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Antioxidant effect of hyaluronan on polymorphonuclear leukocyte-derived reactive oxygen species is dependent on its molecular weight and concentration and mainly involves the extracellular space*

Antyoksydacyjne właściwości hialuronianu wobec ludzkich neutrofilów zależą od jego masy cząsteczkowej i stężenia i są wywierane głównie w przestrzeni zewnątrzkomórkowej

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Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Summary

Introduction: Hyaluronan (HA), a component of the extracellular matrix, may regulate immune cell functions through its interactions with cellular receptors. Besides its effect on cytokine and chemokine production, its antioxidant properties have been described. However, the mechanisms of this are not fully elucidated. The aim of this study was to evaluate the relationship between HA concentration and molecular weight and its antioxidant properties towards human neutrophils. Also assessed was whether the antioxidant effect of HA is connected with a reduction in intracellular oxygen potential, which could indicate its direct effect on neutrophil respiratory burst.

Materials/Methods: The relationship between HA's antioxidant properties and its concentration and molecular weight was assessed by the luminol-enhanced chemiluminescence method (CL). To evaluate the effect of HA on intracellular oxygen potential selectively, the dihydrorhodamine 123 (DHR123) flow cytometric method was used.

Results: Reduction of both HA molecular weight and its concentration decreased its antioxidant properties in the CL method. A selective effect of HA on intracellular oxygen potential measured by the DHR123 method was not shown.

Conclusions: The antioxidant properties of HA are related to both its molecular weight and its concentration. The lack of an antioxidant effect of HA in the DHR123 test compared with a significant reduction in CL values at the same HA concentration suggests that HA acts mainly as a chemical ROI scavenger in the extracellular space.

Key words: **hyaluronan • reactive oxygen intermediates • chemiluminescence • dihydrorhodamine**

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Streszczenie

Wstęp: Hialuronian (HA), składnik macierzy zewnątrzkomórkowej, poprzez interakcje z receptorami może regulować funkcje komórek odpornościowych. Poza wpływem na wytwarzanie cytokin i chemokin, opisano również jego właściwości antyoksydacyjne. Mechanizm tego działania w stosunku do profesjonalnych fagocytów nie został jednak w pełni poznany. Celem pracy była ocena związku między stężeniem HA i jego masą cząsteczkową a jego właściwościami antyoksydacyjnymi wobec ludzkich neutrofilów (PMNL). Zbadano również, czy efekt antyoksydacyjny HA jest związany z redukcją wewnątrzkomórkowego potencjału tlenowego, co mogłoby wskazywać na bezpośrednie oddziaływanie tego glikozaminoglikanu (GAG) na metabolizm tlenowy neutrofilów.

Materiały/Metody: Zależność między antyoksydacyjnymi właściwościami HA a jego stężeniem i masą cząsteczkową oceniono z wykorzystaniem metody chemiluminescencji pośredniej wzmacnianej luminolem (CL). Selektywna ocena wpływu HA na wewnątrzkomórkowy poziom reaktywnych form tlenu (RFT) w neutrofilach została przeprowadzona z wykorzystaniem metody cytometrii przepływowej z 1,2,3 dihydrorodaminą (DHR123).

Wyniki: Redukcja zarówno masy cząsteczkowej HA jak i jego stężenia wpływały na osłabienie właściwości antyoksydacyjnych tego GAG ocenianych testem CL. Nie stwierdzono, aby HA wpływał na wewnątrzkomórkowy potencjał tlenowy PMNL.

Wnioski: Antyoksydacyjne właściwości HA zależą zarówno od stężenia tego GAG, jak i od jego masy cząsteczkowej. Brak antyoksydacyjnego efektu HA w teście z 123DHR w porównaniu z istotną redukcją poziomu RFT w teście CL przy tym samym stężeniu HA wskazuje, że właściwości antyoksydacyjne tego GAG są związane przede wszystkim z jego zdolnością do sekwestracji RFT w przestrzeni zewnątrzkomórkowej.

Słowa kluczowe: **hialuronian • reaktywne formy tlenu • chemiluminescencja • dihydrorodamina**

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Abbreviations: **HA** – hyaluronan; **HMW-HA** – high-molecular-weight hyaluronan; **LMW-HA** – low-molecular-weight hyaluronan; **CL** – luminol-enhanced chemiluminescence method; **DHR123** – dihydrorhodamine 123; **ROIs** – reactive oxygen intermediates; **PMNLs** – polymorphonuclear leukocytes; **GAGs** – glycosaminoglycans; **fMLP** – N-formyl-L-methionyl-L-leucyl-L-phenylalanine; **PMA** – phorbol 12-myristate 13-acetate

Introduction

Reactive oxygen intermediates (ROIs) are produced as a result of cellular oxygen metabolism. An especially intensive generation of ROIs takes place in the course of inflammation after the stimulation of professional phagocytes such as neutrophils and monocytes/macrophages. The generation of ROIs through the NADPH oxidase system in a process called respiratory burst is a crucial effector mechanism of the antimicrobial activity of neutrophils [33]. Their essential function is demonstrated in patients with chronic granulomatous disease (CGD) in which the generation of superoxide anions is dramatically impaired due to defects in the NADPH oxidase system [18]. These patients suffer from recurrent, chronic, life-threatening bacterial infections.

Besides their bactericidal activity, ROIs can work as regulators of cellular functions [13,16,48]. They have been shown to trigger the nuclear factor κB (NF-κB), which promotes inflammatory cytokine production [22,35]. Because of their highly reactive nature and toxicity, an overproduction of ROIs affects several cellular structures, resulting in inappropriate function of the cellular membrane system, enzymes, and transport systems as well as DNA defects. ROIs are attributed to participate in the pathogeneses of chronic wounds, rheumatoid arthritis, ischemia-reperfusion injury, Alzheimer's disease, and aging processes [16,39,48].

Due to the potential toxicity of reactive oxygen intermediates, a large number of antioxidative barriers were created, including enzymes, such as superoxide dismutase,

catalase, glutatione peroxidase, and glutathione reductase, as well as a non-enzymatic system, components of which, such as tocopherols, carotenoids, ascorbic acid, melatonin, and gluthatione, act as chemical scavengers of free radicals [16,48]. The antioxidant properties of glycosaminoglycans (GAGs) of the extracellular matrix have also been described. Increasing literature data indicate their protective effect on cells exposed to the harmful action of free radicals [4–7,52]. Besides direct damage to various cell structures, the toxic effect of ROIs is attributed to an ability of these molecules and of lipid peroxidation products to activate nuclear factor κB (NF-κB) and caspases. This increases the degree of tissue damage and results in significant intensification of inflammation. In the presence of hyaluronan and chondroitin sulfate, a reduction of oxidative DNA damage and NF-κB and caspases activation by inflammatory mediators was noted [4–7,52].

Hyaluronan (HA) is a physiological component of the extracellular matrix. Besides its structural role, it may participate in the regulation of cellular activity. Several studies have demonstrated that the effect of HA on immune cells is dependent on its molecular weight. The high-molecular-weight form (HMW-HA) possesses anti-inflammatory properties which act through the inhibition of phagocytosis, cytokine production, and cytokine gene expression or intensification of the suppressor activity of regulatory T cells [2,14,30,37,49]. In turn, the low-molecular-weight HA fragments (LMW-HA), generated as a result of free radical and enzyme (especially hyaluronidases) activity [40,51], activate immune cells, stimulate cytokine and chemokine production as well as their gene expression and affect matrix metaloprotease (MMPs) and inducible nitric oxide synthase (iNOS) production [12,26,36,45,46,47,50]. The effect of HA on immune cells is connected above all with CD44 receptors.

The aim of this study is to assess the relationship between hyaluronan's antioxidant potency on human neutrophils and its molecular weight and concentration. We focused our attention on whether the antioxidant properties of HA are connected only with its ability of ROI sequestration in the extracellular space or also with its influence on intracellular oxygen potential. Possible alterations in intracellular ROI level could suggest a direct effect of HA on neutrophil respiratory burst.

Materials and Methods

High-molecular-weight hyaluronan (HMW-HA, molecular weight: >2 MDa) and low-molecular-weight hyaluronan (LMW-HA, molecular weight: 234 kDa) were from Lifecore Biomedical (USA). Hyaluronan was dissolved in phosphate-buffered saline (PBS, Biomed Lublin) to a concentration of 5 mg/ml and stored at –20°C until use.

PMNL isolation

Human polymorphonuclear leukocytes (PMNLs) were isolated from heparinized blood of healthy volunteers by the standard Gradisol G (Polfa Kutno) density gradient centrifugation method. Briefly, 5 ml of whole blood was layered on 3 ml of Gradisol G in 10-ml tubes and centrifuged for 25 min at 650×g. Then the lower, PMN-rich pellet was collected into a new 10-ml tube and the cells were washed once with phosphate-buffered saline (PBS, Biomed Lublin). Residual erythrocytes were removed by Pharm Lyse Solution (BD Pharmingen) for 10 min at room temperature, then the cells were washed once with PBS and their number was determined. The PMNLs were suspended at a density of 1×10^6/ml. Cell purity, assessed microscopically with hematoxylin and eosin staining, was >95%. Cell viability determined with the use of the Trypan blue exclusion method was >98%.

CD44 expression on neutrophils

The expression of CD44 receptors on human PMNLs was assessed by immunofluorescence staining of whole blood as follows: 50 µl of heparinized blood was incubated with PE-labeled anti-CD44 monoclonal antibodies (Becton Dickinson) for 30 minutes at room temperature in the dark. After incubation, the erythrocytes were lysed with FASC Lysing Solution (BD Pharmingen) for 10 minutes. After lysis the cells were washed once in Wash Solution (BD Pharmingen) and fixed in 1% paraformaldehyde. The cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson).

HA binding

To evaluate whether human polymorphonuclear cells bind hyaluronan, 100 µl of whole blood was incubated with FITC-labeled hyaluronan (Sigma-Aldrich) for 30 min at 4°C. As a control, cells were incubated with PBS. After incubation the cells were washed once in PBS. Erythrocytes were lysed with FACS Lysing Solution (BD Pharmingen) for 10 minutes at room temperature. After lysis the cells were washed once in Wash Solution (BD Pharmingen) and fixed in 1% paraformaldehyde. The cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson).

The effect of HA on CD11b adhesion molecule expression

The effect of HA on CD11b adhesion molecule expression on human PMNLs was assessed by immunofluorescence staining of whole blood as follows: 100 µl of heparinized blood was incubated with or without HMW-HA or LMW-HA at a concentration of 100 µg/ml at 37°C for 30 min. Then PE-labeled anti-CD11b monoclonal antibodies (Becton Dickinson) were added and the cells were incubated for 30 minutes at 4°C in the dark. After incubation the erythrocytes were lysed with FASC Lysing Solution (BD Pharmingen) for 10 minutes, washed once in Wash Solution (BD Pharmingen), and fixed in 1% paraformaldehyde. The cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson).

Chemiluminescence measurement

To evaluate ROI production applying the luminol-enhanced chemiluminescence method, PMNLs were suspended in phosphate-buffered saline (PBS, Biomed Lublin) at a density of 1×10^6/ml. The cells were incubated with high-molecular-weight hyaluronan or low-molecular-weight hyaluronan (Lifecore Biomedical, USA) at four different concentrations (1, 10, 100, and 500 µg/ml) for 30

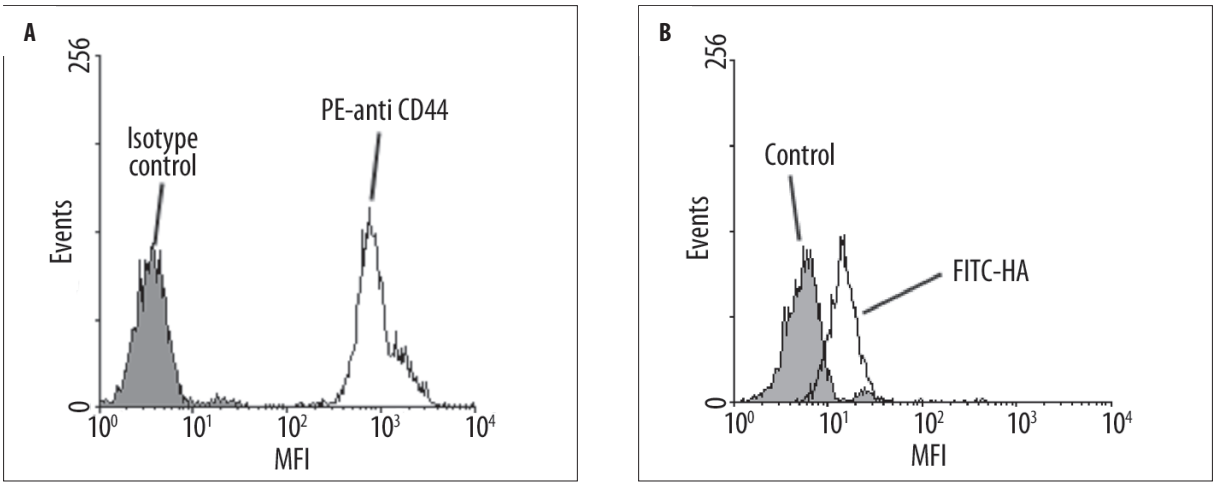

Figure 1. The expression of CD44 receptors on whole blood neutrophils (**A**) and the ability of neutrophils to bind FITC-labeled hyaluronan (FITC-HA) (**B**)

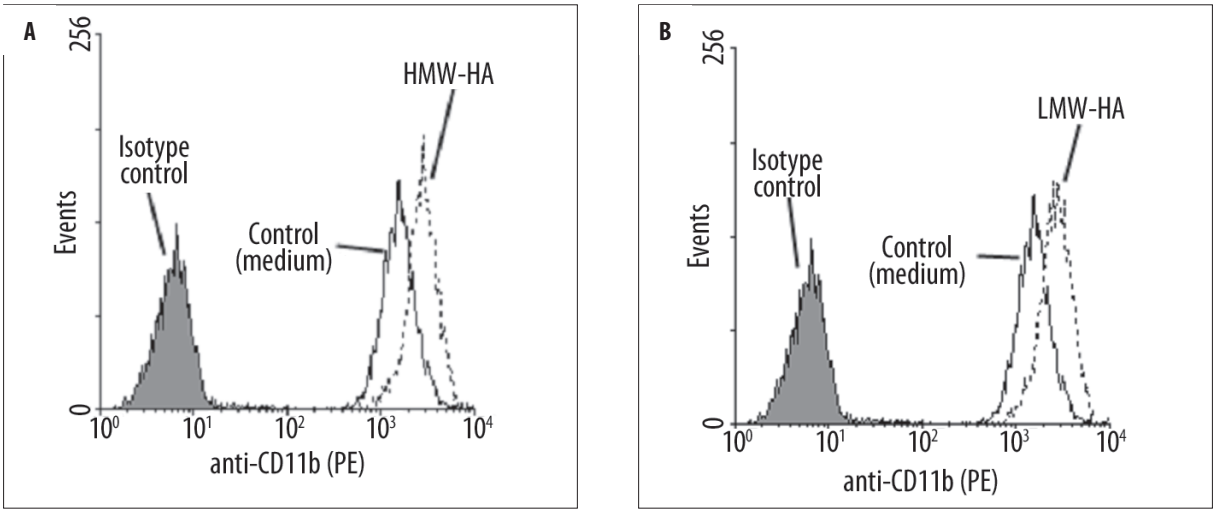

Figure 2. The effect of hyaluronan on the expression of CD11b adhesion molecule. Neutrophils (PMNLs) were incubated for 30 min at 37°C with or without high-molecular-weight (HMW-HA) or low-molecular-weight hyaluronan (LMW-HA) at a concentration of 100 µg/ml and then labeled with PE-labeled anti-CD11b monoclonal antibodies. As a control, PMNLs incubated with PBS (medium) were used

min at 37°C. As a control, PMNLs incubated with medium (PBS) were used. After incubation, 40 µl of the cell suspension was placed into each well of a 96-well microtiter plate. Twenty µl of luminol (Sigma Aldrich) and 30 µl of fMLP (N-formyl-L-methionyl-L-leucyl-L-phenylalanine, 2×10^{-6} M; Sigma Aldrich) or PMA (phorbol-12-myristate-13-acetate, 200 ng/ml; Sigma Aldrich) were added. The measurement was performed at 37°C within 30 min. The results were expressed in relative light units (RLU). The maximum chemiluminescence values (RLUmax) were then analyzed.

To examine the role of CD44 receptor in the antioxidant properties of HA, in some experiments we blocked CD44 with the use of anti-CD44 monoclonal antibodies (BD Pharmingen) at a concentration of 5 µg/ml for 15 min. at 4°C. Then HMW-HA at a concentration of 100 µg/ml was added and the PMNLs were incubated for 30 min at 37°C. After incubation, CL measurements of unstimulated, fMLP-stimulated, and PMA-stimulated neutrophils were performed.

Dihydrorhodamine 123 (DHR123) flow cytometric assay

To measure intracellular ROIs level, a method with dihydrorhodamine 123 (DHR123) was used. PMNLs were suspended in phosphate-buffered saline (PBS, Biomed Lublin) to a density 1×10^6/ml and incubated with HMW-HA or LMW-HA at a concentration of 100 µg/ml for 30 min at 37°C. Then 100-µl samples were transferred to 5 ml polypropylene FACS tubes and incubated for 10 min at 37°C with or without 25 µl of fMLP (2×10^{-6} M) in the presence of 25 µl of DHR123. Then the cells were cooled on an ice-water bath for 5 min and washed once in cold PBS. The supernatant was discarded and the cells were fixed with 1% paraformaldehyde. The cells were then analyzed using a FACSCalibur flow cytometer (Becton Dickinson) within 1 hour.

Statistics

For all parameters, the arithmetic mean and *SD* values were calculated. Statistical analysis was done using Student's t

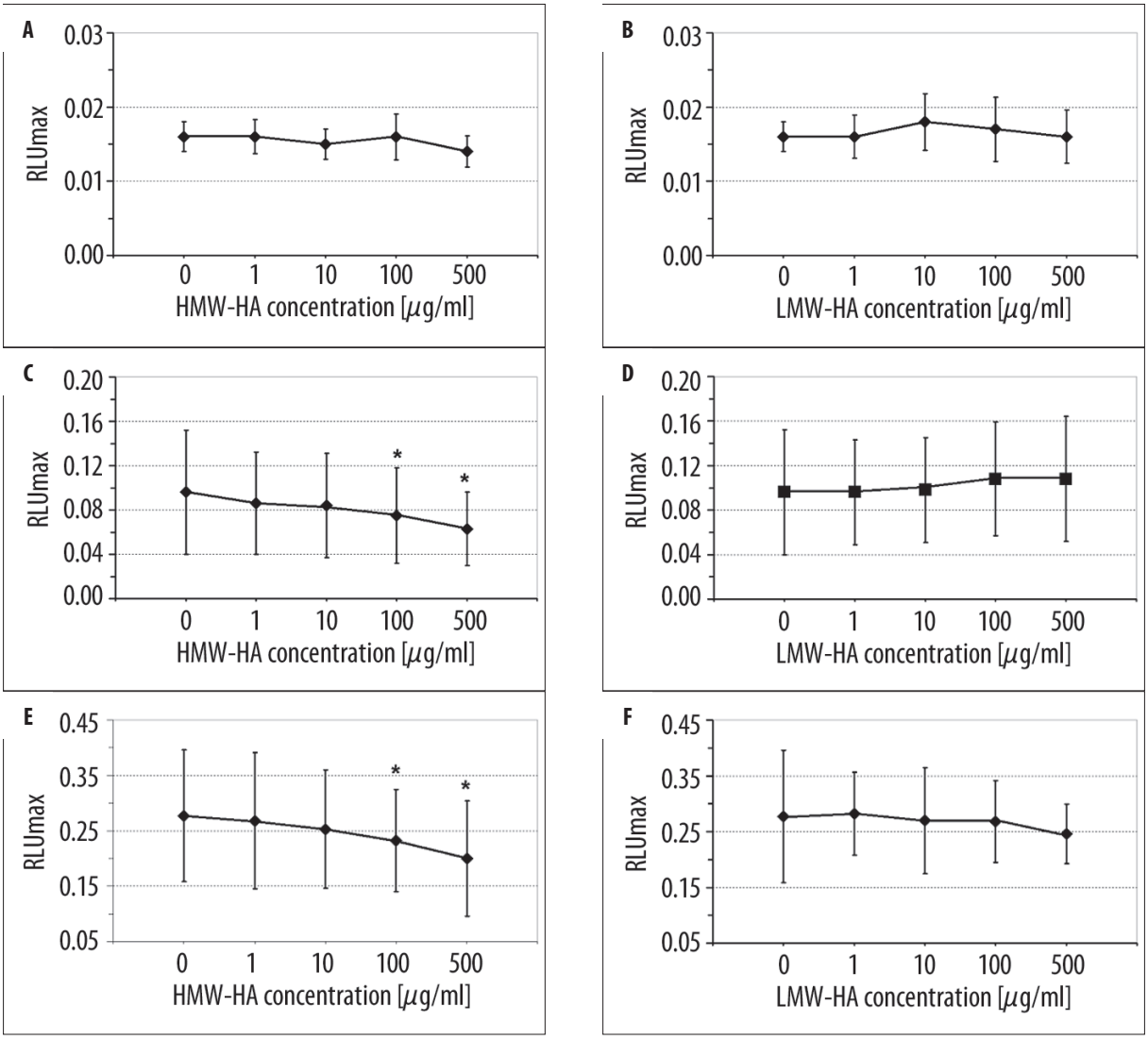

Figure 3. The effect of molecular weight and concentration of hyaluronan (HA) on oxygen potential of unstimulated (**A,B**), fMLP-stimulated (**C,D**), and PMA-stimulated (**E,F**) neutrophils. HA with two different molecular weights (mw): HMW-HA (mw: >2 MDa) and LMW-HA (mw: 234 kDa), and at four different concentrations (1, 10, 100, and 500 μg/ml) was used. As a control, PMNLs incubated in medium (0) were used. The results are expressed as the mean of maximum chemiluminescence intensity (RLUmax) ± *SD*

test and the Willcoxon test. The level of significance was set at ≤0.05.

Results

CD44 expression on PMNLs and their ability to bind hyaluronan

Expression of CD44 receptors on human neutrophils and the ability of PMNLs to bind hyaluronan molecule were evaluated using immunofluorescence staining of whole blood. Flow cytometry analysis showed that neutrophils expressed a high level of CD44 receptors. As hyaluronan is the physiological ligand for this type of receptor, we examined the ability of PMNLs to bind FITC-labeled hyaluronan. The analysis of cells incubated with FITC-HA showed an increase in mean fluorescence intensity (MFI) compared with the control, indicating their ability to bind HA (Figure 1).

The effect of HA on CD11b adhesion molecule expression

Flow cytometric analysis revealed that 30minute incubation of PMNLs with HMW-HA or LMW-HA caused increased CD11b adhesion molecule expression on the surface of human neutrophils compared with PMNLs incubated without HA ($p<0.05$). There was no statistically significant difference between the effects of HMW-HA and LMW-HA (Figure 2).

Chemiluminescence measurement

Comparing the maximum CL values (RLUmax) of PMNLs incubated with HMW-HA or LMW-HA at different concentrations (1, 10, 100, and 500 μg/ml) and the control we found that the antioxidant properties of HA depended on both its molecular weight and its concentration. We did not observe an effect of HMW-HA and LMW-HA on the

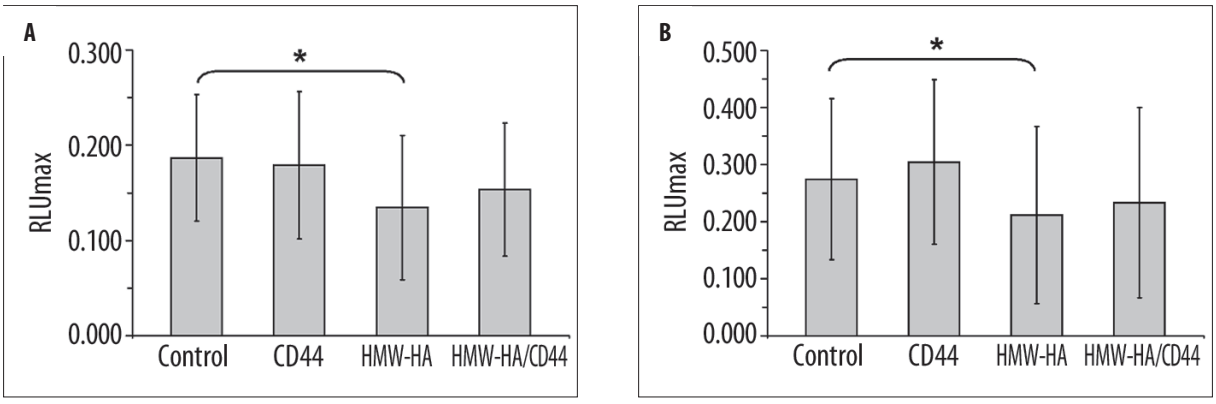

Figure 4. The effect of blocking the CD44 receptor on the antioxidant properties of high-molecular-weight hyaluronan (HMW-HA) towards fMLP-stimulated (**A**) or PMA-stimulated (**B**) neutrophils (PMNLs) measured in the CL assay. PMNLs were incubated for 30 min with or without anti-CD44 antibodies and then HMW-HA at a concentration of 100 µg/ml was added. The results are expressed as mean ± *SD*

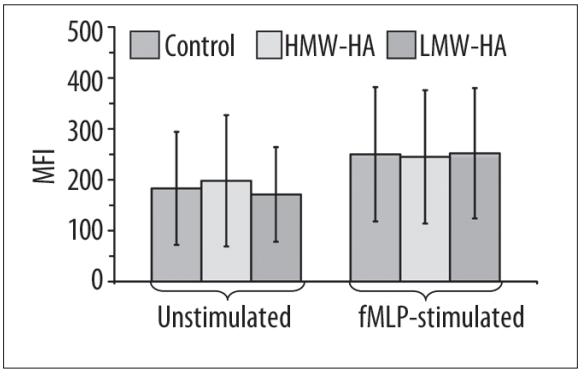

Figure 5. The effect of high-molecular-weight hyaluronan (HMW-HA) and low-molecular-weight hyaluronan (LMW-HA) on the intracellular oxygen potential of unstimulated and fMLP-stimulated PMNLs measured in the DHR123 assay. As a control, PMNLs incubated with PBS were used. The results are shown as the mean fluorescence intensity (MFI) ± *SD*

respiratory burst of unstimulated PMNLs, while after stimulation with fMLP or PMA the effect of HMW-HA on RLUmax values was significant. The ability of HMW-HA to reduce the CL values increased with the concentration of the glycosaminoglycan and became statistically significant at the two highest concentrations of 100 and 500 µg/ml (both $p<0.05$). LMW-HA did not affect the RLUmax values of fMLP- or PMA-stimulated PMNL (Figure 3).

CD44 molecule is considered to be the main hyaluronan receptor. To examine its role in the antioxidant properties of HMW-HA, we blocked CD44 with the use of anti-CD44 monoclonal antibodies. Analyzing the obtained data we observed only a slight, statistically insignificant reduction in the antioxidant properties of HMW-HA towards fMLP-stimulated PMNLs. After PMA stimulation we did not observe any reduction in the antioxidant effect of HMW-HA (Figure 4).

PMNL oxidative burst measurement with the dihydrorhodamine 123 method

The measurement of neutrophil respiratory burst using the dihydrorhodamine 123 (DHR123) flow cytometric method allows a selective examination of intracellular oxygen potential. To assess the effect of HA on PMNL oxygen metabolism by this method, we used HMW-HA or LMW-HA at a concentration of 100 µg/ml, chosen on the grounds of previously obtained CL results. The mean fluorescence intensity of unstimulated or fMLP-stimulated neutrophils was determined. We did not observe any effect of HMW-HA or LMW-HA on PMNL respiratory burst as the MFI values of both unstimulated and fMLP-stimulated neutrophils incubated with HMW-HA or with LMW-HA did not differ from those without HA (Figure 5).

Discussion

Recently, the immunoregulatory properties of hyaluronan (HA) have been widely investigated. Since the discovery of the CD44 receptor, the ability of HA to activate intracellular signal transduction pathways has been proven. Numerous literature data [reviewed in 19,41,44] indicate that HA has an effect on immune cells and suggest its role as a regulator of the inflammatory process. Several studies indicate the antioxidant properties of HA [3–7,9,15,24,27–29,42,52]. The biosynthesis of this GAG is enhanced in inflammatory foci [3,7,20,25,43]. HA can reduce tissue injury caused by reactive oxygen intermediates (ROIs) by creating a mechanical barrier that limits the access of ROIs to the cell surface and reduces DNA damage and NF-κB and caspase activation [4–7,52]. However, the mechanism by which this glycosaminoglycan affects respiratory burst in professional phagocytes has not been clearly explained. Besides its ability to scavenge reactive oxygen intermediates (ROIs) in the extracellular space, its direct effect on oxygen metabolism was suggested [24,42]. This hypothesis is supported by the observation that HA, synergistically with TGF-β2, induces the $p47^{phox}$ NADPH oxidase component in promyelocytic cells [1].

Human neutrophils (PMNLs) constitutively express high levels of CD44 receptor and are able to bind HA [31,38], as confirmed in our study by FACS analysis. CD44 is considered to be a cytotoxic triggering molecule on human PMNLs and to participate in their cytotoxic response and lysis of HA-coated target cells [31] or in HA fragment-stimulated IL-6 production [38]. In our study we also demonstrated that HA is able to interact with PMNLs, as their in-

cubation with this GAG resulted in the induction of CD11b adhesion molecule expression on the neutrophil surface. Therefore we examined whether the antioxidant properties of HA, besides its ability to scavenge ROIs in the extracellular space, are also related to its effect on intracellular ROIs level, which might suggest its direct regulatory activity on PMNL respiratory burst.

To demonstrate this, two different methods of ROIs measurement were used in our study: the luminol-enhanced chemiluminescence method (CL) and the dihydrorhodamine 123 (DHR123) method. As luminol freely diffuses through cellular membranes, the obtained CL values reflect both intracellular and extracellular oxygen potential [23]. In turn, the cytometric measurement of neutrophil respiratory burst with the use of DHR123 allows assessing the intracellular level of ROIs more selectively [10,34], as the oxidation of DHR123 by oxygen metabolites leads to conformational changes within its molecular structure and restricts the rhodamine molecule in passing through cellular membranes [10,34]. In addition, to determine the role of CD44 receptors in HA's antioxidant effect, we performed an experiment with anti-CD44 monoclonal antibodies.

The antioxidant properties of glycosaminoglycans were discovered not only in the case of HA, but also in other molecules of this group, such as heparin and heparan sulfate [8,11]. It is considered that the inhibition of respiratory burst depends on the molecular weights of these GAGs as well as the degree of sulfation [8,11]. The HA molecule is not subject to post-translational modifications (e.g. sulfation, epimerization) [17,21]; however, the relationship between its ability to reduce oxygen potential and its viscoelastic properties correlating with molecular weight has been described [27,28,32]. In the CL method applied in the present study, we confirmed observations [28] that low-molecular-weight hyaluronan (LMW-HA) was not effective in neutralizing oxygen potential generated by PMNLs. Although we used HA concentrations several times lower than those of Moseley [27,28], we noted that high-molecular-weight hyaluronan (HMW-HA) significantly reduced the oxygen potential of stimulated PMNLs in a dose-dependent manner, while low-molecular-weight fragments did not reveal such a property. These results are in contrast to Lym's report [24] indicating that HA concentration is the only factor affecting the antioxidant effect of this GAG. In our study we used low concentrations of HA, which are more likely to be encountered *in vivo* [21]. The results indicate HA to be a component of the antioxidative shield protecting other cells from damage by active oxygen metabolites.

The results of experiments with cell-free sources of ROIs [4,9,27] clearly indicate the ability of HA to scavenge active oxygen metabolites, which leads to depolymerization of its chain structure [51]. To assess the effect of HA on the oxygen potential of professional phagocytes, methods such as the reduction of cytochrome C or the degradation of 2-deoxy-D-ribose are commonly used [24,28,42]. These methods allow estimating ROIs level mostly in the extracellular space. The neutrophil respiratory burst measured by CL applied in our study also does not indicate whether HA acts as a ROIs scavenger only or also affects intracellular oxygen metabolism. However, the results of the DHR123 method clearly show that short-time interaction of fMLP-stimulated neutrophils with HA does not result in a reduction of intracellular ROIs level. It was previously described [40] that some fractions of HA, mostly low-molecular-weight fragments, can be internalized in the cells. Considering the fact that short-time incubation of LMW-HA at the concentration used in the DHR123 method did not cause statistically significant alterations in the CL method, we believe that the hypothetic effect of HA on intracellular ROIs sequestration should be excluded. In conclusion, although PMNLs bind HA, the direct effect of this GAG on intracellular oxygen metabolism was not demonstrated in our study. In addition, after blocking the CD44 receptor we observed only a slight reduction of the antioxidant properties of HMW-HA measured in the CL test. Besides the incomplete blocking effect, this might be evidence for a limited role of this receptor in the antioxidant effect of HA on PMNLs. In our opinion, these findings support the hypothesis about the role of HA mainly as an ROIs scavenger in the extracellular space; however, the alterations in CD11b expression shown in our study indicate the possibility of this GAG to affect other effector functions of PMNLs.

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