Expression of cannabinoid receptor 2 and its inhibitory effects on synovial fibroblasts in rheumatoid arthritis

Huan Gui1,2,*, Xia Liu2,*, Zhi-Wei Wang1, Dong-Yi He3, Ding-Feng Su2 and Sheng-Ming Dai1

Abstract

Objective. Recent studies have suggested immunomodulatory and anti-inflammatory effects of cannabinoid receptor 2 (CB2R) activation, which shows no psychoactivity. However, it is still unclear whether CB2R is expressed in fibroblast-like synoviocytes (FLS) of RA. In this study we investigated whether CB2R is expressed in FLS of RA, and whether CB2R activation modulates the function of RA-FLS.

Methods. Expression of CB2R in synovial tissue and FLS was studied by immunohistochemistry, western blotting and RT-PCR. mRNA expression levels of CB2R, IL-6 and MMPs were analysed by quantitative real-time RT-PCR. The protein concentrations of IL-6 and MMPs in culture supernatants were determined by ELISA. The protein levels of signal transducing molecules were assayed by western blotting.

Results. Both mRNA and protein expression of CB2R were found in synovial tissue and cultured FLS with slightly higher levels in RA patients than in OA patients. In cultured RA-FLS, the expression level of CB2R was up-regulated by stimulation with IL-1β, TNF-α or lipopolysaccharide. In vitro, HU-308, a selective CB2R agonist, inhibited IL-1β-induced proliferation of RA-FLS as well as IL-1β-induced production of MMP-3, MMP-13 and IL-6 in RA-FLS in a dose-dependent manner. HU-308 also suppressed IL-1β-induced activation of extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinase in FLS.

Conclusion. In RA-FLS, proinflammatory mediators up-regulate the expression of CB2R, which negatively regulates the production of proinflammatory cytokines and MMPs. These data suggest that CB2R may be a potential therapeutic target of RA.

Key words: cannabinoid receptor 2, fibroblast-like synoviocytes, interleukin 6, matrix metalloproteinases, rheumatoid arthritis.

Introduction

RA is an immune-mediated inflammatory disease of unknown aetiology that is characterized by chronic inflammatory infiltration of the synovium, leading to eventual cartilage and bone destruction [1]. In spite of significant improvements in the treatment of RA, there is still a need for the identification of new pathways involved in the modulation of inflammation in order to further increase efficacy, particularly in patients in whom the disease does not respond to current therapies.

Recently, discovery of the endocannabinoid system, especially two subtypes of cannabinoid receptors, has elicited a great deal of interest in inflammatory diseases, including multiple sclerosis and RA [2]. Two types of cannabinoid receptors—cannabinoid receptor 1 (CB1R) and cannabinoid receptor 2 (CB2R)—were discovered in the early 1990s [3, 4]. CB1R exists primarily on central and peripheral neurons and is associated with the suppressive role of the CB1R system in inflammation. CB2R, on the other hand, is mainly expressed on immune cells and is considered to exhibit immunomodulatory activity against several inflammatory conditions, including RA [5]. CB2R has been shown to exert anti-inflammatory effects in vitro and in vivo on several inflammation-related cell types, including macrophages, microglia, and fibroblast-like synoviocytes (FLS) [6]. This suggests that CB2R may be a potential therapeutic target for the treatment of RA.
psychoactive effects of cannabinoids. CB2R is predominantly expressed by cells of haematopoietic origin and is thought to mediate cannabinoid-induced immune modulation [5]. This type of distribution supports the prospect that anti-inflammatory and immunosuppressive CB2R-selective drugs without psychoactivity can be developed for the management of chronic inflammatory diseases such as RA.

In 2008 the endocannabinoids anandamide and 2-arachidonoylglycerol were identified in the synovial fluids of RA patients; neither of these were detected in the synovial fluids of normal volunteers [6]. This finding makes the endocannabinoid system an attractive therapeutic target of RA. Fibroblast-like synoviocytes (FLS) are recognized as both propagators of the immune response and the engine of joint damage in RA [7, 8]. However, it remains unclear whether CB2R is expressed in FLS.

In the present study, we revealed the expression of CB2R and its up-regulation by proinflammatory mediators in RA-FLS and demonstrated the inhibitory effects of CB2R activation on the production of proinflammatory cytokine IL-6 and MMPs.

Materials and methods

Reagents

HU-308, a selective agonist for CB2R [9], was purchased from Tocris Bioscience (Bristol, UK), lipopolysaccharide (LPS) from Sigma (St Louis, MO, USA) and human IL-1β and TNF-α from R&D Systems (Minneapolis, MN, USA). Collagenase type II was purchased from Worthington Biochemical (Lakewood, NJ, USA). Recombinant rabbit-polyclonal antibodies to human CB2R, monoclonal antibodies to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and α-tubulin, and HRP-conjugated goat anti-rabbit IgG were purchased from Abcam (Cambridge, UK), Alexa Fluor 488-conjugated donkey anti-rabbit IgG was purchased from Life Technologies (Carlsbad, CA, USA). Trizol, oligo (dT)18 primers, dNTP mixture, ribonuclease inhibitor, and reverse transcriptase M-MLV, premix Ex Taq and SYBR premix Ex Taq were all purchased from TaKaRa Biotechnology (Dalian, China).

Immunohistochemistry

Synovial tissues were obtained at the time of knee joint replacement surgery or synovectomy from RA or OA patients (see supplementary Table S1, available at Rheumatology Online). All the diagnoses of the patients met the ACR criteria for RA [10] or OA [11], respectively. Informed consent was obtained from each patient according to the Declaration of Helsinki. The study design was approved by the ethics committee of Changhai Hospital, Second Military Medical University, Shanghai.

Fresh synovial tissues were fixed in 4% paraformaldehyde for 48 h and then embedded in paraffin. Serial sections (4 μm) were incubated overnight at 4°C with anti-CB2R antibodies and then incubated with HRP-conjugated goat anti-rabbit IgG as a secondary antibody for 1 h at room temperature. The peroxidase activity was visualized with 3,3’-diaminobenzidine and then the sections were counterstained with haematoxylin. Negative controls were stained with isotype-matched non-specific IgG at the same concentration.

For immunocytochemistry, cultured RA-FLS were incubated overnight at 4°C with anti-CB2R antibodies and then incubated with Alexa Fluor 488-conjugated donkey anti-rabbit IgG as a secondary antibody for 1 h at room temperature. 4’,6-diamidino-2-phenylindole (DAPI) solution was applied for 5 min for nuclear staining. The staining was photographed with a CKX41 inverted fluorescence microscope (Olympus, Tokyo, Japan).

Isolation and culture of human FLS

Human FLS were isolated and cultured as previously [12]. After careful removal of the adipose and fibrous tissue, fresh synovial tissue was minced and then digested overnight with 1.0 mg/ml collagenase in serum-free DMEM at 37°C on a horizontal shaker. The cell suspensions were filtered through a stainless steel filter to remove the undigested tissue. The filtrate was centrifuged and the cell precipitate was washed twice with PBS. After that, the cells were resuspended in complete media containing 10% fetal calf serum (FCS), 100 U/ml of penicillin, 100 μg/ml streptomycin and 100 μg/ml amphotericin B. The cells were counted, seeded into culture flasks (2.5 × 10^4 cells/cm²) and cultured overnight in a humidi- fied, 5% CO₂ atmosphere at 37°C. The non-adherent cells were then washed out. Adherent cells were cultured in complete medium, and the culture medium was replaced every week. Upon confluence, cells were dispersed with trypsinization and then transferred to new plastic dishes in a split ratio of 1:3. Passages 3–8 of the FLS were used in subsequent experiments, at which time they were a homogeneous population of fibroblasts. Before each experiment, the FLS were starved for 12 h with DMEM containing 1% FCS.

RT-PCR and quantitative real-time RT-PCR

Total RNA was extracted from cultured FLS with Trizol. RNA was evaluated spectrophotometrically for quantity and purity. After reverse transcription, complementary DNA was used as templates for PCR. PCR amplification was performed using specific primers (Table 1). The constitutively expressed gene encoding GAPDH was used as an internal control in RT-PCR to normalize the amounts of mRNA in each sample. The PCR products were analysed by electrophoresis in 2% agarose gels stained with ethidium bromide and bands were visualized and photographed under ultraviolet excitation. Quantitative real-time RT-PCR was carried out on an Applied Biosystems
Inhibitory effects of HU-308 on IL-1β-induced proliferation of RA-FLS

In this step we tested the biological function of HU-308, a selective CB2R agonist [9], in cultured RA-FLS. IL-1β is present in RA synovial fluid and participates in joint inflammation and joint destruction. As expected, stimulation with IL-1β (1 ng/ml) resulted in an enhanced proliferation rate of RA-FLS. Pre-treatment with HU-308 (1–10 μM) significantly inhibited the IL-1β-induced proliferation of RA-FLS in a dose-dependent manner (Fig. 3).

Inhibitory effects of HU-308 on IL-6, MMP-3 and MMP-13 production in RA-FLS

To determine whether HU-308 can modulate cytokine and MMP production in human RA-FLS, cells were incubated with IL-1β (1 ng/ml) in the presence or absence of HU-308. In IL-1β-stimulated RA-FLS, HU-308 (1–10 μM) inhibited MMP-3 and MMP-13 production with a dose-dependent manner. This suggests that HU-308 can modulate the inflammatory process in RA-FLS.

Table 1: Sequences of specific PCR primers

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB2R</td>
<td>fw: TGG CAG CGT GAC TAT GAC&lt;br&gt;rv: AYA GAG GAA GCT GAT GAA</td>
</tr>
<tr>
<td>IL-6</td>
<td>fw: CCA GGA AAA GAT TCC AAA GAT G&lt;br&gt;rv: GGA AGG TTC AGG TTT TTT TCT G</td>
</tr>
<tr>
<td>MMP-3</td>
<td>fw: TAT GGA CCT CGC CCT GAG TCC&lt;br&gt;rv: AGG TTC AAG CTT CCT GAG G</td>
</tr>
<tr>
<td>MMP-13</td>
<td>fw: GCT GCC TTC TCT TTC TTA A&lt;br&gt;rv: TGC TGC ATT CTT CTT CAG GA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>fw: GAA GGT CGG AGT CAA CGG&lt;br&gt;rv: GAA AGA TGG TGA TGG GAT T</td>
</tr>
</tbody>
</table>

CB2R: cannabinoid receptor 2; fw: forward; rv: reverse; MMP: matrix metalloproteinase; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

Statistical analysis

Data were analysed using SPSS 16.0 software (IBM, Armonk, NY, USA). One-way analysis of variance with Tukey’s post-test for multiple comparisons was used to determine the statistical significance of comparisons. P-values <0.05 were considered statistically significant.

Results

Higher expression level of CB2R in RA than OA

We first investigated the expression of CB2R in the synovium of RA patients. Synovial membranes were obtained from RA and OA patients and stained with anti-CB2R antibodies. Positive staining of anti-CB2R antibodies was evident in all RA tissues as well as OA tissues examined in the lining layer and in the interstitial sublining layer areas (Fig. 1A). Furthermore, the expression level in RA samples was slightly higher than that in OA samples.

We next examined CB2R expression in cultured FLS. As a result, constitutive expression of CB2R in resting FLS was confirmed at the mRNA level by RT-PCR (Fig. 1B) and at the protein level by western blotting (Fig. 1C). Compared with the FLS from OA patients, a slightly higher level of CB2R expression in the FLS from RA patients was also found. The expression of CB2R protein in RA-FLS was further confirmed by IF staining of anti-CB2R antibodies (Fig. 1D).

Up-regulated expression level of CB2R by proinflammatory mediators

To determine whether an inflammatory environment modulates the expression level of CB2R, cultured RA-FLS were stimulated with proinflammatory mediators, such as TNF-α (25 ng/ml), IL-1β (10 ng/ml) or LPS (100 ng/ml). As a result, real-time RT-PCR analysis showed that all of these proinflammatory mediators significantly enhanced the mRNA expression level of CB2R by 3.3–7.5-fold (Fig. 2).

Inhibitory effects of HU-308 on IL-1β-induced proliferation of RA-FLS

In this step we tested the biological function of HU-308, a selective CB2R agonist [9], in cultured RA-FLS. IL-1β is present in RA synovial fluid and participates in joint inflammation and joint destruction. As expected, stimulation with IL-1β (1 ng/ml) resulted in an enhanced proliferation rate of RA-FLS. Pre-treatment with HU-308 (1–10 μM) significantly inhibited the IL-1β-induced proliferation of RA-FLS in a dose-dependent manner (Fig. 3).

Inhibitory effects of HU-308 on IL-6, MMP-3 and MMP-13 production in RA-FLS

To determine whether HU-308 can modulate cytokine and MMP production in human RA-FLS, cells were incubated with IL-1β (1 ng/ml) in the presence or absence of HU-308. In IL-1β-stimulated RA-FLS, HU-308 (1–10 μM) inhibited
the mRNA expression levels of IL-6, MMP-3 and MMP-13 in a dose-dependent manner (Fig. 4A). Moreover, HU-308 also significantly decreased the amounts of IL-6, MMP-3 and MMP-13 proteins released into the culture medium of IL-1β-stimulated RA-FLS in a dose-dependent manner (Fig. 4B).

**Inhibitory effect of HU-308 on IL-1β-induced activation of ERK1/2 and p38 in RA-FLS**

In cultured RA-FLS, IL-1β (1 ng/ml) enhanced intracellular protein levels of both ERK1/2 and phosphorylated ERK1/2 (p-ERK1/2), as well as levels of both p38 MAPK and phosphorylated p38 (p-p38) MAPK. HU-308 (10 μM) blunted the IL-1β-induced activation of ERK1/2 for >2 h and temporarily inhibited IL-1β-induced activation of p38 MAPK for <30 min (Fig. 5).

**Discussion**

The results reported here demonstrate for the first time that RA-FLS constitutively express CB2R, which may be up-regulated by inflammation. Moreover, activation of CB2R inhibits the production of IL-6, MMP-3 and MMP-13 by attenuating the activation of ERK1/2 and p38 MAPK in FLS. These data offer a promising therapeutic target for the development of novel pharmacological approaches to treat RA.

CB2R is expressed by all immune cells, but its expression level varies among immune cell populations and activation states [14]. Recently CB2R has been identified...
molecularly and pharmacologically in numerous other cell types, including articular chondrocytes [15], osteocytes, osteoblasts and osteoclasts [16]. In the present study, immunohistochemical studies showed localization of CB2R in the lining layer and in the interstitial sublining layer of all RA synovial tissues. This finding is consistent with a previous report in which CB2R protein in the homogenates of RA synovial tissues was revealed by western blotting [6]. In RA synovium infiltrated with massive immune cells, the existence of CB2R is not uncommon. In the following experiments we confirmed the expression of CB2R in RA-FLS at both the mRNA and protein levels by RT-PCR, western blotting and immunocytochemical studies. Furthermore, we demonstrated that proinflammatory mediators, e.g. TNF-α, IL-1β and LPS, significantly up-regulated the expression level of CB2R in FLS. We also found that the expression level of CB2R in RA synovial tissues and FLS was higher than that in OA. These data suggest that the up-regulated expression of CB2R may play a counteracting role in the process of RA synovitis, which is also supported by increased concentrations of endocannabinoids in RA synovial fluid [6]. In addition, it has also been demonstrated that the expression level of CB2R can be increased under certain conditions and disease states [14]. For example, increased CB2R has been found in multiple sclerosis [17], allergic contact dermatitis [18] and acute and chronic bladder inflammation [19]. The increasing receptor number is also important for increasing the efficacy of CB2R agonists.

HU-308 is a synthetic cannabinoid, and the affinity of HU-308 binding to CB2R is almost 300 times the affinity to CB1R. Functional studies show it inhibits forskolin-stimulated cyclic adenosine monophosphate (cAMP) production in CB2R-transfected cells, whereas it shows little effect in CB1R-transfected cells. So HU-308 is a specific agonist of CB2R [9].

In bone marrow–derived primary monocytic cultures derived from wild-type mice, HU-308 dose-dependently suppressed the formation of osteoclasts. The deficiency of CB2R completely abolished the inhibitory effect of HU-308 on osteoclast formation [16]. In vivo, HU-308 also attenuated ovariectomy-induced bone loss through suppression of osteoclast formation. No protective effects of HU-308 were observed in ovariectomized CB2R knockout mice [20]. These data further support that CB2R mediates the effect of HU-308, and also imply that selective activation of CB2R may inhibit bone erosion caused by osteoclasts in RA.

Accumulating evidence demonstrates that proinflammatory cytokines such as TNF-α and IL-6 play pivotal roles in the physiopathology of RA, and the biologics of TNF inhibitors and anti-IL-6 receptor monoclonal antibody have shown dramatic efficacy in RA treatment [1, 21, 22]. TNF-α and IL-6 in RA synovitis joints are produced mainly from macrophage-like synoviocytes and FLS. In the present study, the in vitro effect of HU-308 on proinflammatory cytokine production was investigated in RA-FLS. HU-308 significantly decreased IL-1β- and IL-6-induced production of IL-6 from RA-FLS in a dose-dependent manner. In addition, HU-308 significantly inhibited LPS-induced IL-6 and TNF-α production from peritoneal macrophages isolated from wild-type mice, whereas HU-308 failed to suppress LPS-induced cytokine production from CB2R gene knockout macrophages (unpublished data). Together, these results indicate that HU-308 inhibits the inflammatory process by decreasing the production of proinflammatory cytokines in RA synovitis, which is mediated by CB2R.

In a previous study, two synthetic cannabinoids—CP55940 (10 μM) and WIN55212-2 (10 μM)—were reported to attenuate IL-6 and IL-8 secretion from IL-1β-stimulated RA-FLS, and the inhibitory effects were not counteracted by CB1R antagonist AM-281 (80 μM) or CB2R antagonist AM-630 (80 μM). So it was concluded that CP55940 and WIN55212-2 exert a potent anti-inflammatory effect on RA-FLS via a non-CB1R/CB2R-mediated mechanism [23]. Although AM630 was used as a CB2R antagonist, 10 μM of AM-630 was also an inverse agonist at CB2R and a partial agonist at CB1R [24]. So up to 80 μM of AM-630 is not a solely specific antagonist of CB2R, and may also activate CB1R. In some studies, SR 144528 was used as a selective antagonist of CB2R [25], but there was evidence for the inverse agonist property of SR 144528 and the constitutive activation of CB2R.

**Fig. 4** Inhibitory effects of HU-308 on the production of IL-6 and MMPs in IL-1β-stimulated RA-FLS

Cultured RA-FLS were pre-treated with different concentrations of HU-308 for 30 min and then incubated with IL-1β (1 ng/ml) for 3 or 24 h. (A) Expression levels of mRNA assayed by quantitative real-time RT-PCR 3 h after IL-1β stimulation. (B) Protein concentrations in culture supernatants determined by ELISA 24 h after IL-1β stimulation. Data are expressed as the mean (s.d.) (n = 4). *P < 0.05; **P < 0.01 vs IL-1β alone.
in Chinese hamster ovary-expressing cells [26]. At this time there is no pure specific antagonist of CB2R, so we did not use any pharmacological antagonists to interrupt the binding of HU-308 to CB2R in the present study.

In RA, the MMP family is related to the invasiveness of FLS and the erosion of cartilage [27]. In the present study, HU-308 significantly inhibited the IL-1β-induced production of MMP-3 and MMP-13 in RA-FLS. Our results were consistent with the finding that Win-55212-2, a non-selective agonist to CB1R and CB2R, significantly inhibited IL-1β-induced proteoglycan and collagen breakdown in bovine cartilage explants [15]. These data suggest that HU-308 may protect cartilage from damage in RA by inhibiting the production of MMPs. In addition, we also found that HU-308 inhibited IL-1β-induced proliferation of FLS.

The cellular processes that contribute to the pathogenesis of RA are regulated by three families of MAPKs, namely, ERK1/2, JNK, and p38 [7, 28]. In the present study, IL-1β stimulated phosphorylation of ERK1/2 and p38 MAPK, but did not affect JNK in RA-FLS, which is consistent with previous reports that IL-1β stimulated FLS via activating intracellular ERK1/2 and p38 MAPK [29, 30]. In the present study, HU-308 effectively inhibited IL-1β-induced activation of ERK1/2 and p38 MAPK in RA-FLS. This finding was indirectly supported by the finding that HU-308 dose-dependently attenuated TNF-α-induced activation of ERK1/2 and p38 MAPK in vascular smooth muscle cells [31]. However, it was also reported that the activation of CB2R results in activation of ERK1/2 and p38 MAPK in monocytes [32]. These data infer that signalling of CB2R activation may vary substantially depending on cell type and stimulus.

In conclusion, CB2R is expressed in RA synoviocytes, including FLS, and its expression is up-regulated by proinflammatory mediators. Selective activation of CB2R suppresses the production proinflammatory cytokines and MMPs from FLS.

Rheumatology key messages
- Cannabinoid receptor 2 (CB2R) is expressed by RA synovium, including fibroblast-like synoviocytes (FLS), and is up-regulated by inflammation.
- Activation of CB2R inhibits the proliferation and production of proinflammatory cytokines and MMPs in RA-FLS.
- Activation of CB2R inhibits IL-1β-induced activation of extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinase in RA-FLS.
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Supplementary data
Supplementary data are available at Rheumatology Online.

References
Clinical vignette

Polymyalgia rheumatica or lymphoma recurrence? Positron emission tomography/computed tomography is a specific imaging technique that helps differential diagnosis

In November 2011 a 72-year-old retired surgeon was diagnosed with Hodgkin’s lymphoma. Fluorodeoxyglucose-positron emission tomography/computed tomography (FDG-PET/CT) showed cervical lymph node uptake (Fig.1A, arrow), which quickly disappeared after two cycles of adryamicin, bleomycin, vinblastine sulphate, and dacarbazine (ABVD) (Fig. 1B). In October 2012 the patient complained of pain in the girdles of 75 days duration, worsening at night, and accompanied by fatigue and morning stiffness lasting 3 h. He had lost 1 kg in 1 month, but denied fever, headache or vision impairment. Clinical examination revealed active elevation of the arms below 90°, slight pain on passive motion of the shoulders, stiffness of the hips and slight swelling and tenderness of two MCP joints. ESR was 120 mm/h, CRP was 50.6 mg/dl and IgM RF and anti-cyclic citrullinated peptides were absent. The appearance of systemic symptoms raised the suspicion of a lymphoma relapse, in view also of the short course of chemotherapy. A third FDG-PET/CT scan showed involvement of the shoulders (Fig. 1C, arrowheads), trochanteric bursae (open arrowheads) and ischiatic bursae, without large-vessel vasculitis. This pattern is typical of PMR [1]. After the diagnosis of PMR, treatment with 0.2 mg/kg prednisone was started with prompt and complete resolution of symptoms.

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Reference

Fig. 1 Positron emission tomography/computed tomography, coronal view: the three different scans are shown