Cytokine 71 (2015) 165–172

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.journals.elsevier.com/cytokine

Possible chondroprotective effect of canakinumab: An *in vitro* study on human osteoarthritic chondrocytes

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ARTICLE INFO

Article history:
Received 13 June 2014
Received in revised form 9 October 2014
Accepted 28 October 2014

Keywords:
Canakinumab
Osteoarthritis
Human chondrocytes
Tumor necrosis factor-α

ABSTRACT

Canakinumab is a human IgGκ monoclonal antibody that neutralizes the activity of interleukin (IL)-1β blocking interaction with IL-1β receptors. Our study aimed to evaluate the *in vitro* effect of canakinumab on human osteoarthritic (OA) chondrocytes cultivated in the presence or absence of tumor necrosis factor (TNF)-α. Articular cartilage was obtained from the femoral heads of patients with osteoarthritis (OA). Chondrocytes were incubated with two concentrations (1 μg/ml and 10 μg/ml) of canakinumab alone or with TNF-α (10 ng/ml) for 48 h. We evaluated cell viability, release of proteoglycans (PG) and nitric oxide (NO) in culture medium, inducible nitric oxide synthase (iNOS) and metalloproteinanes (MMP)-1,3,13 gene expression, apoptosis, necrosis and morphological feature by transmission electron microscopy (TEM). Canakinumab alone did not have cytotoxic effect. Cell viability was reduced significantly ($p < 0.001$) by TNF-α and restored by canakinumab at both concentrations used. TNF-α determined a significant decrease of PG ($p < 0.001$) and an increase of NO ($p < 0.001$) and MMP-1,3,13 gene expression. Canakinumab significantly increased the PG levels and decreased (1 μg/ml, $p < 0.01$; 10 μg/ml, $p < 0.01$) NO levels in cells cultured with TNF-α. The NO data were confirmed by the immunocytochemistry assay for iNOS. A significant reduction of MMP-1,3,13 gene expression was induced by canakinumab. Our experiments confirmed the pro-apoptotic effect of TNF-α and demonstrated a protective role of canakinumab. The results concerning biochemical data were further confirmed by the morphological findings obtained by TEM. We showed that canakinumab counteracts the negative effects of TNF-α on OA chondrocyte cultures and may have a potential chondroprotective role in OA.

1. Introduction

Osteoarthritis (OA) is considered to be primarily a degenerative joint disease of hyaline cartilage, which secondarily affects subchondral bone and synovial membrane. Recent advances have been made in understanding the pathophysiology of OA and the possible factors involved in the development and progression of this disease [1–3]. An imbalance between the anabolism and the catabolism of the extracellular matrix by an increase in the catabolic activity of chondrocytes is thought to lead to the disruption of the homeostatic state [4].

In vitro and *in vivo* studies have implicated several pro-inflammatory cytokines in the pathogenesis of OA; in particular, interleukin (IL)-1β and tumor necrosis factor (TNF)-α produced by the articular chondrocytes or cells of the synovium are considered the major players [5–8]. IL-1β and TNF-α are increased in the synovial fluid, synovial membrane, subchondral bone, and cartilage in

Abbreviations: OA, osteoarthritis; IL, interleukin-1β; TNF, tumor necrosis factor-α; MMPs, metalloproteinases; NO, nitric oxide; FDA, Drug Administration; EMEA, European Medicines Agency; PG, proteoglycans; iNOS, inducible nitric oxide synthase; TEM, transmission electron microscope; ACR, American College of Rheumatology; DMEM, Dulbecco's Modified Eagle Medium; ELISA, enzyme-linked immune-sorbent assay; An, Annexin (V)-fluorescein isothiocyanate (FITC); PI, propidium iodide; MTT, 3,[4,4-dimethy thiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; DMSO, dimethyl sulfoxide; OD, optical density; EASIA, enzyme amplified sensitivity immunoassay; PBS, phosphate-buffered saline; BSA, bovine serum albumin; NGS, normal goat serum; qPCR, quantitative PCR; ACTB, actin-beta; RQ, relative quantities; ABB, annexin binding buffer; PS, phosphatidylserine; NSAID, nonsteroidal anti-inflammatory drug; DMOAD, disease-modifying OA drug; IL-1 Ra, IL-1 R antagonist; TGFβ, transforming growth factor beta.

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http://dx.doi.org/10.1016/j.cyto.2014.10.023
1043-4666/

OA patients [9]. In response to IL-1β and TNF-α, chondrocytes secrete proinflammatory cytokines, chemokines, neutral metalloproteinases (MMPs), and nitric oxide (NO) [9,10]. Furthermore, these cytokines inhibit chondrocyte proliferation and induce apoptosis [9,11,12]. Several studies have shown that IL-1β and TNF-α block the normal production of cartilage matrix components (collagen type II and aggrecans) [13–15]. These data suggest that blocking IL-1β or TNF-α could be beneficial in counteracting the degradative mechanisms associated with OA pathology [16–19]. In particular, the use of IL-1β inhibitors *in vitro* and *in vivo* in animal models of OA suggests that IL-1β can be considered a most interesting potential new target for OA treatment [20–22].

Thus, therapies to decrease local IL-1β levels, or to block its specific receptors, or to interrupt its signal transduction possess significant clinical value in OA.

Canakinumab (ACZ885, Ilaris) is a human IgGκ monoclonal antibody developed by Novartis for the treatment of immune disorders. Its mode of action is based on the neutralization of IL-1β signaling, resulting in the suppression of inflammation in patients with disorders of autoimmune origin.

This agent was recently approved by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMEA) [23]. The drug is currently being evaluated for its potential in the treatment of rheumatoid arthritis, systemic-onset juvenile idiopathic arthritis, chronic obstructive pulmonary disease, type 1 and 2 diabetes, and ocular diseases [24–27]. Reports from clinical trials suggest that canakinumab is well tolerated in most patients, and no serious adverse effects have been reported [23,28].

This study aimed to evaluate the possible *in vitro* effect of canakinumab on cultures of human OA chondrocytes incubated in the presence or absence of TNF-α. In particular, we evaluated the cell viability, NO, and proteoglycans (PG) in culture medium, the expression of inducible nitric oxide synthase (iNOS) and MMP-1, MMP-3, and MMP-13, and the percentage of apoptosis and necrosis. Finally, we carried out a morphological assessment using a transmission electron microscope (TEM).

2. Materials and methods

2.1. Cell culture

Human articular cartilage fragments were obtained from the femoral heads of five patients with hip OA (mean age of 68 years), defined according to the clinical and radiological criteria of the American College of Rheumatology (ACR) [29], who underwent surgery for total hip prostheses. The study protocol was approved by the Ethics Committee of the Azienda Ospedaliera Universitaria Senese/Siena University Hospital (decision no. 726/07). Written consent was signed by each participant in this study. These samples were sufficient to obtain a large number of cell cultures to perform the study protocol. The cartilage was not macroscopically altered, but a histological study of representative samples showed typical OA changes, such as the presence of chondrocyte clusters, loss of metachromasia, and macroscopic focal fibrillation of the articular surface.

After surgery, the cartilage was dissected aseptically and crushed into small pieces. The fragments were washed in Dulbecco's Modified Eagle Medium (DMEM) with phenol red, containing 2% penicillin/streptomycin solution and 0.2% amphotericin B (GIBCO/Invitrogen, Grand Island, NY, USA). The chondrocytes were isolated from the articular cartilage using sequential enzymatic digestion: 30 min with 0.1% hyaluronidase, 1 h with 0.5% pronase, and 1 h with 0.2% collagenase (Sigma-Aldrich, Milano, Italy) at 37 °C in the wash solution (DMEM + penicillin/streptomycin solution + amphotericin B). The resulting cell suspension was filtered twice using 70 μm nylon meshes, then washed and centrifuged for 10 min at 700*g*. As shown by the Trypan blue viability test, 90% to 95% of the recovered cells were alive.

The primary cultures of chondrocytes so obtained were maintained in an atmosphere of 5% CO_2 in the air at 37 °C for 2 weeks.

2.2. Treatments

The first passage chondrocytes were seeded in 24-well plates at a starting density of 4×10^4 cells/well and overlaid with 2 ml of medium with phenol red containing 10% fetal calf serum, 200 U/ml penicillin, 200 U/ml streptomycin, and 2 mM glutamine (GIBCO/Invitrogen Grand Island, NY USA) until they became confluent. The cells were then incubated with two concentrations (1 μg/ml and 10 μg/ml) of canakinumab (ACZ885, Ilaris, developed by Novartis) alone or in combination with TNF-α (recombinant human TNF-α, DBA Italy) at a concentration of 10 ng/ml for 48 h [30]. After 24 h, for every sample 1 ml of the medium was collected and stored at −80 °C to determine the concentration of IL-1β through an enzyme-linked immune-sorbent assay (ELISA) kit (see below). At the end of the treatment, the remaining media were removed and immediately detected for NO release using Griess's assay and then stored at −80 °C for detection of PG. The chondrocytes were immediately processed for cell viability, immunocytochemistry, detection of MMPs and Annexin V (AnV)-fluorescein isothiocyanate (FITC)/propidium iodide (PI) assay, and morphological assessment using TEM.

2.3. Cell viability

Before and after each experimental condition, we evaluated cell viability by MTT (3,[4,4-dimethy thiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) (Sigma-Aldrich, Milano, Italy) assay based on the ability of the mitochondria of viable cells to convert soluble MTT into an insoluble purple formazan reaction product. MTT (5 mg/ml in DMEM without phenol red) was added to cells in tissue culture and incubated for 4 h. The media were then discarded, and 0.2 ml of dimethyl sulfoxide (DMSO) was added to each well to solubilize the formazan crystals that had formed. The absorbance was measured at 570 nm using a microplate reader (BioTek Instruments, Inc., Winooski, Vermont, USA).

The percentage of cell survival was calculated as follows:

$$\%\ \text{Survival} = \frac{\text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

The experiments were carried out on pre-confluent cell cultures to prevent contact inhibition from influencing the results. Cellular viability was assayed 48 h from the beginning of treatment. Results were expressed as optical density (OD) units per 10^4 adherent cells.

2.4. IL-1β assay

To confirm that TNF-α stimulates the production of IL-1β, we examined the concentration of IL-1β in the culture medium using a commercially available standard sandwich ELISA (Boster Biological technology Co., ELISA Kits, Fremont, CA). Samples were assayed in triplicate following the manufacturer's guidelines. According to the manufacturer, the kit provides a valid measure of the levels of IL-1β. The substrate turnover was determined colorimetrically; the microplate was read at 450 nm using a microplate reader (Bio-Tek Instruments, Inc., USA), and the absorbance rate, proportional to the IL-1β concentration, was measured. The sensitivity of the method was estimated to be <0.15 pg/ml. The results obtained for the different culture supernatants were normalized per 10^6 adherent cells. The IL-1β amount was expressed as pg/mL [31].