Membrane Glucocorticoid Receptor Expression on Peripheral Blood Mononuclear Cells in Patients with Ankylosing Spondylitis

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ABSTRACT. ***Objective.*** To investigate the expression of membrane glucocorticoid receptors (mGCR) on peripheral blood mononuclear cells (PBMC) in patients with ankylosing spondylitis (AS).

Methods. We used high sensitivity immunofluorescence with magnetofluorescent liposomes for the detection of mGCR on PBMC from patients with AS (n = 26) and healthy controls (n = 11).

Results. The frequency of mGCR+ monocytes and B lymphocytes was significantly higher in patients with AS than in controls (monocytes 12.5 ± 9.9% vs 4.8 ± 1.4%, B lymphocytes 8.7 ± 6.3% vs 4.4 ± 3.6%). We did not find mGCR on T lymphocytes. The frequency of mGCR+ cells did not correlate with variables of AS disease activity [C-reactive protein, erythrocyte sedimentation rate, Bath Ankylosing Spondylitis Disease Activity Index (BASDAI), BASDAI 6, or numerical rating scales].

Conclusion. mGCR are upregulated in monocytes and B lymphocytes of patients with AS. This upregulation does not correlate with the humoral or overall disease activity. mGCR are not present on T lymphocytes. Our findings may be related to the limited benefit of low-dose and the efficacy of high-dose (intravenous pulse or intraarticular) glucocorticoid treatment in AS. Drugs binding selectively to mGCR may be a new therapeutic option for AS. (First Release Sept 1 2006; J Rheumatol 2006;33:2249–53)

Key Indexing Terms:
GLUCOCORTICOID NONGENOMIC MEMBRANE RECEPTORS
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Glucocorticoids mostly mediate their therapeutic actions by genomic effects via cytosolic glucocorticoid receptors (cGCR)[1]. However, some effects are too rapid to be mediated by changes at the genomic level[2-4]. Detailed mechanisms of these nongenomic actions are still unclear. Currently, cGCR-mediated nongenomic effects, nonspecific membrane interactions at very high concentrations, and specific interactions with membrane-bound glucocorticoid receptors (mGCR) are considered potentially responsible[1].

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mGCR have been described in amphibian brain and human leukemia/lymphoma cells[5,6]. Using a novel high-sensitivity immunofluorescence technique we recently identified mGCR on human peripheral blood mononuclear cells (PBMC)[7]. This method uses magnetofluorescent liposomes, which can increase fluorescence signal intensity up to 1000-fold compared to conventional methods[8]. We found that mGCR were present on the surface of CD14+ monocytes and CD19+ B cells from healthy donors, and that they were upregulated on monocytes after *in vitro* stimulation and in patients with rheumatoid arthritis (RA)[7]. Since mGCR may be involved in the therapeutic effect discussed above and patients with RA are known to respond well to glucocorticoid treatment[9], we wanted to investigate mGCR expression in a defined patient population that does not usually respond well to systemic glucocorticoid therapy, such as patients with ankylosing spondylitis (AS).

AS is a common rheumatic disease, inflammatory back pain being a main symptom. Every part of the spine can be affected. In some cases peripheral joints are involved. Ninety percent of the patient population is HLA-B27-positive. Physical therapy and nonsteroidal antiinflammatory drugs (NSAID) have been the gold standard for decades. Currently, anti-tumor necrosis factor-α (TNF-α) therapy is a powerful therapeutic option[10]. Surprisingly, only very limited data on glucocorticoid treatment for AS are available[10]. However,

clinical observations have led recently to the conclusion that systemic (low and intermediate dose) glucocorticoid therapy provides only limited if any benefit[11]. However, at higher systemic or local concentrations known to trigger nongenomic effects[1], glucocorticoids are successfully used for intravenous pulse therapy and intraarticular injection into sacroiliac and peripheral joints to treat patients with active AS[12,13].

MATERIALS AND METHODS

Healthy controls. The control group comprised 11 healthy subjects (8 men, 3 women, aged 23 to 44 yrs, mean age 30.0 ± 7.4 yrs). None of the controls had been vaccinated or had any signs of infection for at least 4 weeks prior to study inclusion since both have a possible effect on mGCR expression[7].

Patients with AS. The patient group consisted of 21 patients with AS of variable disease activity (15 men, 6 women, aged 22 to 62 yrs, mean age 37.4 ± 9.2 yrs). There were 26 samples of peripheral blood and 2 of synovial fluid (SF) drawn. All patients met modified New York criteria for AS[14]. Disease duration was 10.7 ± 7.0 years (minimum 0.5, maximum 26.0, median 11.0 yrs). Signs of infection had to have been absent for the last 4 weeks (see above). Three patients had affected peripheral joints. Seventeen of the 21 patients were treated with NSAID/coxibs, 11 with infliximab (anti-TNF therapy), 4 with methotrexate (MTX) or sulfasalazine. No patient was being treated with systemic glucocorticoid therapy. Disease activity was assessed by C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI), the BASDAI for 6 weeks (BASDAI 6), and numerical rating scales for pain (NRS and NRS 6 for 6 weeks).

BASDAI is a self-administrated instrument of six 10-cm horizontal visual analog scales to measure severity of fatigue, spinal and peripheral joint pain, localized tenderness, and morning stiffness (both qualitative and quantitative) over the last week[15]. The final BASDAI score has a range of 0 to 10. For subgroup analysis subjects were divided into 2 disease activity groups (group 1: BASDAI < 4, group 2: BASDAI ≥ 4).

Preparation and incubation of PBMC. After informed consent had been obtained from patients and controls, a sample of maximum 20 ml venous blood was collected in heparinized tubes. In one patient with peripheral manifestation the inflamed and swollen knee joint had to be punctured therapeutically at 2 different timepoints. We also examined SF in parallel to the peripheral blood. PBMC and cells from the SF were isolated by density gradient centrifugation using the Ficoll-Paque™ Plus technique (Amersham Bioscience AB, Uppsala, Sweden).

Cell culture of CCRF-CEM cells. CCRF-CEM lymphoma cells (after about 40 h of reculturing) were used as positive controls as described, since they have been reported to constantly express mGCR[6,7].

Flow cytometric analysis of mGCR. mGCR were detected by high-sensitivity immunofluorescence following the procedures described by Bartholome, *et al*[7]. Antibody stainings were performed in phosphate buffered saline (PBS) containing 0.5% (w/v) bovine serum albumin (BSA; PAA Laboratories, Coelbe, Germany) and 0.02% (v/v) sodium azide (NaN_3; Sigma-Aldrich) at 4°C. To identify different cell types we used phycoerythrin (PE)-conjugated anti-human CD14 (BD PharMingen, San Diego, CA, USA), allophycocyanin (APC)-conjugated anti-human CD19 (BD PharMingen) and peridin-chlorophyll (PerCP)-conjugated anti-human CD3 (BD Biosciences, San Jose, CA, USA). Aliquots of the cell suspension ($1–3 \times 10^6$ cells in 100 μl) were incubated at 4°C for 10 min in presence or absence (control) of digoxigenin-conjugated IgG1 human antibody anti-GCR 5E4, which is directed against the conserved regulatory sequence of human GCR (aa150-176)[16]. Nonspecific binding was minimized by the addition of polyclonal human IgG (Octagam, Octapharma, Langenfeld, Germany). A second control was pretreated with a 50- to 100-fold excess of unlabeled GCR antibody before staining with anti-GCR-Dig conjugate. Cells were washed and incubated on ice for 30 min with anti-Dig magnetofluorescent liposomes[8]. At least 5000 cells per sample were counted and analyzed for surface fluorescence by dot-plot graphs using a FACS-Calibur flow cytometer with Cell-Quest software (Becton Dickinson). The gate for viable cells was determined by exclusion of propidium iodide-positive cells (1 μg/ml added directly before data acquisition). The frequency of mGCR+ cells was calculated from the positive sample by subtracting background signals obtained by blocking.

Statistical methods. Data are expressed as mean ± standard deviation (SD) unless otherwise stated. For statistical comparison between groups we used the Mann-Whitney test. Spearman's test was used for correlation analysis. Probability values of $p < 0.05$ were considered statistically significant.

RESULTS

mGCR expression in PBMC of patients and controls. We found patients with AS had significantly higher frequencies of mGCR+ cells among CD14+ monocytes and CD19+ B lymphocytes compared to controls [monocytes: minimum (min) – maximum (max): 0.3–38.5%; median 9.4%; mean ± SD 12.5 ± 9.9% (n = 26) vs min-max: 3.0–7.2%; median 3.3%, mean ± SD 4.8 ± 1.4% (n = 11), p = 0.017; B lymphocytes: min–max 0.0–23.6%, median 8.1% , mean ± SD 8.7 ± 6.3% vs min–max 0.2–11.4%, median 4.8%, mean ± SD 4.4 ± 3.6% (p = 0.003)]. CD3+ T lymphocytes did not show significant detectable expression of mGCR in either group at any time (data not shown).

Effect of AS disease activity. Disease activity variables (CRP, ESR, BASDAI, BASDAI 6, NRS, and NRS 6) showed a consistent correlation with each other (Table 1). These variables therefore appeared to be reliable indicators for disease activity in our patients. However, we could not find any correlation between any disease activity variable and frequency of mGCR+ monocytes or B cells, respectively.

In addition, the frequency of mGCR+ monocytes did correlate with the frequency of mGCR+ B cells (r = 0.492, p = 0.011; Figure 1).

A BASDAI value of 4 has been proposed to differentiate between low and high disease activity, e.g., for initiation of anti-TNF therapy[17]. Therefore, we divided our patients into 2 disease activity groups (group 1: BASDAI < 4 and group 2: BASDAI ≥ 4). We found every disease activity variable to be significantly higher in group 2 compared to group 1, confirming that a BASDAI of 4 is a reasonable discriminator of disease activity. However, we did not find any differences between the subgroups and there was no correlation with disease activity with regard to frequency of mGCR+ cells.

Subgroups of patients with affected peripheral joints and anti-TNF-therapy. Some authors have indicated that patients with AS with affected peripheral joints form a subgroup in which an increase of systemic inflammatory or overall disease activity can frequently be found[18,19]. We evaluated our 3 patients with peripheral manifestations, but disease activity variables and frequency of mGCR+ monocytes and B cells were similar or even lower compared to the overall group. There was, however, a positive correlation between CRP and frequency of mGCR+ monocytes (r = 1.0, p < 0.001) and a negative cor-

Table 1. Correlation between clinical and laboratory variables of AS disease activity.

	CRP	ESR	BASDAI	BASDAI 6	NRS	NRS 6
Probability values						
CRP	—	< 0.001*	0.029*	0.045*	0.174	0.249
ESR	< 0.001*	—	0.021*	0.019*	0.026*	0.162
BASDAI	0.029*	0.021*	—	< 0.001*	< 0.001*	0.007*
BASDAI 6	0.045*	0.019*	< 0.001*	—	< 0.001*	< 0.001*
NRS	0.174	0.026*	< 0.001*	< 0.001*	—	0.008*
NRS 6	0.249	0.162	0.007*	< 0.001*	0.008*	—
Correlation values						
CRP	—	0.752	0.489	0.492	0.316	0.429
ESR	0.752	—	0.513	0.562	0.497	0.509
BASDAI	0.489	0.513	—	0.890	0.933	0.821
BASDAI 6	0.492	0.562	0.890	—	0.805	0.940
NRS	0.316	0.497	0.933	0.805	—	0.812
NRS 6	0.429	0.509	0.821	0.940	0.812	—

* $p < 0.05$ (statistically significant); CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; NRS: numerical rating scales.

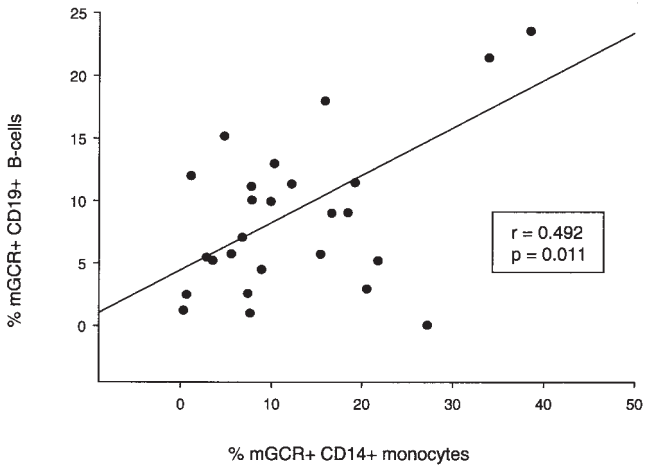

Figure 1. Correlation between the frequency of mGCR+ monocytes (x axis) and B cells (y axis) in patients with AS (n = 26).

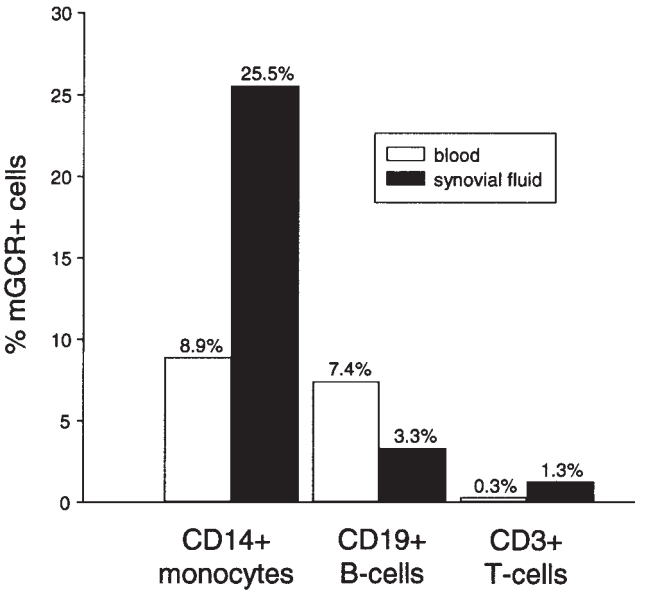

Figure 2. Frequency of mGCR+ cells among monocytes, B cells, and T cells from peripheral blood and synovial fluid of one patient with AS (representative experiment).

relation between BASDAI and the frequency of mGCR+ B cells ($r = -1.0$, $p < 0.001$).

Since 11 patients were treated with anti-TNF therapy with infliximab at the time of our experiments, we wanted to determine if mGCR expression on PBMC could be biased by this therapy. We did not find any difference in the frequency of mGCR+ monocytes and B cells between the groups with and those without anti-TNF therapy. Except for a negative correlation between CRP with mGCR+ B cells, we did not find any correlation between disease activity variables and frequency of mGCR+ cells in the group with anti-TNF therapy.

Comparison of SF and peripheral blood. We twice examined SF at the same time as peripheral blood. In both experiments, we found the frequency of mGCR+ monocytes to be much higher in SF than in the peripheral blood (Figure 2).

DISCUSSION

We analyzed mGCR expression in patients with AS, a disease not usually treated with glucocorticoids. We found the frequency of mGCR+ monocytes and B cells in patients with AS was higher than in healthy subjects. An increased frequency of mGCR+ monocytes had been observed in patients with RA and following immunostimulation with lipopolysaccharide[7]. However, patients with AS also showed an upregulation of mGCR on B cells, which was not the case in patients with RA. Moreover, this upregulation was found to correlate with the upregulation of mGCR on monocytes (Figure 1). This observation supports the hypothesis that upregulation of mGCR could be due to a regulatory process and is not simply reflecting a nonspecific monocyte reaction.

Close correlation of mGCR expression with variables of disease activity has been shown in patients with RA[7]. Our patients with AS did not show any correlation between fre-

quency of mGCR+ monocytes and/or B cells and variables of disease activity. Our explanation for this observation is that the description of disease activity in patients with AS is not as defined and reliable as in patients with RA. It is still a matter of debate which variables should be chosen to assess disease activity in AS[18,20]. To contribute to this discussion we can state that the variables we chose (CRP, ESR, BASDAI, BASDAI 6, NRS) did generally show a consistent correlation (Table 1).

The subgroup of patients with AS with affected peripheral joints is usually characterized by elevated measures of humoral immune and overall disease activity[18,19]. As in patients with RA, we found in this subgroup a positive correlation between CRP and the frequency of mGCR+ monocytes. This is very interesting, but the reason for this observation is unclear. We also found a negative correlation between BASDAI and the frequency of mGCR+ B cells, but given the small number of patients examined (n = 3) further experiments are needed to confirm and explain these findings.

On 2 occasions, we examined mGCR expression on cells prepared from SF from an inflamed joint. The expression of mGCR on monocytes was considerably higher in cells from SF in comparison to peripheral monocytes. Since the inflamed joint is considered to be the "hot spot" of immune activity with highest immune cell activity, we explain our observations as follows: the high activity of the immune system drives mGCR expression, suggesting a correlation between the degree of inflammation/activity of immune cells and expression of mGCR.

Why are higher dosages of glucocorticoids beneficial in RA and AS whereas lower dosages are usually successful only in patients with RA? We assessed similarities and differences between RA and AS with regard to mGCR expression and clinical experience in Table 2. According to our data, higher expression of mGCR on immune cells in both RA and AS could be one reason for the favorable response to high-dose glucocorticoid therapy in both diseases[9,12,21]. Nongenomic effects at high glucocorticoid dosages are considered to be mediated via the cGCR or via nonspecific membrane interactions[1]. However, it is possible that mGCR also contribute to these effects and, therefore, to the therapeutic efficacy of high-dose pulse or intraarticular glucocorticoid treatment.

In contrast to RA, glucocorticoids at lower dosages are known to be less effective in the treatment of AS[11]. The reasons for this lack of efficacy are poorly understood. It is accepted that glucocorticoids at lower doses exert their effects mainly via genomic effects, i.e., effects mediated via cGCR[1]. Recently, Lee, *et al* found the expression of GCR-beta mRNA, a dominant negative regulator of the glucocorticoid response, to be increased in patients with AS, which could explain, at least in part, the lack of efficacy of lower doses of glucocorticoids in these patients[22]. For example, treatment with 25 mg/day prednisolone is highly effective in patients with RA, but provides only limited, if any, benefit in patients with AS.

A third issue of discussion is the different pathogenesis and pathophysiology of each disease. The association of AS with HLA-B27 and its role in antigen presentation, and the association of AS and other spondyloarthropathies with gram-negative infections have led some authors to suggest a disordered elimination of pathogens with subsequent activation of CD8+ T cells[23-25]. However, we did not find any mGCR+ T cells (CD3+). Perhaps it is this lack of mGCR expression on T cells, which are very relevant in the pathogenesis of AS, that explains the lack of efficacy of low-dose glucocorticoids. In RA, monocytes are thought to be pivotal cells involved in the disease pathogenesis[26]. Consequently, consistent, and in cases of high disease activity upregulated, expression of mGCR[7] might contribute to the efficacy of glucocorticoids in patients with RA. We suggest mGCR cause a negative feedback regulation. The regulatory loop might work as follows: activation of the immune system leads to an increased expression of mGCR on immune cells, which are then targeted by glucocorticoids; this interaction may induce apoptosis, which in turn diminishes immune system activity. These considerations are supported by a number of investigations. Gametchu, *et al* showed the expression of mGCR on lymphoma and leukemia cells[6,27,28]. Moreover, they found using purified mGCR-positive mouse lymphoma cells that glucocorticoid treatment induced complete apoptosis, whereas only a partial glucocorticoid-induced apoptosis was found in mGCR-depleted cells[27]. Should these assumptions prove to be correct, they could provide a new approach to glucocorticoid development. Drugs selectively binding to mGCR may be of therapeutic value in the future[29]. First, the functions of mGCR have to be investigated in detail.

Table 2. Similarities and differences between RA and AS with regard to mGCR expression and clinical experience.

	RA	AS
Similarities		
mGCR expression on immune cells	Higher than in healthy persons	
Clinical experience	Good response to high-dose GC therapy	
Differences		
mGCR expression on immune cells	Correlation with disease activity	No correlation with disease activity
Clinical experience	Good response to low-dose GC	Usually no effect of low-dose GC

Patients with AS not treated with glucocorticoids show an upregulation of mGCR on monocytes and B cells. This upregulation does not correlate with humoral and overall disease activity of AS. We suggest that our findings are related to the limited benefit of low-dose and the higher efficacy of high-dose (pulse or intraarticular) glucocorticoid treatment in patients with AS. Drugs selectively binding mGCR may be a new therapeutic option for AS.

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