The anti-inflammatory target A3 adenosine receptor is over-expressed in rheumatoid arthritis, psoriasis and Crohn’s disease


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Abstract
The Gi protein associated A3 adenosine receptor (A3AR) was recently defined as a novel anti-inflammatory target. The aim of this study was to look at A3AR expression levels in peripheral blood mononuclear cells (PBMCs) of patients with autoimmune inflammatory diseases and to explore transcription factors involved receptor expression.

Over-expression of A3AR was found in PBMCs derived from patients with rheumatoid arthritis (RA), psoriasis and Crohn’s disease compared with PBMCs from healthy subjects. Bioinformatics analysis demonstrated the presence of DNA binding sites for nuclear factor-κB (NF-κB) and cyclic AMP-responsive element binding protein (CREB) in the A3AR gene promoter. Up-regulation of NF-κB and CREB was found in the PBMCs from patients with RA, psoriasis and Crohn’s disease. The PI3K-PKB/Akt signaling pathway, known to regulate both the NF-κB and CREB, was also up-regulated in the patients’ PBMCs.

Taken together, NF-κB and CREB are involved with the over-expression of A3AR in patients with autoimmune inflammatory diseases. The receptor may be considered as a specific target to combat inflammation.

A. Ochaion

1. Introduction

Effective anti-inflammatory drugs to treat autoimmune diseases today are mainly monoclonal antibodies against tumor necrosis factor-α (TNF-α) or other inflammatory cytokines. Due to the critical role of these cytokines in regulating normal immune and inflammatory responses, these drugs induce adverse effects and efforts are still directed towards more specific targets which are expressed solely by pathological but not normal cells [1–5].

Recently the A3AR adenosine receptor (A3AR) was identified as a new target to combat RA due to its unique characteristics. The A3AR belongs to the Gi protein associated family of adenosine receptors which includes also the A1, A2A and A2B sub-members. The A3 and the A2B adenosine receptors are negatively associated with cAMP whereas activation of the A2A and A2B stimulates adenylyl cyclase and cAMP formation [6].

The A3AR was found to be over-expressed in cells from synovial and paw tissues of rats with adjuvant induced arthritis (AIA). High receptor expression was also found in PBMCs derived from the AIA rats, reflecting receptor status in the remote inflammatory sites [7–9].

Based on these findings, synthetic highly selective agonists to the A3AR were introduced for the treatment of AIA, CF101, chemically known as 1-deoxy-1-[6-[[3-iodophenyl)methyl]amino]-9H-9-yl]-N-methyl-β-D-ribofuranuronamide (IB-MECA) induced marked amelioration of the clinical and pathological manifestations of AIA. Mechanistically, CF101 decreased the expression levels of PI3K, PKB/Akt, IKK and IκB resulting in down-regulation of NF-κB, inhibition of TNF-α and apoptosis of inflammatory cells. In addition, a direct anti-proliferative effect of CF101 towards auto-reactive T cells was observed [7–11].

Examination of A3AR expression levels in the PBMCs of RA patients revealed receptor up-regulation in both early diagnosed patients which were not yet under treatment and in patients chronically treated with methotrexate (MTX) [9,12].

These data prompted the initiation of a pre-clinical and clinical programs utilizing CF101 as an anti-inflammatory drug candidate. In a Phase I study in healthy subjects, CF101 was found to be safe.
and well tolerated with a linear pharmacokinetic activity [13]. In a Phase IIa study conducted in patients with RA, CF101 administered twice daily for 12 weeks resulted in an improvement of disease signs and symptoms and appeared to be safe and well tolerated. Analysis of A3AR expression levels at baseline showed statistically significant direct correlation with patient responses to CF101, suggesting A3AR utilization as a biomarker to predict patients’ response to the drug prior to treatment initiation [14].

The aim of the present study was to explore whether the A3AR target is over-expressed in additional autoimmune inflammatory diseases.

Table 1

<table>
<thead>
<tr>
<th>Disease</th>
<th>Age (years)</th>
<th>Disease duration (years)</th>
<th>Treatments</th>
</tr>
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<tbody>
<tr>
<td>Rheumatoid arthritis</td>
<td>54.5 ± 2.33</td>
<td>6.67 ± 2.06</td>
<td>MTX, prednisone</td>
</tr>
<tr>
<td>Psoriasis</td>
<td>47.2 ± 2.65</td>
<td>18.6 ± 2.28</td>
<td>Topicals, Dead sea, phototherapy, MTX, immunobiologics</td>
</tr>
<tr>
<td>Crohn’s disease</td>
<td>43.7 ± 3.74</td>
<td>13.07 ± 2.08</td>
<td>Immunosuppressants, anti-inflammatory, immunobiologics, prednisone, antibiotic</td>
</tr>
</tbody>
</table>

Fig. 1. WB analysis of A3AR and NF-κB was performed in PBMCs derived from patients (n = 25 for each disease) with rheumatoid arthritis, psoriasis and Crohn’s. CREB was tested in PBMCs samples utilizing RT-PCR analysis. (A) A3AR; (B) NF-κB; (C) CREB. (Values of units are the mean and SE, \( p < 0.05 \)).
diseases. We thus looked at receptor expression levels in PBMCs derived from patients with RA, psoriasis and Crohn’s disease. We further studied the molecular mechanism involved with receptor over-expression and performed bioinformatics analysis to look at the transcription factors in the A3AR promoter gene regulating receptor expression and functionality.

2. Materials and methods

2.1. Reagents

Rabbit polyclonal antibodies against rat A3AR (developed against amino acids 151–230 with the internal region of the A3AR, Santa Cruz Biotechnology, CA, USA) and the signaling proteins TNF-α (rat anti TNF-α, R&D Systems, CA, USA), phosphorylated PKB/Akt (PKB/Akt phosphorylated at pSer473, Sigma MI, USA), hcB (rabbit polyclonal antibody against a peptide at the C-terminus of hCB-α, Santa Cruz Biotechnology, CA, USA), NF-κB (NF-κB p65-RelA, Chemicon International, CA, USA) were used.

Phytohemagglutinin (PHA) and Wortmannin were purchased from Sigma (Chemical Co., St. Louis, MO, USA).

2.2. Blood sample collection and separation

Blood samples were collected from patients with RA, psoriasis, Crohn’s disease and healthy subjects upon ethical committee approval. The patients signed an informed consent prior to blood withdrawal.

To separate PBMCs, blood (16 ml) was collected in CPT vacutainer (BD, Franklin Lakes, NJ, USA), centrifuged according to manufacturer instructions and washed with PBS.

2.3. In vitro activation of PBMCs with PHA

PBMCs (2 × 10^6/ml) from healthy subjects were incubated with 5 µg/ml PHA in RPMI 1640 supplemented with 10% FBS for 24 h. Wortmannin (200 nM) was added 30 min. prior to PHA (5 µg/ml) stimulation. At the end of the incubation the PBMCs were collected and protein extracts were prepared.

2.4. Western blot analysis of A3AR and additional signaling proteins in PBMCs

Western blot analysis (WB) was carried out according to the following protocol. Samples were rinsed with ice-cold PBS and resus-
analysis was performed by several programs and results were reported as mean ± SD. All data are real transcription factor complexes.

2.8. Statistical analysis

The results were evaluated using the Student’s t-test, with statistical significance set at p < 0.05. Comparison between the mean values of different experiments was carried out. All data are reported as mean ± SD.

3. Results

3.1. A3AR is highly expressed in PBMCs derived from patients with autoimmune inflammatory diseases

Based on former studies which showed that A3AR over-expression in the inflammatory tissues is reflected in the PBMCs, we looked at the A3AR expression levels in PBMCs from patients with autoimmune inflammatory diseases.
RA, psoriasis and Crohn’s disease (n = 25 from each disease) and from healthy subjects (n = 50). Table 1 depicts patients’ characteristics.

Over-expression of the A3AR protein was noted in the PBMCs of the three diseases: RA – 1.8 ± 0.18-, psoriasis – 3.9 ± 0.62-, Crohn’s disease – 3.26 ± 0.57-fold higher than the level in healthy subjects (Fig. 1A).

3.2. Bioinformatics analysis of the A3AR gene promoter

Screening of the entire array of transcription factors with relation to the ADORA3 gene promoter region revealed meaningful high scoring transcription factors which are listed in Table 2.

Mapping of first level interactions clearly enhanced the feasibility of certain transcription factors to form transcription factor

Fig. 2. WB analysis of PI3K, PKB/Akt and IκB in protein extracts of PBMCs derived from patients with (A) rheumatoid arthritis; (B) Psoriasis; (C) Crohn’s disease. (Values of units are the mean and SE, p < 0.05.)
complexes. Several key factors for which binding sites are present on the ADORA3 gene promoter form a tight network of possible interactions. This high level of inter-connectivity is over simplified, as some of the key factors also interact with themselves and with factors which do not bind to binding sites on the promoter itself. In addition we present here a representative set of factors with their interactions: c-fos, c-jun, NF-κB and NF-κB1 (Table 3).

It may be noted that there is an almost complete match between all first levels extended set transcription factors of c-Fos, c-Jun and NF-κB1, including those factors among themselves. The appearance of probable complex forming factors in proximity supports the assumption on their interplay with each other.

3.3. Molecular mechanism involved with A3AR over-expression

NF-κB and CREB, transcription factors found to interact with the ADORA3 gene promoter, are known to play a pivotal role in the pathogenesis of RA, psoriasis and Crohn’s disease (19–24).

A marked increase in the expression levels of NF-κB (protein) and CREB (mRNA) was noted in the PBMCs of all diseases compared to that of healthy subjects (Fig. 1A and C). This was followed by an increase in A3AR expression level (Fig. 1), suggesting a role for NF-κB and CREB in mediating A3AR transcription.

Analysis of the PI3K-PKB/Akt signaling pathway, known to regulate both the NF-κB and CREB expression levels and activity [25–27] was then performed. Up-regulation of PI3K, PKB/Akt and IκB was observed in PBMCs of the three diseases compared to that of healthy subjects (Fig. 2A–C).

Support for the role played by the PI3K signaling pathway in regulating A3AR expression levels came from in vitro experiments utilizing PHA stimulated PBMCs from healthy subjects. It is well established that upon mitogenic stimulation, A3AR expression is up-regulated [12,28]. Indeed, introduction of the PI3K inhibitor Wortmannin to this system prevented the PHA induced up-regulation of A3AR (Fig. 3). A3AR expression levels, resulting in down-regulation of A3AR (Fig. 3).

4. Discussion

In this study we first show that the A3AR is over-expressed in PBMCs derived from RA, psoriasis and Crohn’s disease patients in comparison to low receptor expression in PBMCs from healthy subjects.

These data raised the question whether A3AR up-regulation is a manifestation of the inflammatory condition or does it play a role in mediating disease pathogenesis. Earlier studies showed that A3AR is up-regulated under hypoxic conditions such as cancer or inflammation. Receptor up-regulation was found to be attributed to adenosine, which accumulates in the extra-cellular environment under stressed conditions, known to regulate the expression of its own receptors via an autocrine pathway [9,29,30].
A support for this hypothesis came from in vitro data of the present study showing that in PHA stimulated PBMCs, PI3K and PKB/Akt were up-regulated followed by an increase in A3AR expression levels. A specific PI3K inhibitor reversed PKB/Akt, NF-κB and A3AR expression levels to control values, demonstrating the role of this pathway in mediating A3AR expression levels. Taken together, A3AR up-regulation is most probably a result of the inflammatory condition and is not involved with disease pathogenesis. A support for this hypothesis came from recent data of a Phase IIa clinical study in rheumatoid arthritis patients. A3AR expression levels were analyzed at base line, prior to treatment with the CF101 drug, an A3AR agonist. A statistically significant direct correlation between receptor expression prior to treatment and patient response to the drug was observed, demonstrating that patients with high receptor expression at base line responded positively to the drug [14]. Therefore, A3AR may be suggested as a biological predictive marker in autoimmune inflammatory diseases.

References


