Synovial fluid expression of autoantibodies specific for RAGE relates to less erosive course of rheumatoid arthritis

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Objectives. The receptor for advanced glycation end products (RAGE) is expressed by many cells in joints of rheumatoid arthritis (RA) patients and interacts with a variety of pro-inflammatory ligands that are enriched in inflamed joint. The RAGE-ligand interaction leads to a sustained inflammatory response. Also, secreted form of the receptor, called soluble RAGE (sRAGE), the levels of which are decreased in RA patients, modulates inflammatory responses. We sought to determine whether RA patients display increased occurrence of autoantibodies against RAGE and whether such an autoantibody production is related to disease characteristics.

Methods. Matching samples of blood and synovial fluid were collected from 50 patients with RA with acute joint effusion. Blood from 43 healthy individuals and synovial fluid samples from 32 patients with non-inflammatory joint diseases were used for comparison. Anti-RAGE antibody levels were analysed using an ELISA.

Results. RA patients displayed significantly higher blood and synovial fluid levels of anti-RAGE antibodies, both of IgG as well as of IgM class as compared with healthy controls and with patients with non-inflammatory joint diseases. Patients with seropositive RA had significantly less IgG antibodies in their synovial fluid as compared to seronegative patients. Furthermore, the presence of IgG class of anti-RAGE antibodies locally in the joint was found to be related to less aggressive, i.e. non-erosive disease.

Conclusion. These results suggest that RAGE-specific B cell response protect patients with RA from destructive course of the disease.

KEY WORDS: Rheumatoid arthritis, RAGE, Autoantibodies, HMGB1.

Introduction

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease that leads to joint inflammation resulting in the destruction of bone and cartilage and resulting in functional impairment. The pathogenesis of the disease is complex and far from elucidated but it is clear that autoimmune reactions are part of the disease mechanisms. The question, to what extent autoimmunity in RA might be protective, is at present less well investigated.

The receptor for advanced glycation end products (RAGE) is a multi-ligand member of the immunoglobulin superfamily being expressed as a cell surface molecule and interacting with a diverse class of ligands [1, 2]. RAGE is expressed by many of the cells that participate in the development of RA, including macrophages, neutrophils and T cells. RAGE is expressed on macrophages and T cells within synovial tissues of RA patients as well as on synovial fluid macrophages [3–5]. Moreover, synovial fibroblasts that account for ~50% of the cellular constituents of the synovial lining layer constitutively express RAGE [6] and functional RAGE has also been detected in articular chondrocytes [7].

Soluble RAGE (sRAGE), a truncated form of the receptor, is composed of only the extra-cellular ligand-binding domain lacking the cytosolic and transmembrane domains (i.e. the part that transfers a signal into the cell) [1]. This soluble form of the receptor has the same ligand binding specificity and therefore, has been suggested to compete with cell-bound RAGE for ligand binding, therefore, functioning as a ‘decoy’ abrogating cellular activation. We have demonstrated previously, that patients with RA have lower levels of sRAGE in their blood and synovial fluids in comparison with healthy controls and patients with traumatic/degenerative joint disease [8]. Furthermore, the synovial sRAGE levels were higher in RA patients receiving methotrexate treatment as compared with patients without disease-modifying anti-rheumatic drugs [8].

In the case of RA, there is a wide diversity of RAGE ligands present in the inflamed joints [9, 10], as well as in the circulation, that could lead to the binding and consumption of sRAGE during the inflammatory process. Alternatively, the production of autoantibodies against RAGE/sRAGE could result in decreased sRAGE levels and thereby modulate the course of the disease.

Our aim was to investigate the immune response to RAGE/sRAGE in patients with RA and to assess whether there is an association between sRAGE levels, anti-RAGE antibody levels and disease characteristics.

Materials and methods

Patients and controls

Blood and synovial fluid samples were collected from 50 RA patients (mean age 62±14 yrs, mean disease duration 9.6±8.7 yrs) who met the American College of Rheumatology criteria for RA [11]. Synovial fluids from 32 patients (mean age 42±18 yrs) with non-inflammatory joint diseases (NID) were used as controls. Patients in the NID group were diagnosed to have the following diseases: osteoarthritis, five patients; rupture of meniscus, four patients; rupture of anterior cruciate ligament, 21 patients; and contusion of the knee joint, two patients. All NID patients were examined by an orthopaedic surgeon at the time of arthroscopy/arthrocentesis and chronic inflammatory joint diseases were excluded.

Twenty-seven out of 50 RA patients received disease-modifying anti-rheumatic drugs (DMARDs). Methotrexate predominated and was used by 20 patients, either as a monotherapy (15 patients), or in combination with anti-tumour necrosis factor-α targeted therapy (three patients) or sulphasalazin (two patients). One patient received anti-tumour necrosis factor-α agent in combination with azathioprin and cyclosporine A, while six patients received monotherapy with other DMARDs (parenteral or oral gold salt compounds, two patients; cyclosporine A, one patient; sulphasalazin, three patients). The remaining 23 patients received non-steroidal anti-inflammatory drugs alone and were considered...
as having no DMARD treatment at the time of blood sampling. The reason for the relatively high number of RA patients without DMARD treatment was due to a considerable number of subjects being non-erosive and RF-negative. However, there was no significant correlation between these variables, possibly due to subgroup analysis and hence small patient numbers.

Blood samples from 43 healthy adults (mean age 51 ± 11 yrs) were collected to determine the presence of autoantibodies against RAGE in a healthy population.

The clinical investigation was approved by the Ethical Committee of Göteborg University and informed consent was obtained from all the patients.

**Clinical and laboratory assessment**

Clinical examinations were performed by the rheumatologist in all RA patients and disease activity variables were recorded. The serum concentration of C-reactive protein was measured with a standard nephelometric assay; with normal range 0–5 mg/l. White blood cell counts in the blood were assessed using a microcell counter (F300; Sysmex, Norderstedt, Germany). The white blood cell count in the synovial fluid was also assessed in 19 RA patients.

Radiographs of the hands and feet were obtained from all RA patients. Criteria for the erosive disease included the presence of one or more bone erosions, defined as loss of cortical definition of the joint and recorded in proximal interphalangeal joints, metacarpophalangeal joints, carpal joints, wrist joints and metatarsophalangeal joints. Twenty-seven patients out of 50 had erosive disease. The presence of rheumatoid factor of any of the immunoglobulin isotypes was considered positive. Twenty-seven patients had seropositive RA.

**Collections and preparation of patient samples**

Synovial fluid was collected from RA patients who attended the Department of Rheumatology at Sahlgrenska University Hospital in Göteborg with acute knee joint effusion. Blood samples from the same patients were simultaneously obtained from the cubital vein and transferred into the sodium citrate containing tubes. Synovial fluid from NID patients who attended the Department of Orthopaedics at Malmö University Hospital was obtained by arthrocentesis.

The collected blood and synovial fluid samples were centrifuged at x2000 g for 10 min, aliquoted and stored at −70 °C until use.

**Analyses of anti-RAGE antibodies and of sRAGE**

Anti-RAGE antibodies of IgG and IgM type were measured in the blood and synovial fluid samples by an ELISA. Briefly, microtitre plates (Maxisorp F96, Nunc, Denmark) were coated with 100 μl human highly purified recombinant soluble RAGE in a concentration 0.5 μg/ml (lacking Fc-gamma portion, custom service from R&D Systems, Minneapolis, MN, USA) in phosphate buffered saline (PBS) and incubated overnight at 4 °C. After removal of the un-adsorbed antigen, plates were washed and unsaturated binding sites were blocked for 2 h with 0.1% human serum albumin (blocking solution) in PBS at room temperature (RT). The blood and synovial fluid samples were diluted 1:50 and 1:200 using blocking solution and incubated 3 h at RT. The amount of bound antibodies was quantitated with biotinylated goat anti-human IgG (γ-chain specific) and goat anti-human IgM (μ-chain specific) (Sigma, St Louis, MO, USA) in a concentration 0.5 μg/ml for 2 h, followed by 1 h incubation with 0.5 μg/ml streptavidin – horseradish peroxidase conjugate and 45 min H₂O₂ –ABTS substrate. The absorbance was recorded at 405 nm in a spectrophotometer at different time points. The values from 1:50 dilutions were chosen for analysis after background subtraction.

The chosen cut-off level was based on mean absorbance value plus two standard deviations from healthy population regarding blood and from NID patients regarding synovial antibody levels.

For competition assay, blood and synovial fluid samples were pre-incubated 30 min in vitro with or without different concentrations of human recombinant sRAGE and an ELISA assay was performed as described above.

The levels of sRAGE in sera and synovial fluid of patients described above were determined using a specific sandwich ELISA kit (R&D Systems, Minneapolis, MN, USA) and the original data have been previously published [8].

**Statistical analysis**

Non-parametric methods were used for statistical comparisons since data showed a non-normal distribution. Statistical differences with respect to anti RAGE antibody levels between independent groups were calculated using the Kruskall–Wallis test followed by the Mann–Whitney U-test. The Wilcoxon signed rank test for paired samples was used to compare differences between variables in matched pairs. Correlations between different variables in patients were assessed with the Spearman rank correlation test. Fisher’s exact probability test was used to assess differences between groups with regard to disease characteristics. G-squared test was used to calculate differences in the frequencies of positive samples between RA patients and control groups. All values are expressed showing the median and the mean ± standard error of the mean. Patients’ age and disease duration are reported as the mean ± s.d. P < 0.05 is considered significant.

**Results**

**The presence of anti-RAGE antibodies in blood and synovial fluid**

We investigated antibodies to sRAGE in the synovial fluid and in the blood stream of 50 patients who had RA. Blood samples from 43 healthy controls and synovial fluid from 32 patients with NID were assessed as controls. Antibodies to RAGE we detected more frequently in RA patients in comparison with healthy controls and with NID patients (Table 1). The frequency of anti-RAGE IgM and anti-RAGE IgG antibodies in RA patients was 2–5-fold higher in their blood in comparison with healthy control subjects. RA patients also displayed significantly higher antibody titres than healthy subjects (Fig. 1A). This applied both for serum IgM (0.44 ± 0.04 vs 0.28 ± 0.02, P = 0.0002) as well as IgG isotypes of antibodies (0.15 ± 0.03 vs 0.08 ± 0.02, P = 0.024). Synovial fluid anti-RAGE antibodies of IgM and IgG isotypes were found more often in RA patients; whereas in NID patients the frequency was lower (Table 1). The corresponding antibody levels in the synovial fluid of RA patients were higher than that of NID patients (IgM: 0.33 ± 0.03 vs 0.22 ± 0.03, P = 0.0048; IgG: 0.09 ± 0.03 vs 0.03 ± 0.01, NS) (Fig. 1B). There was a significant positive correlation between IgG (r = 0.46, P = 0.002) and IgM (r = 0.53, P = 0.0002) anti-RAGE antibody levels in the matching samples of blood and synovial fluid in RA patients.

| Table 1. Frequency of anti-RAGE antibodies (percentage) of IgM and IgG class in sera and in synovial fluids of RA patients in comparison with control groups |
|-----------------|----------------|-----------------|
|                  | Rheumatoid arthritis patients | Healthy controls | NID patients |
| Sera             | IgM              | 26%**           | 5%             |
|                  | IgG              | 18%             | 9%             |
| Synovial fluid   | IgM              | 14%             | 9%             |
|                  | IgG              | 20%*            | 3%             |

**P < 0.004 as compared with healthy controls. *P < 0.02 as compared with NID patients.”**
Correlation between anti-RAGE antibody levels and clinical features of RA

We investigated further the association between antibody responses to RAGE with main characteristics of the disease. Stratification of patient data by radiological imaging showed that 27 patients fulfilled the criteria for erosive disease and 23 patients had no erosions on recent radiographs. There was no difference in patients' age between these two groups (61.5 ± 13.7 yrs vs 62.6 ± 16.1 yrs). No statistically significant differences in synovial fluid and blood antibody levels were found between these two groups regarding IgM anti-RAGE antibody. However, patients with erosive RA had 4-fold lower IgG anti-RAGE levels locally in their synovial fluid than patients with non-erosive disease (0.04 ± 0.02 vs 0.16 ± 0.05, respectively, \( P < 0.05 \)) (Fig. 2A). Since occurrence of rheumatoid factor might interfere with the ELISA for antibody determination, we assessed the relationship between the presence of RF and antibody levels. No statistically significant differences regarding IgM antibody levels in patients having either seropositive or seronegative RA were found. Interestingly, seropositive RA patients had significantly less antibodies of IgG subclass in their synovial fluid than patients having seronegative disease (0.04 ± 0.02 vs 0.16 ± 0.05, \( P < 0.02 \)) (Fig. 2B). This finding precludes any major RF interference in this setting. No association between anti-RAGE antibody levels and disease duration or acute-phase reactant C-reactive protein was found.

The relationship between soluble RAGE levels and anti-RAGE antibodies

We further investigated whether decreased levels of sRAGE detected previously in RA patients can be a result of increased autoantibody production. In an overall RA patient population, no correlation was seen between sRAGE levels and anti-RAGE antibody levels, neither in synovial fluid nor in the blood. The similar results were obtained in healthy population as well as in patients with non-inflammatory joint disease. In RA patients stratified by the occurrence of RF, the circulating and synovial IgM anti-RAGE antibodies correlated significantly with sRAGE levels in corresponding compartment in seronegative RA patients (\( r_s = 0.44, \ P < 0.05 \) and \( r_s = 0.54, \ P < 0.02 \), respectively). The same relationship was true for IgM anti-RAGE antibody levels in patients that had non-erosive disease (blood \( r_s = 0.51, \ P < 0.02 \) vs synovial \( r_s = 0.59, \ P = 0.0055 \)). Interestingly, a significant negative association was found between circulating IgM autoantibody levels and erosive course of RA (\( r_s = 0.51, \ P < 0.02 \)).

Fig. 1. Synovial fluid and circulating antibodies of both IgM (Fig. 1A) and IgG (Fig. 1B) class specific for sRAGE/RAGE in patients with RA and in patients with degenerative/trophic joint diseases (NID). In addition, blood levels of anti-RAGE antibodies were assessed in healthy controls. Box plots show the 25th and 75th percentiles. Horizontal lines in bold within boxes indicate medians, lighter lines represent means. Vertical bars indicate the 5th and 95th percentiles.
The effect of methotrexate treatment on anti-RAGE antibody levels in RA patients

At the time of sampling, all patients were receiving anti-inflammatory treatment. We decided to investigate whether DMARD treatment had an effect for antibody response of RA patients. Methotrexate is the most used DMARD in RA treatment and predominated in our RA patient population. In order to obtain a more homogeneous population, a subgroup of patients \( n = 15 \) receiving methotrexate treatment was analyzed and compared with patients without any DMARD treatment \( n = 23 \). The patients’ data are presented in Table 2.

Both groups were comparable with respect to age, sex and the presence of rheumatoid factor. However, as expected, patients receiving DMARD treatment had significantly longer disease duration \( 12.3 \pm 9.2 \) yrs vs \( 8.0 \pm 8.8 \) yrs, \( P < 0.05 \) and more often erosive disease \( 11/15 (73\%) \) vs \( 7/23 (30\%) \), \( P < 0.02 \).

We did not find any significant differences between anti-RAGE antibody levels in methotrexate treated patients in comparison with patients receiving non-steroidal anti-inflammatory drugs. However, there was a trend towards higher IgG anti-RAGE expression in methotrexate treated patients in both, in blood as well as in synovial fluid (Fig. 3).

The anti-RAGE antibodies present in RA patients’ blood and synovial fluid bind sRAGE in vitro

To further ascertain the specificity of detected anti-RAGE IgG and IgM in patients’ blood and synovial fluid, we performed in vitro competition assay. Blood and synovial samples were pre-incubated with recombinant sRAGE (at concentrations ranging from 0.3 \( \mu \)g/ml to 15 \( \mu \)g/ml) before antibody detection by ELISA (Fig. 4). Our results show dose dependent decrease in absorbance values regarding both IgG and IgM class of anti-RAGE antibodies indicating that in vitro added sRAGE inhibited antibody detection by ELISA.

The presence of sRAGE/anti-RAGE antibody complexes in RA patients and control individuals

We next examined whether subjects with lower sRAGE levels display more sRAGE/anti-RAGE antibody complexes in their circulation and locally in synovial fluids than subjects with higher detectable sRAGE levels. The representative samples with highest as well as lowest sRAGE levels from RA, NID and healthy group were analyzed employing ELISA. We did not find any significant differences between groups regarding sRAGE/anti-RAGE antibody complexes (data not shown).
Discussion

Rheumatoid arthritis (RA) is a chronic autoimmune disease being accompanied by the production of a number of autoantibodies [12]. Most of them are not specific for RA occurring also in other diseases, while others display a higher degree of specificity [12]. The pathogenic role for cell-bound RAGE has been implicated in various inflammatory diseases, including RA [9]. The expression of this receptor depends largely on the presence of its ligands and ligand-receptor interaction generates positive feedback loop further amplifying the inflammatory responses and increasing receptor expression [10]. Soluble RAGE, a truncated form of the receptor, retaining its extracellular ligand-binding domain with binding affinity equal to that of cellular RAGE, has been, therefore, suggested to counteract the inflammatory response acting as a decoy.

In our previous report, lower circulating sRAGE levels were detected in patients that have RA in comparison with healthy population or patients with traumatic/degenerative joint diseases [8]. In RA, there is a wide diversity of RAGE ligands present in the inflamed joints [9, 10, 13], as well as in the circulation [14], that could lead to the binding and consumption of sRAGE during the inflammatory process. As reported here, RA patients have also increased levels of autoantibodies directed against endogenous RAGE that might result in complex formation in vivo followed by their elimination and resulting in decreased sRAGE levels. In our in vitro competition assay, we demonstrate that anti-RAGE antibodies found in RA sera and synovial fluid bind sRAGE since antibody detection dose-dependently decreased upon exposure to sRAGE.

The potential of RAGE-specific antibodies to modulate the disease activity is presently not understood. The data showing link between low synovial IgG anti-RAGE levels and the presence of erosivity suggests that these autoantibodies may have protective properties. The presence of anti-RAGE IgM antibodies correlates positively with synovial and circulating sRAGE levels in less severe manifestations of RA, namely in non-erosive and seronegative disease. In addition, significant negative association between circulating IgM anti-RAGE antibody and sRAGE levels in erosive course of RA was found, suggesting that anti-RAGE antibodies might participate in the down-regulation of local inflammatory responses. These assumptions are supported by our recent findings, where we demonstrate that sRAGE acts as a pro-inflammatory and chemotactic molecule [15]. Considering these findings and our present data we believe that the higher level of antibodies against sRAGE/RAGE in more benign RA may reflect a protective autoimmune response. The higher antibody reactivity to RAGE found in RA patients treated with methotrexate, one of the most used and potent anti-rheumatic drugs, is consistent with these observations.

In conclusion, antibodies directed against RAGE/sRAGE might be implicated in the pathogenesis of RA, at least in terms of balancing the inflammatory environment.

Further studies are needed to provide insights into the clinical significance of anti-RAGE antibodies whether they are beneficial or detrimental and to determine whether these antibodies in RA have any predictive role for the disease progression.

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