INTRODUCTION: CD44 mediates the endocytosis of hyaluronan (HA) and HA-retained aggrecan fragments. Most of the internalized HA is destined for complete catabolism intracellularly via the action of lysosomal hyaluronidases. The hyaluronidases are represented by a family of β-endoglucosidases that degrade HA by cleaving internal β-1, 4 linkages. Six hyaluronidase genes have been identified in humans [1]. Cartilage has been shown to express mRNA encoding HYAL1, HYAL2 and HYAL3 and of these three hyaluronidases, hyaluronidase 2 (HYAL2) is the predominant mRNA transcript expressed in human chondrocytes [2]. Recently, studies in rat fibroblasts have demonstrated that HYAL2 binds to CD44 and may be co-internalized with CD44 into endosomes [3]. Moreover, when HYAL2 was overexpressed in the rat fibroblasts, proteolytic fragmentation of CD44 was induced but, the overexpressed HYAL2 had no enzymatic activity as a hyaluronidase. Other contrasting studies suggest that HA in the medium of cells may be degraded if HYAL2 and CD44 are both overexpressed [4]. We have recently observed substantial CD44 fragmentation associated with human osteoarthritic (OA) chondrocytes [5]. The cleavage of CD44 in chondrocytes involves a sequential two step mechanism including the action of a membrane-bound metalloprotease followed by intracellular cleavage by a γ-secretase. Although there is controversy as to whether HYAL2 exhibits hyaluronidase activity, CD44 and HYAL2 may still interact and affect other cellular properties—proteolytic activities such as CD44 fragmentation. The aim of this study was to investigate whether changes in CD44 modulate HYAL2 retention or, whether HYAL2 modulation affects CD44 cleavage in chondrocytes.

MATERIALS AND METHODS:

Cells: Human articular chondrocytes were isolated from knee cartilage obtained following joint replacement surgery, within 24 hours after surgery and with institutional approval. Primary bovine chondrocytes were isolated from the articular cartilage of metacarpophalangeal joints of 18-24 month old adult bovine steers. Cells were liberated from full-thickness slices of articular cartilage by sequential 0.4% pronase and 0.025% collagenase P digestion. The chondrocytes were grown as high-density monolayers (2.0x10^4 cells/cm²) in 1:1 DMEM/Ham’s F12 medium containing 10% FBS. The immortalized human chondrocyte cell line, C28/I2 was grown in DMEM containing 10% FBS.

Treatments of cells: Human chondrocytes were treated with various concentrations of IL-1β for 24 hours. Bovine chondrocytes were transfected with control-siRNA or CD44-siRNA using Amaxa nucleofection, followed by incubation with 1.0 ng/ml IL-1β for 24 or 48 hours. In some experiments, bovine chondrocytes were separately incubated with 10 μM GM6001 (general MMP inhibitor), 10 μM DAPT (γ-secretase inhibitor) for 24 hours, with or without 1.0 ng/ml IL-1β. After all cell treatments, the conditioned media and cell lysates were collected separately. The medium was concentrated 10-fold. Equivalent volumes of the concentrated conditioned medium or equivalent protein of cell lysates were analyzed by western blot analysis.

Co-immunoprecipitation (Co-IP): C28/I2 cells were lysed in a solution containing 10 mM Tris, 2 mM EDTA, 1% Triton X-100, protease and phosphatase inhibitor cocktails. Some C28/I2 cells were incubated with 0.25% trypsin for 15 min, followed by inactivation prior to cell lysis. Protein-G Dynabeads were incubated with anti-human CD44 antibody (BU52) diluted in PBS/Tween-20 for overnight with rotation. The washed beads-antibody complexes were then incubated with cell lysate for overnight with rotation, followed by five washes. Eluted and flow-through protein fractions were analyzed by western blotting; immunoblotted with HYAL2 antibodies.

RESULTS: When human OA chondrocytes were treated with IL-1β, a proportional increase in the 54 kD HYAL2 bands was observed in the medium fraction (Fig. 1). In contrast, the 54 kD bands were much weaker in the cell lysates and displayed no apparent change in protein levels with increase in IL-1β (Fig. 1). To determine if CD44 played a role in HYAL2 retention, bovine chondrocytes were transfected with CD44-siRNA. This approach results in a ~50% knock-down of CD44 mRNA and protein expression. Compared to control siRNA, CD44 knock-down treatment resulted in enhanced accumulation of HYAL2 in the media at both 24 and 48 hrs (Fig. 2A). This suggests that CD44 may function to retain HYAL2. Thus, one possibility for the IL-1β-mediated increase in medium HYAL2 (Fig 1) could be proteolytic shedding of CD44. To explore this possibility, chondrocytes were treated with inhibitors of CD44 proteinase including GM6001 and DAPT. Blocking CD44 proteolysis diminished IL-1β-mediated increase in medium HYAL2 (Fig 2B). To determine whether CD44 and HYAL2 physically interact with each other, human C28/I2 cell lysates were immunoprecipitated (IP) with anti-human CD44 antibody and immunoblotted (IB) using the anti-HYAL2 antibody. A 54 kD band was observed in association with pull-down of CD44 (Fig. 3). Pretreatment of C28/I2 cells with trypsin decreased the co-IP HYAL2 band (Fig. 3).


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