Biochimie 92 (2010) 204–215

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

Molecular size hyaluronan differently modulates toll-like receptor-4 in LPS-induced inflammation in mouse chondrocytes

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ARTICLE INFO

Article history:
Received 26 June 2009
Accepted 20 October 2009
Available online 29 October 2009

Keywords:
Hyaluronan
Lipopolysaccharides
Cytokines
Chondrocytes
NF-kB factor

ABSTRACT

Hyaluronan (HA) action depends upon its molecular size. Low molecular weight HA elicits pro-inflammatory responses by modulating the toll-like receptor-4 (TLR-4) or by activating the nuclear factor kappa B (NF-kB). In contrast, high molecular weight HA manifests an anti-inflammatory effect via CD receptors and by inhibiting NF-kB activation. Lipopolysaccharide (LPS) –mediated activation of TLR-4 complex induces the myeloid differentiation primary-response protein (MyD88) and the tumor necrosis factor receptor-associated factor-6 (TRAF-6), and ends with the liberation of NF-kB/Rel family members. The aim of this study was to investigate the influence of HA at different MWs (low, medium, high) on TLR-4 modulation in LPS-induced inflammatory response in mouse chondrocyte cultures.

Messenger RNA and related protein levels were measured for TLR-4, MyD88, and TRAF-6 in both untreated and LPS-treated chondrocytes, with and without the addition of HA (two doses for each MW). NF-kB activation, TNF-α and IL-1β levels, matrix metalloprotease-13 (MMP-13), and inducible nitric oxide synthase (iNOS) gene expression were also evaluated.

LPS increased all the parameters studied as well as NF-kB activation. Low MW HA upregulated TLR-4 expression, increased MyD88 and TRAF-6 and the inflammation mediators in untreated chondrocytes, and it enhanced the LPS effect in LPS-treated cells. Medium and high MW HA exerted no activity in untreated cells and only the latter reduced the LPS effects. Specific TLR-4 blocking antibody was utilised to confirm TLR-4 as the target of HA action.

These findings suggest that the regulatory effect exerted by HA (at any MW) on NF-kB activation may depend upon the interaction between HA and TLR-4 and HA may thereby modulate pro-inflammatory activity via its different state of aggregation.

1. Introduction

Cartilage consists of an extensive extracellular matrix and provides mechanical stability and resistance to load. Cartilage homeostasis is orchestrated and finely tuned by the chondrocytes via communications with their surrounding matrix environment [1].

The degradation of the extracellular matrix in articular cartilage is a key event that leads to joint destruction in many erosive diseases, including rheumatoid arthritis, osteoarthritis and septic arthritis. Chondrocytes respond to a variety of stimuli, such as pro-inflammatory cytokines and mechanical loading, by elaborating degradative enzymes and catabolic mediators [1]. Cartilage erosion is also associated with an increased expression of mediators of inflammation, for example nitric oxide (NO), interleukin-1beta (IL-1β), and tumor necrosis factor alpha (TNF-α) [2]. NO is involved in the stimulation of metalloproteinases (MMPs) mRNA expression

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; EDTA, ethylenediaminetetraacetic acid; FBS, foetal bovine serum; GAGs, glycosaminoglycans; HA, hyaluronan; HMWHA, high molecular weight hyaluronan; HRP, horseradish peroxidase; IL-1beta, interleukin-1 beta; iNOS, inducible nitric oxide synthase; LMWHA, low molecular weight hyaluronan; LPS, lipopolysaccharide; MMPs, metalloproteases; MMWHA, medium molecular weight hyaluronan; MyD88, myeloid differentiation primary-response protein; MW, molecular weight; NF-kB, nuclear factor-kB; NO, nitric oxide; OD, optical density; PAMPs, pathogen-associated molecular pattern; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PGs, proteoglycans; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TBP, tris buffered phosphate; TBP, tributylphosphine; TBS, tris buffered saline; TLR-4, toll-like receptor-4; TNF-α, tumor necrosis alpha; TRAF-6, tumor necrosis factor receptor-associated factor-6.

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doi:10.1016/j.biochi.2009.10.006

and activity, and MMP-13 seems, in particular, to play a key role in extracellular matrix degradation [3,4]. It is widely accepted that IL-1β and TNF-α are pro-inflammatory cytokines that are deeply involved in articular cartilage destruction as well as in the inflammatory response in arthritis. Biologics that inhibit the signalling cascade mediated by both of these cytokines have been effective in treating erosive pathologies by reducing both inflammation and cartilage destruction [5,6]. However, blocking IL-1β and/or TNF-α does not lead to total protection of the joint structure, indicating that other signalling pathways that mediate joint catabolism have still to be elucidated [5,6].

Toll-like receptors (TLRs) are critical components in the innate immune response based on their ability to recognize pathogen-associated molecular patterns (PAMPs) [7]. These receptors are key sensors of microbial products and are expressed in the sentinel cells of the immune system, in particular dendritic cells and macrophages, where they sense a range of chemical produced by viruses, bacteria, fungi and protozoa [7,8]. The activation of signalling pathways by TLRs, through the various molecular components of the microbes, represents one of the body's earliest signals that it has been invaded by a foreign microorganism. Thirteen TLRs have been identified so far; of these, TLR1, 2 4, 5, 6 and 11 are displayed on the cell surface, while TLR3, 7, 8 and 9 are localized intracellularly [9]. After ligand binding, the TLRs dimerize and undergo the conformational change required for the recruitment of downstream signalling molecules. The latter include the adaptor molecule myeloid differentiation primary response protein 88 (MyD88), Il-1R-associated kinases (IRAKs), transforming growth factor-beta (TGF-β) activated kinase (TAK-1), TAK-1 binding protein (TAB1 and TAB 2), and tumor necrosis factor (TNF)-receptor-associated-factor-6 (TRAF-6) [10]. Tumor necrosis factor associated factors (TRAFs) are intracellular adaptor proteins that are proximal signal transducers for the TNFR superfamily [11]. Many of the physiological effects of TRAF-6 are mediated by activation of the IkB kinase complex and MAPK members which then regulate transcription of genes via NF-kB and AP1. The role of TRAF-6 in TLR signalling, seems to be particularly selective between signalling pathways stimulated by TLR-4 activation [12].

Hyaluronan (HA) is a major non-sulphated glycosaminoglycan of the extracellular matrix that has been shown to undergo rapid degradation at inflammation sites resulting in the accumulation of lower molecular weight HA fragments [13,14]. It has been reported that low molecular weight degradation products of HA may elicit various pro-inflammatory responses, such as the activation of murine alveolar macrophages as well as the stimulation and invasion of macrophages into affected joints in rheumatoid arthritis [15,16]. Other reports have shown that low molecular weight HA oligosaccharides induced a complete and irreversible phenotypic and functional maturation of human dendritic cells, while high molecular weight HA had no such effect [16].

Lipopolysaccharide (LPS)-mediated activation of the TLR-4 complex was found to induce specific signalling pathways, involving a series of protein mediators, such as MyD88 and TRAF-6, that led to the liberation of NF-kB/Rel family members into the nucleus [17]. However, activation of the TLR-4 receptor complex is not limited to LPS, and other pro-inflammatory stimuli such as Heat-Shock Protein 70 [18] and HA have been described as alternative ligands [19,20].

Interestingly, the effect of HA on the inflammatory response appears to be related to its molecular size, i.e. larger hyaluronan has anti-inflammatory activity while smaller hyaluronan has pro-inflammatory activity [21–23].

Starting from the above data the aim of this study was to investigate whether different MWs of HA (low, medium and high) have any influence on TLR-4 modulation in LPS-induced inflammation in mouse chondrocyte cultures.

2. Methods

2.1. Materials

HA sodium salt at low MW (50 kD, HYA-50K-1 SelectHA™50K), and at medium MW (1000 kD, HYA-1000K-1 SelectHA™1000K) were obtained from NorthStar Bioproducts (East Falmouth, USA), while high MW HA (5000 kD, HEALON) was purchased from Pharmacia Corporation, (Kalamazoo, USA). LPS from salmonella enteritidis was obtained from Sigma–Aldrich S.r.l. (Milan, Italy). Mouse TNF-α, IL-1β, inducible nitric oxide synthetase (iNOS), TLR-4, MyD88, TRAF-6 and MMP-13 monoclonal antibodies and Horseradish peroxidase-labeled goat anti-rabbit antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against TLR-4/MD-2 complex to block TLR-4 receptors and inhibit LPS-induced cytokine production were also supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS), L-glutamine, penicillin/streptomycin, trypsin-EDTA solution and phosphate buffered saline (PBS) were obtained from Gibco Brl (Grand Island, NY, USA). All cell culture plastics were obtained from Falcon (Oxnard, CA, USA). RNase, proteinase K, protease inhibitor cocktail, sodium dodecylsulphate (SDS) and all other general laboratory chemicals were obtained from Sigma–Aldrich S.r.l. (Milan, Italy).

2.2. Cell cultures

Normal mouse knee chondrocytes (DPK-CACC-M, strain: C57BL/6J, Dominion Pharmakine, Bizkaia, Spain) were cultured in 75 cm^2 plastic flasks containing 15 ml of DMEM to which was added 10% FBS, L-glutamine (2.0 mM) and penicillin/streptomycin (100 U/ml, 100 μg/ml), and were incubated at 37 °C in humidified air with 5% CO_2. Experiments were performed using chondrocyte cultures between the third and the fifth passage.

2.3. LPS stimulation and HA treatment

Chondrocytes were cultured in six-well culture plates at a density of 1.3×10^5 cells/well. Twelve hours after plating (time 0) the culture medium was replaced with 2.0 ml of fresh medium containing LPS at concentrations of 2.0 μg/ml. Four hours later, LMWHA, MMWHA or HMWHA was added using two different doses of 0.1 and 0.2 mg/ml for each MW. A separate set of plates was first treated with LPS and 2 h later with a specific antibody against TLR-4/MD-2 complex. HA was added 2 h after the antibody treatment. In LPS-stimulated chondrocytes treated only with the antibody, this was administered 5 min before LPS stimulation. Finally, the cells and medium underwent biochemical evaluation 24 h later.

2.4. RNA isolation, cDNA synthesis and real-time quantitative PCR amplification

Total RNA was isolated from chondrocytes for reverse-PCR real time analysis of TNF-α, IL-1β, iNOS, TLR-4, MyD88, TRAF-6 and MMP-13 (RealTime PCR system, Mod. 7500, Applied Biosystems, USA) using an Omnizol Reagent Kit (Euroclone, West York, UK). The first strand of cDNA was synthesized from 1.0 μg total RNA using a high capacity cDNA Archive kit (Applied Biosystems, USA). β-actin mRNA was used as an endogenous control to allow the relative quantification of TNF-α, IL-1β, iNOS, TLR-4, MyD88, TRAF-6 and MMP-13. PCR RealTime was performed by means of ready-to-use assays (Assays on demand, Applied Biosystems) on both targets and endogenous controls. The