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Potential antipsoriatic effect of chondroitin sulfate through inhibition of NF-κB and STAT3 in human keratinocytes

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ABSTRACT

Chondroitin sulfate (CS) is a natural glycosaminoglycan, formed by the 1–3 linkage of D-glucuronic acid to N-acetylgalactosamine, present in the extracellular matrix. It is used as a slow acting disease modifying agent in the treatment of osteoarthritis, and part of its beneficial effects are due to its antiinflammatory properties that result from an inhibitory effect on NF-κB signaling pathway. This ability raises the hypothesis that CS might be effective in other chronic inflammatory processes such as psoriasis, in which a deregulation of NF-κB is a key feature. In addition, psoriasis is characterized by an upregulation of STAT3 signaling pathway that is related to the epidermal hyperplasia. In the present study we report the pharmacological modulation of the NF-κB and STAT3 signaling pathways by CS in normal human keratinocytes. CS inhibited NF-κB activation and the release of some of the key psoriatic cytokines such as TNFα, IL-8, IL-6 and CCL27. Moreover, it impaired STAT3 translocation to the nucleus and significantly reduced STAT3 transcriptional activity by a mechanism that was independent from STAT3 phosphorylation. Our results confirm the interest of CS as a candidate for future drug research in the therapeutics of psoriasis given the need of more effective and safer oral medications for these patients.

1. Introduction

Psoriasis is a chronic inflammatory skin disorder characterized by inflammation in dermis and epidermis, keratinocyte hyperproliferation, leukocyte infiltration and dilatation and growth of blood vessels. Although the etiology of psoriasis supports a role for genetic and environmental factors, immune-mediated inflammatory processes, involving both innate and adaptive immunity effector mechanisms drive this pathology [1]. Among all the signaling pathways involved in skin inflammation, the transcription factor NF-κB has been described as a crucial player in the pathogenesis of psoriasis. Thus, activation of the NF-κB pathway in psoriatic skin leads to the transcription of numerous genes including cytokines, such as TNFα, chemokines and growth factors, which are involved in the characteristic cutaneous symptomatology of this disease [2]. Activation of NF-κB induces the production of proteins, such as TNFα, which are, in turn, able to stimulate the signal transduction pathway to activate NF-κB, thus conforming a vicious cycle [2]. In fact, TNFα is one of the most important cytokines associated with innate immunity. It controls and regulates the expression of numerous genes, and in doing so leads to cutaneous responses in psoriasis. The intracellular events from the TNFα receptor to NF-κB activation in epidermal keratinocytes have been extensively studied [3,4] and recently developed anti-inflammatory therapies based on blocking TNFα signaling have been shown to be effective in the treatment of psoriasis and could become a highly promising option for this pathology [1,5].

Together with the infiltrated cells, keratinocytes contribute to the chronification of the inflammatory state by producing chemokines, such as IL-8 and CCL27/CTACK (cutaneous T cell-attracting chemokine) in psoriatic plaques, setting up a positive feedback loop which enhances cell recruitment [6,7]. IL-8 stimulates chemotaxis and degranulation of neutrophils, induces angiogenesis and modulates several functions of keratinocytes, including HLA-DR expression and proliferation [6]. CCL27/CTACK is a chemokine constitutively expressed by keratinocyte and highly upregulated in inflammatory skin diseases. This chemokine induces

Abbreviations: CS, chondroitin sulfate; EMSA, electrophoretic mobility shift assay; NHK, normal human keratinocyte; OA, osteoarthritis; STAT, signal transducers and activators of transcription; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; CCL27/CTACK, cutaneous T cell-attracting chemokine.

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inflammation by promoting Th1 and Th2 lymphocyte migration into the skin [7–9] and can be induced in cultured keratinocytes by TNFα, which is also known to induce activity of the transcription factor NF-κB [9,10].

Other cytokines and growth factors upregulated in psoriasis, such as IL-6 and the IL-20 family cytokines, signal through activation of the signal transducer and activator of transcription 3 (STAT3) [11–13]. In fact, increased phosphorylation of STAT3 (pSTAT3) has been observed in lesional skin of psoriatic patients, and transgenic mice with keratinocytes expressing constitutively active STAT3 develop skin lesions strikingly similar to human psoriatic plaques and constitute a mouse model for this pathology [14]. Moreover, STAT3 regulates the expression of genes that mediate survival (survivin, bclxl, mcl-1), proliferation (c-fos, cmyc, cyclinD1), invasion (MMP-2) and angiogenesis (VEGF) through collaboration with other transcription factors, including NF-κB [15].

Chondroitin sulfate (CS) is a natural glycosaminoglycan (GAG) present in the extracellular matrix surrounding cells, especially in the cartilage, skin and blood vessels, where it forms an essential component of proteoglycans. It is formed by the 1–3 linkage of D-glucuronic acid to N-acetylgalactosamine and the disaccharide units are attached by β 1–4 galactosamine links. The galactosamine residues are sulfated either in position 4 (Δdi-4S), 6 (Δdi-6S) or 4 and 6 (Δdi-4,6S). The sulfate groups along with the carboxyl groups of glucuronic acid are ionized, conferring a negative charge that draws water into tissues and hydrates them [16,17].

CS has been shown to be a safe drug with a positive global effect on osteoarthritis (OA) structural changes [18] and improvement of symptoms [19]. The European League Against Rheumatism (EULAR) supports the usefulness of CS in the management of knee osteoarthritis and grants the highest level of evidence to this product coupled with a very low level of toxicity, confirming CS as one of the safest drugs for osteoarthritis treatment [20]. The beneficial effect of CS on patients with OA results from different effects on articular tissues, primarily its immune-modulatory profile, mediated by the inhibition of NF-κB nuclear translocation and the decrease in the production of proinflammatory cytokines such as IL-1β and TNFα [17], among others. These properties raise the hypothesis that CS might be effective in other chronic inflammatory processes in which a deregulation of NF-κB is a key feature such as psoriasis [5].

Preliminary clinical evidence suggesting the possible antipsoriatic effect of CS has been reported. In a series of 11 patients with knee OA and concomitant psoriasis, the use of CS as a symptomatic treatment resulted in a marked clinical and histological improvement of the psoriatic lesions [21]. Furthermore, in a double-blind, placebo-controlled clinical trial, the daily administration of CS for 3 months to patients with knee OA, reduced the incidence of concomitant plantar psoriasis [22]. In light of this evidence, we sought to elucidate the inhibitory effect of CS on several biomarkers of psoriatic skin. In the present study, we show that CS effectively downregulates NF-κB and STAT3 signaling pathways in human primary keratinocytes, limiting, thus, the release of proinflammatory cytokines and chemokines, which have been implicated in the pathogenesis of psoriasis.

2. Materials and methods

2.1. Isolation, culture and stimulation of primary human keratinocytes

All protocols and procedures were approved by the University of Valencia Ethical Committee and were carried out according to the Declaration of Helsinki Principles. Primary human keratinocytes were isolated from foreskins of healthy young donors essentially as described previously [23]. Briefly, skin samples were treated with a dispase solution and trypsinized. The resultant keratinocytes were grown at 37 °C in a humidified atmosphere with 5% CO_2 in a serum-free low-Ca^{2+} (<0.1 mM) defined keratinocyte-SFM (Invitrogen, Carlsbad, CA). The medium was renewed every other day.

For all experiments, cells were seeded at passage numbers 1–3 and treated upon reaching 60–80% confluence. The day before the experiments, medium was replaced to growth factor-free keratinocyte medium (KBM), and the cells were incubated for another 24 h before stimulation. Prior to the addition of the stimulus, KBM was renewed and the cells were subjected to 24 h pretreatment with CS (CS Bio-ActiveTM, Bioibérica S.A., Barcelona, Spain), dissolved in sterile water and added to the culture medium in a ratio of 1% vehicle. Reference molecules such as dexamethasone (1 μM) and the proteasome inhibitor MG-132 (1 μM) (Sigma–Aldrich, St. Louis, MO) or the Jak2 Inhibitor "1,2,3,4,5,6-hexabromocyclohexane" (50 μM) (Calbiochem, San Diego, CA) were preincubated during 30 min in the same conditions. Cells were stimulated with either IL-6 (50 ng/ml) or TNFα (10 ng/ml) from R&D Systems (Abingdon, UK), or 12-*O*-tetradecanoylphorbol-13-acetate (TPA, 1 μg/ml) from Sigma–Aldrich (St. Louis, MO). After the desired stimulation time, supernatants were collected to assess cytokine production and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed in the remaining keratinocytes to assess the possible effects of CS on cell viability [24]. TNFα, CCL27, (R&D Systems, Abingdon, UK) and IL-6, IL-8 (eBioscience, San Diego, CA) levels were assessed using ELISA assays following standard manufacturer protocols. In some experiments, cells were collected in order to obtain the protein extract to assess protein expression.

2.2. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as described previously [25]. Cells were washed in ice-cold PBS and then treated with a lysis buffer pH 7.4 (10 mM HEPES, pH 8, 1 mM EDTA, 1 mM EGTA, 10 mM KCl, 1 mM dithiothreitol, 5 mM NaF, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 0.1 μg/ml aprotinine, and 0.5 mM phenylmethyl sulfonyl fluoride) for 15 min, followed by addition of Nonidet P-40 10%. After vigorous vortexing, nuclei were pelleted by centrifugation at 13000 × *g* for 1 min at 4 °C. Nuclear pellets were incubated at 4 °C for 30 min in lysis buffer supplemented with 400 μM NaCl. After centrifugation at 13000 × *g* for 5 min at 4 °C, supernatants were collected. Protein concentration of the nuclear extracts was determined by Bio-Rad DC Protein Assay (Richmond, CA). Double-stranded oligonucleotides containing the consensus NF-κB sequence (Promega Corp., Madison, WI) were end-labeled using T4 polynucleotide kinase (GE Healthcare, Wessling, Germany) and [γ-^{32}P] ATP, followed by purification on G-25 microcolumns (GE Healthcare, Wessling, Germany).

Binding to the consensus sequence was achieved by incubating 10 μg of nuclear extract, 100000 cpm of the labeled probe and 2 μg of poly(dI-dC) in binding buffer (40 mM HEPES pH 8.1, 0.2 mM EDTA, 100 mM NaCl, 2 mM dithiothreitol, 10% (v/v) glycerol) for 20 min at room temperature. Complexes were then analyzed by nondenaturing 6% polyacrylamide gel electrophoresis in 45 mM Tris–borate buffer (pH 8.3) containing 1 mM EDTA, followed by autoradiography of the dried gel using a Typhoon Imager-9400 (GE Healthcare, Wessling, Germany).

2.3. Western blotting

Cell cultures were washed in ice-cold PBS and lysed [25]. The lysates were then sonicated and centrifuged. Supernatants were collected and protein concentrations were determined by