

## RAGE activation induces invasiveness of RA fibroblast-like synoviocytes *in vitro*

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## ABSTRACT

*Ligands for the receptor for advanced glycation endproducts (RAGE) are increased in RA synovial fluid (SF), serum and synovium. Since RAGE is present on fibroblast-like synoviocytes (FLS), the present study investigates whether the RAGE ligands HMGB-1 and AGEs are able to stimulate the characteristic, pathological invasive behaviour of these cells.*

*FLS were obtained during joint replacement surgery. FLS were seeded in serum free medium with HMGB-1 or glycated albumin (BSA-AGE) on transwell filters coated with Matrigel. The lower compartment contained medium with serum as a chemoattractant. After three days, the percentage of invading cells was determined and compared to the control invasion.*

*Stimulation with HMGB-1 increased invasiveness to 125% compared to the control ( $p = 0.001$ ). Addition of anti-RAGE antibody reduced this back to baseline (98%,  $p = 0.002$ ). Stimulation with BSA-AGE, another RAGE ligand, increased invasiveness to 150% compared to the control ( $p = 0.003$ ). Addition of anti RAGE was again able to reduce the increased invasiveness back to baseline (95%,  $p = 0.008$ ).*

*HMGB-1 and BSA-AGE stimulated the invasiveness of RA-FLS by activation of RAGE. As such, RAGE may be an interesting target for therapy directed at the inhibition of synoviocyte activation.*

## Introduction

In rheumatoid arthritis (RA), the levels of several ligands of the receptor for advanced glycation endproducts (RAGE) are increased in synovial tissue, synovial fluid (SF) and serum. The first identified group of RAGE ligands, AGEs, are elevated in the synovial tissue of RA patients, and also in the serum and synovial fluid of RA patients compared to OA patients and controls (1, 2). AGEs are formed by non-enzymatic glycation of exposed amino groups such as on lysine of arginine residues on proteins. AGE formation is accelerated in conditions of hyperglycaemia and/or oxidative stress (3). Levels of a second RAGE ligand, HMGB-1 are increased in synovial fluid of RA

patients compared to patients with osteoarthritis (OA) (4, 5). *In vivo*, intra-articular injection of HMGB-1 results in arthritis in about 80% of the animals of different mouse strains (6). Levels of S100A12, a third RAGE ligand, are increased in synovial tissue and serum of RA patients compared to control. S100A12 is found in higher amounts in patients with active arthritis than in patients in clinical remission (7).

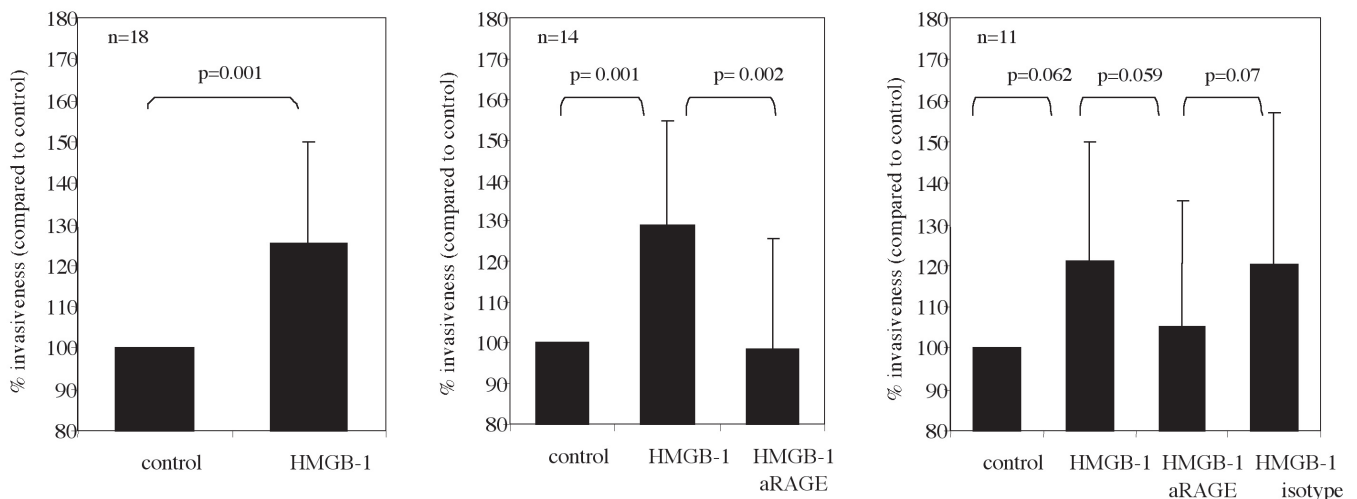
In the rheumatoid joint, RAGE is present on a variety of cell types such as fibroblast-like synoviocytes (FLS), macrophages, T-cells, and certain B-cells (1, 8). One of the RAGE ligands, HMGB-1 induces cytokine production by macrophages and can itself be released by macrophages after stimulation with cytokines such as TNF- $\alpha$  (5). However, the FLS in the inflamed arthritic synovium (pannus tissue) show characteristics similar to cells in pathologies such as tumour growth and diabetes, in which RAGE triggering leads to cell activation, proliferation and migration. Therefore, our current study explores if RAGE triggering by HMGB-1 or AGEs can lead to enhanced invasion of RA-FLS directly.

## Material and methods

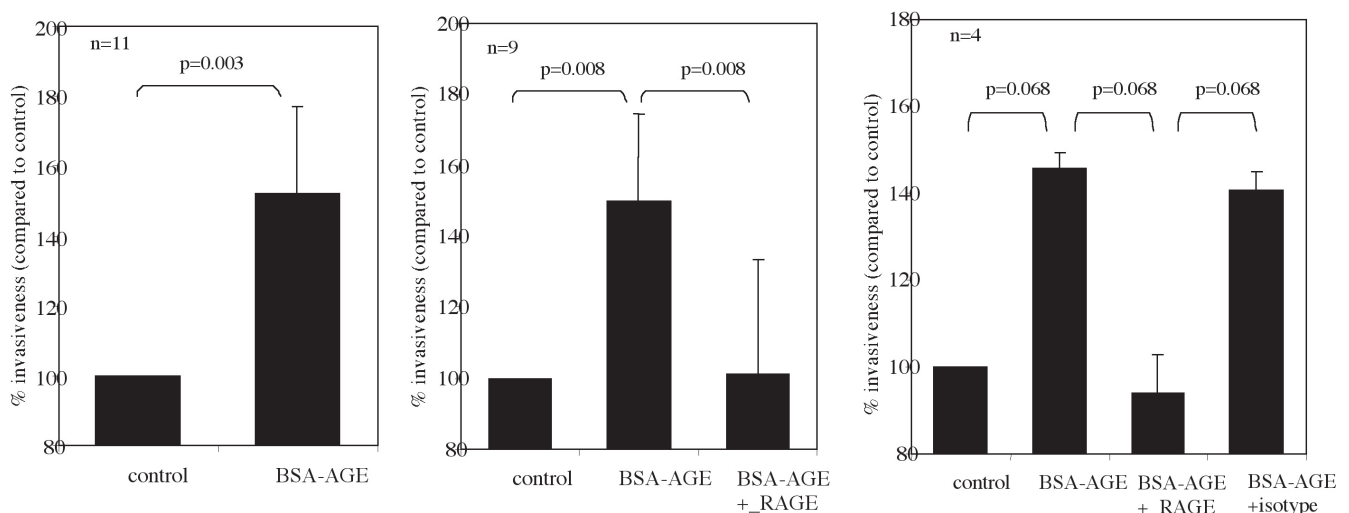
### Synovial fibroblasts

Synovial fibroblasts were obtained during joint replacement surgery or synovectomy after informed consent. Tissue was harvested by an orthopedic surgeon and collected in sterile phosphate buffered saline (PBS). Fat and connective tissue were removed and tissue was digested with collagenase (CLS2, Worthington Biochemical Corporation) for at least two hours at 37°C. Cells were then separated from the undigested tissue using a 200 $\mu$ m filter (B-Braun Medical, Oss, the Netherlands) and cultured in 162 cm<sup>2</sup> culture flasks (Costar, Cambridge, NY, USA) with Iscove's Modified Dulbecco's medium (IMDM; Biowittaker) supplemented with glutamax (Biowittaker, Versiers, Belgium) and 100U/ml penicillin and streptomycin (Biowittaker, Versiers, Belgium) and 10% foetal calf serum (FCS, GibcoBRL) at a humid atmosphere of 5% CO<sub>2</sub> in air at 37°C. On reaching confluence, cells were de-

Competing interests: none declared.



**Fig. 1.** Invasiveness of fibroblast-like synoviocytes (FLS) from rheumatoid arthritis (RA) patients, as determined by their ability to invade a Matrigel matrix. FLS were significantly more invasive when HMGB-1 was added ( $p = 0.001$ ) (A). When anti RAGE antibody was added, the stimulating effect of HMGB-1 was significantly inhibited ( $p = 0.002$  vs HMGB-1) (B). An isotype control antibody had no effect on HMGB-1 stimulation ( $p = 0.258$ , compared to HMGB-1) (C).



**Fig. 2.** Invasiveness of fibroblast-like synoviocytes (FLS) from RA patients, as determined by their ability to invade a Matrigel matrix. Glycated bovine serum albumin (BSA-AGE) significantly increased the invasiveness of the FLS ( $p = 0.003$ ) (A). The stimulating effect on invasiveness of BSA-AGE was inhibited by anti RAGE antibody ( $p = 0.008$ ) (B). An isotype control antibody had no effect on BSA-AGE stimulation ( $p = 0.273$ , compared to BSA-AGE) (C).

tached with 0.25% trypsin and split in a 1:3 ratio. For all experiments, third passage synovial fibroblasts were used.

#### Invasion experiments

Invasion assays with FLS were performed using transwells (with 6.5 mm diameter polycarbonate filters with 8.0  $\mu$ m pore size) coated with 37.5  $\mu$ g/well matrigel (Becton Dickinson, USA) to achieve a barrier for the cells to invade through. 4 mg/ml bovine serum albumin (BSA, Sigma-Aldrich) was incubated in IMDM with 10 mM ribose (Sigma-Aldrich) at 37°C for 3

days to induce glycation (resulting in BSA-AGE) and subsequently dialysed to remove excess ribose. Cells were seeded in the inner compartment of the transwells in serum-free medium with or without high mobility group box 1 (HMGB-1; 500 ng/ml) or BSA-AGE and in the presence or absence of 2.5  $\mu$ g/well anti-RAGE antibody (Santa Cruz, CA, USA). The outer compartment was filled with medium containing 10% FCS and 10% human serum to provide a chemoattractant. After three days, the cells were fixed with 4% formaldehyde and stained with a crystal violet solu-

tion (1% in PBS). Non-invading cells were removed by cleaning the inner compartment with a cotton bud and the number of cells that had invaded the matrigel and grown through the transwell membrane was counted using a light microscope. Experiments were all performed in triplicate.

#### Statistical analysis

Wilcoxon tests were performed in order to test differences between conditions.  $P$  values  $< 0.05$  were considered to indicate statistically significant differences.

## Results

To investigate whether HMGB-1 had an effect on the invasive behaviour of RA-FLS, 500 ng/ml HMGB-1 was added in the inner compartment of the transwells. Stimulation with HMGB-1 increased invasiveness to 125% compared to control ( $n = 18$ ,  $p = 0.001$ ) (Fig. 1). This was reduced back to baseline when an anti-RAGE antibody was added (98%,  $n = 14$ ,  $p = 0.002$ ).

A similar effect was seen after stimulation with glycated albumin (BSA-AGE). Stimulation of FLS with BSA-AGE increased invasiveness to 150% ( $n = 11$ ,  $p = 0.003$ ). This increased invasiveness could again be inhibited by an antibody against RAGE ( $n = 9$ ,  $p = 0.008$ ). Addition of an isotype control had no effect on BSA-AGE induced invasiveness (140%,  $n = 4$ ,  $p = 0.273$ ).

Together these data indicate that FLS in RA can be triggered by activation of RAGE by HMGB-1 and AGEs. This points to a broader mode of action of HMGB-1 in the pathogenesis of RA than the previously recognized proinflammatory effects on inflammatory cells.

## Discussion

In this study we show that RAGE triggering stimulates invasiveness of RA-FLS. A role for RAGE in RA pathology is suggested by the increased levels of its ligands in RA pathology. HMGB-1, S100/calgranulins and AGEs all accumulate in RA synovial tissue, fluid or in serum. Levels of these RAGE ligands correlate with disease severity (2, 4, 6, 9, 10). Previously, RAGE has mostly been implicated in the inflammatory disease processes due to its activation of macrophages and phagocytes (5, 6, 11, 12). Vice versa, inflammatory processes and cells of the immune system potentially play a stimulating role in the formation of RAGE ligands. S100A12, a member of the S100 family, has increased levels in serum and synovial fluid of patients with active RA and is expressed in the sub-lining layer of the synovium, associated with granulocytes. Two other S100 family members, S100A8 (MRP8) and S100A9 (MRP14)

are overexpressed in the lining layer of inflamed synovial tissue in RA (13). A second RAGE ligand, HMGB-1 has increased levels in RA synovial fluid and upon injection can induce arthritis in several mouse strains (4, 6). Activation of macrophages can lead to increased release of HMGB-1, which in turn can activate macrophages itself (5). Finally, production of a third group of RAGE ligands, the AGEs is accelerated by oxidative stress, a process occurring during inflammation (3). Pentosidine, one of the AGEs, has elevated levels in articular cartilage, serum, synovial fluid and urine of RA patients associated with disease severity (9, 14).

Besides our study, other studies have shown that RAGE can have effects other than the effects on the inflammatory processes of RA. Studies by Hou *et al.* and Owen *et al.* (8,15) show that  $\beta$ 2-microglobulin modified by AGEs induced the release of monocytes chemoattractant protein (MCP-1) by FLS and production of collagen type I by skin fibroblasts respectively, which, similar to our data, indicate a stimulatory effect of RAGE activation on FLS. Together with our data, this suggests that RAGE not only affects inflammation in the arthritic joint, but might influence the altered, aggressive behaviour of FLS.

In conclusion, HMGB-1 and BSA-AGE stimulated the invasiveness of RA-FLS. HMGB-1 and BSA-AGE activated invasiveness could be inhibited completely by an antibody against RAGE, indicating that the HMGB-1 and BSA-AGE effect was RAGE mediated. In combination with the increased HMGB-1 and AGE levels in the SF of RA patients, RAGE may be an interesting target for therapy directed at the inhibition of cartilage and bone invasion by pannus tissue.

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