Expression patterns of CD44 and CD44 splice variants in patients with rheumatoid arthritis

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Abstract

Objectives

It has been suggested that CD44 is involved in the pathogenesis of rheumatoid arthritis (RA). By alternative splicing, numerous CD44 isoforms can be generated which may play different roles the inflammatory process. We therefore studied the expression of various CD44 splicevariants in the circulation and synovial tissue of patients with RA and correlated expression with clinical features.

Methods

Expression of distinct CD44 splice variants was analysed by FACS in peripheral monocytes of 46 RA patients and 36 healthy controls. Expression of CD44 splice variants in synovial tissue of RA and OA patients was analysed by immunohistochemistry and the effects of blocking CD44v4 on RA-fibroblast like synoviocytes (FLS) were studied.

Results

On monocytes, the expression of CD44 and CD44v3 was significantly lower in patients with erosive disease than in those without radiographic progression ($p<0.05$ for CD44 and $p<0.01$ for CD44v3). CD44v6 on monocytes was significantly associated with the clinical disease activity index ($r=0.34$, $p<0.05$) and CRP-levels ($r=0.37$, $p<0.02$).

Immunhistochemical analyses revealed that most variants were expressed to a significantly higher extent in RA than in OA synovial membranes. Particularly the variants CD44v4, CD44v6 and CD44v7-8 were highly expressed in the RA lining and also abundantly in the endothelium.

Blocking CD44v4 in RA-FLS reduced the proliferation to 68±8% ($p<0.02$) when compared to control experiments and led to a reduction in IL-1ß mRNA expression ($p<0.05$).

Conclusions

Expression of CD44 splice variants is generally increased in the synovial lining of RA patients when compared to OA. The inverse association of CD44v3 expression on monocytes with the development of erosive disease and the functional impacts of CD44v4 blockade in RA-FLS suggests a pathogenetic role of this splice variants which needs to be further investigated.

Key words

rheumatoid arthritis, CD44, splice variants, synovium, monocytes

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Introduction

Rheumatoid arthritis (RA) is characterised by chronic inflammation of the synovial membrane that leads to bone and cartilage destruction. Several proinflammatory cytokines, such as tumour necrosis factor (TNF), Interleukin (IL)-1 and IL-6, propagate the disease process (1) and therapies targeting these molecules have garnered groundbreaking therapeutic effects (2).

The inflammatory response involves intensive communication between different cell populations and also complex cell-matrix interactions. Among many other proteins, adhesion molecules are pivotally involved in these communications. In RA, many of these molecules are highly upregulated both on synovial and peripheral blood cells (2). These cell surface receptors and ligands, which are partly induced by proinflammatory cytokines, are regarded as important contributors to the inflammatory process by virtue of enhancing cellular traffic and activating and perpetuating the disease process.

Among the many families of adhesion molecules the CD44 family is a very prominent one. CD44 is a type I transmembrane glycoprotein whose major ligand is hyalorunic acid or hyaluronan, respectively and, hence, these molecules belong to the hyaluronan receptor family (3). CD44 is involved in various activities of peripheral blood mononuclear cells (PBMC) and other cells of the inflammatory system and regulates cell adhesion, homing and transendothelial migration of mononuclear cells as well as cell-cell and cell-matrix interactions (3). Proinflammatory cytokines can induce shedding of CD44 and this soluble form of CD44 can be detected in the serum, plasma as well as in the synovial fluid (4).

In murine models CD44 modulation has revealed some promising therapeutic effects (5, 6).

The CD44 gene contains variable exons (v2-v10) that can undergo alternative splicing to generate numerous different CD44 isoforms. So far, 33 different splice variants of CD44 have been described (7), which subserve distinct functions (8). For example, CD44v6 appears to mediate the invasive behaviour of cancer cells (9). The role of these splice variants in rheumatic diseases is not completely understood, although it has been shown that CD44v3 and CD44v6 are overexpressed on synovial fibroblasts which carry an increased invasive capacity *in vitro* (10). In contrast, it has been described that the overexpression of CD 44v8-v9 correlates inversely with the invasive capacity of fibroblast-like synoviocytes (11). CD44v6 has also been detected in the synovial fluid of OA patients and was shown to be increased in an animal model linear to the time course of OA (12, 13).

The expression of CD44 on peripheral blood mononuclear cells of RA patients has not been extensively investigated, especially not on monocytes. Monocytes/macrophages are not only generally important cells involved in inflammatory responses, but subserve special functions in the synovial membrane and RA pathogenesis; monocyte/macrophages can enter the synovial membrane and are recruited into the lining layer as type A synoviocytes (14), contributing locally to the inflammatory events and degradation of cartilage; moreover, they serve as precursors of osteoclasts that are generated within the synovial membrane (15, 16), thus playing a pivotal role in bone destruction. Interestingly, CD44 deficient arthritic mice overexpressing TNF exhibit an enhanced osteoclastogenesis and joint destruction, suggesting that decreased expression of CD44 rather than its overexpression may importantly contribute to the destructive propensity of the synovial membrane in RA (17). Thus, CD44 may play a dual role in the context of arthritis and joint destruction, on the one hand acting in a proinflammatory way, and on the other hand possibly portending anti-inflammatory and anti-osteoclastogenic activities, a duality that might be brought about by the different splice variants of the molecule. In contrast to our knowledge of CD44 in RA and its effects on bone metabolism (18), very little is known about the role of its splice variants in this chronic inflammatory disease. Indeed, it has not been investigated which splice variants, or their

deficiency, affect osteoclastogenesis either in an enhancing or downregulating manner.

To further elucidate a potential role of CD44 and its splice variants in RA we studied their expression on the surface of cells of the RA synovial membrane as well as on monocytes isolated from peripheral blood of RA patients.

Patients and methods

Patients

CD44 and distinct CD44 splice variants were studied on monocytes isolated from peripheral blood of 46 RA patients (mean age: 56.1±2.1 years; mean disease duration: 9.4±1.0 years) and 36 matched healthy controls (HC) by fluorescence activated cell sorting (FACS) analysis.

In the RA patients, clinical variables, laboratory surrogates of inflammation (CRP and erythrocyte sedimentation rate (ESR)), and the presence or absence of radiographic erosions as detected by x-rays of the hands and feet were determined. Disease activity was assessed by the Clinical Disease Activity Index, CDAI (9.5±1.3) (19). 74% of the patients were rheumatoid factor positive, 70% were treated with methotrexate, 21% with other DMARDs than methotrexate (leflunomide, sulfasalazine and azathioprine) and 9% with TNF blockers (with or without methotrexate). 46% of the patients were receiving glucocorticoids at doses of 5 to 25 mg prednisone/day.

All patients provided informed consent according to the declaration of Helsinki for the analyses of their blood and/or tissues and the study was approved by the ethics committee of the Medical University of Vienna.

Isolation of PBMC and staining of CD44 splice variants on monocytes

PBMC were isolated according to standard procedures by centrifugation on LSM 1077 lymphocyte separation medium (PAA Laboratories GmbH, Linz, Austria). Staining of PBMC was performed with fluorescein isothiocyanate (FITC) conjugated mouse anti-human monoclonal antibodies to CD44 and the splice variants CD44v4, CD44v6 and CD44v7 (Bender Med Systems, Vienna, Austria) and biotinylated monoclonal mouse anti-human antibodies against CD44v3 and CD44v5 (Bender Med Systems, Vienna) that were labelled with Streptavidin/RPE-Cy-5. The expression of CD44v10 and CD44 v3-10 was quantified using primary antibodies against CD44v3-v10 (polyclonal rabbit anti-human) and CD44v10 (polyclonal goat anti-human, both from Chemicon International, Temecula, CA, USA) that were conjugated with secondary polyclonal goat anti rabbit IgG FITC (CD44v10) and swine anti rabbit FITC (CD44v3-10) antibodies. For all stainings adequate isotype control stainings were performed.

The mean fluorescence intensity (MFI) was determined after gating on the monocyte population. The results obtained for RA patients were compared with those of HC and further correlated with clinical disease activity, presence of erosive disease, therapy and laboratory variables.

Immunohistochemistry and histomorphometry

Synovial membrane cryosections of 8 RA (20) and 6 osteoarthritis (OA) patients were additionally studied. The specimens were obtained from patients who underwent synovial biopsy, arthroscopic synovectomy or joint replacement surgery. We assessed the expression of the following splice variants on cryosections: CD44, CD44v3, CD44v4, CD44v5, CD44v6 and CD44v7-8 using mouse anti-human monoclonal antibodies (Serotec, Oxford, UK).

Since synovial membrane specimens from HC were not available, we used specimens of 6 OA patients as "non-inflammatory" controls.

Immunohistochemistry was performed essentially as described (21). Briefly, tissue sections were incubated with one of the above monoclonal antibodies at a dilution of 1:100–1:3000 for 60 minutes with isotype-matched monoclonal antibodies serving as controls.

Subsequently, biotinylated horse anti-mouse IgG was applied as second antibody, and incubated for 30 minutes; thereafter, sections were incubated with the Vectastain ABC reagent (Vector, Burlingame, CA, USA) for another 30 minutes. Diaminobenzidine (Sigma, St. Louis, MO, USA) was used for colour development resulting in brown stainings. Finally, sections were counterstained with haematoxyline or Meyer's Haemalaun solution (Merck, Darmstadt, Germany).

To investigate which cells expressed CD44 and its splice variants, tissue sections were stained as described above followed by incubation with monoclonal antibodies against CD44 and various cell lineage-specific antibodies, including anti-CD68 (monocytes/macrophages, Dako, Glostrup, Denmark), clone ASO2 (fibroblasts, Dianova, Hamburg, Germany), anti-CD34 (endothelial cells, Novocastra, Newcastle upon Tyne, UK), anti-CD3 (T cells, Becton Dickinson, Mountain View, CA, USA) and anti-CD20 (B cells, Dako, Glostrup, Denmark). All antibody clones were mouse anti-human.

Again, isotype-matched, unrelated monoclonal antibodies served as controls. Sections were incubated with the primary antibody diluted between 1:40 and 1:300 in TRIS –buffered saline for 60 minutes at room temperature. After rinsing, alkaline phosphatase-labelled, affinity-purified rabbit anti-mouse immunoglobulins (Dako, Glostrup, Denmark) were applied as secondary antibody for 30 minutes, followed by incubation with the APAAP complex (Dako, Glostrup, Denmark). Colour was developed using Fast Blue (Sigma, St. Louis, Mo, USA) as substrate. This reaction provided blue cell staining, allowing to discern if specific cells expressed CD44 or a variant by their double staining.

In addition to their expression on specific cell populations, we also assessed the expression of CD44 and its splice variants in the different synovial compartments, the synovial lining, the sublining, the infiltrates in the sublining and endothelial cells of blood vessels. The expression was rated blindly (*i.e.* without knowing the diagnosis) for each compartment in a semi quantitative fashion on a five point scale from 0 (absence) to 4 (high expression) by two independent observers as previously described (22, 23); quantification was performed in at least three random microscopic fields.

RA-FLS proliferation assay

For these experiments we used FLS from synovial tissue samples obtained from RA patients undergoing joint surgery. Briefly, synovial samples were minced, digested using collagenase solution and resulting FLS were washed and cultured to passages three to five as described previously (14).

To test the effect of CD44v4 blockade on RA-FLS proliferation, we used a [^{3}H]-thymidine incorporation assay. Briefly, the FLS were plated onto 96 well plates in RPMI 1640 medium supplemented with 10% foetal calf serum (FCS). After 24 hours, the medium was changed and FLS further incubated in the presence of either a monoclonal CD44v4 antibody (clone VFF-11, Bender, Austria) at a concentration of 50 μg/ml (24) or an isotype control antibody (Bender) with 1 μCi [^{3}H]-thymidine (Perkin Elmer, Waltham, MA, USA)/well added. After a further incubation period of 72 hours, the cells were harvested, lysed and the [^{3}H]-thymidine incorporation quantified using a 2450 microplate counter micro beta^2 (Perkin Elmer).

TNF and IL-1β mRNA expression in RA-FLS

FLS from 6 RA patients were incubated for 96 hours in RPMI 1640 supplemented with 10 % FCS in the presence of either anti-CD44v4 (50 μg/ml) or the isotype control in 24 well-plates. Subsequently, the cells were harvested and lysed followed by RNA separation.

For real time-PCR 50 ng total RNA was reversely transcribed to cDNA with a commercially available first-strand cDNA-synthesis kit (Sensiscript, Qiagen, Hilden, Germany) using oligo-dT primer (Invitrogen) and RNase inhibitor (Invitrogen) for 1 hour at 37°C.

In brief, 2μl aliquots were amplified in a 20 μl reaction mixture by quantitative PCR. The IL-1β and TNF primers were purchased ready to use from SA Biosciences Corp. (Frederick, MD, USA). Reactions were run on Light Cycler 480 Real-Time PCR System (Roche, Basel, Switzerland) Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as housekeeping gene to standardise for the overall cDNA content. Analyses were performed in triplicates. To calculate the relative expression, we normalised to the GAPDH and compared to the mean control values.

Statistical evaluation

Statistical evaluations were performed using SPSS for Windows v 14.0 (SPSS Inc.) and Graph Pad Prism 4.0. Data were evaluated using normality test, equal variance test and Student's *t*-test. Data not normally distributed were analysed using non-parametric methods. All data are presented as mean ± standard error of the mean (SEM) if not mentioned otherwise. *P*-values less than 0.05 were considered significant.

Table I. MFI of CD44 splice variants on monocytes.

Variant	HC	RA	Tendency* (RA *vs*. HC)	*p*-value
CD44	166 ± 11	161 ± 11	=	0.86
CD44v3	220 ± 73	197 ± 50	↓	0.56
CD44v4	134 ± 14	136 ± 13	=	0.89
CD44v5	199 ± 65	159 ± 44	↓↓	0.52
CD44v6	68 ± 7	71 ± 8	=	0.81
CD44v7	85 ± 8	83 ± 7	=	0.88
CD44v3-10	2943 ± 373	4629 ± 504	↑↑↑	**<0.01**
CD44v10	1056 ± 289	1457 ± 329	↑↑	0.84

*Tendencies of MFI on monocytes between HC and RA were rated as follows:
= when discrepancy was lower or equal than 10%; ↓ for discrepancies between 10 and 20%; ↓↓ or ↑↑ for discrepancies between 21 and 50 % and ↑↑↑ for discrepancies higher than 50%.
Statistically significant *p*-values are indicated in bold letters.

Results

Comparison of the expression of CD44 and its splice variants on monocytes of RA patients and HC

First, we analysed the expression of CD44 and its splice variants on the cell surface of monocytes of RA patients and HC by FACS. CD44 was expressed to a similar degree whereas CD44v3-v10 showed a significantly higher expression level in RA patients (MFI: 4629±504) when compared to HC (MFI: 2943±373, $p<0.01$, Table I). However, no significant differences were seen for any of the other splice variants.

Expression pattern of CD44 splice variants in subpopulations of RA patients

We then evaluated whether the expression of CD44 splice variants was different among subsets of RA patients taking into account clinical disease activity, radiographic changes, rheumatoid factor status, levels of acute phase reactants or therapy.

Recent x-rays of the hands and feet were available for 35 of the 46 RA patients. In 30 patients (=86%) radiographic erosions were found, as evaluated by independent radiologists who were not involved in this study. In these patients, the expression of CD44 on monocytes was lower than in patients without erosions (MFI: 158±12 *vs*. 230±29, $p<0.05$). While the MFI of CD44 on healthy controls' monocytes (166±11) was comparable with that of patients with erosions, it was significantly lower on healthy monocytes when compared with those from RA patients without bony damage ($p<0.05$).

An analysis of the CD44 splice variants revealed that expression of variant CD44v3 was highly decreased on monocytes of erosive when compared with non-erosive RA patients (MFI: 160±54 *vs*. 569±159, $p<0.01$), and again its expression on cells from patients with non-erosive RA was found to be significantly higher also when compared to HC (569±159 *vs*. 220±73 , $p<0.0001$).

However, therapy with disease modifying anti-rheumatic drugs (DMARDs), regardless of the type, did not appear to have an influence on CD44 or variant expression (not shown).

Correlation with disease activity

Disease activity, as assessed by the CDAI, was associated only with CD44v6 expression on monocytes (r=0.34, $p<0.02$, respectively, Fig. 1). In line with this finding, CD44v6 expression on monocytes was also associ-

ated with increased CRP levels (r=0.37, p<0.02) the daily dose of glucocorticoids (r=0.41, p<0.01), both characteristics of more active disease.

Expression of CD44 and distinct splice variants in the rheumatoid synovium

Expression of CD44 and several of its splice variants in RA and OA synovial membranes was investigated by immunohistochemistry and the data obtained were evaluated blindly in a semiquantitative manner for the synovial lining, sublining infiltrates and endothelial cells of the microvessels (Table II).

CD44 was found to be moderately expressed in the RA synovial lining (score: 2.6±0.3, [score range: 0-4]; Fig 2A) while the expression in the OA lining layer tended to be lower (1.6±0.4, p<0.1, Fig. 2B). CD44 was significantly higher expressed in RA-microvessels (1.6±0.5) when compared to the microvessels in the OA tissue specimens (0.6±0.4, p<0.02).

Moreover, low expression of CD44 was seen in sublining cells of RA patients (0.8±0.3, Fig. 2A), in contrast to OA synovial tissue where sublining cells lacked CD44 expression (Fig. 2B).The findings obtained for the distinct synovial regions are summarised in Table II.

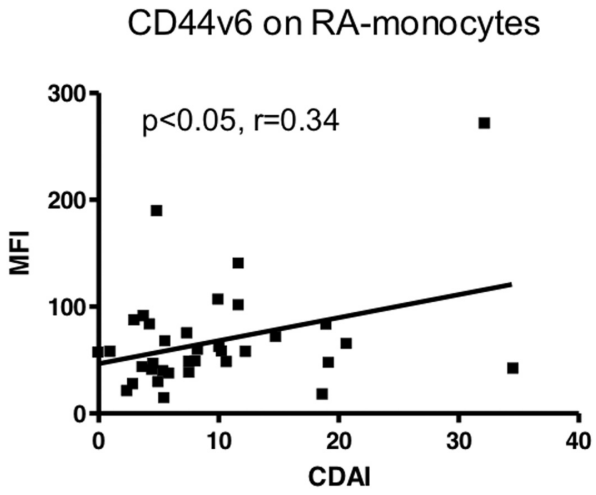

Fig. 1. Correlation of disease activity with CD44v6 expression. Disease activity as assessed by the CDAI showed a significant correlation with the MFI of CD44v6 on monocytes.

Table II. Semiquantitaive assessment of expression of several CD44 splice variants in the synovium of OA and RA patients.

Variant	Synovial lining		Endothelium		Synovial infiltrate	
	OA	RA	OA	RA	OA	RA
CD44	1.6 ± 0.4	2.6 ± 0.3	0.6 ± 0.4	1.6 ± 0.5 **	n.e.	0.8 ± 0.3
CD44v3	2.7 ± 0.6	3.0 ± 0.4	0.5 ± 0.3	2.5 ± 0.2	n.e.	0.8 ± 0.7
CD44v4	0.6 ± 0.3	2.9 ± 0.3***	1.6 ± 0.7	2.5 ± 0.3	0.3 ± 0.3	0.1 ± 0.1
CD44v5	0.8 ± 0.2	2.2 ± 0.7	1.4 ± 0.5	0.4 ± 0.32	n.e.	n.e.
CD44v6	1.0 ± 0.7	2.4 ± 0.4*¥	1.0 ± 0.3	2.9 ± 0.9 ***	n.e.	n.e.
CD44v7-8	1.5 ± 0.5	2.8 ± 0.6	1.3 ± 0.9	3.9 ± 0.1 ***	n.e.	n.e.

Expression was rated on a 5 point scale from 0 (absence) to 4 (high expression).
*p<0.05 *vs*. OA
**p<0.02 *vs*. OA
***p<0.005 *vs*. OA
¥: inhomogenous expression within the synovial lining of one cryosection. n.e.: not expressed.

- Lining

Most of the CD44 splice variants investigated were more strongly expressed in the synovial membrane of RA patients when compared with OA patients (see Table II) CD44v4 was significantly higher expressed in the RA lining (2.9±0.3, Fig. 2C), but was nearly undetectable in OA sections (0.6±0.3, p<0.005, Fig. 2D).

Interestingly, the expression of CD44v6 in the synovial lining of RA showed considerable variation among different patients and also within the specimens of individual patients (mean of 2.4±0.4, Fig. 2E), suggesting focal expression of this variant. In OA however, we observed a significantly lower, but homogenous expression of CD44v6 (1.0±0.7, p<0.05 *vs*. RA, Fig. 2F).

Double stainings for CD44v6 and CD68 in RA specimens confirmed the patchy distribution of CD44v6 since some monocytes/macrophages expressed this variant whereas others did not (Fig. 3A)**.** T-cells, fibroblasts and B-cells expressed CD44v6 all co-expressed CD44v6 partly (data not shown). CD44v7-8 was highly expressed in the RA synovial lining layer with the majority of cells staining positive (2.8±0.6, Fig. 2G) while expression in the OA lining was considerably lower (1.5±0.5, Fig. 2H).

Double stainings for CD44v7-8 and various cell lineage markers revealed that cells expressing these variants were mainly macrophages (Fig. 3B) and to a lesser extent synovial fibroblasts (Fig. 3C), and T-cells (Fig. 3D) while expression in B-cells was not observed (Fig. 3E).

- Microvessels

When focusing on the microvessels and the endothelium, we found a moderate expression of most of the variants in RA specimens. Interestingly, the splice variant CD44v6, which, as described above, was inhomogenously expressed in the lining layer, was found to be uniformly expressed in the microvascular endothelium (2.9±0.9, Fig. 2I); expression was also significantly higher than in OA endothelium (1.3±0.9, p<0.005). CD44v7-8 turned out to be intensely expressed in RA endothelial cells (3.9±0.1, Fig. 2J), but rarely in OA endothelium (1.0±0.3, p<0.005; Fig. 2H). In the microvessels of OA specimens, only a low expression of all variants tested was detectable varying from 1.6±0.7 when staining for CD44v4 to 0.5±0.3 (CD44v3, Table II).

- Synovial sublining infiltrates

The synovial infiltrates present in the sublining area of both RA and OA specimens were found to express the distinct CD44 splice variants tested either

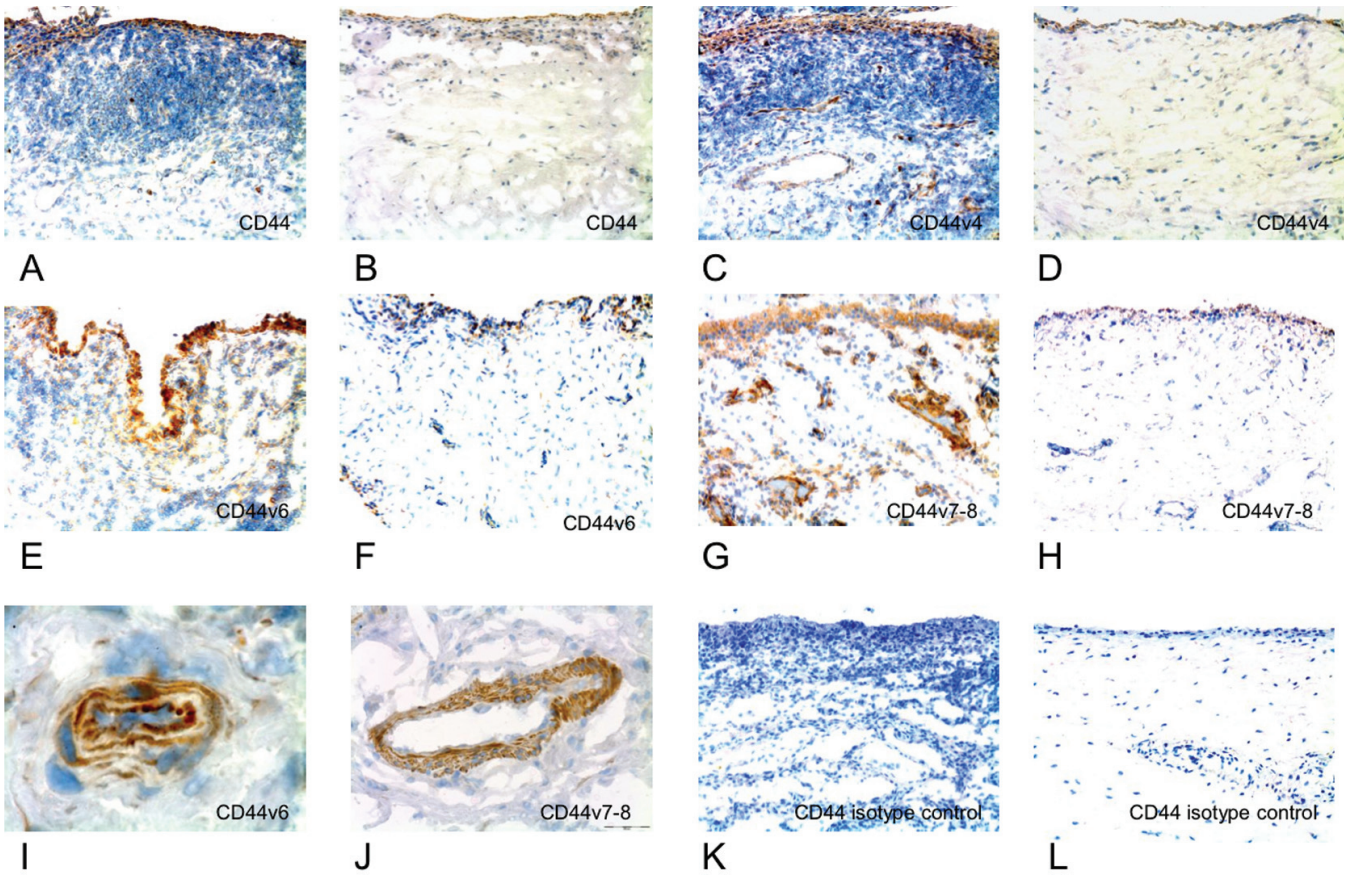

Fig. 2. Expression of CD44 and distinct splice variants in the RA and OA synovial tissue: CD44 was strongly expressed in the lining layer of RA synovial membranes and to a weaker degree also by some cells within the sublinig area (**A**), whereas expression in the OA synovial tissue was generally weaker (**B**). The splice variant CD44v4 was expressed in the RA synovial lining (**C**), but could hardly be detected in OA synovial tissue (**D**). Pronounced but inhomogeneous expression of CD44v6 was seen in the RA synovial lining,(**E**) while expression was scarce in OA specimens (**F**). The splice variant CD44v7-8 was highly overexpressed in RA synovial tissue (**G**) and to a lesser degree also in OA tissue (**H**). Particularly pronounced expression of both CD44v6 (**I**) and CD44v7-8 (**J**) was observed in endothelial cells of the RA synovium.
Representative isotype matched control stainings of RA-tissue (**K**) and OA-tissue (**L**).
Magnifications of pictures shown are 100 x (**A**-**H**, **K**, **L**) and 400 x (**I**, **J**).

at a very low degree or not at all (see Table II).

Taken together, most of the CD44 variants investigated were significantly upregulated in the lining layer and endothelial cells of the synovial membrane of RA patients when compared to specimen of OA patients. In contrast to the other variants, CD44v6 showed scattered expression in the RA synovial lining while low but homogeneous expression was seen in OA synovial membranes.

Effect of CD44v4 blockade on RA-FLS proliferation and cytokine gene expression

When analysing the proliferation of FLS isolated from RA patients in the presence of a CD44v4 antibody, we observed a significantly reduced proliferation rate when compared to the control experiments after 3 days of culture: as depicted in Figure 4 A the proliferation of RA-FLS was significantly reduced to 68±9% ($p<0.02$) in the presence of the CD44v4 antibody compared to control experiments that were set to 100%.

An analysis of the mRNA expression following incubation with an anti-CD44v4 antibody revealed that the blockade of the splice variant CD44v4 indeed had a relevant effect on IL-1β expression: the relative expression of IL-1β mRNA in RA-FLS incubated in the presence of the CD44v4 antibody was significantly reduced to 0.006±0.001 when compared to expression in the presence of an isotype-matched control antibody (0.011±0.002, $p<0.05$, Fig. 4B). In contrast, the relative expression of TNF mRNA was not affected by the CD44v4 antibody (Fig. 4C).

Discussion

The role of CD44 and its splice variants has been intensively studied in the field of oncology with special focus on malignant tumours and tumour biology. These studies suggest dual effects of CD44 and its splice variants, on the one hand promoting the invasiveness of tumours but on the other hand also exhibiting protective effects by acting as tumour suppressive co-factor (25).

The RA pannus shares features with malignant tumours by disrespecting local tissue boundaries, invading bone and cartilage locally, and showing increased vascularisation. Nevertheless, only few investigations have been performed on the expression and role of CD44 splice variants in RA. The increased expression of CD44 in the synovial membrane of RA patients shown here is in

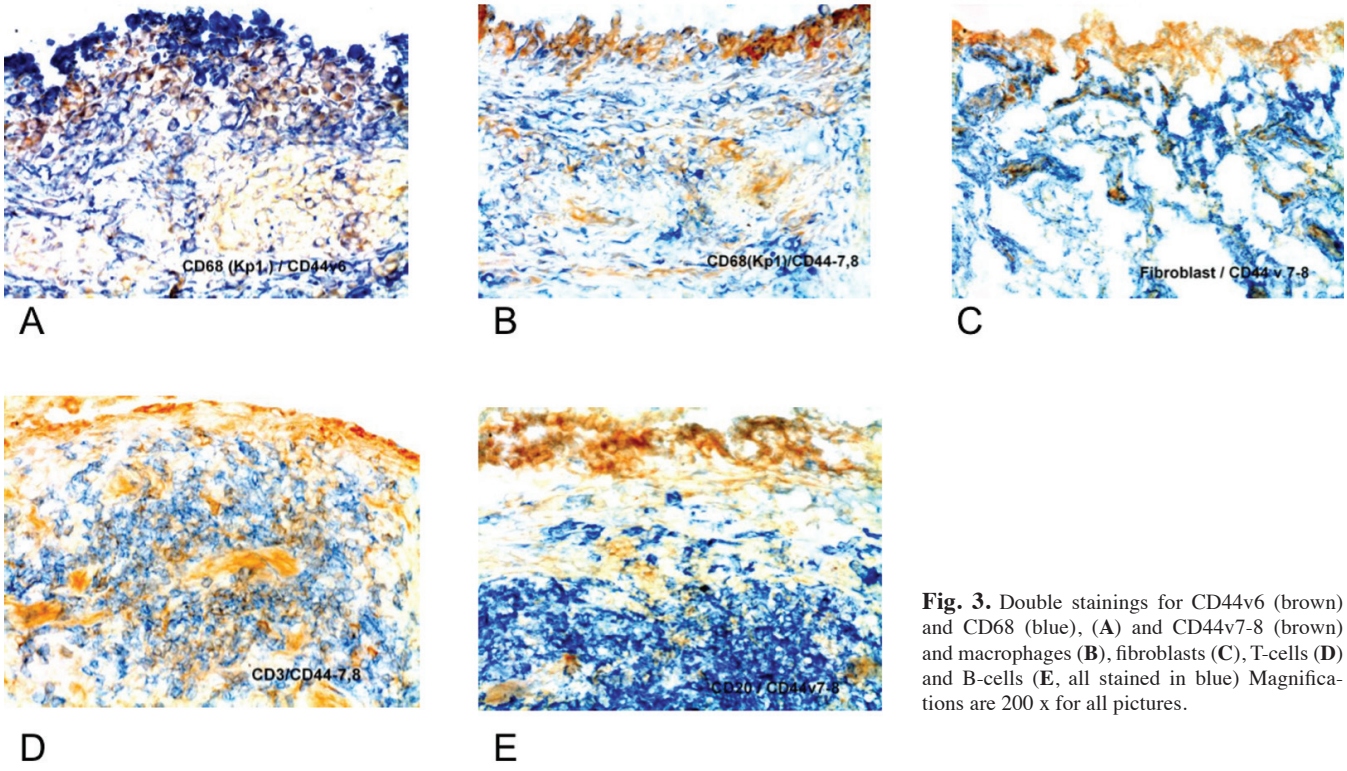

Fig. 3. Double stainings for CD44v6 (brown) and CD68 (blue), (**A**) and CD44v7-8 (brown) and macrophages (**B**), fibroblasts (**C**), T-cells (**D**) and B-cells (**E**, all stained in blue) Magnifications are 200 x for all pictures.

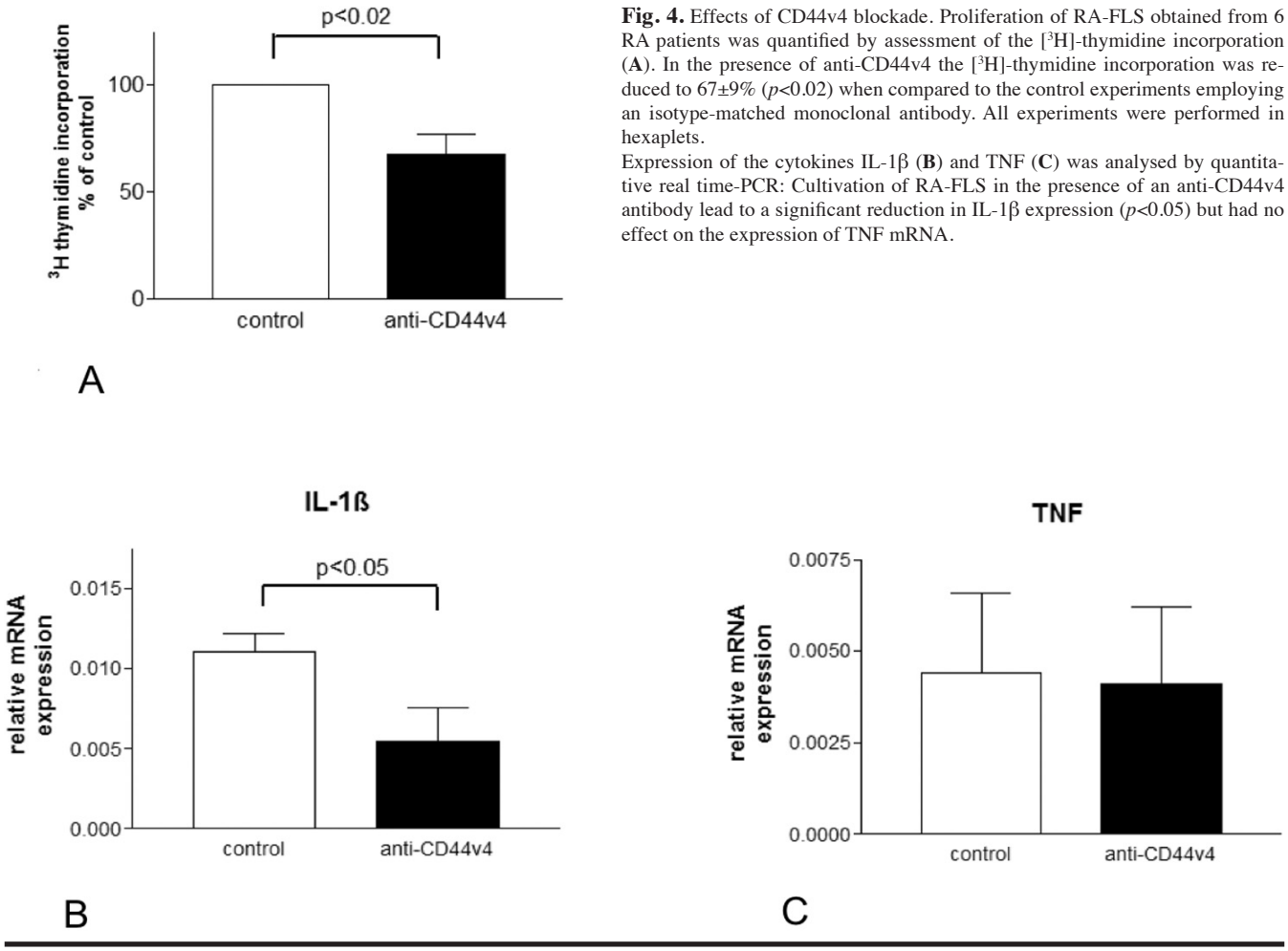

Fig. 4. Effects of CD44v4 blockade. Proliferation of RA-FLS obtained from 6 RA patients was quantified by assessment of the [^{3}H]-thymidine incorporation (**A**). In the presence of anti-CD44v4 the [^{3}H]-thymidine incorporation was reduced to 67±9% (p<0.02) when compared to the control experiments employing an isotype-matched monoclonal antibody. All experiments were performed in hexaplets.

Expression of the cytokines IL-1β (**B**) and TNF (**C**) was analysed by quantitative real time-PCR: Cultivation of RA-FLS in the presence of an anti-CD44v4 antibody lead to a significant reduction in IL-1β expression (p<0.05) but had no effect on the expression of TNF mRNA.

line with previous findings (26) and has been interpreted as a consequence of the proinflammatory milieu in the joint of RA patients.

Among the results of our study, three findings deserve particularly detailed discussion.

Firstly, and presumably most strikingly, we observed decreased expression of both CD44 and CD44v3 on monocytes of RA patients with bone erosions when compared to RA patients without radiographic alterations, who showed significantly increased expression when compared to HC.

This observation is reminiscent of findings of an increased destructive propensity of TNF induced arthritis in CD44 deficient mice (17). The increased expression of CD44 and CD44v3 on monocytes could be interpreted as counter regulatory mechanism to inhibit bone erosion. Alternatively, the increase of CD44 expression observed in peripheral monocytes of non-erosive patients may reflect an initial event representing a (beneficial) consequence of the inflammatory cytokine milieu, while downregulation of CD44 expression may lead to an increased propensity of monocytes to invade and destroy bone.

This observation is especially remarkable, since Hayer *et al*. have shown that disruption of the CD44 gene in combination with an inflammatory milieu, as conveyed by chronic overexpression of TNF, led to massive osteoclast activity and bone erosion, which by far exceeded that of CD44 expressing TNFtg mice (17). In conjunction with this finding, our data suggest that CD44 deficiency itself has no effect on bone resorption or osteoclast activity, leading to the speculation that CD44 might act in an osteoprotective manner, at least in inflammatory TNF-driven arthritis. However, de Vries *et al*. have shown that the formation of osteoclasts can be increased in the absence of CD44 depending on the conditions and the microenvironment (27); this further supports the hypothesis that CD44 can exert an amplifying role in bone biology.

With respect to CD44v3, data are broadly missing in the literature, although there is some evidence that in RA synovial fibroblasts CD44v3 expression is crucial for invasive behaviour.

Secondly, the expression of the splice variant CD44v6 on monocytes was positively correlated with disease activity and acute phase reactant levels in RA. This finding is reminiscent of a recent publication by Crispin and coworkers, who found an increased expression of CD44v3 and CD44v6 on T cells in SLE which correlated with disease activity (28). Whether CD44v6 expression on distinct PBMC subtypes would be a useful biomarker in inflammatory autoimmune diseases needs to be further investigated.

Moreover, the synovial lining as well as endothelial cells were found to overexpress CD44v6. The expression within the synovial RA lining, however, was not homogeneous and seemed to depend on the intensity and grade of synovial inflammation. These observations suggest an upregulation in active disease both on circulating PBMC as well as at sites of inflammation. Furthermore, our finding is corroborated by data of Wibulswas *et al*. who described that CD44v6 is expressed on fibroblast like synoviocytes isolated from RA patients that exhibited highly invasive capacities (29).

The correlation of CD44v6 on monocytes with glucocorticoid likewise suggests a correlation with disease activity, since glucocorticoids are usually applied in patients with active disease. Indeed, recently similar findings have been reported on T cells of SLE patients (28).

There are several studies that quantified sCD44v6 in patients suffering from malignancies, such as breast cancer (30) head and neck cancer (31) and colon cancer (32). Since the levels of sCD44v6 were associated with larger tumour size and lymph node metastases it was even postulated that sCD44v6 levels may allow distinguishing between benign and malignant effusions and (33) and that CD44v6 could confer prognostic information in vulvar carcinoma (34).

Taking all these data into account, one could speculate that CD44v6 might pivotally be involved in the active, invasive process of RA. However, further mechanistic studies are necessary to corroborate this notion.

The third noteworthy observation was the strong expression of CD44v7-8 in the rheumatoid synovial membrane, especially in endothelial cells of RA when compared with OA patients. Synovial fibroblast-like cells from RA patients have been shown to express CD44v7-8 *in vitro* and it was suggested that this contributes to their transformed phenotype (29, 35). In line, our double staining experiments revealed that CD44v7-8 was highly expressed by macrophages and although to a lesser extent, also by fibroblast-like cells of the synovial lining.

Interestingly, both these splice variants, CD44v6 and CD44v7-8, are also highly upregulated in atherosclerotic lesions and blockade of CD44v6 was shown to decrease endothelial cell proliferation (36). Furthermore, microvessels formed within aneurysms stain highly positively for CD44v6, an observation that is well in line with our immunohistochemical analyses in the RA synovium. Both splice variants have also been found expressed in inflammatory bowel disease, and the level of expression may be associated with disease activity as determined by endoscopy and pathological features (37).

Finally, the finding of an increased expression of the splice variant CD44v4 has been described in tumours, where this splice variant serves as a ligand mediating breast cancer cell transendothelial migration (38) but has rarely been investigated in RA before (39).

Excessive proliferation of synovial fibroblasts is a hallmark of the disease (40); given our findings of an increased expression of CD44v4 in the RA synovial membrane and a reduced proliferation when inhibiting this splice variant, it is tempting to speculate that CD44v4 is involved in synovial fibroblast expansion.

Moreover, blockade of CD44v4 on cultured RA-FLS was associated with a reduction in IL-1β mRNA expression corroborating the potential beneficial effects of targeting this CD44 variant in RA and related inflammatory conditions; interestingly however, the expression of TNF mRNA remained unchanged and therefore such therapy might have additional benficial effects in patients treated with TNF blocking agents.

Taken together, the results of our studies suggest that splice variants of CD44 may be importantly involved in various parts of the disease process. The variants CD44v4, CD44v6 and CD44v7-8 on monocytes and/or synovial tissue exhibited significantly different expression in RA when compared to HC or OA, suggesting their involvement in the inflammatory process, and possibly also the destructive one. Whether targeting these splice variants might be a possible option for future interventions has to be further investigated.

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References

1. SMOLEN JS, STEINER G: Therapeutic strategies for rheumatoid arthritis. *Nat Rev Drug Discov* 2003; 2: 473-88.
2. SMOLEN JS, ALETAHA D, KOELLER M, WEISMAN MH, EMERY P: New therapies for treatment of rheumatoid arthritis. *Lancet* 2007; 370: 1861-74.
3. NAGANO O, SAYA H: Mechanism and biological significance of CD44 cleavage. *Cancer Sci* 2004; 95: 930-5.
4. KITTL EM, HABERHAUER G, RUCKSER R *et al*.: Serum levels of soluble CD44 variant isoforms are elevated in rheumatoid arthritis. *Rheumatol Int* 1997; 16: 181-6.
5. MIKECZ K, BRENNAN FR, KIM JH, GLANT TT: Anti-CD44 treatment abrogates tissue oedema and leukocyte infiltration in murine arthritis. *Nat Med* 1995; 1: 558-63.
6. MIKECZ K, DENNIS K, SHI M, KIM JH: Modulation of hyaluronan receptor (CD44) function *in vivo* in a murine model of rheumatoid arthritis. *Arthritis Rheum* 1999; 42: 659-68.
7. <http://www.genecards.org/cgi-bin/carddisp.pl?gene=CD44&search=CD44&asd=33> hwgoc-bcpgCsCas. In.
8. SKOTHEIM RI, NEES M: Alternative splicing in cancer: noise, functional, or systematic? *Int J Biochem Cell Biol* 2007; 39: 1432-49.
9. HERRERA-GAYOL A, JOTHY S: Adhesion proteins in the biology of breast cancer: contribution of CD44. *Exp Mol Pathol* 1999; 66: 149-56.
10. WIBULSWAS A, CROFT D, PITSILLIDES AA *et al*.: Influence of epitopes CD44v3 and CD44v6 in the invasive behavior of fibroblast-like synoviocytes derived from rheumatoid arthritic joints. *Arthritis Rheum* 2002; 46: 2059-64.
11. TOLBOOM TC, HUIDEKOPER AL, KRAMER IM, PIETERMAN E, TOES RE, HUIZINGA TW: Correlation between expression of CD44 splice variant v8-v9 and invasiveness of fibroblast-like synoviocytes in an *in vitro* system. *Clin Exp Rheumatol* 2004; 22: 158-64.
12. FUCHS S, ROLAUFFS B, ARNDT S, TIBESKU CO, PREHM P: CD44H and the isoforms CD44v5 and CD44v6 in the synovial fluid of the osteoarthritic human knee joint. *Osteoarthritis Cartilage* 2003; 11: 839-44.
13. TIBESKU CO, SZUWART T, OCKEN SA, SKWARA A, FUCHS S: Increase in the expression of the transmembrane surface receptor CD44v6 on chondrocytes in animals with osteoarthritis. *Arthritis Rheum* 2005; 52: 810-7.
14. KIENER HP, WATTS GF, CUI Y *et al*.: Synovial fibroblasts self-direct multicellular lining architecture and synthetic function in three-dimensional organ culture. *Arthritis Rheum* 2010; 62: 742-52.
15. REDLICH K, HAYER S, RICCI R *et al*.: Osteoclasts are essential for TNF-alpha-mediated joint destruction. *J Clin Invest* 2002; 110: 1419-27.
16. GOLDRING SR, GRAVALLESE EM: Pathogenesis of bone lesions in rheumatoid arthritis. *Curr Rheumatol Rep* 2002; 4: 226-31.
17. HAYER S, STEINER G, GORTZ B *et al*.: CD44 is a determinant of inflammatory bone loss. *J Exp Med* 2005; 201: 903-14.
18. NAOR D, NEDVETZKI S: CD44 in rheumatoid arthritis. *Arthritis Res Ther* 2003; 5: 105-15.
19. ALETAHA D, NELL VP, STAMM T *et al*.: Acute phase reactants add little to composite disease activity indices for rheumatoid arthritis: validation of a clinical activity score. *Arthritis Res Ther* 2005; 7: R796-806.
20. ARNETT FC, EDWORTHY SM, BLOCH DA *et al*.: The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988; 31: 315-24.
21. SCHETT G, TOHIDAST-AKRAD M, SMOLEN JS *et al*.: Activation, differential localization, and regulation of the stress-activated protein kinases, extracellular signal-regulated kinase, c-JUN N-terminal kinase, and p38 mitogen-activated protein kinase, in synovial tissue and cells in rheumatoid arthritis. *Arthritis Rheum* 2000; 43: 2501-12.
22. CROTTI TN, SMITH MD, WEEDON H *et al*.: Receptor activator NF-kappaB ligand (RANKL) expression in synovial tissue from patients with rheumatoid arthritis, spondyloarthropathy, osteoarthritis, and from normal patients: semiquantitative and quantitative analysis. *Ann Rheum Dis* 2002; 61: 1047-54.
23. TAK PP, VAN DER LUBBE PA, CAULI A *et al*.: Reduction of synovial inflammation after anti-CD4 monoclonal antibody treatment in early rheumatoid arthritis. *Arthritis Rheum* 1995; 38: 1457-65.
24. BLAHETA RA, POWERSKI M, HUDAK L *et al*.: Tumor-endothelium cross talk blocks recruitment of neutrophils to endothelial cells: a novel mechanism of endothelial cell anergy. *Neoplasia* 2009; 11: 1054-63.
25. HERRLICH P, MORRISON H, SLEEMAN J *et al*.: CD44 acts both as a growth- and invasiveness-promoting molecule and as a tumor-suppressing cofactor. *Ann N Y Acad Sci* 2000; 910: 106-18; discussion 118-120.
26. HAYNES BF, HALE LP, PATTON KL, MARTIN ME, MCCALLUM RM: Measurement of an adhesion molecule as an indicator of inflammatory disease activity. Up-regulation of the receptor for hyaluronate (CD44) in rheumatoid arthritis. *Arthritis Rheum* 1991; 34: 1434-43.
27. DE VRIES TJ, SCHOENMAKER T, BEERTSEN W, VAN DER NEUT R, EVERTS V: Effect of CD44 deficiency on *in vitro* and *in vivo* osteoclast formation. *J Cell Biochem* 2005; 94: 954-66.
28. CRISPIN JC, KEENAN BT, FINNELL MD *et al*.: Expression of CD44 variant isoforms CD44v3 and CD44v6 is increased on T cells from patients with systemic lupus erythematosus and is correlated with disease activity. *Arthritis Rheum* 2010; 62: 1431-7.
29. WIBULSWAS A, CROFT D, BACARESE-HAMILTON I, MCINTYRE P, GENOT E, KRAMER IM: The CD44v7/8 epitope as a target to restrain proliferation of fibroblast-like synoviocytes in rheumatoid arthritis. *Am J Pathol* 2000; 157: 2037-44.
30. MAYER S, ZUR HAUSEN A, WATERMANN DO *et al*.: Increased soluble CD44 concentrations are associated with larger tumor size and lymph node metastasis in breast cancer patients. *J Cancer Res Clin Oncol* 2008; 134: 1229-35.
31. KAWANO T, YANOMA S, NAKAMURA Y *et al*.: Evaluation of soluble adhesion molecules CD44 (CD44st, CD44v5, CD44v6), ICAM-1, and VCAM-1 as tumor markers in head and neck cancer. *Am J Otolaryngol* 2005; 26: 308-13.
32. AMIRGHOFRAN Z, JALALI SA, HOSSEINI SV, VASEI M, SABAYAN B, GHADERI A: Evaluation of CD44 and CD44v6 in colorectal carcinoma patients: soluble forms in relation to tumor tissue expression and metastasis. *J Gastrointest Cancer* 2008; 39: 73-8.
33. AFIFY A, PANG L, HOWELL L: Diagnostic utility of CD44 standard, CD44v6, and CD44v3-10 expression in adenocarcinomas presenting in serous fluids. *Appl Immunohistochem Mol Morphol* 2007; 15: 446-50.
34. HEFLER LA, CONCIN N, MINCHAM D *et al*.: The prognostic value of immunohistochemically detected CD44v3 and CD44v6 expression in patients with surgically staged vulvar carcinoma: a multicenter study. *Cancer* 2002; 94: 125-30.
35. BAUER S, JENDRO MC, WADLE A *et al*.: Fibroblast activation protein is expressed by rheumatoid myofibroblast-like synoviocytes. *Arthritis Res Ther* 2006; 8: R171.
36. KRETTEK A, SUKHOVA GK, SCHONBECK U, LIBBY P: Enhanced expression of CD44 variants in human atheroma and abdominal aortic aneurysm: possible role for a feedback loop in endothelial cells. *Am J Pathol* 2004; 165: 1571-81.
37. CAMACHO FI, MUNOZ C, SANCHEZ-VERDE L, SAEZ AI, ALCANTARA M, RODRIGUEZ R: CD44v6 expression in inflammatory bowel disease is associated with activity detected by endoscopy and pathological features. *Histopathology* 1999; 35: 144-9.
38. ZEN K, LIU DQ, GUO YL *et al*.: CD44v4 is a major E-selectin ligand that mediates breast cancer cell transendothelial migration. *PLoS One* 2008; 3: e1826.
39. CROFT DR, DALL P, DAVIES D, JACKSON DG, MCINTYRE P, KRAMER IM: Complex CD44 splicing combinations in synovial fibroblasts from arthritic joints. *Eur J Immunol* 1997; 27: 1680-4.
40. HUBER LC, DISTLER O, TARNER I, GAY RE, GAY S, PAP T: Synovial fibroblasts: key players in rheumatoid arthritis. *Rheumatology* (Oxford) 2006; 45: 669-75.