The CD44v7/8 Epitope as a Target to Restrain Proliferation of Fibroblast-Like Synoviocytes in Rheumatoid Arthritis

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CD44 is a receptor for the glycosaminoglycan hyaluronan. It exists in a large range of isoforms because of variability in the pattern of glycosylation (both N- and O-linked) and of multiple splice variants. Human fibroblast-like synoviocytes derived from patients with rheumatoid arthritis express certain CD44 splice variants and we have investigated the functional implications of their expression. We found that the rate of proliferation of fibroblast-like synoviocytes expressing the CD44v7/8 epitope (average doubling time 55 hours) exceeds those obtained from the same synovial specimen but lacking this particular epitope (69 hours). Antibodies against CD44v7/8, but not against other exons, inhibit cell proliferation with concomitant induction of the cell cycle inhibitors GADD45, GADD153 and the cyclin-dependent protein-kinase inhibitors p21Waf/Cip. These data show that expression of CD44v7/8 contributes to the transformed phenotype of fibroblast-like synoviocytes. More importantly, they reveal the presence of a target that might be amenable to pharmacological intervention in the treatment of rheumatoid arthritis. (*Am J Pathol* 2000, 157:2037–2044)

CD44, originally discovered as the lymphocyte homing receptor, is a widely distributed cell surface receptor and hyaluronan is its major ligand.¹ CD44 is heterogeneous in size because of various forms of glycosylation and the variable expression of 10 exons (splice variants).² CD44 splice variants have obtained great attention when it was shown that inclusion of exons v4-7 (CD44 pMeta-1) induces metastatic transformation in a rat pancreatic tumor cell line³ and that antibodies against v6 could subsequently prevent this.⁴ Further studies in rodents showed other functional implications of CD44 splice variants. In mice they facilitate migration of Langerhans cells to

lymph nodes (exons v4 to v6)⁵ and in rats they are instrumental in fibroblast growth factor-mediated mesenchymal cell proliferation during limb bud development (exons v3 and v6).⁶ Human tumors frequently express CD44 splice variants and although in certain cases this coincides with a less favorable prognosis, no functional implication has been discerned yet.^{7–11} Fibroblast-like synoviocytes obtained from patients with rheumatoid arthritis (RA) also appear to have a transformed phenotype, their number is greatly increased (hyperplasia),¹² they grow in soft agar,¹³ invade cartilage in SCID mice,¹⁴ and have elevated levels of c-myc expression.¹⁵ We have noticed expression of CD44 splice variants in cultures of fibroblast-like synoviocytes when derived from patients with RA. In particular expression of the epitope CD44v7/8 was prominent, whereas the metastasizing splicing combination CD44v4-7 was completely absent.¹⁶ In this article we demonstrate that CD44v7/8 expression is indeed manifest in the synovial membrane of these patients but not in membranes of nondiseased joints. We show that cells selected for its expression have a proliferative advantage over cells that are obtained from the same joint but lack expression of this epitope. Antibodies against the CD44v7/8 epitope selectively annul this advantage by raising the level of expression of cell cycle inhibitors.

Materials and Methods

Isolation of Fibroblast-Like Synoviocytes

Synovial membrane specimens were obtained from knee and hip joints from patients with RA undergoing joint replacement surgery. Control tissues were obtained from knee joints of patients undergoing amputation for sarcomata of the lower limb. The intimal surface of the synovial membrane was dissected, cut into small dices, and cells were dissociated through treatment with collagenase (2 mg/ml) (Worthington, Biochemical Corp., Lakewood, NJ)

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for 1 hour at 37°C. Dissociated tissue was sheared using a sterile syringe, filtered using a fine sterile gauze, and then washed and resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution (Gibco BRL, Paisley, UK) and kept in culture for 1 week as described by Croft et al.¹⁶ When confluent, cells were passaged using a trypsin-ethylenediaminetetraacetic acid solution. After the third passage the populations were on average 98% VCAM-1-positive and devoid (<1%) of monocyte or macrophage markers and therefore mainly consist of fibroblast-like synoviocytes (FLSs).

Immunocytochemistry

Cultured Cells

Cells were transferred to Permanox Lab-Tek chamber slides (Nunc) at a density of 2×10^4 cells/well and cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were fixed in methanol for 4 minutes followed by 1 minute in acetone, both kept at -20°C. After air-drying, the cells were washed twice in phosphate-buffered saline (PBS) and then incubated in 10% FBS/PBS for 20 minutes to saturate nonspecific binding sites. The cells were washed with PBS three times after each of the following steps. Hydrogen peroxide (3%) was applied for 5 minutes to quench endogenous peroxidase activity. All antibodies were diluted to their optimal concentration in 10% FBS/PBS. Anti-CD44v7/8 (clone VFF-17), anti-Ki67 (both from Serotec, Kidlington, UK), or anti-VCAM-1, clone BBIG-V1(4B2), (R&D Systems, Abingdon, UK) were applied to each well and incubated for 1 hour or overnight in the case of anti-Ki67. A negative control was performed by incubating cells with 10% FBS/PBS in the presence of mouse IgG1 antibodies (5 µg/ml; Sigma, Poole, UK). To visualize antibody binding, after three washes in PBS for 5 minutes, anti-mouse IgG biotin (Sigma) was added for 30 minutes, followed by avidin-peroxidase (Sigma) for 30 minutes, and the slides were stained using aminoethylcarbazole (AEC staining kit; Sigma) as substrate. The reaction was stopped in water. Except for the cells stained with anti-Ki67, nuclei were counterstained with Mayer's hematoxylin for 5 minutes. The slides were mounted with Fluoromount-G and examined by light microscopy.

Synovial Membranes

The tissues were snap-frozen in liquid nitrogen and stored at -74°C until use. Ten-micrometer cryostat sections of these tissues were transferred to glass slides and allowed to air dry. The histology of the synovial lining layer was checked by staining the sections with 1% (w/v) toluidine blue in acetate buffer for 5 minutes. The slides were washed with running water, mounted with Fluoromount-G, left to dry, and examined by light microscopy using an Olympus PM-10AD. Fields of interest were photographed on Fujichrome T 64ASA film. For detection of CD44 splice variant expression, the sections were incubated with 10% (v/v) FBS in PBS to eliminate nonspecific

background. They were next incubated for 1 hour at room temperature with mouse monoclonal anti-CD44v7/8 (clone VFF-17) antibodies diluted in PBS containing 10% FBS. A negative control was performed by incubating cells with 10% FBS/PBS in the presence of mouse IgG1 antibodies (5 µg/ml; Sigma). The sections were washed three times with PBS after each of the following steps. Anti-mouse IgG-fluorescein isothiocyanate was next applied and incubated for 30 minutes at room temperature. The sections were finally washed with water. The sections were mounted with Fluoromount-G and covered with glass coverslips, left to dry, and examined by a confocal microscope using a Leica TCS4D.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and Southern Blotting

Total cellular RNA was isolated using Tri-reagent according to the manufacturers instructions. Total RNA (2 µg) was reverse-transcribed using a Ready to Go T-Primed First-Strand cDNA synthesis kit (Amersham Pharmacia Biotech, Amersham, UK) for 60 minutes at 37°C. This kit utilizes the Moloney murine leukemia virus reverse transcriptase and an oligo (dT) primer to generate first strand cDNA. The completed first-strand reaction mix was heated (5 minutes at 90°C) to inactivate the reverse transcriptase and stored at -20°C. Semiquantitative PCR was performed on cDNA (equivalent to 10 ng of RNA) with oligonucleotide primers that were designed using primer-select software (DNA Star) based on published sequences.⁷ In the case of CD44, the primers are upstream and downstream of the variant exon insertion point. CD44s: sense, CAG ACC TGC CCA ATG CCT TTG ATG GAC C; antisense, CAA AGC CAA GGC CAA GAG GGA TGC C. For GAPDH the primers are as follows: sense AAG GTG AAG GTC GGA GTC AAC; antisense GGC AGA GAT GAT GAC CCT TTT GGC. [α -³²P]dATP was added to the reaction mixture to detect the PCR products formed. The number of amplification cycles varied between 25 to 40, with each cycle consisting of 30 seconds at 94°C, 30 seconds at 62°C (annealing), and 30 seconds at 72°C. PCR products were electrophoresed on a 6% polyacrylamide gel and radioactivity was visualized using a phosphorimager (Fuji Bas 1000). For Southern blotting, the PCR products, this time not labeled with [α -³²P]dATP, were separated on a 1.5% agarose Tris borate-ethylenediaminetetraacetic acid gel, transferred onto nylon membrane (Schleicher and Schuell, London, UK), and hybridized with either CD44v7- or v8-specific probes in a Southern blot protocol. ³²P-labeled variant exon-specific probes were generated from a variant CD44 template (containing variants v3-10) using exon-specific primers and a Megaprime DNA labeling kit (Amersham Pharmacia Biotech). The primers that were used are as follows: CD44 v7 sense, GCC TCA GCT CAT ACC AAC; antisense, CCT TCT TCC TGC TTG ATG ACC TCG TC CAT CCA ATG. CD44 v8 sense ATG GAC TCC AGT CAT AGT ACA ACG C; antisense GTT GTC ATT GAA AGA GGT CCT GTC.

Western Blotting

Fibroblast-like synoviocytes from two patients with RA were cultured for 1 week after dissociation from the synovial membrane. The cells were trypsinized, counted (10^6 cells), and replated in 25-cm² flasks (Nunc, Life Technologies, Paisley, UK). The next day the cells were washed in PBS, solubilized in Laemmli sample buffer, and proteins were separated on a 10% (w/v) sodium dodecyl sulfate-acrylamide gel. After transferring protein on polyvinylidene difluoride Immobilon membrane (Millipore, Watford, UK), the blot was incubated overnight at 4°C with mouse monoclonal antibodies against CD44 (clone 5F12 CL4, NeoMarkers, New Market, UK) or against the variable exons CD44v7/8 (clone VFF-17, Serotech). A second, peroxidase-conjugated, antibody was applied for 1 hour at room temperature and antibody binding was visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech).

Selection of CD44v7/8-Expressing Cells

Cells expressing CD44v7/8 were selected from a total population of 10^6 cells in a panning protocol using Dynabeads M-450 bound to goat anti-mouse IgG (Dyna, Bromborough, UK) according to the manufacturer's protocol. Anti-CD44v7/8 was used at 5 µg/ml after extensive repeated washings in PBS supplemented with 1% (w/v) bovine serum albumin using Microcon-30 (Millipore) to remove azide. To remove antibody-coupled beads after selection, the cell suspension was treated with trypsin/ethylenediaminetetraacetic acid (Sigma) for 4 minutes after which FBS was added to a final concentration of 10%. Cells were washed and cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. Selection was assessed in an immunocytochemistry protocol before using the cells for further experimentation.

Cell Proliferation Measurements

Cells were plated in 24-well plates at 3×10^3 cells/well and left to adhere for 4 hours. Either CD44v7/8-enriched populations were compared with negatively selected cells (4 days of culture) or the effects of anti-CD44v3, anti-CD44v6, anti-CD44v7/8, or anti-VCAM-1 (10 µg/ml), was assessed in a total population (throughout a period of 11 days). The choice of the exon-specific antibodies in this assay is based on our evidence that the predominant splicing combinations in fibroblast-like synoviocytes from patients with RA are: CD44v3, CD44v6, and CD44v6-10. The variably spliced exons v4 or v5 have never been detected.¹⁶ For counting, cells were harvested and each cell suspension was resuspended in Isoton II (Coulter Electronics, Hialeah, FL). The cell suspension was then measured in Coulter counter type ZBI with a 100-µm orifice (Coulter Electronics). Cell numbers are expressed as cells per well or as percentage of control population.

³H-Thymidine Incorporation

One-week-old cells were seeded into 96-well plates at 2×10^3 cells/well in 100 µl of medium. Cells were left to adhere for 4 hours after which antibodies against CD44v3, CD44v6, CD44v7/8, or VCAM-1 at 10 µg/ml were added. Cultures were continued for another 3 days. ³H-thymidine (Amersham Pharmacia Biotech) was added at 0.5 µCi/well and left for 16 hours. This time period is appropriate for cells with a doubling time of around 60 hours (such as the fibroblast-like synoviocytes). Cells were then washed in PBS and fixed in 10% (w/v) trichloroacetic acid. DNA was solubilized in 0.25 mol/L NaOH for 1 hour and counted in the presence of UltimaGold (Packard Bioscience, Meriden, CT) in a scintillation counter (Beckman).

High-Density Oligonucleotide Array

Cells were incubated with or without dialyzed anti-CD44v7/8 (10 µg/ml) for 5 days. Cell numbers were counted, to check for growth inhibition, and the cells were lysed in denaturing solution (ClonTech, Basingstoke, UK) after which RNA was isolated and treated with DNase I (ClonTech). cDNA was obtained with the use of Moloney murine leukemia virus reverse transcriptase in the presence of [α -³²P]dATP. The ³²P-labeled cDNA was hybridized to a high-density oligonucleotide array (Atlas Human Cell Cycle Array; ClonTech) according to the manufacturer's procedures. Radioactivity was quantified and visualized using a phosphorimager (Molecular Dynamics Storm II, Amersham Pharmacia Biotech).

Detection of Apoptosis

FLSs were cultured with or without dialysed anti-CD44v7/8 or anti-VCAM-1 mAbs. At day 5, cell supernatant was collected and the cells harvested by trypsinization. To record DNA histograms, the cells, together with the culture supernatant, were centrifuged ($150 \times g$ at room temperature for 5 minutes) and then washed once with PBS. They were fixed in cold 70% (v/v) ethanol in PBS and kept at 4°C until further processing. The cells were washed twice in phosphate-citrate buffer. RNase (100 µg/ml) was added to ensure that only DNA is stained. Propidium iodide (50 µg/ml) was subsequently added and the suspension analyzed for subG1 apoptotic cells using a flow cytometer (Becton Dickinson, Cowley, UK).

Statistical Analysis

All values given are the means $\pm$ SE. Statistical analyses were performed using analysis of variance or Student's unpaired *t*-test. *P* values <0.05 were considered to be significant.

Figure 1. Expression of splice variant CD44v7/8 in synovial membrane of patients with RA. Synovial specimens were snap-frozen, sectioned, and were either stained with 1% (w/v) toluidine blue in acetate buffer for 5 minutes to control tissue integrity (histology), or incubated with matching control-mouse IgG (neg control) or antibodies against exons CD44v7/8 (CD44v7/8). A second anti-mouse IgG1 antibody, labeled with fluorescein isothiocyanate was used to visualize binding of primary antibody. This figure is representative of two controls (normal) and four arthritic (RA) specimen tested.

Results

Abundant Expression of CD44v7/8 in Synovial Membranes of Patients with RA

Synovial sections were stained, using anti-CD44v7/8 antibodies, and analyzed under the microscope. In tissues obtained from nondiseased joints, no signal above background could be observed. Little staining was detected in tissue treated with nonspecific mouse IgG1-antibodies (negative control). Strong staining of the intimal layer and deeper regions of the synovial membrane was visible in tissues obtained from patients with RA (Figure 1). To test whether or not detection of CD44v7/8 is a mere consequence of a general elevated expression of CD44, we performed a semiquantitative RT-PCR experiment in which we amplified the cDNA, obtained from freshly prepared synovial cells, at various cycle numbers (Figure 2A). The level of expression of standard-CD44 (CD44s) does not vary greatly between cells derived from diseased and nondiseased joints. However, high molecular

weight CD44 splice variants (500 to 1,000 bp) are exclusively present in cells derived from patients. When probed for the presence of exon 7 and 8 in a Southern blot protocol, we confirmed that these exons are indeed exclusively expressed in diseased joints (RA) (Figure 2B). We next tested in a Western blotting protocol whether or not the anti-CD44v7/8 specifically detects alternative splice-variants. In these experiments we used cells derived from patients with RA. The antibody directed against CD44 detects a whole range of isoforms with molecular weights from Mr 85 kd to >200 kd, the variability being due to different glycosylations and the presence of multiple splicing combinations of CD44. The CD44v7/8 exon-specific antibody detects a single protein of Mr 100 kd (Figure 2C). This finding is entirely in agreement with our previous study where we found that the CD44v7/8 epitope is most likely present in a single CD44v6-10 splicing combination (see also 2B where there is only one PCR product (1.2 kb) that contains both v7 and v8).¹⁶ From these findings we conclude that the antibody detects a CD44 splice variant containing exons

Figure 2. A: RT-PCR analysis of CD44 mRNA expression in freshly dissected fibroblast-like synoviocytes from nondiseased (normal) and diseased (RA) joints. RNA was isolated and subjected to RT-PCR using CD44 and glyceraldehyde-3-phosphate-specific primers in the presence of [α -³²P]dATP. PCR products were separated on an acrylamide gel and radioactivity was visualized using a phosphorimager. Note the appearance of high molecular weight CD44 splice variants (500 to 1,000 bp), at 30 rounds of amplification, in RA-derived tissue but not in normal tissue. This figure represents three controls (Normal) and four arthritic (RA) specimens tested. **B:** Southern blotting with exon-specific probes reveals the presence of variant exon v7 and v8 in patients with RA. Nonlabeled RT-PCR products were blotted onto nylon membrane and probed with ³²P-labeled v7- and v8-specific oligonucleotides. Radioactivity was visualized using a phosphorimager. Note the absence of v7 and v8 in nondiseased joints (normal) and their presence in patients with RA. There is only one splicing combination (~1.2 kb) that contains both v7 and v8. This autoradiograph is representative of three controls (normal) and four arthritic (RA) specimens tested. **C:** Immunoblotting reveals the presence of a single CD44 isoform (Mr 100 kd) containing the v7/8 epitope. Cells obtained from two patients with RA were lysed and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein was transferred onto a polyvinylidene difluoride membrane and probed with antibodies that recognize the core protein of CD44 (CD44) or those that recognize variable exons v7/8 (CD44v7/8). Antibody binding was visualized after addition of a second, peroxidase-conjugated anti-mouse IgG antibody in an enhanced chemiluminescence protocol. Note the presence of numerous isoforms of CD44 in patients with RA but only a single protein band that contains both exons v7 and v8.

Figure 3. A: Cells staining positive for CD44v7/8 also express the fibroblast-like synoviocyte marker VCAM-1. Cells were kept in culture for 1 week after isolation from the synovial tissue and were stained, in separate dishes, for the expression of CD44v7/8 and VCAM-1. Antibody binding was visualized using a peroxidase-labeling technique. A high percentage of the cells express CD44v7/8 and all of the cells express VCAM-1, meaning that the cells must co-express both markers. This immunostaining is representative of 20 specimens of patients with RA tested. **B:** The proportion of fibroblast-like synoviocytes expressing CD44v7/8 increases with time in culture. Cells were stained with antibodies against CD44v7/8 and the percentage of positive cells were counted per 100 cells viewed. Nuclei were counterstained with Mayer's hematoxylin for 5 minutes. This graph is representative of six experiments performed with different RA synovial specimens.

v7/8 and that synovial membranes from arthritic joints, but not from nondiseased joints, abundantly express this splice variant.

Enrichment of CD44v7/8-Expressing Fibroblast-Like Synoviocytes in Culture

Expression of CD44v7/8 is maintained in cultured cells obtained from synovial tissues from RA patients but again could not be detected from those obtained from nondiseased joints. Because the majority of the cells also express VCAM-1 in these cultures, we identify them as fibroblast-like synoviocytes (Figure 3A). We do not, however, exclude that in the synovial tissue other cells also express CD44 splice variants, this we are currently studying in more detail. While assessing the percentage of cells expressing CD44v7/8 in the cell culture dishes we observed, with time, an increase in the percentage of cells bearing this epitope (Figure 3B). This phenomenon was unlikely to be a consequence of a switch in CD44 splice-variant expression, for instance as a consequence of the culture conditions, because, as mentioned earlier, we did not observe the acquisition of these splice variants in cultures of cells obtained from nondiseased joints.¹⁶ We therefore argued that the enrichment of cells expressing the CD44v7/8 epitope could be a consequence of a proliferative advantage.

Cells Expressing CD44v7/8 Have a Proliferative Advantage

To address this matter we next studied co-expression of the splice variants with the Ki67 epitope, a nuclear antigen present in proliferating cells.¹⁷ We observed a high level of co-expression between Ki67 and CD44v7/8

Figure 4. Expression of CD44v7/8 correlates positively with expression of the proliferation marker Ki67. **Top:** Cells were simultaneously stained with CD44v7/8 and Ki67 (note the nuclear localization of this antigen). **Bottom:** Ki67-positive cells were scored as the number of Ki67-stained cells per 250 of CD44v7/8-positive (+ve) or negative (-ve) cells viewed ($n = 3$). A higher proportion of Ki67-positive cells means a higher proportion of proliferative cells.

whereas a much lower co-expression was observed in nonexpressing cells (Figure 4). This difference in co-expression was significant and persisted in the presence of lower levels of fetal bovine serum (down to 0.3%, v/v;

Figure 5. CD44v7/8-selected cells have an increased proliferation rate. **Top:** Immunostaining of cells expressing CD44v7/8 after panning with an anti-CD44v7/8 antibody. Positive (+ve) mean cells that are retained on magnetic beads and negative (-ve) means cells that are not. The result shown is representative of six experiments. **Bottom:** Increase in cell number of CD44v7/8 negatively and positively selected fibroblast-like synoviocytes throughout a period of 4 days. Cell numbers were counted using a Coulter cell counter. The difference in proliferation rate is significant with a $P < 0.01$ ($n = 6$).

data not shown). To further analyze the relation between cell surface expression of CD44v7/8 and cell proliferation, we selected fibroblast-like synoviocytes expressing this epitope using a panning protocol with antibodies (anti-CD44v7/8) coupled to magnetic beads. Using this method, with a 1-week-old culture, we routinely obtained a nearly 100% CD44v7/8-positive population (+ve) (Figure 5, top). The percentage of cells still expressing CD44v7/8 in the negatively selected population (-ve) was estimated at 20%. Fibroblast-like synoviocytes enriched for expression of CD44v7/8 have a higher proliferation rate, we observed a 3.3-fold increase in cell number after 4 days compared to a 2.6-fold increase of low expressers ($n = 4$) (Figure 5, bottom). From these data, we calculate an average doubling time of 55 hours ($96 \times \log[2]/\log[3.3]$) for the positive population and an average doubling time of 69 hours ($96 \times \log[2]/\log[2.6]$) for the negatively selected cells. This difference in proliferation rates would predict an enrichment of CD44v7/8-positive cells during culture which is slightly slower than the one presented in Figure 3. One should, however, bear in mind that we did not entirely deplete the population of CD44v7/8-expressing cells and these cells contribute to the total population proliferation rate. These cell-doubling times are within the range published earlier using similar culture times and conditions, in which it was shown that fast proliferating fibroblasts had an average doubling time of 42 hours and slow proliferating cells a doubling time of 72 hours.¹⁸

Antibodies against CD44v7/8 Inhibit Proliferation by Elevation of Expression of Cell Cycle Inhibitors

To determine whether or not the expression of the CD44v7/8-epitope plays a role in the regulation of the cell cycle, we next investigated cell proliferation and DNA synthesis. For this purpose we took fibroblast-like synoviocytes with a 1-week culture history and added antibodies against the variable exons CD44v3, CD44v6, or CD44v7/8, or antibodies against VCAM-1. The latter was included because the fibroblast-like synoviocytes express very high levels of this adhesion molecule¹⁹ for which no implication in proliferation had been reported and could therefore serve as a negative control for antibody treatment. After a culture period of 4 days, only the presence of anti-CD44v7/8 antibodies reduced DNA synthesis and likewise after 11 days reduced cell proliferation (Figure 6). We interpret these findings as indicating that expression of CD44v7/8 confers a proliferative advantage on the fibroblast-like synoviocytes. To discern a molecular mechanism, through which the antibodies against CD44v7/8 inhibit cell proliferation, we used a high-density oligonucleotide array and analyzed the profile of gene expression after treatment throughout a 5-day time period. In the antibody-treated cells we detected a significant elevated expression of cyclin-dependent kinase inhibitor p21Waf1/Cip1²⁰ and of growth arrest and DNA damage-inducible protein GADD45²¹ and GADD153²² (Figure 7 and Table 1). Because expression of GADD gene

Figure 6. Antibodies against CD44v7/8 reduce cell proliferation and ³H-thymidine incorporation of fibroblast-like synoviocytes in culture. **Top:** Cell numbers of fibroblast-like synoviocytes cultured for a period of 11 days with or without the presence of antibodies against CD44 exon v3, v6, v7/8, or against VCAM-1 ($n = 6$). In the case of anti-CD44v7/8 the reduction was highly significant with a $P < 0.0001$. **Bottom:** ³H-thymidine incorporation after incubation of fibroblast-like synoviocytes for a period of 4 days with or without antibodies against CD44 exon v3, v6, v7/8, or against VCAM-1 ($n = 4$). Only the difference between control and treatment with anti-CD44v7/8 is significant with a $P < 0.01$.

products is associated with either growth arrest or programmed cell death (apoptosis), we next analyzed the integrity of DNA at day 5 but found no signs of fragmentation (data not shown). We take this finding to mean that expression of these cell-cycle inhibitory genes, within the time span of the experiment, is not linked to cell death.

Discussion

Cells in the synovial membrane of patients with RA abundantly express the splice variant CD44v7/8. In a Western blot protocol, antibodies against CD44v7/8 recognize a single protein of Mr 100 kd. When brought into culture, the fibroblast-like synoviocytes bearing this splice variant have a proliferative advantage over those that do not, but are derived from the same tissue. The expression of CD44v7/8 must play a role in the enhanced proliferation of the cells because antibodies that recognize the CD44v7/8 epitope annul the proliferative advantage. This finding shows that, in contrast to many studies analyzing their role in human tumor development, aberrant expression of CD44 splice variants alters cellular behavior in

Figure 7. Treatment with antibodies against CD44v7/8 up-regulates expression of cell cycle inhibitors. Gene expression of fibroblast-like synoviocytes cultured in the presence or absence of antibodies against CD44v7/8 was analyzed in a Southern blotting protocol using a high-density oligonucleotide array. Radioactivity is quantified and visualized with a phosphorimager. The data are representative of three separate experiments with cells obtained from three different synovial specimen (RA).

fibroblast-like synoviocytes. Surprisingly, the binding to hyaluronan plays no role in the altered cell proliferation because addition of an anti-CD44 antibody that blocks its binding (clone 5F12 CL4) did not affect ³H-thymidine incorporation (data not shown). Our working hypothesis is that CD44 containing the alternative exons v7/8 recruits a growth factor receptor into an adhesion complex and this results in an intracellular signal that suppresses ex-

pression of cell cycle inhibitors. The presence of antibodies against CD44v7/8 abrogates this recruitment followed by an elevation of expression of cell cycle inhibitors. We know that the two exons are part of a larger splicing complex, most likely CD44v6-10, because we have never detected their exclusive presence in CD44 mRNA.¹⁶ We are currently cloning the various CD44-splicing combinations to overexpress them and study complex formation with cell membrane proteins.

The observation that antibodies against CD44v7/8 inhibit cell proliferation is of interest for therapeutic reasons. First of all there is evidence that hyperplasia of the fibroblast-like synoviocytes plays a role in the development of a chronic erosive environment within the synovial joint, most likely irrespective of the role of the immune system therein.^{23,24} We base this idea on the findings that 1) the degree of synovial hyperplasia correlates with the degree of joint erosion,^{12,24} 2) H2-c-fos transgenic mice develop destructive arthritis,²⁵ and 3) our finding that fibroblast-like synoviocytes produce excessive amounts of interleukin-6 independent of the presence of inflammatory mediators such as tumor necrosis factor- α and interleukin-1.²⁶ High levels of interleukin-6 are instrumental in the reduction of cartilage production²⁷ and in its breakdown.²⁸ Secondly, in a recent study using a mouse colitis model, it was shown that antibodies against the CD44v7 epitope caused a full regeneration of the intestine.²⁹ This effect was explained by moderating an overshooting Th1 reaction. An excessive Th1 response occurs in RA and is part of the chronic inflammatory response.³⁰ It remains the question, however, if fibroblast-like synoviocytes in the synovial membrane are equally sensitive to the blocking of the CD44v7/8 epitope. Results from *in situ* studies have given good evidence for a contribution of local proliferation of fibroblast-like synoviocytes to synovial hyperplasia, both in humans¹⁵ and in experimental models of arthritis.³¹ Given these considerations, the CD44v7/8 epitope could be an interesting target for pharmacological intervention in the treatment of RA.

Table 1. Expression Levels of Cell Cycle Genes in Control and anti-CD44v7/8-Treated Fibroblast-Like Synoviocytes

GenBank accession no.	Name of gene	Intensity	
		Untreated	Treated
Housekeeping genes for normalization of experimental values			
M11886	HLA Class 1	24,150 ± 603	25,550 ± 580
X56932	23-kd HBP	68,050 ± 2,416	68,030 ± 456
Genes whose expression is significantly altered			
U09579	P21Waf/Cip	4,008 ± 280	6,707 ± 309*
M60974	GADD45	152 ± 33	2,411 ± 250*
S40706	GADD153	1,900 ± 45	2,324 ± 61*
Genes whose expression in unchanged			
U18291	CDC16Hs	858 ± 15	1,176 ± 78
L05624	MEK1	392 ± 94	457 ± 34
D89667	c-myc BP	1,200 ± 278	1,350 ± 248
X66360	PCTAIRE-2	531 ± 46	487 ± 51

Radioactivity levels of spots of the Atlas Array blots were calculated using a PhosphorImager and presented in arbitrary units corrected for background. For normalization of total cDNA, intensity values of housekeeping genes were also estimated. The data are an average of three separate experiments with cells obtained from three different synovial specimens (RA)

*indicates significant differences between control and treated sample.

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