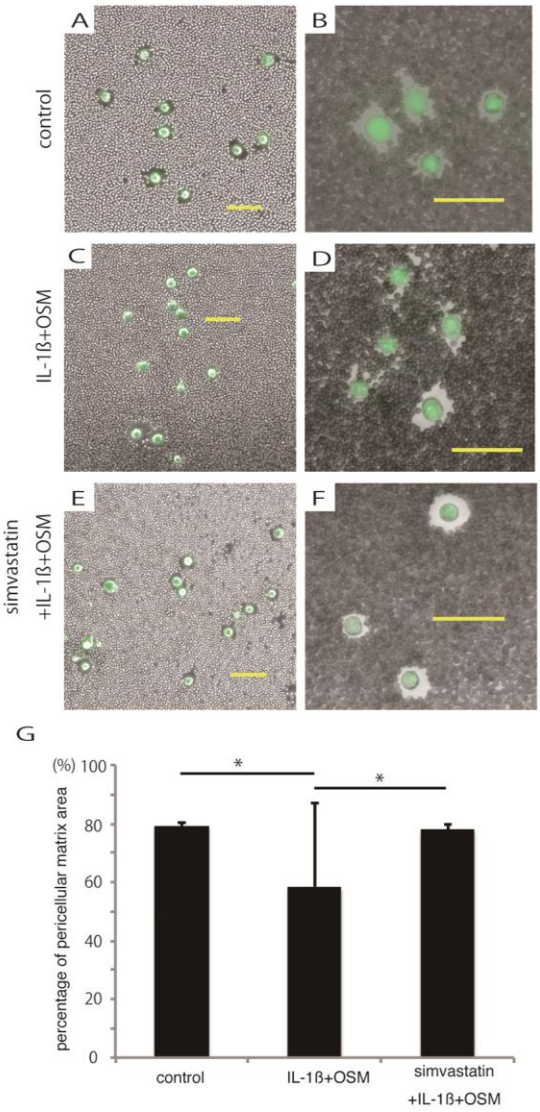


3.2. Effect of simvastatin on pericellular matrix retention.

One of the typical phenotypic characteristics of bovine chondrocytes in culture is the retention of large pericellular matrix (Fig. 2, A and B). These pericellular matrices are composed, at a minimum, of hyaluronan-aggrecan aggregates anchored to the cell surface by hyaluronan binding to CD44 [1-3]. When bovine chondrocytes were grown under conditions that induce CD44 proteolytic cleavage [5], namely treatment

with IL-1β + OSM, the cells lost their capacity to retain a pericellular matrix (Fig. 2, C and D). However, pre-incubation with simvastatin blocked the effect of IL-1β + OSM, resulting in chondrocytes with pericellular matrices of nearly equivalent size as control cells (Fig. 2, E and F). This was documented by morphometric analysis of matrix and cell areas taken from fifty cells randomly selected of each experimental group. As shown in Fig. 2G, the pericellular matrix area of control chondrocytes represented, on average, 80% of the total particle-exclusion area. This percentage of the total area diminished significantly in the IL-1β + OSM-treated group but, returned to control percentage when the cells were pre-incubated with simvastatin prior to IL-1β + OSM treatment (Fig. 2G). These data indicated that the simvastatin rescue was dependent on reversing IL-1β+ OSM effects on CD44, HA or aggrecan; resulting in the improved retention pericellular matrix.

Figure 2

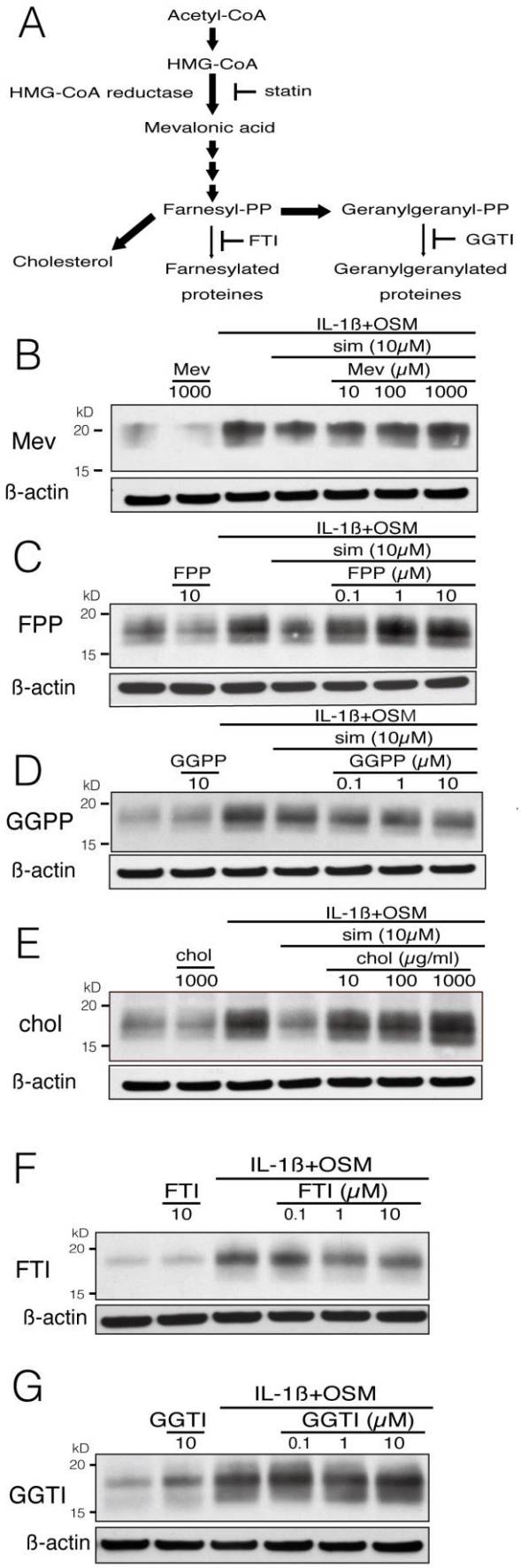


3.3. Reversible effect of simvastatin by the additional treatment with intermediates of the mevalonate pathway

Statins inhibit HMG-CoA reductase, which catalyzes the rate-limiting step in the generation of mevalonate. Mevalonate or mevalonic acid (Mev) once generated is converted to cholesterol via the production of a number of intermediates, all of which

are depleted following statin treatment (Fig. 3A). The effect of statins can be reversed by the introduction of Mev as well as the isoprenoids farnesyl-pyrophosphate (FPP), squalene and cholesterol [22]. Thus, to determine the primary mechanism of simvastatin inhibition of CD44 fragmentation, chondrocytes were treated with Mev or one of three of the downstream isoprenoids, FPP, GGPP or cholesterol, in combination with simvastatin. As shown in Fig. 3B, C and E, co-supplementation with Mev, FPP, and cholesterol reversed the inhibitory effect of simvastatin on CD44 degradation observed in IL-1β + OSM-treated bovine articular chondrocytes. This suggested that the simvastatin-inhibitory effect on CD44 fragmentation was due to depletion of cholesterol and cholesterol precursors. On the other hand, addition of the isoprenoid GGPP (Fig. 3D) representative of a side reaction wherein GGPP is transferred as a prenyl group to proteins, had no effect of reversing to the inhibition of simvastatin treatment. Moreover, addition of the chemical inhibitor of the GGPP transfer, geranylgeranyltransferase (GGTI-298), also had no effect of inhibition of CD44 fragmentation (Fig. 3G). However, FPP, in addition to being a precursor to cholesterol may also be used in the transfer prenyl groups to proteins. As shown in Fig. 3F, an increasing dose of the FPP inhibitor (FTI) resulted in a proportional inhibition of CD44 degradation. Thus, these results suggest that the mechanism for simvastatin inhibition of CD44 fragmentation was due primarily to inhibiting the production and accumulation of cholesterol with the additional smaller contribution due to farnesyl transfer to proteins.

Figure 3


A
Acetyl-CoA
HMG-CoA
HMG-CoA reductase
statin
Mevalonic acid
Farnesyl-PP
Geranylgeranyl-PP
FTI
GGTI
Cholesterol
Farnesylated proteines
Geranylgeranylated proteines
B
IL-1ß+OSM
sim (10μM)
Mev
1000
Mev (μM)
10 100 1000
kD
20
15
Mev
ß-actin
C
IL-1ß+OSM
sim (10μM)
FPP
10
FPP (μM)
0.1 1 10
FPP
ß-actin
D
IL-1ß+OSM
sim (10μM)
GGPP
10
GGPP (μM)
0.1 1 10
GGPP
ß-actin
E
IL-1ß+OSM
sim (10μM)
chol
1000
chol (μg/ml)
10 100 1000
chol
ß-actin
F
IL-1ß+OSM
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10
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FTI
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G
IL-1ß+OSM
GGTI
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GGTI (μM)
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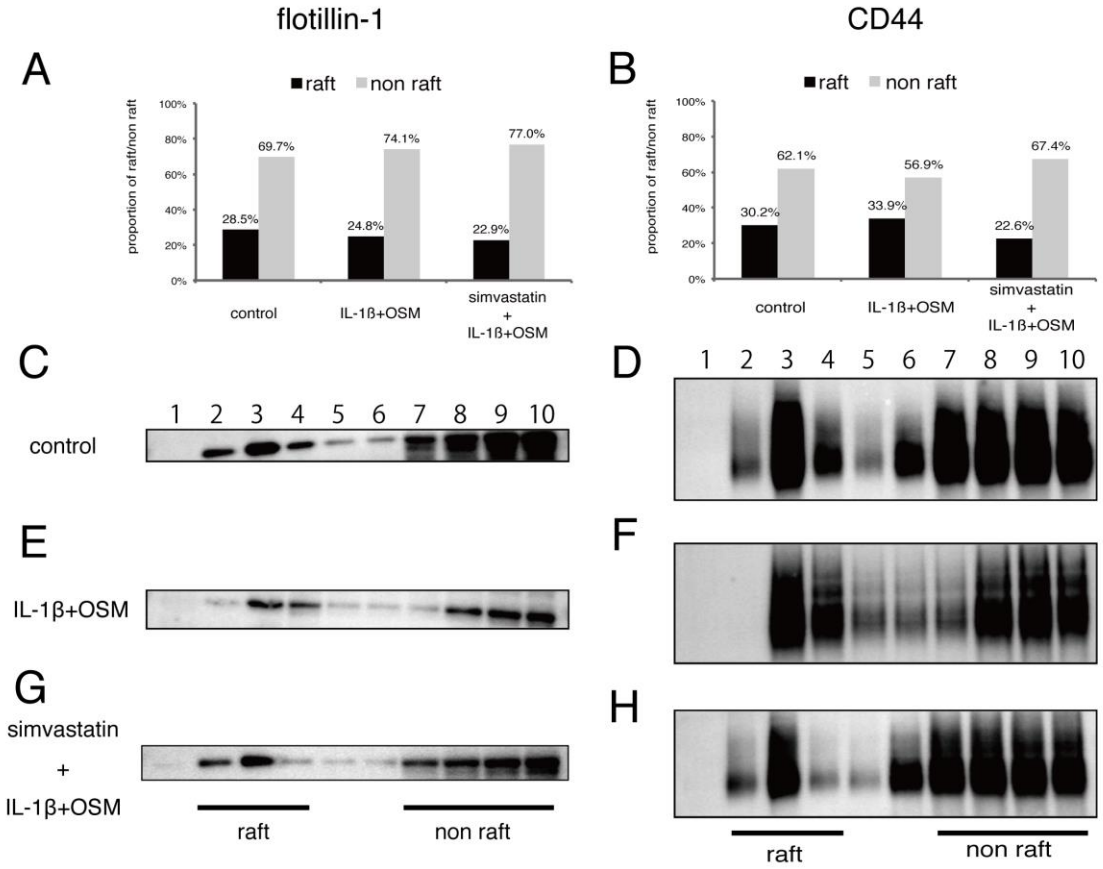
ß-actin

3.4 Simvastatin treatment limits CD44 distribution into lipid rafts.

A previous study demonstrated that CD44 transit into lipid rafts was a required prerequisite for the shedding of this receptor by membrane-type MMPs [5]. The cholesterol chelator, methyl-β-cyclodextrin (MβCD), blocked CD44 cleavage coincident with the removal of membrane cholesterol and dissolution of lipid rafts. We hypothesized that statins such as simvastatin would have similar effects as MβCD because of its role as a cholesterol-lowering drug. To verify this hypothesis we examined the distribution of CD44 in cholesterol-rich lipid raft and non-raft subdomains of chondrocyte plasma membranes following treatment with simvastatin. Cellular membranes of HCS-2/8 cells were isolated and subdomains separated by sucrose density-gradient centrifugation. The position of lipid raft compounds in the sucrose gradients was identified by the distribution of the raft-marker flotillin-1 as viewed by western blot analysis [23]. As shown in Fig. 4C, in untreated control chondrocytes flotillin-1 was distributed predominantly in the low density fractions of the sucrose gradient (fractions 2 to 4). These fractions were determined to represent Triton X-100-insoluble fractions, another feature indicative of lipid rafts. CD44 isolated from control chondrocytes was detected in the similar low-density fractions (Fig. 4D) as well as a population of CD44 in non-raft fractions (fractions 7-10). Upon the stimulation of HCS-2/8 chondrocytes with IL-1β + OSM, a higher proportion of the total CD44 was shifted into the lower density lipid raft fractions (from control to IL-1β + OSM: 30.2% to 33.9%), with a notable decrease of CD44 proteins in the more soluble non-raft (62.1% to 56.9%) (Fig. 4B). In contrast, simvastatin treatment led to a marked decrease of flotillin-1 (from control to simvastatin: 28.5% to 22.9%; Fig. 4A) and CD44 (30.2% to 22.6%; Fig. 4B) distribution in the low-density lipid raft fractions compared with control and proportional increase into the non-raft fractions

of the gradient, flotillin-1 (from control to simvastatin: 69.7% to 77.0%; Fig. 4A), CD44 (62.1% to 67.4%; Fig. 4B). Consistent with these results, a previous study reported that lovastatin treatment decreased the presence of lipid rafts in HEK-293 cells [24]. These data suggest that IL-1β + OSM stimulation promoted the translocation of CD44 into lipid rafts. Simvastatin inhibition of cholesterol biosynthesis, the subsequent depletion of cholesterol in the plasma membrane and dissolution of lipid rafts, is likely one mechanism for the inhibitory effects of simvastatin on CD44 fragmentation.

Figure 4

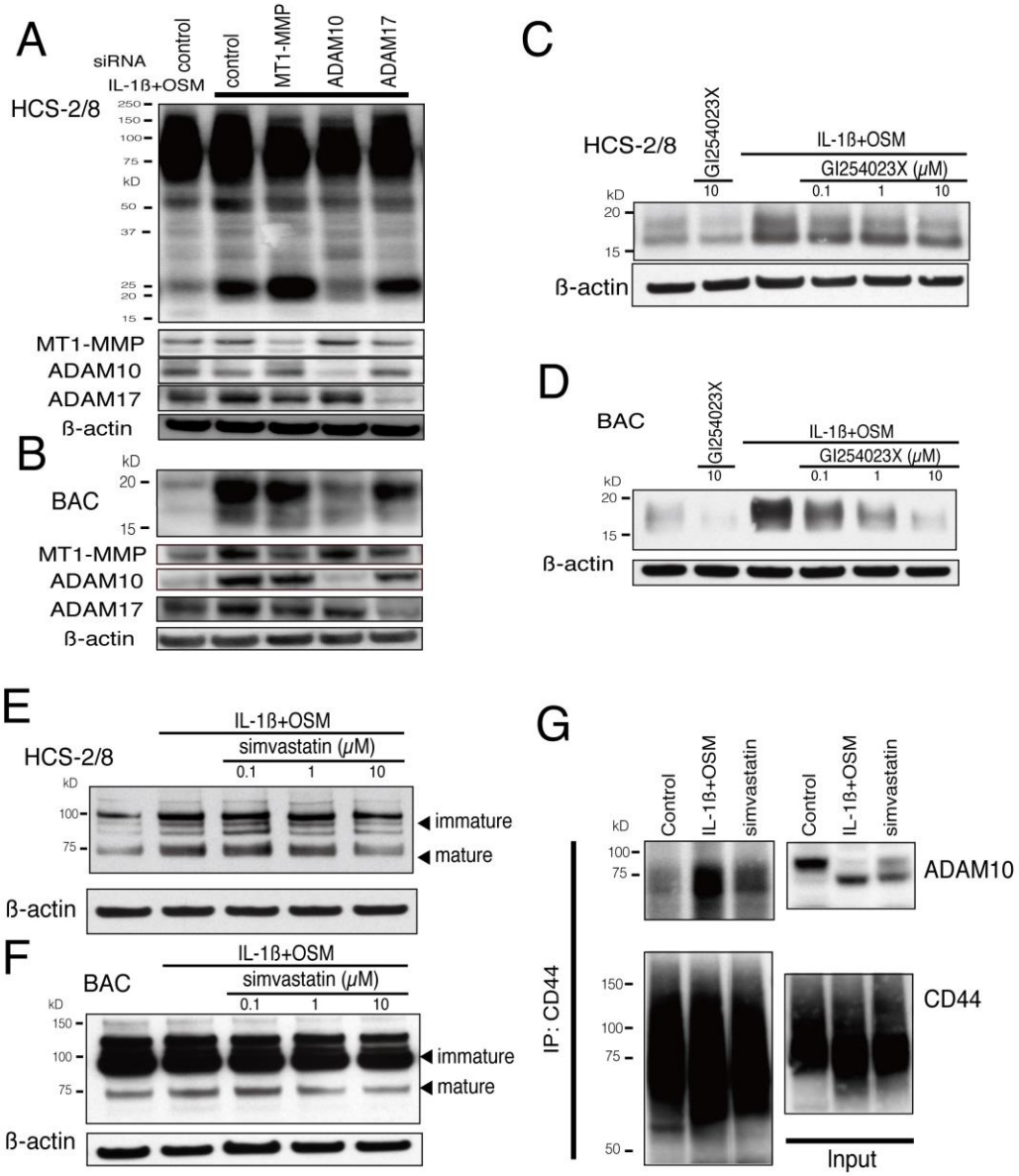


3.5 ADAM10 regulates the cleavage of the extracellular domain of CD44

In previous reports, the first proteolytic cleavage of CD44 occurs by the action of membrane-type MMPs such as MT1-MMP (MMP14), ADAM17 or ADAM10 [7,25]. To determine which MMP was responsible for the shedding of CD44 in chondrocytes, a RNA interference approach (siRNA) was used to individually knockdown MT1-MMP, ADAM10 and ADAM17 expression in HCS-2/8 cells in the presence of the γ-secretase inhibitor DAPT. Validation of substantial siRNA knockdown of ADAM10, MT1-MMP and ADAM17 as determined by western blotting are shown in Fig. 5A, B. When HCS-2/8 cells were stimulated with IL-1β + OSM, only the knockdown of ADAM10, but not that of MT1-MMP, ADAM17, markedly inhibited CD44 cleavage and the generation of CD44-EXT (Fig. 5A). The same results were obtained in BAC (Fig5B). To confirm the role for ADAM10, we used the ADAM10 inhibitor GI254023X (Fig. 1A). Increasing doses of GI254023X inhibited CD44 cleavage in both HCS-2/8 cells and BAC (Fig. 5C, D). These results indicated that ADAM10 is the principal sheddase of CD44 in this study. In the next step, we confirmed the activity of ADAM10 under the condition of simvastatin treatment. ADAM10 protein in cell lysates of chondrocytes was revealed as two bands at 90kD and 70kD, corresponding to the immature form (prodomain-containing) and mature form (prodomain-lacking), respectively. Simvastatin decreased 70 kD mature form of ADAM10 in both HCS-2/8 (Fig. 5E) and BAC (Fig. 5F). To further validate these findings, we examined the potential of a physical interaction between CD44 and ADAM10 using co-immunprecipitation. Cells treated with IL-1ß + OSM displayed enhanced ADAM10 co-immunoprecipitation with CD44 as compared to untreated control cells (Fig. 5G; upper left blot). Simvastatin treatment reduced the level of ADAM10 that co-immunoprecipitated with CD44. These results suggested that ADAM10 has central role in CD44 fragmentation and simvastatin has the potential as

ADAM10 inhibitor. In addition, simvastatin-mediated inhibition of cholesterol biosynthesis and the dissolution of lipid rafts is likely responsible for preventing interactions between CD44 and ADAM10, thereby blocking CD44 cleavage.

Figure 5



3. Discussion

To the best of our knowledge, we are the first to report the protective effects of statins on pericellular matrix retention and the inhibition of CD44 fragmentation in

chondrocytes. In cartilage, chondrocytes are highly dependent on the integrity of the pericellular matrix for nutrient support and external signal recognition [26]. The HA / aggrecan-rich pericellular matrix of chondrocytes is anchored to the cell surface via the interaction between HA and CD44 [3]. Thus, loss of the CD44 through proteolytic cleavage events will have detrimental effects on this integrity and contribute to the dysregulation of chondrocyte metabolism.

CD44 cleavage or shedding is most often associated with tumor invasion and metastasis [27,28]. Takahashi *et al*. demonstrated that CD44 fragmentation was also a prominent feature of freshly isolated human OA chondrocytes, where substantially higher levels of CD44 fragmentation were observed in comparison to normal human chondrocytes isolated from donor ankle cartilage [5]. In normal bovine articular chondrocytes, CD44 fragmentation is barely detectable unless the cells are treated with agents that induce an OA-like phenotype such as IL-1β, HA oligosaccharides or phorbol 12-myristate 13-acetate [5]. This suggests that CD44 fragmentation is a sensitive marker of changes in cell-associated protease activity, be it in malignant cells, or highly catabolic OA chondrocytes. Simvastatin treatment may thus provide a safe, effective approach to dampen these membrane proteases and provide an environment more conducive to repair.

In this study, we determined that simvastatin has the potential to inhibit CD44 fragmentation and our data suggests that there are two main mechanisms for this effect. First, simvastatin decreases ADAM10 activity that has an important role in CD44 fragmentation. Second, simvastatin inhibits the interaction between CD44 and ADAM10 due to a decrease in the proportion of CD44 existing in lipid raft.

CD44 cleavage occurs in a variety of tissues and is mediated by membrane-type MMPs including MT1-MMP, ADAM10 and ADAM17 [7,25] although many recent

studies point to ADAM10 as a major sheddase [7,29]. In our study, only siRNA-mediated knockdown of ADAM10 blocked CD44 cleavage (Fig. 5A, 5B) and ADAM10 inhibitor GI254023X also inhibited it in HCS-2/8 cells and BAC (Fig. 5C, 5D). ADAM10 is known to cause ectodomain cleavage of a wide variety of cell surface proteins including epidermal growth factor, L1 adhesion molecule, E-cadherin, and Notch [30]. Again, although CD44 and pericellular matrices were the focus of this study, it is likely that many other chondrocyte cell surface proteins and receptors are affected by the enhanced protease activity and re-distribution of ADAM10.

Newly-synthesized ADAM10 protein includes a pro-domain that renders the enzyme catalytically inactive. The ADAM10 pro-domain is removed by a furin-like protease as the protein traverses through the trans Golgi network, resulting in activation of the membrane protease [31]. IL-1β + OSM increased the formation of mature ADAM10. In addition, we showed that simvastatin inhibited the generation of the mature form of ADAM10, ADAM10 activity as well as the interaction of CD44 and ADAM10.

We demonstrated by co-immunoprecipitation analysis that the ADAM10 associated with CD44 in control, untreated chondrocytes was predominately the 90 kD immature isoform whereas following IL-1β + OSM treatment, CD44 complexes contained the mature 70 kD isoform of ADAM10. Co-treatment with simvastatin reduced the percentage of mature/immature ADAM10 associated with CD44, reduced the overall level of ADAM10 complexed to CD44 and reduced the level of proteolytic cleavage of CD44 (Fig. 5G). Future studies will determine whether simvastatin exerts similar effects on the cleavage of other important cell surface proteins of chondrocytes.

In this study, we also demonstrated that simvastatin-mediated inhibition of CD44 fragmentation was reversible by the addition of mevalonate isoprenoid derivatives

namely, Mev, FPP and cholesterol (Fig. 3B, 3C, 3E) but not GGPP (Fig. 3D). The reversible effect of Mev proved that the protective effect of simvastatin caused by inhibition of mevalonate pathway. Cholesterol and FPP reversible effects indicate that cholesterol lowering plays and important role in this model because the mevalonate pathway has the specific character that FPP can be converted either to cholesterol or to GGPP (Fig. 3A). In addition, FTI (but not GGTI) inhibited CD44 fragmentation (Fig3F, 3G). Farnesyl transferases use FPP for post-translational modifications of the small GTP-binding proteins of Ras [11]. Ras has been shown to induce CD44 cleavage in cancer cells [32] and has been implicated in MMP-13 expression in chondrocytes [33]. On the other hand, previous studies demonstrated that statin inhibition of IL-1β-stimulated MMP-13 occurred through a mechanism involving the depletion of the GGPP isoprenoid and was not dependent on FPP or cholesterol [34,35]. Although role of Ras is controversial from several studies in chondrocytes, the results of this study suggest that cholesterol and FPP are likely responsible for protective effect of simvastatin in IL-1β + OSM stimulated chondrocytes.

One of the likely mechanisms of action of simvastatin in these studies was the dissolution of cholesterol-rich lipid rafts. Lipid rafts are very small (10-200 nm), highly dynamic membrane domains thought to recruit or exclude various signaling molecules such as G proteins, tyrosine kinases, and phosphatases [36]. The cholesterol chelator MβCD is often used disrupt lipid rafts and provide a loss-of-function approach to identify the role of lipid rafts in cellular events. For example, a study by Fujita *et al*. demonstrated by immunoelectron microscopy using quick-freeze-fractured specimens, that the distribution of GM1 ganglioside displayed clustering in the plasma membrane under normal conditions but this clustering was

lost upon treatment with MβCD [37]. MβCD has been shown previously to block the movement of CD44 into lipid rafts in a variety of cell types [38,39] including chondrocytes [5,9]. In this study, reduction in cholesterol biosynthesis by simvastatin resulted in similar effects as MβCD, including the dissolution of lipid rafts from 28.5% to 22.9% and the shifting CD44 into non-raft membrane domains from 62.1% to 67.4% (Fig. 4A 4B).

The transit of CD44 into lipid rafts is also required for CD44-mediated HA endocytosis [9,40]. When chondrocytes are treated with IL-1β + OSM, it is possible that the movement of CD44 into lipid rafts serves to provide for enhanced HA endocytosis and turnover. Given that following IL-1β + OSM, ADAM10 also becomes localized within lipid rafts, the end result is the sequestering or concentrating of enzyme and substrate within a confined membrane domain. Whether CD44 cleavage by ADAM10 is a part of the mechanism of HA endocytosis or an unfortunate circumstance, remains to be determined. Interestingly, in some cancer cells CD44 cleavage by ADAM10 occurs in non-raft membrane domains. In a study by Murai *et al*. the depletion of membrane cholesterol triggered the shedding CD44 mediated by ADAM10 [41]. Such differing results may depend on the changes in the function of CD44, especially CD44 functions that are dependent on receptor organization within particular membrane subdomain. A report by Babina *et al*. showed that CD44 raft affiliation was increased during migration of non-invasive breast cell lines but decreased during the migration of highly-invasive breast cancers [42]. As noted above, when chondrocytes become catabolically activated, CD44 function shifts from anchoring the HA/aggrecan-rich pericellular matrix to enhanced CD44-mediated HA endocytosis [43]. Also differing from many cell types, the CD44 in chondrocytes interacts primarily with the actin adaptor protein, ankyrin-3 instead of

ERM adaptors [6]. A recent report suggested that CD44 cleavage requires CD44 dimerization, an event mediated by changes in ERM/Merlin interactions [44]. Clearly, multiple mechanisms and cellular environments support the cleavage of CD44. In this study, when chondrocytes were treated with IL-1β + OSM, CD44 and the mature form of ADAM10 displayed enhanced co-localization, shown by co-immunoprecipitation (Fig. 5G) and a substantially increased level of CD44 cleavage (Fig. 1B, 1C, 1E, 1F). Given that both these events are reversed upon treatment with simvastatin strongly suggests that CD44 cleavage by ADAM10 occurs in a cholesterol-rich lipid raft environment in chondrocytes.

In addition to diminishing cell surface protease activity, statins have been reported to have other anti-catabolic effects on chondrocytes, including preventing collagen breakdown by MMPs (MMP-1, 3, 9, 13) and decreasing pro-inflammatory and inflammatory mediator levels such as IL-1β, TNF-α, IL-6. [16,34,35,45,46]. Pro-anabolic effects have also been reported. For example, Hatano *et al*. [18] demonstrated that mevastatin increased mRNA expression of bone morphogenetic protein-2, aggrecan, and collagen type 2 as well as increasing proteglycan synthesis. In this study we report a reduction in CD44 cleavage by simvastatin. It may be a combination of the pleiotropic, protective effects of statins that provides for the regrowth and assembly of pericellular matrices such as those shown in Fig. 2E, 2F and 2G.

Statins are widely used therapeutically and there is already substantial evidence that they reduce morbidity and mortality in patients with hyperlipidemic cardiovascular disease [47,48]. However, with regards to statin use in clinical studies of human knee OA, oral statin use was not associated with improvements in knee pain, function or structural progression over a 4-year study period [49]. On the other hand,

Akasaki *et al*. [19] demonstrated that intra-articular injection of mevastatin attenuated histological degradation in an OA rabbit model. These differing results suggest that to obtain the protective effects of statins in joint cartilage, the method of statin administration may be an important issue.

Three in vitro cell types were used in this study as representative models of “chondrocytes” namely, HCS-2/8 cells, human OA chondrocytes and BAC. HCS-2/8 cells were established from a human chondrosarcoma and can proliferate permanently as a cell line. Nonetheless, the HCS-2/8 cells synthesize an extracellular matrix consisting of cartilage-specific proteoglycan and type II collagen. The human OA chondrocytes, although derived from normal-looking cartilage regions from patient material removed during knee replacement surgery, likely display an altered cellular metabolism. Interestingly, in all three cell types, HCS-2/8 cells, human OA chondrocytes and BAC, treatment with simvastatin diminished the proteolytic fragmentation of CD44 (Fig. 1C, 1E, 1F).

In conclusion, we demonstrated that treatment of human HSC-2/8 chondrosarcoma chondrocytes as well as human OA chondrocytes and primary bovine articular chondrocytes with simvastatin inhibited CD44 cleavage induced by inflammatory cytokines. Simvastatin reduced cholesterol-rich lipid rafts and the translocation of CD44 into these lipid rafts. ADAM10 was found to be the principal sheddase in these chondrocytes and simvastatin reduced the co-localization of CD44 and ADAM10. We propose that CD44 cleavage in chondrocytes serves as a useful marker of cell surface protease activity in general. All of the protective effects of statins may, including a reduction in CD44 shedding, combine to promote chondrocyte repair as

was visualized in this study by the re-assembly of a HA / aggrecan-rich pericellular matrix.

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Figure 1

Simvastatin inhibits CD44 fragmentation induced by IL-1β + Oncostatin M stimulation (A) CD44 cleavage requires that CD44 transit into lipid raft (horizontal arrow). CD44 can be cleaved by 2 steps. The first is a metalloproteinase mediated cleavage that generates an extracellular domain fragment (ECD) and a C-terminal fragment (CD44-EXT). Next, cleavage of CD44-EXT by γ-secretase generates CD44-ICD. These cleavages can be blocked by inhibitors (GM6001; general MMP inhibitor, GI254023X; ADAM10 inhibitor, DAPT; γ-secretase inhibitor). (B-F) Cell lysates were analyzed by Western blotting using anti-CD44 cytotail antibody. (B) Upon treatment of bovine articular chondrocytes (BAC) with 0.5 ng/ml IL-1β and 10 ng/ml Oncostatin M (OSM) for 48h, CD44 fragmentation was enhanced. The enhanced fragmentation included the 18-20 kD doublet CD44-EXT bands and the 15 kD CD44-ICD bands. (C) In the presence of 5 μM γ-secretase inhibitor DAPT, pre-incubation with indicated dose of simvastatin for 48h inhibited the IL-1β +OSM induced CD44 fragmentation in bovine articular chondrocytes (BAC). (D) The histogram depicts mean values of relative level of CD44 EXT band ± S.D. from three independent experiments; * $p < 0.05$. (E) (F) Pre-incubation with indicated dose of simvastatin and 5 μM DAPT for 48h inhibited the IL-1β +OSM induced CD44 fragmentation in (E) HCS-2/8 cells and (F) human articular chondrocytes (HAC). Panel F is representative of experiments from using chondrocytes from three OA patients.

Figure 2

Pericellular matrices were visualized by the exclusion of erythrocyte particles from the cell surface.

(A) (B) Bovine articular chondrocytes were incubated in the absence or (C) (D) presence of 0.5 ng/ml IL-1β and 10 ng/ml OSM. (E) (F) Chondrocytes were treated with simvastatin in the presence of IL-1β + OSM. These are shown as two-color overlays of phase contrast images and green fluorescence due to calcein-AM to provided contrast. All bars are 50μM. (G) The percentage of pericellular matrix area was calculated that the area of pericellular matrix divided the area of pericellular matrix plus cell area. Fifty cells were selected randomly in each group for morphometric analysis. Bars represent the mean ± SD. * = $P < 0.05$ by one way ANOVA test.

Figure 3

Reversible effect of additional treatment with intermediates of mevalonate pathway isoprenoids on CD44 fragmentation

(A) Statins inhibit cholesterol synthesis at the level of mevalonic acid (Mev) formation and decrease in the downstream biosynthesis of cholesterol and other intermediate metabolites, including the isoprenoids farnesyl pyrophosphate (FPP), squalene and geranylgeranyl pyrophosphate (GGPP). In this study, Mev, cholesterol, FPP and GGPP as representative intermediates or side products of the cholesterol pathway. Selective inhibitors of farnesylation (FTI) and geranylgeranylation (GGTI) were used to target distinct side reactions. (B – G) CD44 fragmentation in bovine articular chondrocytes (BAC), was detected by Western blot with anti-CD44 cytotail antibody. In all experiments, chondrocytes were treated with 5 μM γ-secretase inhibitor DAPT, and chondrocytes were stimulated with IL-1β + Oncostatin M (OSM) as indicated. (B-E), In BAC, CD44 fragmentation enhanced by IL-1β + OSM was detected by Western blot analysis of CD44-EXT band formation following simvastatin treatment

in the presence of varying concentrations of Mev (B), FPP (C), GGPP (D) and water soluble cholesterol (F) for 48h. (F) (G) BAC were pre-incubated with indicated dose of FFI (F) or GGTI (G) in combination with 0.5 ng/ml IL-1β and 10 ng/ml OSM at 24 h.

Figure 4

Simvastatin treatment limits CD44 distribution into lipid rafts

(A) (B) This observation was verified by calculation of the raft/non-raft affiliation. (C) (D) HCS-2/8 cells were incubated in the absence or (E) (F) presence of 0.5 ng/ml IL-1β and 10 ng/ml OSM or (G) (H) treated with simvastatin in the presence of IL-1β + OSM. Cell lysates were subjected to sucrose gradient centrifugation to isolate lipid rafts and fractions 1 to 10 (top to bottom) were analyzed by Western blot. Fractions 2 to 4 were designed as lipid rafts indicated by immunodetection of the marker protein flotillin-1. CD44 was detected in raft fractions as well as non-raft (soluble) fractions using the anti-CD44 cytotail antibody.

Figure 5

ADAM10 regulates the cleavage of the extracellular domain of CD44

(A) HCS-2/8 cells and (B) bovine articular chondrocytes (BAC) were transfected with control, MT1-MMP, ADAM10, or ADAM17 siRNAs. These cells were then treated with 0.5 ng/ml IL-1β and 10 ng/ml and γ-secretase inhibitor 5μM DAPT for 24h. Lysates were subjected to Western blot analysis to confirm efficacy of siRNA treatments on enzyme expression and on CD44 cleavage. (C) HCS-2/8 cells and (D) BAC were incubated in the absence or presence of 0.5 ng/ml IL-1β + 10 ng/ml OSM and 5μM DAPT for 24h with or without GI254023X treatment for 24h. Lysates were probed for CD44-EXT domains to reveal CD44 cleavage. (E) HCS-2/8 cells and (F)

BAC were incubated in the absence or presence of 0.5 ng/ml IL-1β + 10 ng/ml OSM and 5 μM DAPT for 24h with o r without simvastatin pre-treatment for 48h. The lysates were subjected to immunoblot analysis with anti-ADAM10 antibody. (G) HCS-2/8 cells were detergent-extracted using 10 mM Tris, pH 7.5, with 2 mM EDTA, 1% Triton X-100, and protease and phosphatase inhibitors. The lysates were then either analyzed by Western blotting directly (input) or immunoprecipitated using the CD44 anti-cytotail antibody (IP:CD44). Antibody-antigen complexes were captured on protein-G magnetic microbeads. Equivalent volume aliquots of all eluded fractions were processed for Western blot analysis using anti-ADAM10 antibody and anti-CD44 cytotail antibody.