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

J. Grisar¹, M. Munk¹², C.W. Steiner¹, L. Amoyo-Minar¹, M. Tohidast-Akrad³, P. Zenz⁴, G. Steiner¹³, J.S. Smolen¹³

¹Division of Rheumatology, Department of Internal Medicine III, and ²Department of Ophthalmology, Medical University of Vienna, Vienna, Austria; ³Ludwig Boltzmann Institute of Rheumatology and Balneology, Cluster for Rheumatology, Balneology and Rehabilitation, Vienna, Austria; ⁴Orthopaedic Centre, Otto Wagner Spital, Vienna, Austria.

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 in the inflammatory process. We therefore studied the expression of various CD44 splice variants 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). Immunohistochemical 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-1β 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 variant which needs to be further investigated.

Key words
rheumatoid arthritis, CD44, splice variants, synovium, monocytes
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 hyaluronic 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 CD44v3, CD44v4, 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 CD44v3-10 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 [³H]-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 μg/ml [³H]-thymidine (Perkin Elmer, Waltham, MA, USA)/well added. After a further incubation period of 72 hours, the cells were harvested, lysed and the [³H]-thymidine incorporation quantified using a 2450 microplate counter micro beta² (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, lysed and the [³H]-thymidine incorporation quantified using a 2450 microplate counter micro beta² (Perkin Elmer).

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.

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.

Table I. MFI of CD44 splice variants on monocytes.

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

*Tendencies of MFI on monocytes between HC and RA were rated as follows:
= when discrepancy was lower or equal than 10%; i for discrepancies between 10 and 20%; i1 for discrepancies between 21 and 50 % and i11 for discrepancies higher than 50%.
Statistically significant p-values are indicated in bold letters.

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 (rs=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<0.01, 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.

- 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 CD44v4 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. 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.** Semiquantitative assessment of expression of several CD44 splice variants in the synovium of OA and RA patients.

<table>
<thead>
<tr>
<th>Variant</th>
<th>Synovial lining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OA</td>
</tr>
<tr>
<td>CD44</td>
<td>1.6±0.4</td>
</tr>
<tr>
<td>CD44v3</td>
<td>2.7±0.6</td>
</tr>
<tr>
<td>CD44v4</td>
<td>0.6±0.3</td>
</tr>
<tr>
<td>CD44v5</td>
<td>0.8±0.2</td>
</tr>
<tr>
<td>CD44v6</td>
<td>1.0±0.7</td>
</tr>
<tr>
<td>CD44v7-8</td>
<td>1.5±0.5</td>
</tr>
</tbody>
</table>

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

1: inhomogenous expression within the synovial lining of one cryosection. n.e.: not expressed.
at a very low degree or not at all (see Table II).

Taken together, most of the CD44 variants investigated were significantly up-regulated in the lining layer and endothelial cells of the synovial membrane of RA patients when compared to specimens 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 4A 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-1\(\beta\) (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-1\(\beta\) 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 TNF/β 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 the observation ofWiebulswas 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 immuno-histochemical 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 beneficial 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.

Acknowledgements
The authors thank Hans-Peter Kiener and Karolina von Dalwigk for providing the RA-FLS and Young Sook Steiner for drawing the blood samples.

References
9. KWOZC wpc-bcpGCaas. In.