Rheumatol Int (2008) 28:631–635
DOI 10.1007/s00296-007-0489-0

ORIGINAL ARTICLE

Suppression of human macrophage interleukin-6 by a nonpsychoactive cannabinoid acid

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Received: 22 May 2007 / Accepted: 6 November 2007 / Published online: 27 November 2007

Abstract Interleukin-6 (IL-6) is a multifunctional cytokine which contributes to inflammation and tissue injury in several diseases. Thus, inhibition of IL-6 production may be a useful strategy for treatment of patients with diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). A synthetic nonpsychoactive cannabinoid, ajulemic acid (AjA), prevents joint damage in experimental arthritis. Results of experiments presented here indicate that addition of AjA (3–30 μM) to human monocyte derived macrophages in vitro reduces steady state levels of IL-6 mRNA and the subsequent secretion of IL-6 from LPS stimulated cells. Although AjA binds to and activates PPARγ, its anti IL-6 effects are PPARγ independent. These studies provide evidence to support the view that AjA may prove to be an effective, safe antiinflammatory agent.

Keywords Interleukin-6 · Inflammation · Cannabinoid acid · Macrophages · Rheumatoid arthritis

Introduction

Interleukin-6 (IL-6) may be considered the prototypic multifunctional cytokine. Indeed, this is reflected in the many names assigned to it before its final designation in 1987 [1]. Human IL-6 is a 26kDa glycoprotein produced by several cell types including macrophages and lymphocytes. Macrophages, with T and B lymphocytes, and dendritic cells comprise an immune compartment that in joints of patients with rheumatoid arthritis (RA) replaces what was the subintimal layer of synovium [2]. IL-6 is able to degrade cartilage and erode bone [3], and it is responsible for the production of acute phase reactants such as C-reactive protein [4].

In patients with RA, concentrations of IL-6 in serum correlate with disease activity and extent of joint damage, and reduction in disease activity after treatment with disease-modifying antirheumatic drugs (DMARDs) is associated with reductions in serum concentrations of IL-6 [5]. Several small clinical trials suggest efficacy of treatment of RA patients with antibody to IL-6 or its receptor [6]. In addition, IL-6 induces spinal cord injury in patients with immune mediated transverse myelitis [7], and it has been implicated in the immunopathogenesis of systemic lupus erythematosus [8]. Thus, inhibition of IL-6 production may be a useful strategy for treatment of patients with RA and other immune mediated diseases characterized by inflammation and tissue injury.

The *Cannabis* plant has been a source of medicinal preparations since the earliest written records on pharmacobotany [9]. A major obstacle to broad acceptance of cannabinoids as therapeutic agents is their psychoactive effects. A class of cannabinoids, the carboxyl tetrahydrocannabinols, which are metabolites of tetrahydrocannabinol (THC), shows promise as therapeutic agents that are free of cannabimimetic central nervous system activity [10]. These compounds, called cannabinoid acids, include all the carboxylic acid metabolites of the cannabinoids and their synthetic analogs. One analog, 1'1'-dimethylheptyl-THC-11-oic acid, termed ajulemic acid (AjA), is a potent antiinflammatory and analgesic agent in several animal models [11, 12]. In addition, AjA is not psychoactive in mice. In fact, AjA suppresses THC-induced catalepsy in mice (10).

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Moreover, administration of 80 mg/day AjA for 1 week to patients with neuropathic pain relieved symptoms and did not induce behavioral changes [13].

Oral administration of AjA to rats with adjuvant arthritis prevents joint tissue injury in this animal model [14]. AjA binds to and activates the nuclear receptor peroxisome proliferator activated receptor gamma (PPARγ) in vitro [15]. Therefore, we investigated the influence of AjA on IL-6 production by and release from human monocyte derived macrophages (MDM). We also examined the role of PPARγ in the mechanism of action of AjA.

Materials and methods

Reagents

AjA was obtained from Organix (Woburn, MA). Its purity was monitored on high-pressure liquid chromatography by comparison with material synthesized previously [16]. The sample was 97% chemically pure, and was >99% chirally pure in the R, R enantiomer. AjA was dissolved in DMSO, then diluted with minimal essential medium (MEM) 2% fetal bovine serum (FBS) to achieve appropriate concentrations. The concentration of DMSO was kept constant at 0.3%. GW9662 and troglitazone were from Biomol Laboratories Inc.

Establishment of monocyte derived macrophages (MDM)

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation in the usual manner [17]. PBMC (10^7/ml) in RPMI-2% FBS were incubated overnight at 5% CO_2 and 37°C in 6 or 12 well tissue culture plates. Non-adherent cells were removed and media was replaced with RPMI containing 10–15% FBS. Cultures were maintained for 4–6 days in a 5% CO_2 humidified incubator at 37°C. Media was then replaced with RPMI-2% FBS or other defined media according to experimental protocol. Cells were exposed or not to AjA (3–30 μM) for 60 min, then stimulated with 10 ng/ml LPS. Cells were collected at 4 h for assessment of IL-6 gene expression, and supernatants were collected at 18–24 h for measurement of secreted IL-6.

Measurement of IL-6 mRNA by the hybridization/colorimetric assay

Quantikine mRNA (R&D Systems) is a colorimetric microplate method used to quantify cytokine-specific mRNA at low levels [18]. RNA samples were hybridized with mRNA-specific biotin-labeled "capture" probes in a microtiter plate. After the hybridization reaction was complete, samples were transferred to a streptavidin coated microtiter plate, and the RNA/probe complex was captured. Unbound material was washed away, and anti-digoxigenin alkaline phosphatase conjugate was added. Unbound conjugate was washed away, and substrate solution was added, followed by the addition of an amplification solution. Development of color is in proportion to the amount of IL-6 mRNA in the original sample. The reaction was stopped, and the color intensity was measured with a standard plate reader at 490 nm with background correction at 650 nm. The minimal detectable level was 1.6 amol/ml. Conditions were run in triplicate.

Measurement of IL-6 protein

Supernatants were collected and analyzed for IL-6 by ELISA (R&D). Standards and diluted samples were incubated for 2 h at room temperature and then washed. Substrate solution was added to the samples for 30 min. Samples were then read on a microplate reader at 450 nm with background correction at 540 nm. The minimal detectable level was 3.1 pg/ml. Conditions were run in triplicate.

Viability of cells

The integrity of the MDM at the end of experiments was assessed by exclusion of trypan blue. Cells were diluted in 0.5% trypan blue. Nonviable cells lost their ability to exclude trypan blue, and stained blue. In no instance did the proportion of nonviable cells exceed 5%.

Statistical analysis

Data were analyzed and compared by paired Student's test or by Student's *t*-test difference of means of independent groups.

Results

Release of IL-6 from MDM stimulated with LPS in vitro was suppressed by AjA in a dose-dependent manner (Fig. 1). In a series of five experiments, IL-6 secretion was reduced $69.1 \pm 6.0\%$ (mean $\pm$ sd) by 10 μM AjA ($P = 0.001$ versus control cells not exposed to AjA). Similarly, reduction by AjA of IL-6 gene expression was significant and dose dependent (Fig. 2).

We then tested the possibility of PPARγ dependence by exposure of human MDM to the irreversible PPARγ antagonist GW9662. AjA suppressed IL-6 release from LPS stimulated cells whether or not PPARγ activity was blocked by GW9662. In addition, the known PPARγ activator troglitazone [19] did not suppress IL-6 release (Table 1).

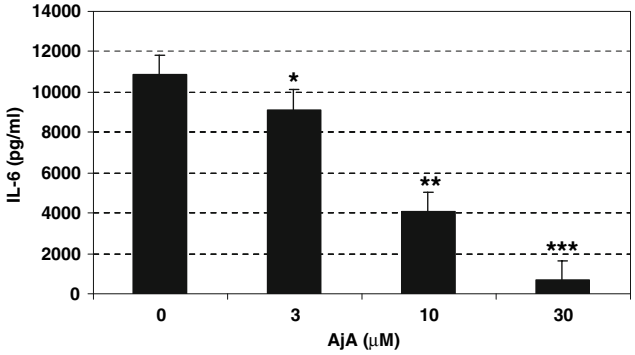

Fig. 1 IL-6 release from human MDM. Cells exposed to AjA for 60 min then stimulated 18 h with 10 ng/ml LPS. IL-6 in supernatants measured by ELISA. Values are means of three experiments. All samples assayed in triplicate. Error bars are SD for three experiments. *$P = 0.03$, **$P = 0.01$, ***$P = 0.005$, all versus 0 μM AjA

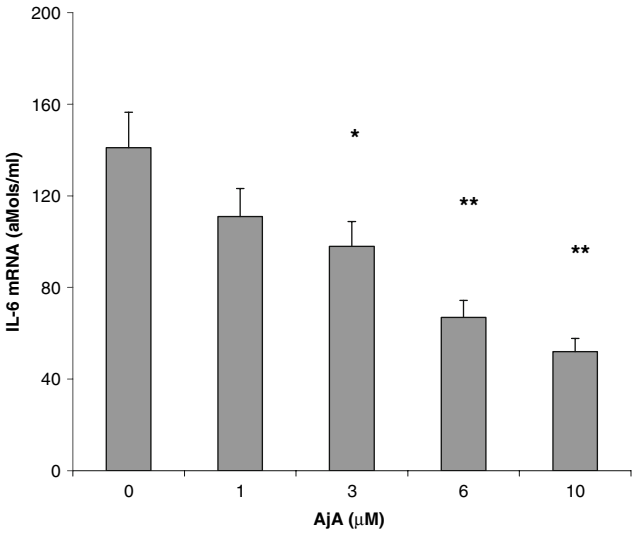

Fig. 2 IL-6 gene expression in human MDM. Cells exposed to AjA for 60 min, then stimulated 4 h with 10 ng/ml LPS. Steady state levels of cellular IL-6 mRNA measured by the hybridization/colorimetric assay. Error bars are SD for three experiments. *$P < 0.03$, **$P = 0.02$, all versus 0 μM AjA

The results suggest that the action of AjA on IL-6 secretion from human MDM is PPARγ independent.

Discussion

Blockade of inflammatory cytokines such as tumor necrosis factor-α and interleukin 1β (IL-1β) is an effective strategy for the treatment of RA [20]. Interleukin-6 is a cytokine which can help maintain synovial inflammation and facilitate bone erosion [21,22], and blockade of IL-6 action appears to be beneficial in treatment of patients with active RA [6]. In addition, a functional IL-6 gene is necessary for development of type-II collagen induced arthritis in mice

Table 1 Influence of PPARγ on IL-6 Release from Human MDM stimulated with LPS[a]

Condition	IL-6 (pg/ml)
Untreated control	830.9
Troglitazone (10 μM)	937.4
AjA (10 μM)	2.5
GW9662 (10 μM) + AjA (10 μM)	5.0

[a] Values are means of triplicate samples. Cells treated 60 min with each agent before stimulation with 10 ng/ml LPS for 18 h. Supernatant IL-6 measured by ELISA. Mean baseline IL-6 = 3.1 pg/ml. Results representative of two experiments

[23], and blockade of IL-6 prevents establishment of antigen-induced arthritis in mice [24].

Results of experiments presented here indicate that addition of AjA to human MDM in vitro reduces steady state levels of IL-6 mRNA and the subsequent secretion of IL-6 from activated cells. Similar results were obtained with human PBMC and synovial fibroblasts (not shown). However, the influence of AjA on MDM IL-6 was much more consistent, perhaps because of the greater homogeneity of the cell system. Experiments done with MDM in which PPARγ activity was blocked indicate that suppression of IL-6 by AjA does not depend on PPARγ activation. In addition, the known selective PPARγ agonist, troglitazone, does not suppress IL-6 release. Other PPARγ ligands such as prostaglandin J_2 (PGJ2) can also affect cell function in a PPARγ-independent manner [25]. PPARs were first cloned as nuclear receptors that mediate the effects on gene transcription of synthetic compounds called peroxisome proliferators. Up-regulation of PPARγ reduces expression of several mediators of inflammation including IL-6 [26]. However, responses of cells to PPAR ligands can be due to activation of PPAR or can be PPAR independent [27], actions which appear to be cell and stimulus, and perhaps, ligand specific. It is not unlikely that PPARγ activation by AjA contributes to the therapeutic effect of the cannabinoid. However, it appears from the studies presented here that the anti IL-6 action of AjA exhibited in vitro is not due to PPARγ activation.

The precise mechanism whereby AjA suppresses IL-6 production by activated human MDM is not clear. Suppression by AjA of activation of the transcription factor NFκB (Stebulis et al. unpublished) might be important to suppression of inflammatory cytokines by AjA. T lymphocytes activated by IL-6 in the synovium of patients with RA can induce human monocyte/macrophages to express inflammatory cytokines and chemokines by NFκB dependent and independent mechanisms [28]. In addition, T lymphocytes in the joints of RA patients are resistant to apoptosis [29]. IL-6 rescues T cells from entering apoptosis in vitro [30], and suppression of T cell apoptosis in vivo leads

to autoimmune arthritis in mice [31]. It is of interest then, that AjA induces apoptosis of T cells [32], and suppresses activation of NFκB (J. Stebulis et al. unpublished).

Inflammation is a well-orchestrated process designed to combat infection and prevent tissue injury. Like other cytokines, IL-6 can be defined as a modulating factor that balances initiation and resolution of inflammation. In diseases characterized by chronic inflammation, it appears that suppression of IL-6 prevents tissue injury [33]. It is likely that AjA acts in vivo on several targets to suppress immune mediated inflammation and tissue injury.

Of course, it is difficult to correlate results of experiments done in vitro with studies done in vivo in animal models. However, the evidence from animal studies [12] and experiments done in vitro which indicate that AjA suppresses monocyte IL-1β production [17], enhances T cell apoptosis [32], and suppresses production of matrix metalloproteinases [34], and results presented here, suggest that AjA may have value for the treatment of joint inflammation in patients with RA, osteoarthritis, and SLE. Successful therapy of joint tissue injury and of autoimmune disease will require modification of several aspects of host defense responses with agents that can be given safely for long periods of time. Nonpsychoactive cannabinoid acids meet those criteria.

Acknowledgments This study was supported by National Institute of Health Grants DA3691, AI1056362, and T32AR07572 (RP, Trainee), and a grant from the FAO Schwarz Research Foundation (FA, Trainee).

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