Influence of Epitopes CD44v3 and CD44v6 in the Invasive Behavior of Fibroblast-Like Synoviocytes Derived From Rheumatoid Arthritic Joints

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Objective. To investigate the functional implications of CD44 splice variant expression in fibroblast-like synoviocytes (FLS) obtained from patients with rheumatoid arthritis (RA).

Methods. FLS were isolated from synovial tissue obtained from both diseased and nondiseased joints. The expression of splice variants containing exons v3 and v6 was analyzed using immunocytochemistry with exon-specific antibodies and reverse transcription-polymerase chain reaction followed by Southern blotting. The invasive capacity of the cells was studied in a transwell invasion assay.

Results. FLS obtained from RA joints expressed various CD44 splicing combinations containing the variant exons v3 and/or v6. These cells were highly invasive, whereas cells from normal tissues, which lacked expression of CD44 splice variants, were not. Variant exons CD44v3 and CD44v6 were instrumental in matrix invasion in vitro, with cells enriched for CD44v3 and v6 exhibiting greater invasion and anti-bodies that specifically recognize CD44v3 and v6 abrogating this capacity to invade. Invasive cells showed a reduced expression of CD44v7/8, and antibodies against this epitope had no significant effect on cellular infiltration of the matrix. The antibodies had no effect on cell migration into the porous section of the transwell.

Conclusion. FLS obtained from patients with RA express CD44 splice variants and are highly invasive, whereas cells obtained from healthy tissue do not express these variants and are not invasive. Expression of the epitopes CD44v3 and CD44v6 is instrumental in the invasive capacity but not in cell migration. This finding highlights a functional implication for the expression of CD44 splice variants at the level of matrix degradation.

One of the hallmarks of rheumatoid arthritis (RA) is progressive destruction of bone and cartilage, and fibroblast-like synoviocytes (FLS) are key players in this process (1). Indeed, FLS derived from RA joints are different from FLS obtained from nondiseased joints. For example, FLS from RA joints grow in soft agar (2), invade cartilage (3), and express elevated levels of vascular cell adhesion molecule 1 (VCAM-1) (4) as well as members of the integrin family (5). In addition, FLS derived from RA joints, but not those derived from nondiseased tissues, express various alternative splicing combinations of the adhesion molecule CD44 (6).

CD44 is a widely distributed cell surface molecule. It is a member of the link family of proteins and acts predominantly as a receptor for the glycosaminoglycan hyaluronan (7). CD44 splice variants have received great attention since it was shown that inclusion of exons v4–v7 (CD44pMeta-1) induces metastatic transformation of a rat pancreatic tumor cell line (8). We have previously demonstrated that expression of the CD44v7/8 epitope, as part of larger splicing combina-
tions, contributes to the transformation of FLS by conferring a proliferative advantage on these cells (9). In the present study, we focused on the expression and functional implications of CD44 splice variants containing the variable exons v3 and v6. We found that these exons are instrumental in the highly invasive behavior of FLS obtained from patients with RA.

PATIENTS AND METHODS

Materials. Transwells (polycarbonate membranes with a diameter of 6.5 mm, thickness of 10 μm, and pore size of 8 μm) were purchased from Costar (Corning, High Wycombe, UK). Growth factor–reduced Matrigel matrix was obtained from Becton Dickinson (Cowley, UK). Anti-CD44s (F10-44-2) anti-CD44v3 (3G5), anti-CD44v6 (2F10), anti-CD44v7/8 (VFF-17), anti-CD31, anti-VCAM-1, and anti-CD68 were from Serotec (Kidlington, UK). Horseradish peroxidase–labeled anti-mouse IgG was obtained from Dako (Ely, UK). Fluorescein isothiocyanate (FITC)–labeled anti-mouse IgG, an aminothiocarbazole staining kit, nonspecific IgG, and hydrogen peroxide were obtained from Sigma-Aldrich (Poole, UK). Enhanced chemiluminescence reagent was obtained from Amersham Pharmacia Biotech (Little Chalfont, UK). Immobilon-P transfer membrane was obtained from Millipore (Watford, UK). High-performance liquid chromatography–analyzed n-hexane was obtained from JT Baker (Scientific & Chemical Supplies, Bilston, UK). Tissue culture plastics were from Nunc (Life Technologies, Paisley, UK).

Isolation of primary FLS. Synovial membrane specimens were obtained from the knee and hip joints of patients with RA undergoing joint replacement surgery. Nondiseased joints were obtained from patients undergoing amputation surgery. Tissue treatment was essentially as described elsewhere (6,9). After 1 week of culture, cells were prepared for analysis of the expression of VCAM-1, CD68, and CD31, which are markers for FLS, monocytes, and vascular endothelial cells, respectively. If the cell number was sufficient and contamination limited (i.e., <2%), cells were used for experimentation. If not, they were left for another week (equivalent of passages 3–4) before being used.

Synovial biopsy. Biopsy specimens were obtained with a Tru-Cut needle (14 gauge, 15.2 cm, 20 × 1.5 mm) from patients diagnosed as having RA according to the American College of Rheumatology (formerly, the American Rheumatism Association) criteria (10) and with established disease (2–5 years’ duration). The specimens were frozen in n-hexane and maintained at −70°C.

Immunocytochemistry. Synovial membrane. Immunocytochemistry was performed as described previously (9). The histologic features of the synovial lining layer were evaluated by examining serial sections that had been stained with 0.1% (weight/volume) toluidine blue. Splice variant expression was studied using antibodies against exons CD44v3 and CD44v6. Antibody binding was visualized by FITC-labeled anti-mouse IgG, and the sections were examined using a Leica TCS4D confocal microscope with a 25× objective (Leica, Cambridge, UK).

Cells in culture. Cultured cells, uniquely obtained from synovial tissue at the time of joint replacement surgery, were transferred to glass coverslips (no. 1) and fixed in methanol for 4 minutes, followed by acetone for 1 minute (both maintained at −20°C). The slides were further treated as described previously (9).

Cells in matrix. The inserts were removed and fixed with methanol for 10 minutes followed by acetone for 1 minute (both maintained at −20°C). The inserts were incubated in 10% (volume/volume) fetal bovine serum (FBS) in phosphate buffered saline (PBS) for 20 minutes, washed, and then incubated with antibodies against human CD44v3, CD44v6, and CD44v7/8 (for 1 hour). Cells were counterstained with 0.5 μg/ml of propidium iodide (PI).

The inserts were analyzed using a Leica TCS4D confocal microscope with a 25× objective. Optical sections were scanned at 10-μm intervals from the bottom of the filter. The relative contribution of splice variant–positive cells was calculated as the number of FITC-positive cells divided by the number of PI-positive cells (total cell number) in each section. The proportion of splice variant–positive cells on the polycarbonate filter (migration compartment) was calculated from section 10 μm and in the invasion compartment from sections 20, 30, and 40 μm.

Reverse transcription–polymerase chain reaction (RT-PCR) and Southern blotting. The RT-PCR procedure was performed exactly as described elsewhere (6,9). A detailed description of the various CD44 splice variant primers is available from the authors (e-mail Dr. Kramer at: i.kramer@iecn-telecom.univ-bordeaux.fr).

Western blotting. Western blotting was performed as described previously (9). Prior to Western blotting, gel electrophoresis for anti-CD44v3 was performed on 6–10% gradient gels to improve resolution of the high molecular weight bands. The content of the CD44v6 splice variant was enriched prior to gel electrophoresis using an immunoprecipitation protocol (causing the immunoglobulin band in the Western blot). Cells (2.6 × 10⁶) were lysed in 200 μl of 120 mM NaCl, 50 mM HEPES (pH 7.4), 10 mM NaF, 1 mM EDTA, 40 mM β-glycerol phosphate, 1% (v/v) Nonidet P40, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1 mM Na₃VO₄. After clearing the insoluble components, 5 μg of antibody against CD44v6 was added. After turning end-over-end for 120 minutes at 4°C, antibodies were precipitated through the addition of 30 μl of protein G-Sepharose. Antibody staining was visualized with horseradish peroxidase–conjugated anti-mouse IgG antibody and enhanced chemiluminescence reagent as described (9).

In vitro invasion assay. The invasion assay was adapted from a procedure described previously (11). Matrigel was diluted 2-fold in PBS, and 120 μl was layered on the top of the polycarbonate filter insert. FLS (4 × 10⁵ cells/ml) were incubated with dialyzed antibodies against CD44s, CD44v3, CD44v6, or CD44v7/8 for 20 minutes (37°C), after which 100 μl was loaded on inverted filter inserts (4 × 10⁴ cells) and left for 1.5 hours at 37°C. The inserts were washed twice in 500 μl of 0.5% (v/v) FBS in Dulbecco’s modified Eagle’s medium (DMEM) and transferred to a 24-well plate with 500 μl of 0.5% FBS in DMEM in the lower compartment and 150 μl of the same medium in the upper compartment. After 4 days at 37°C, the assay was terminated by fixing the cells in 100% methanol at −20°C for 15 minutes.
Nuclei were stained with 0.5 μg/ml PI in PBS and the filters were viewed with a Leica TCS4D confocal microscope with a 25× objective. Optical sections were scanned at 10-μm intervals from the filter bottom. The number of cells in each optical section was counted using the ImageTool program developed at the University of Texas Health Science Center at San Antonio (available online at: http://ddsdx.uthscsa.edu/dig/itdesc.html). Migration is presented as the number of cells in sections 10–40 μm (cells in porous filter plus Matrigel) divided by the total number of cells counted in all sections. Invasion is presented as the number of cells counted in sections 20–40 μm (cells in Matrigel only) divided by the total number of cells counted in all sections.

**Statistical analysis.** All values are reported as the mean ± SEM. Statistical analyses were performed using
RESULTS

Expression of CD44v3 and CD44v6 in FLS from patients with RA. Immunostaining with anti-CD44v3 (Figure 1A) and anti-CD44v6 antibodies produced diffuse staining of the intimal and deeper subintimal regions of synovial membranes obtained from RA patients undergoing joint replacement surgery. In synovial samples from nondiseased joints, staining failed to reach levels above those detected for tissues treated with nonspecific mouse IgG antibodies (data not shown). Splice variants were also observed in biopsy samples from the joints of RA patients who had progressive destruction at 2–5 years after diagnosis (Figure 1B). CD44 splice variant expression was maintained when FLS were maintained in culture (Figure 1C). Furthermore, the detection of CD44v3 and CD44v6 was not a consequence of a generally elevated expression of CD44, since we could not detect different levels of expression between RA and nondiseased tissues in a semiquantitative RT-PCR or flow cytometry protocol (6,9).

In order to obtain information about the nature of the CD44 splicing combinations that harbor these epitopes, we probed complementary DNA from synovial fibroblasts (selected on the basis of VCAM-1 expression) for the presence of variant exons in a Southern blot protocol. We repeatedly detected the splicing combinations CD44v3, CD44v6, CD44v3v6v8v9, and CD44v6v7v8v9v10 (Figure 1D). Western blotting revealed several protein bands with molecular weights from 54 kd to >200 kd when anti-CD44v3 antibodies were applied and 3–4 bands (depending on the patient), ranging from 54 kd to 120 kd when anti-CD44v6 antibodies were applied (Figure 1E). Together, these data confirm that multiple isoforms of CD44 are expressed at both the transcript and protein level in the joints of patients with RA and that they are present in both similar and distinct splicing combinations.

Inhibition of FLS invasion of Matrigel by antibodies against CD44v3 and CD44v6. Cultures of FLS, verified for the absence of cells expressing CD68 and CD31, were subjected to a transwell in vitro invasion assay. We could not detect any invasion of Matrigel with FLS derived from normal synovial specimens; these cells also failed to express CD44 splice variants. FLS derived from patients with RA were consistently highly invasive (Figure 2A) to a level comparable with that of v-fos–transformed fibroblasts 208F (11), which we used as a positive control (data not shown). Invasion was not affected by a growth factor gradient of epidermal growth factor, platelet-derived growth factor, or transforming growth factor β1.

In order to verify the role of CD44 and its splice variants in the characteristic invasive behavior, cells were incubated for 20 minutes prior to the assays with antibodies against CD44v3 and CD44v6. In the presence of anti-CD44v3 or anti-CD44v6, invasion into Matrigel was inhibited by 62% (Figure 2B). No significant effect was observed with the addition of antibodies against CD44v3 and CD44v6. Results are representative of 3 separate experiments performed in duplicate. P values were determined by Student’s unpaired t-test, comparing the mean ± SEM of 12 optical fields for each treatment with the control in 1 representative experiment.
invasion and cell survival (12), we screened for CD44 expression levels on FLS maintained in culture in the presence of anti-CD44v3 and anti-CD44v6 antibodies for 4 days. We could detect no significant change in cell surface expression in a flow cytometry protocol (data not shown).

Enrichment of the expression of CD44v3 and CD44v6 in FLS invading Matrigel. If the expression of CD44v3 and CD44v6 is instrumental in cell invasion, then one would expect the number of CD44v3- and CD44v6-positive cells to be enriched in the Matrigel relative to the number of CD44v7/8-expressing cells. The percentages of FLS expressing CD44v3 or CD44v6 were much higher in the Matrigel than in the porous polycarbonate filter section. In contrast, the percentage of CD44v7/8-expressing cells in Matrigel had declined (Figure 3A). When presented as CD44v3:CD44v7/8 or CD44v6:CD44v7/8 ratios in either of the 2 compartments, a manifest shift in favor of v3 and v6 was observed (Figure 3B).

**DISCUSSION**

Our results demonstrate that antibody-mediated blockade of the function of CD44v3 or CD44v6 splice variants selectively limits the ability of FLS to invade in Matrigel without having an effect on cellular migration. This suggests that these variants facilitate the process of matrix degradation required during these events. This finding is unique for cells derived from human tissues. Although human tumors often express CD44 splice variants, and in certain cases this is associated with a poor prognosis, a functional implication for their expression has so far not been discerned.

We postulate that FLS exhibit a “transformed” behavior, but only to a limited extent, such that it does not obscure phenotypic changes accompanied by the expression of CD44 splice variants. On the other hand, metastatic human tumors have established multiple mechanisms that facilitate invasion, which together render the contribution of CD44 splice variants redundant. Although overexpression of standard CD44 (CD44s), regardless of the presence or absence of splice variants, is involved in tumor invasion (12), this does not appear to be the case with FLS. Addition of splice variant–specific antibodies does not reduce the expression levels of CD44s, and cells obtained from diseased tissue do not express a significantly higher level of CD44s compared with those derived from healthy tissue (6).
From these findings and those of our previous study (9), it appears that different splicing combinations of CD44 govern different functions. Expression of the CD44v7/8 epitope provides a proliferative advantage but an invasive disadvantage, whereas expression of variants containing v3 or v6 is linked with an increased invasive capacity. Splice variants are also present in earlier stages of the disease in RA patients who have progressive destruction but are not yet candidates for joint replacement. We are currently assessing whether the early expression of these epitopes predicts a highly invasive arthritis.

At least 2 important questions remain to be addressed: How do distinct splicing combinations govern such different cellular functions, and are splice variants a potential target for pharmacologic intervention? With respect to the first question, increasing numbers of reports indicate that CD44 interacts with both cellular and extracellular factors and that different splicing combinations may regulate such interactions (13–15). With respect to the question of pharmacologic intervention, our studies and those of other investigators who have shown evidence of a role of CD44 in inflammation (16) and cartilage destruction (3) suggest that CD44 splice variants present novel targets for restraining the proliferative and destructive behavior of FLS.

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