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Serglycin is involved in inflammatory response in articular mouse chondrocytes

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Serglycin is expressed by a variety of cell types and mediates different functions in both normal and pathological conditions by interacting with different biological molecules, such as the CD44 receptor. Many studies suggest that serglycin has a crucial role in inflammatory response, but there are limited data on the functions of this proteoglycan in chondrocytes.

In this study we investigated the effect of serglycin knockdown induced by a specific serglycin small interfering RNA (SRGN siRNA) in normal mouse chondrocytes stimulated with lipopolysaccharide (LPS).

LPS administration in normal chondrocytes increased the expression of serglycin mRNA and related protein and the production of the pro-inflammatory mediators TNF-alpha, IL-1beta, IL-6, iNOS and MMP-9, through NF-kB activation. In addition, a marked increased expression of CD44 after LPS stimulation was observed. Notably, the CD44 expression and the inflammatory response were significantly reduced by SRGN siRNA treatment in LPS treated chondrocytes. Similar results were obtained in normal mouse chondrocytes exposed to LPS, using a specific blocking antibody against CD44.

These results indicate that serglycin produced in LPS-induced inflammation in normal mouse chondrocytes is able to modulate inflammation by interacting with CD44 receptor, suggesting a possible key role in the cartilage inflammation.

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1. Introduction

Serglycin is a small proteoglycan, consisting of a core protein containing serine/glycine repeats. Glycosaminoglycans (GAGs) linked to protein core of serglycin may be chondroitin sulfate (CS), heparan sulfate (HS) and heparin. The type and extension of sulfation of GAGs chains vary between different species and cell types, and are determinant for the serglycin biological functions [1]. Serglycin is mainly expressed in hematopoietic cells, where it is involved in the intracellular storage of different compounds for secretion, apoptosis, immune responses, and blood coagulation [1].

Serglycin is present in intracellular secretory granules, but can be secreted into the extracellular matrix [2]. Different inflammatory stimuli, such as tumor necrosis factor-alpha (TNF-α) in endothelial cells and adipocytes [3,4], lipopolysaccharide (LPS) in macrophages [5] and interleukin 1β (IL-1β) in smooth muscle cells [6], stimulate the expression of such PG.

Many of the functions of serglycin are due to its ability to interact with numerous inflammatory mediators such as proteases, cytolytic enzymes, grow factors, cytokines and chemokines [7].

Toyama-Sorimachi et al. [8] have showed that serglycin is a ligand for the cluster determinant 44 (CD44), the main receptor for hyaluronan, that is widely distributed on several cell types. The binding is especially mediated through CS chains of serglycin, suggesting a pivotal role of these chains in the CD44 stimulation induced by serglycin.

CD44 participates to important processes such as cellular adhesion, hyaluronate degradation, angiogenesis, lymphocyte recruitment and homing. Therefore it has been proposed that CD44 plays a number of roles in both physiological and pathological processes including metastatic spread of tumors and chronic inflammation [9]. CD44 signal transduction cascade includes

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different members of the PKC family, which let IκBα phosphorylation and the consequent NF-kB translocation into the nucleus [10,11]. NF-kB activation induces, in turns, the transcription of different genes including cytokines and other detrimental inflammatory mediators [12].

Chondrocytes play an active role in the inflammatory process that leads to the cartilage damage and degradation, through the secretion of inflammatory cytokines, such as IL-1β, interleukin 6 (IL-6) and TNF-α [13], proteolitic enzymes and catabolic mediators such as nitric oxide (NO) [14].

Chondrocytes may respond to a variety of stimuli, including LPS, a classical pathogen-associated molecular pattern (PAMPs), that induces the innate immune response through the stimulation of Toll-like receptor-4 (TLR-4). The activation of such receptor triggers a signaling pathway that activates NF-kB, with consequent increased expression of pro-inflammatory mediators that modulate the inflammatory response [15].

As recent studies have showed that serglycin is also synthesized by other cell types, including chondrocytes [16], we aimed this study in order to evaluate the effect of LPS stimulation on serglycin expression in normal and serglycin knockdown mouse chondrocyctes. The pro-inflammatory effect of serglycin was also investigated by using a specific CD44 blocking antibody.

2. Materials and methods

2.1. Cell culture

Normal knee chondrocytes (DPK-CACC-M, strain: C57BL/6 J; Dominion Pharmakine, Spain) were cultured in 75-cm^2 plastic flasks containing DMEM/F12 with 10% FBS, L-glutamine (2.0 mM) and penicillin/streptomycin (100 U/ml, 100 mg/ml) (Sigma-Aldrich, Italy), and were incubated at 37 °C in humidified air with 5% CO_2. At confluence, chondrocytes were trypsinized, subdivided, and replated. In the present experiments chondrocytes from passage 4 to 7 were used.

2.2. Cell treatment

For siRNA experiments, cells were seeded in six-well plates (2×10^5 cells/well), so as to be confluent to about 80% after 24 h. Then, chondrocytes medium was replaced with OPTIMEM (Gibco/Brl, USA) and cells were transfected with SRGN siRNA (50 pmol/well) using the RNAiMAX in according to manufactory protocol (Invitrogen, USA). Scrambled siRNA (Invitrogen, USA) was used under the same conditions in other wells, as a negative control. After 24 h the medium was replaced with 1% FBS chondrocyte medium without antibiotics and then added with 25 μg/ml anti-CD44 specific blocking antibody (R&D Systems, USA). After 1 h cells was treated with LPS from *Salmonella enterica* serotype typhimurium (1 μg/ml) (Sigma-Aldrich, Italy). Finally, the chondrocytes and medium were collected for biochemical evaluation 24 h after the last treatment.

2.3. RNA isolation, cDNA synthesis, and quantitative reverse transcription-PCR amplification (qRT-PCR)

Total RNA was extracted from the samples using Trizol reagent (Invitrogen, USA) and retro-transcripted using a High Capacity Retrotranscript cDNA Synthesis Archive kit (Applied Biosystems, USA) following the manufacturer's protocols. qRT-PCR reactions were performed in order to evaluate gene expression of serglycin, CD44, TNF-α, IL-1β, IL-6, iNOS, MMP-9 and β-actin by means of ready-to-use assays (Assays on demand, Applied Biosystems, USA). Reactions were carried out by using a Taqman PCR master mix (Applied Biosystems, USA) and a Real Time PCR system model 7500 (Applied Biosystems, USA). After normalization against β-actin, used as endogenous control, the mean value of untreated chondrocyte target levels was chosen as the calibrator and the results were expressed according to the $2^{-\Delta\Delta Ct}$ calculation, as fold change relative to normal controls.

2.4. Protein extraction and western blot assay

For SDS–PAGE and western blotting, both chondrocytes and culture supernatants were collected. In order to evaluate CD44 and iNOS protein levels, cells were washed in PBS and the proteins were extracted by using RIPA buffer with 1 nM PMSF and protease inhibitor cocktail (Sigma-Aldrich, Italy). To assay serglycin expression, supernatants were centrifuged at 3000 rpm for 5 min and concentrated with Amicon Ultra 10 K centrifugal filter devices (Merk Millipore, USA). The concentrated samples were treated with 0.02 units of chondroitinase ABC from *Proteus Vulgaris* (Sigma-Aldrich, Italy) in 50 mM Tris-HCl 50 mM sodium acetate, pH 7.5 at 37 °C for 2 h. Aliquots of the protein (10 μg) were mixed to Laemmli buffer (Biorad, USA) with β-mercaptoethanol and separated on a mini gel (10–12%). Then the proteins were electroblotted to PVDF membranes (Amersham, UK). The blots were blocked with 5% bovine serum albumin (BSA) in TBS, 0.1% Tween-20 (TBS-T), for 1 h and were then incubated with a rabbit polyclonal anti-serglycin (1 μg/ml) (Abcam, UK), a rabbit polyclonal anti-CD44 (1 μg/ml) (R&D Systems, USA) and a rabbit polyclonal anti-iNOS (diluted 1:500) (Abcam, UK) in 5% BSA, TBS-T, at 4 °C overnight. The membranes were washed and then incubated with a goat anti-rabbit antibody (Pierce Biotechnologies, USA) in TBS-T. After washing with TBS-T, the proteins were made visible using enhanced chemiluminescence (ECL; Amersham, UK). Protein band density quantification was calculated using Odissey software and comparing β-actin protein as an endogenous control for the normalization of CD44 and iNOS proteins.

2.5. NF-kB p50/65 transcription factor assay

NF-kB activation was assayed by a commercial kit (Merk Millipore, USA) consisting in the evaluation of NF-kB p50/65 subunits DNA binding activity on nuclear extracts, according to the manufacturer's protocol. Chondrocyte nuclear extracts were obtained by using NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce Biotechnologies, USA).

2.6. IL-1β, TNF-α, IL-6 and MMP-9 ELISA kits

Culture cell medium was centrifuged at 3000 rpm for 5 min in presence of 1 nM PMSF and protease inhibitor cocktail. The supernatants were assayed for IL-1β, TNF-α, IL-6 and MMP-9 determination by using specific commercial ELISA kits (R&D Systems, USA), according to the manufacturer's protocol.

2.7. Protein determination

The amount of total protein was determined using Coomassie Plus-Bradford Assay™ Kit (Bio-Rad Lab., USA) using bovine serum albumin as a standard.

2.8. Statistical analysis

All data were analysed by one way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test. The statistical significance of differences was set at $P < 0.05$. Data are expressed as the mean ± S.D. values of at least seven experiments for each test.

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