Correlation between expression of CD44 splice variant v8-v9 and invasiveness of fibroblast-like synoviocytes in an *in vitro* system

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Abstract

Objectives

Rheumatoid arthritis is characterized by inflammation, hyperplasia of the synovial membrane, pannus formation and degradation of cartilage and bone. Fibroblast-like synoviocytes are thought to be involved in the invasion and subsequent degradation of cartilage. Two processes play a role in cellular invasion: cellular migration and degradation of the extracellular matrix. The adhesion molecule CD44 and chemokine receptors are instrumental in migration and invasion. Both components have been reported to play a role in tumour metastasis but also appear to be implicated in the destruction of synovial joints in rheumatoid arthritis. CD44, an ubiquitously expressed receptor for the glycosaminoglycan hyaluronan, contains 9 exons that are alternatively spliced and this gives rise to the expression of multiple splice variants, each exhibiting different functional capacities.

Methods

In this report we describe an analysis of the expression of chemokine receptors and CD44 splice variants in diseased synovial tissues using the Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). We have correlated our findings with the clinical diagnosis of rheumatoid or osteoarthritis, with invasion into the extracellular matrix in vitro*, and with the rate of proliferation of fibroblast-like synoviocytes.*

Results and conclusions

We conclude that fibroblast-like synoviocytes from both osteo- and rheumatoid arthritis express a number of different chemokine receptors and CD44-splice variants, but none of these correlate with a particular diagnosis. However, elevated expression of CD44v8-9 was found to correlate negatively with the invasive capacity of fibroblast-like synoviocytes.

Key words

Fibroblast-like synoviocytes, CD44, chemokine receptors, rheumatoid arthritis, invasion.

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Introduction

Rheumatoid arthritis (RA) is a disabling disease characterized by inflammation, hyperplasia of the synovial membrane, pannus formation and degradation of cartilage and bone. Fibroblast-like synoviocytes (FLS) are thought to be involved in the invasion and degradation of cartilage. These cells show characteristics of transformation, including increased proliferation (1), production of type I collagen (2), anchorage-independent growth (3), expression of oncogenes (4-7) and invasion in normal human cartilage in both *in vitro* (8) and *in vivo* systems (9) (co-implanted in SCID mice).

Invasion of the cartilage requires cellular migration and degradation of the extracellular matrix. Candidate molecules involved in migration are CD44 and chemokine receptors.

CD44 is an 80,000 – 95,000 kD transmembrane glycoprotein and was originally discovered as a lymphocyte homing receptor (10-12). Subsequently, CD44 was observed to have functions associated with migration and cell-matrix interactions in many different cell types. CD44 has the ability to bind components of the extracellular matrix, of which hyaluronic acid is the most important component (10, 13, 14). In humans, different CD44-splice variants have been described that confer different functions on the cells that express them. For instance, CD44 variants containing the variable exons v5, v6 and v8-v10 are often found in tumours and are related to a higher rate of metastasis (15).

Several studies have indicated a role for various isoforms of CD44 in the pathogenesis of RA. Previously, it was shown that serum levels of soluble CD44v5 (sCD44v5) and soluble CD-44v6 (sCD44v6) were elevated in patients with rheumatoid factor-positive, erosive RA compared to patients with other inflammatory rheumatic diseases and that the level of sCD44v5 correlated with the inflammatory activity of the disease (16, 17). In another study it was demonstrated that only FLS derived from patients with RA or osteoarthritis (OA) expressed CD44 splice variants and that FLS from non-inflamed tissue did not show CD44 splice variant expression (18). Recently Wibulswas *et al*. showed that FLS from patients with RAexpressed the splice variants v3 and v6 and that antibodies against these splice variants inhibited invasion of Matrigel by FLS *in vitro* (19).

Chemokine receptors are G-protein coupled receptors that play a role in leukocyte trafficking and development (20). It is a large family consisting of 19 members, CCR1 – CCR11, CXCR1 – CXCR6, CX3CR1 and XCR1.

CXCR4 and CCR7 are highly expressed in human breast cancer cells and signalling through these receptors induces chemotactic and invasive responses *in vitro*. In malignant melanoma, the same chemokine receptors are expressed as in breast cancer cells, but also CCR10 is highly expressed, which is implicated in the preference of melanoma cells to metastasise to the skin (21).

On gastric carcinomas CXCR1 and CXCR2, which are receptors for IL-8, are overexpressed and addition of IL-8 to a gastric carcinoma-cell line stimulates invasion *in vitro* (22).

It has already been shown that chemokine receptors play a role in the pathogenesis of RA. Patients with RA expressed CCR3 and CCR5 on both peripheral blood and synovial fluid monocytes, while peripheral blood monocytes from patients with RA also expressed CCR4 (23). CXCR4 was expressed on CD4+ memory T cells in the synovium and its ligand SDF1 was found in the same region as the T cells (24, 25). Addition of SDF1 inhibited activation induced apoptosis of T cells (24), which indicates a role for SDF1-CXCR4 interaction in T cell accumulation in the rheumatoid synovium.

Addition of the chemokines CCL2/ monocyte chemotactic protein-1, CCL5/RANTES and CXCL12/SDF1 enhanced IL-6 and IL-8 production by FLS from patients with RA and their corresponding receptors CCR2, CCR5 and CXCR4 were expressed by FLS. CCL2, CCL5 and CXCL12 were expressed by FLS, which indicates that chemokines play a role in FLS activation in RA in a paracrine or autocrine

manner (26). However, the expression level of these chemokines was not compared between patients with RA or OAor normal controls.
In this report, we investigated the expression of CD44-splice variants and expression of chemokine receptors in FLS from patients with RAand OAand correlated their expression with a diagnosis of RA or OA, with invasiveness and with the rate of proliferation.

Materials and methods

Synovial tissue samples

Synovial tissue was obtained from 29 patients with RA and 25 patients with OA at joint replacement surgery. All patients with RAmet the criteria of the American College of Rheumatology. Tissue was harvested by the orthopaedic surgeon and collected in sterile phosphate buffered saline (PBS). After removal of connective tissue and fat, tissue was digested with collagenase IA (1 mg/ml; Sigma, St. Louis, MO, USA) for 2 hours at 37ºC. Cells were separated from tissue debris by filtration through a 200 µm filter (NPBI, Emmer-Compascuum, The Netherlands) and cultured in 75 cm^2 culture flasks (Cellstar, Greiner, Alphen aan de Rijn, The Netherlands) with Iscove's Modified Dulbecco's medium (IMDM; Biowhittaker, Verviers, Belgium) supplemented with glutamax (GibcoBRL, Paisley, UK) and penicillin and streptomycin (Boehringer, Mannheim, Germany) and 10% foetal calf serum (FCS; GibcoBRL) at 37ºC in the presence of 5% CO_2. When cells had grown to confluence, they were detached with 0.25% trypsin and split in a 1:3 ratio. Cells obtained after 1 or 2 passages were used for RNA isolation and the *in vitro* invasion assay. Cell cultures consisted of more than 95% FLS as judged by Giemsa staining and light microscopy.

RNA isolation and cDNA synthesis

Prior to RNA isolation, FLS were plated in 6-well flat-bottom plates at a density of 100,000 cells per well. RNAwas isolated using RNAzol B (Campro, Veenendaal, The Netherlands) and according to the manufacturer's protocol. RNA pellets were resuspended in diethyl-pyrocarbonate treated water.
cDNA was synthesized from 1 µg of total RNA with oligo dT (Gibco BRL) and mouse Murine Leukemia Virus reverse transcriptase (mMLV-RT; GibcoBRL). In short, 11µl water containing 1µg RNA and 0.5 µg oligo dT was incubated at 72ºC for 10 minutes and then put on ice. 1.2 mM dNTPs (GibcoBRL), 10U RNAsin (Promega, Madison, WI, USA), 5mM $MgCl_2$ (Perkin-Elmer, Branchburg, NJ, USA), 50 mM KCl, 10 mM Tris-HCl pH = 8.3 (PCR buffer II, Perkin Elmer) and 20U mMLV-RT were added to a final volume of 20 µl. This mixture was incubated at 37ºC for 1 hour and 95ºC for 5 minutes.

Reverse transcriptase polymerase chain reaction CD44 splice variants

CD44 splice variants were tested using the primers listed in Table I (Life Technologies, Breda, The Netherlands). PCR amplifications were performed using 1 µl cDNA, 1.5 mM MgCl2 (Perkin-Elmer), 2.5 mM 10x PCR buffer II (Perkin-Elmer), 0.2 mM dNTPs (GibcoBRL) 0.2 µM of primers for the exons to be investigated and 1.5 U Taq polymerase (Promega) in a total volume of 30 µl. The cDNAwas amplified in 40 cycles; 0.5 minute denaturation at 94ºC, 0.5 minute annealing at 64ºC, and 1 minute extension at 72ºC. The first cycle started with 5 minutes denaturation at 94ºC and the last cycle was followed by an extra extension at 72ºC for 10 minutes.
The PCR products were analysed on a 2% agarose gel (Roche, Mannheim, Germany) and detected using ethidiumbromide and UV-light.
β-actin was used as an internal control.

Reverse transcriptase polymerase chain reaction chemokine receptors

Total RNA was treated with DNAse (Promega) according to the manufacturer's protocol.
Expression of chemokine receptors was tested using the primers listed in Table I (Life Technologies). PCR amplifications were performed using 1 µl cDNA, 2.5 mM $MgCl_2$ (3 mM $MgCl_2$ for CCR10, CXCR1 and XCR1), 2.5 mM 10x PCR buffer II (Perkin-Elmer), 0.25 mM dNTPs (GibcoBRL), 0.3 µM of primers for the chemokine receptor to be investigated and 1.15 U Taq polymerase (Promega) in a total volume of 30µl. The cDNA was amplified in 40 cycles; 1 minute denaturation at 94ºC, 1 minute annealing at 58ºC (or 60ºC for CXCR1 and XCR1) and 1 minute extension at 72ºC. The first cycle started with 5 minutes annealing at 94ºC and the last cycle was followed by an extra extension at 72ºC for 10 minutes.
The PCR products were analysed on a 2% agarose gel (Roche, Mannheim, Germany) and detected using ethidium bromide and UV-light.
β-actin was used as an internal control.

In vitro *invasion assay*

Invasiveness of FLS was measured as described previously (8). Briefly, Transwells (6.5 mm diameter, 8.0 µm pore width; Costar, Cambridge, NY, USA) were coated with paraffin to avoid meniscus formation. Hereafter, the transwells were pre-incubated with 100 µl IMDM for 30 minutes at 37ºC. Transwells were coated overnight with 100 µl of 0.375 mg/ml Matrigel (Matrigel basement membrane matrix; Becton Dickinson, USA) in IMDM under sterile conditions in a laminar flow cabinet. The next day the Matrigel-coated wells were incubated with 100 µl IMDM for 1 hour at 37ºC. Cells were harvested as described above and after removal of the medium, 200 µl of 100,000 FLS/ml in IMDM was seeded in the inner compartment of the transwell system. In the outer compartment, 900 µl IMDM/ 10% FCS/10% human serum was pipetted and the cells were incubated for 3 days at 37ºC and 5% CO_2.
After 3 days, the cells were fixed with 2% glutaraldehyde in PBS for 30 minutes at room temperature. After removal of the glutaraldehyde and subsequent washing with PBS, the cells were stained with a crystal violet solution for 30 minutes at room temperature. The cells were thoroughly washed with PBS and the cells that did not invade through the transwell membrane were removed together with the matrix by cleaning the inner wells of the transwell system with a cotton bud. The number of cells that had grown through

Table I. Sequences of PCR primers.

	Sense	Antisense
CD44s	CAGACCTGCCCAATGCCTTTGATGGACC	CAAAGCCAAGGCCAAGAGGGATGCC
CD44 v3	GCAGGCTGGGAGCCAAATGAAGAAAATG	ATCTTCATCATCATCAATGCCTGATCCAG
CD44 v4	TTCAACCACACCACGGGCCTTTGAC	AGTCATCCTTGTGGTTGTCTGAAGTAG
CD44 v5	GTAGACAGAAATGGCACCACTGCTTATG	TGTGCTTGTAGAATGTGGGGTCTCTTC
CD44 v6	ATCCAGGCAACTCCTAGTAGTACAAC	TGTCCCTGTTGTCGAATGGGAGTC
CD44 v7	GCCTCAGCTCATACCAACCATCCAATG	CCTTCTTCCTGCTTGATGACCTCGTC
CD44 v8	ATGGACTCCAGTCATAGTACAACGC	GTTGTCATTGAAAGAGGTCCTGTC
CD44 v9	GCAGAGTAATTCTCAGAGCTTCTC	TTGATGTCAGAGTAGAAGTTGTTGG
CD44 v10	TAGGAATGATGTCACAGGTGGAAG	TGATAAGGAACGATTGACATTAGAG
CCR-1(30)	AGAAGGTGAACGAGAGG	AGCCTGAAACAGCTTCC
CCR-2(31)	GCGGAATCTTCTTCATCATCCTC	CCTCTTCTTCTCGTTTCGACACC
CCR-3(32;33)	TGGCGGTGTTTTTCATTTTC	CCGGCTCTGCTGTGGAT
CCR-4(33)	GAAGAAGAACAAGGCGGTGAAGAT	ATGGTGGACTGCGTGTAAGATGAG
CCR-5(30;33)	TGCTACTCGGGAATCCTAAAACT	TTCTGAACTTCTCCCCGACAAA
CCR-6(34)	ATTTCAGCGATGTTTTCGACTC	GGAGAAGCCTGAGGACTTGTA
CCR-7(35)	GGCTGGTCGTGTTGACCTA	CCATTGTAGGGCAGCTGGAA
CCR-8(30)	AGTATGCACATCTTGGATGG	TGTAGTCTACGCTGGAGGA
CCR-9(36)	TGATCATGTGCATCAGCGTG	TTGGAGATGAACATGGCATA
CCR-10(37)	GTTTCCTGGGCCATTAC	ACAGCGTCGTTGGCCTTC
CXCR-1(33)	GGGGCCACACCAACCTTC	AGTGCCTGCCTCAATGTCTCC
CXCR-2(22)	CATGGAGAGTGACAGCTTTGA	ACTTGTTGATTTCCAGGGATT
CXCR-3(38)	ACCTAGCTGTAGCAGACACG	CATAGCAGTAGGCCATGACC
CXCR-4(30)	GAACTTCCTATGCAAGGCAGTCC	CCATGATGTGCTGAAACTGGAAC
CXCR-5(39; sense only)	ATGAACTACCCGCTAACGCTG	AGGAGGTCGGCCACGGCCAG
XCR-1(36)	GACCCTGTTCCGCTCACG	AATGCTCCTTCCAGGCCC
CX3CR-1(40)	ACCAGCAAGAAGCCCAAGAGT	CGCCTAGGCTGATGGTGAC

the matrix and the transwell membrane were counted under a light microscope. All experiments were carried out in duplicate.

Rate of proliferation

FLS were seeded at a density of 5000 cells per well (12-well flat-bottom plates) and cultured in 1 ml of IMDM/10% FCS. After days 3, 7, 10, 14, and 17 the cells were trypsinized and counted in a counting chamber. The cells were stained with trypan blue to exclude dead cells.

From the data obtained, growth curves were established and the rate of proliferation was determined from the steepest slope.

Table II. Expression of chemokine receptors as measured by RT-PCR.

	RA		OA		
Chemokine receptor	Positive	Negative	Positive	Negative	Mann-Whitney
CCR1	2	8	2	8	p = 1
CCR2	3	7	1	9	p = 0.481
CCR3	5	5	3	7	p = 0.481
CCR4	1	9	1	9	p = 1
CCR5	2	8	1	9	p = 0.739
CCR6	0	10	1	9	p = 0.739
CCR7	4	6	4	6	p = 1
CXCR4	3	7	5	5	p = 0.481

This technique had been performed before by our group, and it was compared with [^{3}H]thymidine incorporation to validate its accuracy. No differences were found between these techniques (8).

Statistical analysis

Expression of CD44 splice variants and chemokine receptors was tested using RT-PCR and data were analysed with SPSS 10.0. P-values were estimated using the Mann-Whitney U-test for unpaired groups and linear regression analysis. This study is designed to reveal large differences between the groups with a confidence of 95% and a power of 80%.

Results

Expression of chemokine receptors

Expression of chemokine receptors was measured by RT-PCR in 20 patients (10 patients with RA and 10 patients with OA) (Table II). CCR1,

Table III. (**A**) Expression pattern of CD44 v8-v9 as measured by RT-PCR after enlargement of the groups of samples. (**B**) Correlation between expression of CD44 v8-v9 and *in vitro* invasion.

A	RA		OA		
Splice variant	Positive	Negative	Positive	Negative	Mann-Whitney
CD44 v8-v9	19	10	20	5	p = 0.240

B Splice variant	Mean number of invasive cells (± standard deviation)	Mann-Whitney
CD44 v8-v9 positive	3489 (± 1882; n=39)	p=0.022
CD44 v8-v9 negative	5043 (± 2445; n=15)	

CCR2, CCR3, CCR4, CCR5, CCR6, CCR7 and CXCR4 were expressed in some but not all samples of FLS examined. However, no difference could be found between RAand OA. CCR3 was expressed most frequently in FLS. No correlation existed between expression of chemokine receptors and invasiveness *in vitro* or rate of proliferation (data not shown).

Expression of CD44 splice variants

29 patients with RA and 25 patients with OA were tested for the expression of CD44 splice variants. No significant differences in the expression of CD44 splice variants could be observed between patients with RA and OA. However, in the group of patients with RA, there tended to be more patients negative for expression of CD44 v8-v9 than in the group of patients with OA (p = 0.24) (Table III A).

Negative correlation between expression of CD44 v8-v9 and in vitro *invasiveness*

It was shown before that FLS from patients with RA are more invasive than FLS from patients with OA. Invasive cells were shown to have enhanced expression of CD44v3 but a reduced expression of CD44v7/8. For tumour cells the inverse was shown, cell expressing CD44 v8-v9 are involved in metastasis (15). We therefore further analysed the correlation between the expression of the splice variant v8-v9 and the invasiveness of FLS in an *in vitro* invasion system.

Samples positive for the expression of CD44 v8-v9 were significantly less invasive (mean number of invasive cells: 3610, n = 39) than samples negative for the expression of CD44 v8-v9 (mean number of invasive cells: 5043, n = 15; p = 0.022; Table III B). We evaluated the association between CD44 expression and invasiveness within one population and found that FLS from patients with RA are more invasive than FLS from patients with OA. Moreover, we performed a regression analysis to use all data. Indeed, the diagnosis of RAand OA and expression of CD44 v8-v9 are independent regression terms of invasive behaviour of FLS (p = 0.001). Together, these data indicate that expression of the splice variant containing v8-v9 negatively correlates with matrix invasion by FLS.

We also tested whether a correlation existed between the rate of proliferation of FLS and expression of the splice variants v8-v9 but could not observe a correlation (p=0.820; n=22).

Discussion

In this study, it is shown that FLS that express CD44 splice variant v8-v9 are less invasive in an *in vitro* transwell invasion assay than FLS that do not express this splicing combination. The invasion of FLS was measured using an *in vitro* invasion assay as described previously. Using this assay it was shown that FLS from patients with RA were significantly more invasive than FLS from patients with OA. It was also shown that the expression of MMP-1, MMP-3 and MMP-10 (which could mediate degradation of extracellular matrix) correlated with invasiveness (8). Here, it is shown that expression of CD44 splice variants v8-v9 correlates with a lower rate of invasion.

No correlation could be found between expression of CD44 splice variants and diagnosis. This could be due to culture artefacts. Because this is most likely a random process, the correlation found between expression of CD44 v8-v9 and invasiveness of FLS does not seem to be explained by culture artefacts.

These data confirm earlier findings published by Wibulswas, who showed that the cells that had entered Matrigel showed a significant reduced expression of CD44v7/8 (19), but contradict the findings obtained with cells from patients suffering from liver metastases and colorectal carcinoma (15), where expression of CD44 v8-v10 was found to be an independent prognostic marker. Apparantly, the mechanisms of invasive behaviour of FLS and metastasis are different with respect to the role of CD44 v8-v9 therein. With respect to the proliferation rate of cell populations, we did not find a positive correlation with CD44v8-v9 expression. Although CD44v7-v8 expressing FLS have a proliferative advantage over non-expressers (27), such an advantage does not explain why the cell populations obtained from RA patients are more proliferative than those obtained from patients with OA.

An explanation for the decreased invasion of FLS that express CD44 v8-v9 could be that additional glycosylation sites are present in the polypeptides encoded by the variant exons. The extra sugars attached to these sites regulate the binding of hyaluronan to CD44 and subsequently alter the functional activity of the CD44 protein (28, 29). However, this should be studied more extensively in FLS.

In serum from patients with rheumatoid factor-positive, erosive RAsoluble forms of CD44 v5 (sCD44v5) and CD44 v6 (sCD44v6) have been shown to be elevated compared to patients with other inflammatory rheumatic diseases. Serum levels of sCD44v5 was correlated to the inflammatory activity of the disease (16,17). Recently, Wibulswas *et al*. showed that FLS from patients with RA expressed the splice variants v3 and v6 and that antibodies

against these splice variants inhibited invasion of FLS *in vitro* (19). It is not known whether patients who have high expression of CD44 v8-v9 have low expression of CD44 v3 and v6.

It was previously reported that FLS from patients with RA had a higher expression of CD44 v7-v8 than FLS from normal controls and that cells expressing this splice variant had a proliferative advantage above cells that did not express CD44 v7-v8 (27). However, in our study no correlation between the expression of CD44 v7-v8 and a diagnosis of RA or OA could be found and also no correlation between the expression of this splice variant and the rate of proliferation was observed (results not shown).

In this population all patients expressed CD44 and different splice variants. Therefore it would be interesting to see what splice variants are expressed at the sites of joint destruction. However, no monoclonal antibodies against all different exons exist to perform such a study.

Another family of proteins that could be involved in the invasion of Matrigel by FLS is the chemokine receptor family. In this study, we addressed the question whether their expression correlated with a particular diagnosis, invasion or proliferation. Low levels of mRNA for the chemokine receptors CCR1-CCR7 and CXCR4 in FLS from patients with both RA and OA were detected. CCR3 was most abundantly expressed. However, no correlation was discerned between the expression of chemokine receptors and a diagnosis of RA or OA, nor did we find any correlation between the expression of chemokine receptors and invasion in an *in vitro* invasion system or between their expression and the rate of proliferation of FLS.

This indicates that chemokine receptors play no apparent role in the invasion of FLS in our model. So far, this is the first report that adresses the expression of chemokine receptors in FLS from patients with RAor OA.

In summary, in this study an association of the expression of CD44 v8-v9 and the invasive properties of FLS from patients with RAin an *in vitro* invasion system was observed, while no association could be found for other CD44 splice variants or chemokine receptors. These results suggest that CD44 v8-v9 does not favour invasion.

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