The effect of different molecular weight hyaluronan on macrophage physiology

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Abstract

OBJECTIVES: Hyaluronan, a linear glycosaminoglycan, is an abundant component of extracellular matrix. In its native form, the high-molar-mass hyaluronan polymers have an array of structural and regulatory, mainly anti-inflammatory and anti-angiogenic, functions. In contradiction, the biological effects of fragmented low molecular weight hyaluronan are suggested to be pro-angiogenic and pro-inflammatory. METHODS: The effects of highly purified pharmacological grade hyaluronan of defined molecular weights 11, 52, 87, 250 and 970 kilodaltons were tested on mouse macrophage cell lines RAW 264.7 and MHS. The surface expression of CD44 and Toll-like receptor 2, surface receptors for hyaluronan, was determined by flow cytometry. Activation of macrophages was determined based on nitric oxide and tumour necrosis factor alpha production, inducible nitric oxide synthase expression, and the activation of the nuclear factor kappa B transcriptional factor. RESULTS: Both macrophage cell lines expressed CD44 and Toll-like receptor 2, which were significantly increased by the pre-treatment of macrophages with bacterial lipopolysaccharide. Hyaluronan of any molecular weight did not activate production of nitric oxide or tumour necrosis factor alpha in any mouse macrophage cell lines. Correspondingly, hyaluronan of any tested molecular weight did not stimulate nuclear factor kappa B activation. Similarly, hyaluronan of any molecular weight neither exerted stimulatory nor inhibitory effects on macrophages pre-treated by lipopolysaccharide. CONCLUSION: Interestingly, the data does not support the current view of low molecular weight hyaluronan as a pro-inflammatory mediator for macrophages. Further studies are necessary to clarify the effects of different molecular weight hyaluronan on phagocytes.

INTRODUCTION

Hyaluronan (HA) is a high-molecular-weight (HMW) linear glycosaminoglycan found in the extracellular matrix (ECM) and can reach a size of 6 to 8 MDa. Currently, it is suggested that pharmaceutically produced HA alone or in combination with other drugs possesses significant therapeutic potential (Dvorakova et al. 2008; Frankova et al. 2006; Taylor & Gallo 2006). Under inflammatory conditions, HA undergoes degradation, and low-molecular-weight HA (LMW) is formed (Stern et al. 2006; Valachova et al. 2008). HA directly affects cell functions through binding to different cell surface receptors, particularly CD44 and Toll-like receptors (TLR) 2 and 4, which have a different affinity to HA of various MW (Johnson et al. 2000; Pure & Cuff 2001; Termeer et al. 2003). Thus, the cell response to HA of different MW is dependent on the complex interaction of multi-
valent binding events affected mainly by the quantity, density and activation state of cell surface HA receptors (Dvorakova et al. 2008; Pure, 2001; Taylor & Gallo, 2006). In general, HMW-HA is widely reported to possess anti-angiogenic, anti-inflammatory, and immuno-suppressive properties. Contradictory to HMW-HA, the biological effects of fragmented HA are suggested to be angiogenic, inflammatory and immunostimulatory (Noble & Jiang, 2006; Taylor & Gallo, 2006; Termeer et al. 2003). However, under physiological conditions, a high turnover of HA occurs that is not accompanied by any inflammatory reactions. Thus, the role of LWM HA as a mediator of inflammatory reactions is still a matter of controversy.

Macrophages and polymorphonuclear leukocytes play a critical role in the controlling of acute and chronic tissue inflammation through the release of a variety of inflammatory mediators, including cytokines, growth factors, reactive oxygen species (ROS), and nitric oxide (NO) (Frankova et al. 2006; Gallova et al. 2004; Lojek et al. 2008; Pekarova et al. 2009; Perecko et al. 2008). It has been reported that LMW-HA may stimulate macrophages recruited at the sites of inflammation to induce gene expression and to produce important mediators of inflammation and tissue injury (inducible NO synthase (iNOS), matrix metaloproteases, production of the interleukin-1β, interleukin-12, and tumour necrosis factor (TNF)-α) (Ariyoshi et al. 2005; Horton et al. 1998; Horton et al. 2000; McKee et al. 1997; Wang et al. 2006). However, the ability of cells to respond to HA of different sizes varies with cell type and HA MW. Therefore, the potential of different highly defined MW of HA to modulate inflammatory cell physiology needs to be clarified.

In the presented study, the potential of HA to induce an inflammatory reaction was tested in vitro. The effects of HMW and LMW-HA of different size 11, 52, 87, 250 and 970 kDa were tested on mouse macrophages.

MATERIALS AND METHODS

Materials. The stock solution of lipopolysaccharide (LPS) from Escherichia coli serotype 026:B6 was 1 mg/ml in phosphate buffered solution. Alexa Fluor 488-labelled anti-mouse/human Toll-like receptor 2 (TLR2, CD282) (Clone: T2.5), PE-labelled anti-mouse/human CD44 (Clone: IM7), Alexa Fluor 488-labelled Mouse IgG1 κ Isotype Control and PE-labelled Rat IgG2a κ Isotype Control antibodies were purchased from Biolegend (Biolegend, USA). Mouse monoclonal antibody against the murine iNOS (Clone: 54) was purchased from Transduction Laboratories (Transduction Lab, USA). Horseradish peroxidase-conjugated anti-mouse IgG antibody was purchased from Millipore (Millipore, USA). All reagents without specification were purchased from Sigma-Aldrich (Sigma-Aldrich, USA). Hyaluronan. Hyaluronan of Streptococcus Equi biotechnological origin and pharmaceutical grade (Contipro C, Dolni Dobrouc, Czech Republic) was used. To prepare HA of a particular MW 1% water solution of 0.97–2.33 MDa hyaluronan was degraded by acid hydrolysis utilizing HCl at pH 3.8, 100°C for different time periods (8 hrs for MW 11.54 kDa, 60 min for 51.6 kDa, 40 min for 88.1 kDa, and 7 min for 249.5 kDa). Each product was isolated by ethanol precipitation, followed by repeated washing with isopropanol and centrifugation. The precipitate was dissolved in demi-water (1% solution) and the spray dried. MW and polydispersity were determined by SEC-MALS analysis employing the Agilent 1100 Series chromatography system (Santa Clara, CA, USA) equipped with the following column system: PL aquagel-OH Mix and PL aquagel-OH 30 (300 × 7.5 mm, 8 µm; Polymer Laboratories). Eluent (0.1 M sodium phosphate buffer pH 7.5) was monitored using a DAWN-EOS multi-angle laser light scattering photometer (18-angle, Wyatt Technologies Corporation) and refractive index detector Optilab rEX (Wyatt Technologies Corporation). Data acquisition and MW calculations were performed using Astra V software, Version 5.3.2.15. The flow rate of the mobile phase was maintained at 0.8 ml/min. The specific refractive index increment (dn/dc) of 0.155 mg/ml was used for sodium HA. The polydispersity reached 1.2–1.29 in all samples. The presence of endotoxins was tested using the Pyrogen® Recombinant Factor C Endotoxin Detection System (Cambrex, USA), which did not detect any significant amount of endotoxin (less than 0.01 EU ml⁻¹). Stock solutions of HA (10 mg/ml) of 11, 52, 87, 250 and 970 kDa were prepared in Dulbecco’s Modified Eagle Medium (DMEM, PAN - Biotech GmbH, Germany).

Cell lines. RAW 264.7 cells from the American Type Culture Collection (ATCC, USA) were maintained in DMEM supplemented with a 10% fetal bovine serum (Ultra Low Endotoxin Foetal Bovine Serum, PAA Laboratories GmbH, Austria) and gentamycin (0.045 mg/L) (SERVA electrophoresis, Germany) and harvested as described previously (Pekarova et al. 2009). MHS cells (ATCC, USA) were maintained in RPMI (PAN – Biotech GmbH, Germany) supplemented with a 10% fetal bovine serum (Ultra Low Endotoxin Foetal Bovine Serum, PAA Laboratories GmbH) and gentamycin (0.045 mg/L). Cells (10⁶ per well) were seeded on a 6-well cell culture microplate and cultivated for 4 hours before treatment by different MW HA, LPS, and their combinations.
Cell viability. The viability of cells, incubated on 12-well plates (0.5 x 10^6 per well) with HA of different MW for 24 hours, was tested using the determination of protein concentration in total cellular lysate using BCA protein assay (Pierce, USA), according to the manufacturer's instructions.

Determination of nitric oxide. The production of nitric oxide (NO) was estimated indirectly by a microtiter plate reader (SLT Rainbow spectrophotometer, TECAN, Germany) as the accumulation of nitrites in the cell culture medium using Griess colorimetric reagent (Sigma-Aldrich, USA), according to the method described previously (Pekarova et al. 2009). The nitrite accumulation was also determined by using 2,3-diaminonaphtalene fluorometric reagent (DAN, Molecular Probes) with a sensitivity as low as 10 nM. Briefly, 100 µl of the sample were incubated with DAN (20 µl of a 0.5 mg/ml solution in 0.62 M HCl) in darkness at RT for 10 minutes. Finally, 10 µl of 2.8 M NaOH were added to each sample. The fluorescence was measured with an Infinite M200 fluorometer (Tecan, Germany) with an excitation of 365 nm and emission of 450 nm wavelengths, and the nitrite concentration was calculated using sodium nitrite as a standard (Jaworski et al. 2008). Determination of nitric oxide (NO) was estimated indirectly by a microtiter plate reader (SLT Rainbow spectrophotometer, TECAN, Germany) as the accumulation of nitrites in the cell culture medium using Griess colorimetric reagent (Sigma-Aldrich, USA), according to the method described previously (Pekarova et al. 2009). The nitrite accumulation was also determined by using 2,3-diaminonaphtalene fluorometric reagent (DAN, Molecular Probes) with a sensitivity as low as 10 nM. Briefly, 100 µl of the sample were incubated with DAN (20 µl of a 0.5 mg/ml solution in 0.62 M HCl) in darkness at RT for 10 minutes. Finally, 10 µl of 2.8 M NaOH were added to each sample. The fluorescence was measured with an Infinite M200 fluorometer (Tecan, Germany) with an excitation of 365 nm and emission of 450 nm wavelengths, and the nitrite concentration was calculated using sodium nitrite as a standard (Jaworski et al. 2008).

Detection of inducible nitric oxide synthase (iNOS) by Western blot. The expression of iNOS protein was detected by Western blot analysis, as described previously (Konopka et al. 2008), using a mouse monoclonal antibody against the murine iNOS as a primary detection antibody and horseradish peroxidase-conjugated anti-mouse IgG antibody as a secondary antibody. Luciferase reporter construct and transient transfection of RAW 264.7. The reporter construct pBIX-LUC was kindly donated by Dr. Kalle Saksela (Institute of Molecular Medicine, University of Tampere, Finland) (Saksela & Baltimore, 1993) and transiently transfected into the RAW 264.7 and MHS cells using an electroporation system (Gene Pulser II, Bio-Rad Laboratories, USA) (Hyzdalova et al. 2008). Cells (10 x 10^6) were mixed with 10 µg of reporter plasmid and electroporated (270 V and 1050 µF). Subsequently, the cells were seeded on 12-well plates at a concentration 1 x 10^6 per well and cultured for 24 h in DMEM with 10% FBS. Further, transiently transfected cells were treated with different concentrations of LPS (0.1, 0.25, 0.5 µg/ml) to determine the sensitivity of the system or with HA (0.5 mg/ml). Cells were lysed with 100 µl of lysis buffer after 6 h. Activation of the reporter construct was determined by luciferase activity measurement using Luciferase Reporter Gene Assay (Roche, USA). The cell lysate 40 µl was mixed with 40 µl of luciferase substrate, and luminescence was quantified by a Lumino-meter LM-01T (Immunootech, Prague, Czech Republic) (Konopka et al. 2008).

Detection of proinflammatory cytokine TNF-α by ELISA. The production of TNF-α was detected by ELISA according to the manufacturer's instructions for the TNF-α DuoSet ELISA Development System (R&D Systems, Inc., USA). The absorbance was measured at 450 nm against a reference wavelength of 620 nm.

Determination of cell surface receptors CD44 and TLR2 expression. Both types of cell lines were incubated with LPS (5 ng/ml) for 4 hours, non-treated cells were used as a control. After the incubation time, the cells were released from the plastic by PBS with EDTA by a cell scraper, washed, counted and resuspended in PBS with 0.1% BSA. Four hundred thousand cells in 50 µl were incubated with PE labelled anti-CD44 and Alexa Fluor 488 labelled anti-TLR2 monoclonal antibodies or murine immunoglobulin of the same isotype for 30 min at 4°C. Further, cells were washed with PBS, resuspended in PBS, placed on ice and analyzed within 2 h by a flow cytometer FACScalibur (Becton Dickinson, USA), as described previously (Gallova et al. 2004).

Statistical analysis. All data are reported as means ± standard error of mean (SEM) from at least 3 independent experiments. Nonparametric Friedman ANOVA test, Statistica software (StatSoft, Inc., USA), was applied to compare differences among the control and treated groups. A p value ≤ 0.05 was considered significant (*).

RESULTS

Determinations of cell viability excluded any significant effect of evaluated HA on the viability of RAW 264.7 and MHS macrophage cell lines (data not shown). The HA mediated activation of RAW 264.7 and MHS macrophages was determined by an evaluation of NO production based on nitrite accumulation, the end product of NO metabolism, in cell culture media. The exceptional high sensitivity of employed RAW 264.7 and MHS cell lines to inflammatory stimuli was documented by a dose dependent increase of NO production in response to increasing doses of LPS from doses as low as 1-5 ng/ml (Fig. 1 A, D). However, the incubation of macrophages with 0.5 mg/ml HA of different MW did not induce any significant production of NO after 24 hours (Fig. 1 B, E). Further, the incubation of macrophages with 0.5 mg/ml HA of different MW together with LPS (1 ng/ml) did not induce any significant increase in LPS stimulated production of NO after 24 hours of incubation (Fig. 1 C, F). Similarly, the incubation of cells with HA of different MW in final concentrations of 0.1 mg/ml or 1 mg/ml did not reveal any significant activation of NO production by these macrophage cell lines (data not shown). These data were confirmed by analysis of iNOS protein expression responsible for NO production in macrophages. No HA of different MW (0.5 mg/ml) either triggered iNOS expression in unstimulated RAW 264.7 and MHS macrophages or modulated the already increased expression of iNOS in LPS (1 ng/ml) stimulated macrophages (data not shown). Corresponding to NO production, different MW-HA did not significantly potentiate basic
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Figure 1: Nitric oxide production by RAW 264.7 and MHS macrophages. The dose-dependent accumulation of nitrites in cell culture media from RAW 264.7 (A) and MHS (D) cells stimulated with different concentrations of LPS for 8 hours. The effect of tested MW HA (0.5 mg/ml) on nitrites accumulation in cell culture media from unstimulated RAW 264.7 (B) and MHS (E) cells after 24 hours. The effect of tested MW HA (0.5 mg/ml) on nitrites accumulation in cell culture media from LPS (1 ng/ml) stimulated RAW 264.7 (C) and MHS (F) cells for 24 hours. The data represent mean ± SEM (n = 3).

Figure 2: The effect of tested MW HA on TNF-α production by RAW 264.7 (A) and MHS (B) unstimulated macrophages. The data represent mean ± SEM (n = 3).

Figure 4: The effect of LPS (0.1; 0.25 and 0.5 µg/ml) and tested MW HA (0.5 mg/ml) on NF-κB activation in transiently transfected cells RAW 264.7 (A) and transiently transfected cells MHS (B). The data represent mean ± SEM (n = 4).
TNF-α production by unstimulated RAW 264.7 and MHS macrophages (Fig. 2 A, B).

The lack of both RAW 264.7 and MHS macrophage responsiveness to HA could not be explained by the absence of the main suggested surface receptors for HA CD44 and TLR2. Both cell lines expressed CD44 receptor abundantly on their surface, which was further increased by the pre-incubation of cells with LPS (Fig. 3). TLR2 was also expressed on the surface of the tested cell lines (Fig. 3). The expression of both CD44 and TLR2 increased after the pre-incubation of cells with LPS (Fig. 3).

To determine the activation of the main signalling pathways involved in inflammatory activation of macrophages, we tested the effects of HA of different MW on RAW 264.7 and MHS macrophages transfected with the NF-κB activation reporter system. LPS effectively activated transcription factor NF-κB in a dose dependent manner in both transiently transfected cells RAW 264.7 (Fig. 4 A) and MHS (Fig. 4 B), whereas different MW HA did not significantly affect NF-κB activity.

DISCUSSION

Neither the selected LMW-HA nor HMW-HA was found to posses any tendency to significantly activate or decrease the LPS induced activation of two different mouse macrophage cell lines in vitro.

Interestingly, the results obtained are controversial in regard to other studies showing that HA significantly stimulates macrophage cell lines RAW 264.7 and MHS as employed in our experimental setup. McKee et al. (1997) with co-authors found that approximately 200 kDa HA induced and markedly potentiated interferon-γ to induce iNOS expression and activity in MHS cells. The activation of macrophages was dependent on the activation of NF-κB. Interestingly, they showed that alveolar macrophages isolated from control mice responded to this HA minimally, whereas inflammatory alveolar macrophages stimulated with HA fragments exhibited a marked induction of iNOS mRNA. However, in our study, pre-incubation of macrophages with pro-inflammatory stimuli LPS did not increase the responsiveness of cells to HA of any MW. Wang et al. (2006) showed that RAW 264.7 were significantly stimulated by 500-800 kDa HA to produce TNF-α through the activation of p38 mitogen activated protein kinase, extracellular signal-regulated kinase, and c-Jun N-terminal kinase that was dependent on CD44 and TLR4 (Wang et al. 2006). Similarly, Habara et al. (2002) observed HA mediated stimulation of NO production by RAW 264.7 cells. Further, the activation of NF-κB through p38 and extracellular signal-regulated kinase activation in RAW 264.7 by LMW-HA dependent on CD44 was observed by Ariyoshi et al. (2005). Horton and colleagues observed 200 kDa HA dependent activation of matrix metalloproteinase production (Horton et al. 1999) and plasminogen activator inhibitor 1 by MHS macrophages (Horton et al. 2000). Interestingly, in conformity to our observations HA 800 and 2700 kDa of an unspecified origin inhibited production of TNF-α and other pro-inflammatory mediators, although with a different cell line, human monocyctic U937 (Yasuda, 2007). However, this leukaemia cell line has a significantly different phenotype than the macrophages employed.

In our study, we confirmed that both cell lines abundantly expressed on their surface some of the receptors for HA CD44 and TLR2. Both cell lines expressed CD44 receptor abundantly on their surface, which was further increased by the pre-incubation of cells with LPS (Fig. 3). TLR2 was also expressed on the surface of the tested cell lines (Fig. 3). The expression of both CD44 and TLR2 increased after the pre-incubation of cells with LPS (Fig. 3).

The experimental setup of the above-mentioned controversial studies was similar to ours; thus, it is difficult to suggest any potential reason for the observed differences. The only major difference was the origin and the purity of the tested HA. To reduce maximally any non-specific effects, a highly purified bacterial origin pharmaceutical grade HA of 11, 52, 87, 250 and 970 kDa with a very low polydispersity characteristic, a marker of highly specific MW in the particular sample, was
tested in this study. All other studies mentioned above employed HA of human or animal origin in which HA was isolated from human umbilical cords (Horton et al. 1999; Horton et al. 2000; McKee et al. 1997; Wang et al. 2006) or rooster comb (Ariyoshi et al. 2005; Habara et al. 2002). In these studies, the authors used different methodological approaches, such as blocking of the HA receptors (Ariyoshi et al. 2005), blocking of the non-specific effects of endotoxin by polymyxin B (Horton et al. 1999; Horton et al. 2000; McKee et al. 1997), using chondroitinase (McKee et al. 1997), and the degradation of tested HA by hyaluronidase (Wang et al. 2006) to prove that the observed effects were specific to HA interaction with cells. However, since we used exactly the same cell lines directly obtained from the American Type Culture Collection, the origin and purity of the tested HA samples is the only potential source of the observed differences. It could be speculated that HA obtained from different suppliers could contain traces of biologically active impurities such as hyaloadherines or other ECM proteins tightly joined to HA. Further, biologically important differences in HA preparations accountable to different isolation procedures of animal and bacterial origin of HA or different procedures for preparation of LMW-HA could be speculated. However, currently there are no reports available to support these suggestions.

In conclusion, our data does not support the current view on LMW-HA as a pro-inflammatory mediator. More experiments should be conducted to resolve the question of the effect of different MW HA on macrophages, crucial players in innate immunity.

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REFERENCES


