ORIGINAL ARTICLE

# Hyaluronan-binding receptors: possible involvement in osteoarthritis

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**Abstract** Our objectives were to compare the expression of the hyaluronan receptors CD44 and RHAMM in knee synovial tissue of patients with and without advanced osteoarthritis (OA). Both receptors were detected immunohistochemically; the staining appeared more intense in the tissues from the patients with advanced OA. Expression of CD44 and RHAMM were each significantly increased ($p < 0.05$) in synovial tissue from patients with OA, as determined by means of Western-blot analysis. The findings suggested that changes in levels of the HA-binding proteins might be implicated in the development or progression of OA.

**Keywords** CD44 · Hyaluronan-binding proteins · Osteoarthritis · RHAMM

## Introduction

The glycosaminoglycan hyaluronan (HA) is capable of altering the expression of inflammatory mediators in many cell types, depending on the molecular mass and concentration [1, 2]. Changes in concentration and molecular mass of HA reportedly occurred in synovial fluid from osteoarthritic joints [3, 4], and may play a role in the chronic low-level inflammatory condition that characterizes the disease. We were interested in the possibility of a connection between osteoarthritis (OA) and HA at the level of the proteins that specifically bind HA.

CD44 is a major membrane-bound HA receptor with a mass of about 85 kDa, depending on variations in splicing and glycosylation; it occurs in both normal and arthritic synovial tissues [5]. RHAMM occurs on the surface of synovial cells, and intercellularly; the constitutive form is 85 kDa, but smaller isoforms have been identified [6].

Our objectives were to compare the expression of CD44 and RHAMM in synovial tissues of patients with and without OA. We planned to interpret evidence of increased expression of proteins in tissues from patients with OA as support for the proposition that the change in protein levels might be implicated in the development or progression of the disease.

## Materials and methods

### Synovial tissue

The study patients came from a single-site, knee-only orthopaedic practice (DDW). Synovial tissue specimens (six per patient) were obtained from eight patients undergoing total knee arthroplasties for advanced OA (Kellgren-Lawrence grade 4) (age range and average age 51–77 and 63 years, respectively), and from eight patients who were undergoing meniscectomy or ligament reconstruction and who had no radiologic evidence of OA and no arthroscopic evidence of synovial inflammation (age range and average age 14–45 and 27 years, respectively) (control patients). All patients with osteoarthritis used nonsteroidal anti-inflammatory agents and/or oral analgesics during the month prior to biopsy; two patients, one in each of the two studies (see below), had received viscosupplementation

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more than 6 months prior to biopsy. Four control patients used oral analgesics during the month prior to biopsy.

The specimens were taken from relatively flat areas (to minimize variations in the number of synovial lining cells attributable to irregularities of the synovial surface) of the suprapatellar pouch or fat pad using a full bite of an arthroscopic basket punch (No. 012013, Acufex, Smith & Nephew, Andover, MA). In preliminary studies we established that the specimens averaged approximately 20 mg, 0.12 cm$^2$, ±15%. Specimens used for immunohistochemical studies were fixed immediately in 4% formalin, and the others were stored at −80°C until analyzed by Western blot. All experimental procedures were approved by the Institutional Review Board for Human Research at our institution.

Immunohistochemistry

The specimens were processed into paraffin blocks, sectioned at 6 μm, dewaxed, and steamed 20 min in sodium citrate buffer for antigen retrieval. Endogenous peroxidases and background binding were blocked with 0.3% $H_2O_2$ and horse serum, respectively. Sections were exposed overnight at 4°C to mouse-anti-human CD44 (Neomarkers, Freemont, CA) 1:100, and rabbit-anti-human RHAMM (Genscript, Piscataway, NJ) 1:200; phosphate buffered saline was used as a negative control. The sections were incubated in biotinylated pan-specific (anti-mouse and anti-rabbit) secondary antibody for 30 min at room temperature, and the protein was visualized using a commercial kit (ABC reagent, Vector Labs, Burlingame, CA). Adjacent sections were stained with hematoxylin and eosin.

Western blots

The tissues were sonicated in Laemmli buffer (BioRad, Hercules, CA) and the protein levels were then measured (BCA, Pierce Biotechnology, Rockford, IL). For electrophoresis, 1 M dithiothreitol (5 μL) was added and samples (20 μg of protein) were loaded on 10% SDS-polyacrylamide. Three samples from a patient with OA (each from a different biopsy specimen) and three samples from a control patient (also from different specimens) were loaded on each gel; additional lanes contained molecular-weight markers, a positive control (human tonsil tissue lysate for CD44) (ProSci, Inc., San Diego, CA); U373 whole-cell lysate for RHAMM (kind gift of Dr. Omar Skalli), or blanks. The gels were run at 40 mA for 2 h and the proteins were transferred to nitrocellulose (verified by Ponceau-S staining).

Background staining was blocked by bovine serum albumin (BSA) and the blots were incubated overnight at 4°C in either mouse-anti-human CD44 (Neomarkers,

1:500) or rabbit-anti-human RHAMM raised against the peptide sequence GIKHFDPSKAFHHESKC (GenScript, 1:1,000); the blots were also incubated with a primary antibody against alpha-tubulin (Neomarkers, 1:5,000) as a loading control. The proteins were labeled by incubating in horseradish-peroxidase-conjugated anti-rabbit and anti-mouse IgG (GE Biosciences, Piscataway, NJ, 1:5,000, 2 h, room temperature), and detected by chemiluminescence (ECL Plus, GE Biosciences). The maximum optical density (OD) (ImageQuant, GE Healthcare, Piscataway, NJ) was used to characterize the relative protein concentration. To compare CD44 and RHAMM concentrations in patients with and without OA, we used the OD ratio of the receptor and tubulin bands. The values from the individual specimens were averaged, and the result was used in the statistical evaluations.

The data was evaluated using the Mann–Whitney test at $p < 0.05$.

Results

CD44 and RHAMM were detected immunohistochemically in the intimal and subintimal layers of the synovial tissue from each of three patients with OA and from three control patients; typical results are shown in Fig. 1. The staining appeared more intense in the tissues from the patients with advanced OA, but was not quantitated. The histological appearance of the synovium as visualized in the hematoxylin and eosin sections was identical to that previously reported for normal synovium [7] and advanced osteoarthritic synovium [8] (data not shown). In the patients with osteoarthritis the microscopic appearance of the synovium varied markedly with location in the joint. Consequently, in the Western-blot studies (below) we made no attempt to correlate the local inflammatory appearance of the tissue with local HA receptor expression. Instead, we made multiple measurements of the expression in each joint and averaged the results.

Expression of CD44 in synovial tissue was significantly increased in patients with advanced OA (Fig. 2). The six biopsy specimens obtained from each patient were analyzed separately, using tubulin as a loading control; the CD44 levels varied within each joint (Fig. 2a). The average CD44/tubulin OD determinations obtained from the six specimens from each patient was used to compare the OA and control patients; average CD44 expression in the patients with OA was four times greater than that of the controls (Fig. 2b). We analyzed the staining patterns in relation to location within the joint from which the biopsies were obtained but found no common result among the patients.

RHAMM was quantified using the OD of the RHAMM band in relation to the tubulin band on the same gel, and the

data was analyzed similarly to that obtained for CD44. Average RHAMM concentration was more than three times greater in patients with OA (Fig. 3). Again, there was no pattern among the patients regarding receptor expression and biopsy site.
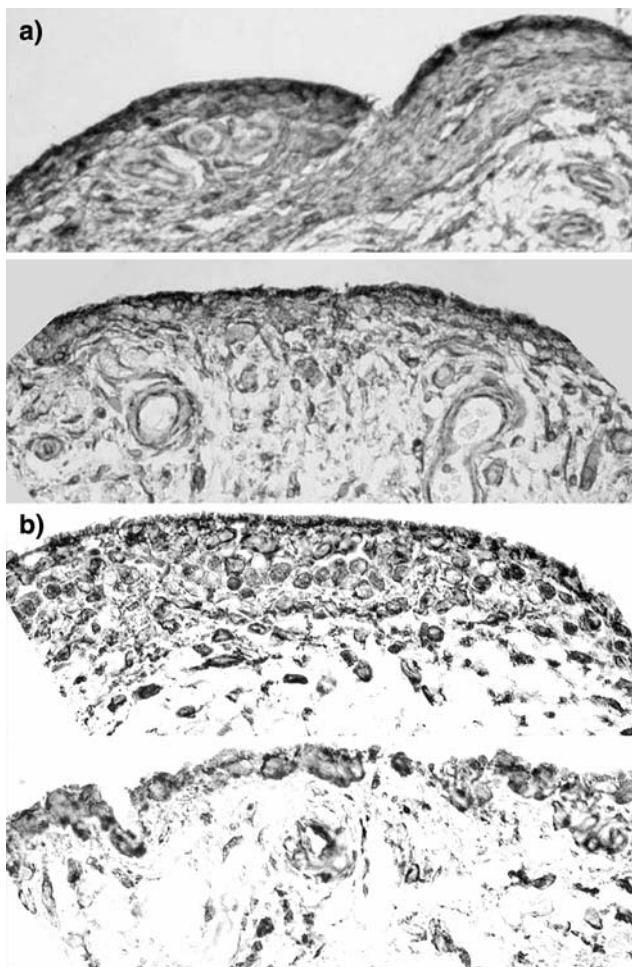


**Fig. 1** Immunolocalization of hyaluronan receptors in synovial tissues from patients with and without osteoarthritis. **a** CD44 (×200). **b** RHAMM (×400). *Top panes* OA, *bottom panes* controls

## Discussion

The importance of HA in determining the unique visco-elastic properties of synovial fluid has long been recognized. The biological signaling properties of HA, in contrast, have only recently come into focus, and the possible link between joint disease and HA-mediated signaling pathways in synovial cells had not been explored. In search of evidence to support the hypothesis that progression of OA was mediated at the level of expression of HA binding proteins, we compared their expression in synovial tissue from patients with or without OA.

The immunohistochemical results suggested that the expression of CD44 and RHAMM were increased in association with the presence of advanced OA (Fig. 1), and these observations were confirmed by means of quantitative Western-blot analysis. Levels of expression of both HA-binding proteins were significantly increased in tissues of patients with OA (Figs. 2, 3). RHAMM had previously been found in OA tissue, but this is the first study in which the HA-binding proteins in tissues from patients with a standardized degree of OA were quantitated and directly compared with those from patients without OA.

Bioethical considerations prevented us from obtaining true control data (tissues of normal age-matched subjects). We attempted to minimize this limitation by choosing control patients who had no arthritis or signs of inflammation. There are no reports of an age dependence of the HA-binding proteins in any tissue, but the possible influence of age still remains.

It might be argued that more than five patients in each group should have been studied. However, the results showing increased expression of each receptor were consistent and clear, and would not likely be different if the groups were larger.

Quantitative, controlled measurements of HA receptor expression in patients with advanced OA have not been previously reported, however, the results of limited measurements have been published. For example, Fuchs et al.
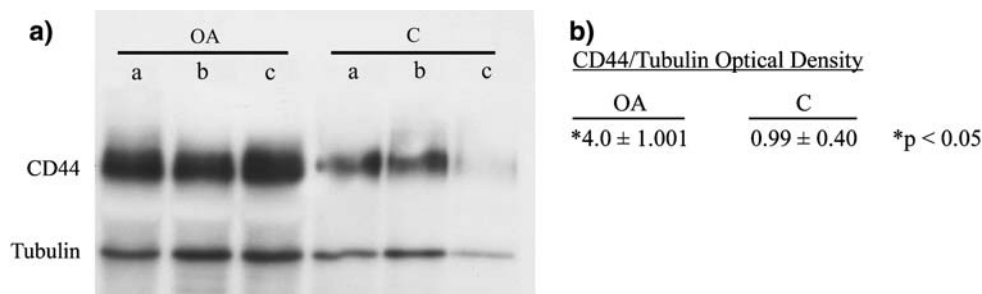


**Fig. 2** Western-blot analysis of *CD44* in synovial tissue from patients with and without advanced osteoarthritis. **a** *a*, *b*, *c*, results from tissue specimens taken at different locations within the joint of a patient with osteoarthritis (*OA*), and corresponding results from a control patient (*C*). **b** Mean optical density ± SE. $N = 5$ in each group

**b)**
CD44/Tubulin Optical Density

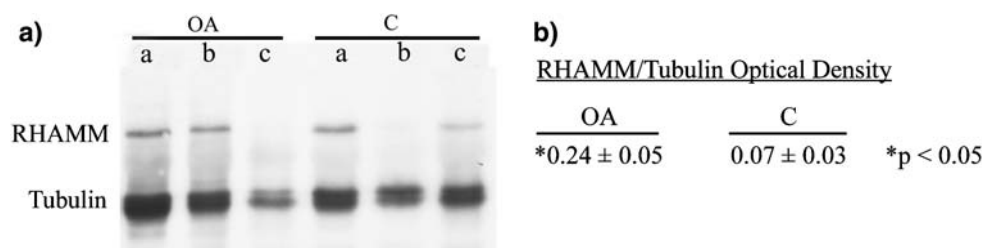| OA | C | |
|---|---|---|
| *4.0 ± 1.001 | 0.99 ± 0.40 | *p < 0.05 |

**Fig. 3** Western-blot analysis of *RHAMM* in synovial tissue from patients with and without advanced osteoarthritis. **a** *a*, *b*, *c*, results from tissue specimens taken at different locations within the joint of a patient with osteoarthritis (*OA*), and corresponding results from a control patient (*C*). **b** Mean optical density ± SE. $N = 5$ in each group

[9] reported disease-related changes in CD44; however, they studied only one CD44 isoform, used a less accurate method (immunocytochemistry), did not employ controls, found expression in only half the patients, and did not measure RHAMM.

RHAMM staining in control tissue was barely detectable immunohistochemically (Fig. 1b); in the Western blots, its expression level was near the noise level of the measurement (Fig. 3b). In patients with OA, RHAMM was prominent in comparison with control tissue (Figs. 1b, 3b), but its staining was still less intense than that of CD44 (Figs. 1a, 2). One possibility was that, in patients with OA, CD44 expression was greater than that of RHAMM; another possibility was that the difference in staining was due to a relative difference in the binding of the antibodies to their antigens.

Although pertinent details regarding how signaling via HA-binding proteins helps to mediate progression of OA is presently unavailable, there is ample literature support for the proposition that such signaling is an intimate part of the inflammatory response. CD44 mediates inflammatory signaling [10], homing of lymphocytes [11, 12], HA cable formation [13], and binding of MMPs [14–16]. Large molecular mass HA bound to clustered CD44 receptors promotes cellular quiescence [17]. The potential inflammation-related regulatory roles of RHAMM have not been delineated as clearly as CD44, but may include its association with signaling mediators such as calmodulin [18, 19] and ERK kinases [20].

In summary, CD44 and RHAMM were over-expressed in patients with OA. This finding supports the theory that the manifestation or perpetuation of the low-grade inflammatory changes associated with OA may be related to signaling involving HA receptors and/or binding proteins.

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