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THE EFFECTS OF HYALURONIC ACID ON MACROPHAGE FC RECEPTOR BINDING AND PHAGOCYTOSIS ARE INDEPENDENT OF THE MODE OF DEPOLYMERIZATION

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In order to determine whether exposure of hyaluronic acid to oxygen radicals caused an alteration in its properties, independent of the change in molecular weight induced, we examined its effect upon macrophage Fc receptor binding. High molecular weight hyaluronic acid (Healon-Pharmacia) caused a dose dependent inhibition of binding between the concentrations of 0.2-1 mg/ml. At a concentration of 0.3 mg/ml both oxygen radical depolymerized and enzymatically degraded hyaluronic acid caused an inhibition of Fc receptor binding at molecular weights of 1×10^6, 1.5×10^6 and 2×10^6. Oxygen radical degraded hyaluronic acid caused a stimulation of Fc receptor binding at molecular weights of 2×10^5 and 3.5×10^5, and enzyme degraded hyaluronic acid causes stimulation at a molecular weight of 2.5×10^6. Thus this "biological property" of hyaluronic acid is dependent upon molecular weight solely and not upon the mode of depolymerization.

KEY WORDS: Hyaluronic acid, oxygen radicals, macrophages, phagocytosis.

INTRODUCTION

Hyaluronic acid is of considerable biological importance for a number of reasons. Firstly it is the predominant macromolecular extracellular component of synovial fluid, vitreous humour and Wharton's jelly, as well as being one of the major components of the extracellular matrices of many other tissues.[1] The physiological importance of these matrices is underscored by the fact that, for example, cartilage nutrients are derived from the synovial membrane and therefore must traverse the hyaluronate rich synovial fluid matrix.[2] Secondly hyaluronic acid is susceptible to depolymerization by oxygen radicals,[3,4] particularly the hydroxyl radical,[5,6] with resultant loss of intrinsic viscosity of solutions of hyaluronic acid and decrease in

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molecular weight.[5] Other molecular alterations in the hyaluronate molecule as a result of oxygen radical exposure have been suggested. These include the possibility of branch formation as a result of repolymerization,[7] specific β1–3 glycosidic bond cleavage during oxygen radical depolymerization,[8,9] and the formation of a resonance stabilized carbonyl radical centred on the fifth carbon of the glucuronic acid component.[9]

Finally, despite its relatively simple chemical structure hyaluronic acid has a variety of complex biological actions. It inhibits chondrocyte proteoglycan synthesis,[10] it can inhibit cell–cell interactions,[11] and it has been shown to delay and thus possibly regulate cellular differentiation.[12] Indeed a number of cells exhibit receptors for hyaluronic acid.[13,14] Brandt[15] observed inhibition of neutrophil phagocytosis of urate crystals with hyaluronic acid of high molecular weight. Subsequently, Forrester and Balazs[16] demonstrated that hyaluronic acid can inhibit the phagocytosis of latex beads by elicited rat peritoneal macrophages. This effect was seen at high molecular weights (4×10^6–2.8×10^6) and at hyaluronate concentrations between 0.1 and 0.2 mg/ml. In addition, however, Forrester and Balazs observed that hyaluronic acid of molecular weight 9.0×10^5 had a stimulatory effect upon macrophage phagocytosis at concentrations between 0.2–0.5 mg/ml. Hyaluronic acid of this size can be expected following depolymerization with oxygen radicals[7] and is found in the synovial fluid of a considerable proportion of patients with inflammatory arthropathies.[17] Oxygen radical induced depolymerization of hyaluronic acid probably occurs *in vivo*, and has been postulated to account for the finding of decreased average molecular weight of the hyaluronic acid of inflamed synovial fluids.[3–5]

We therefore sought to establish whether the appearance of biological properties of hyaluronic acid, that are molecular weight dependent, is determined by the mode of depolymerization (i.e. oxygen radical induced vs enzymatic degradation). An important biological property of hyaluronic acid, suitable for testing, is its effect on phagocytosis. Specifically we sought to determine whether the biphasic effect of hyaluronic acid, dependent on molecular weight, was still present when hyaluronic acid was exposed to oxidants.

METHODS

1. Adherent monocytes

10 ml samples of venous blood, anticoagulated with lithium heparinate, were obtained from healthy Red Cross blood donors. These were then diluted with an equal volume of Hanks balanced salt solution (HBSS), placed in a 30 ml siliconized glass centrifuge tube and underlayed with 7.5 ml Ficoll Paque (Pharmacia, South Seas, Sydney). The tubes were then centrifuged at 400 g for 35 min at room temperature. The mononuclear cell fraction was then aspirated from the Ficoll Paque-HBSS interface. They were then washed three times in HBSS and the final cell pellet was resuspended in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin. They were then transferred to 24-well tissue culture plates (16 mm diam) Costar (Cambridge, Mass, U.S.A). At the bottom of each well had been placed a small circular glass coverslip that previously had been washed in acid (10% H_2SO_4) and 70% ethanol. Approximately 4×10^6 cells were added to each well. The cells were then incubated for 1 hour at 37°C in a 5% CO_2 incubator. Non-adherent cells were then washed off with HBSS.

2. Fc receptor assay

(a) Preparation of antibody coated red blood cells. A mixture was made consisting of 50 μl packed washed red blood cells, 200 μl anti-D serum (Commonwealth Serum Laboratories, Sydney, Australia) and 2 ml HBSS. This mixture was placed in a 37°C incubator for 1 hour. During the incubation, the tube was inverted every 20 minutes. The red cells were then washed three times in sterile normal saline and finally made up in 25 ml RPMI.

(b) Fc receptor assay 500 μl of the antibody coated erythrocytes were added to each washed monocyte monolayer. This was then incubated for 1 hour after which each well was washed three times with HBSS to remove non-bound erythrocytes. The cells were fixed by the addition of 3% gluteraldehyde in Hanks BSS for 30 min at room temperature (or 12 hrs at 4°C).

(c) Staining and counting The coverslips were stained with Giemsa stain. After 60 seconds the coverslips were washed in water and allowed to stand in distilled water for 30 minutes, the water being changed every 10 minutes. They were dried and mounted. The microscopic appearance of these preparations showed readily identifiable adherent monocytes to which were attached red blood cells, either touching or partially or completely ingested. Since the end point in this investigation was receptor attachment, no attempt was made to distinguish attachment from complete phagocytosis. For each coverslip five high power fields were counted or enough to guarantee at least 100 monocytes and the corresponding number of erythrocytes. This was then expressed as a binding ratio. The results for each assay were expressed as the Binding Index.

$$\text{Binding Index} = \frac{\text{ratio RBC per monocyte with HA}}{\text{ratio RBC per monocyte without HA}}$$

3. Hyaluronic acid preparation

(a) Oxygen radical exposed hyaluronic acid Purified high molecular weight hyaluronic acid (MW $> 2 \times 10^6$) was the gift of the Pharmacia (South Seas) Company. Hyaluronic acid at a concentration of 1 mg/ml in 50 mM phosphate buffer, pH 7.4, was exposed to oxygen radicals generated by the autoxidation of ferrous ions. Ferrous sulphate was added in concentrations between 50–1000 μM and ethylenediaminetetraacetic acid was present in a molar ratio to ferrous ions of 1:1.

Average molecular weights of the depolymerized samples were determined by fractionating alliquots upon Sepharose 4B-CL columns (10 × 0.5 cm) and the Kav of the peak uronic acid value was measured.[17] The corresponding molecular weight was determined by reference to data for polysaccharides given by Fisher.[19] The ferrous ion concentration and the corresponding average molecular weights of the products are shown in the Table.

(b) Enzyme exposed hyaluronic acid A preparation of Healon was exposed to testicular hyaluronidase (Sigma Type IV-S, EC 3.2.1.35), 0.1 mg/ml for 5 mins at 60°C and the reaction was stopped by decreasing the pH to 8.0 and dropping the temperature to 4°C. This was then fractionated on a Sephacryl S1000 column. Pooled fractions were dialyzed extensively against water, ethanol precipitated, and then dried

TABLE 1
Molecular weights obtained after exposure of hyaluronic acid to testicular hyaluronidase and the ferrous ion atuoxidation system

Oxygen radical depolymerized Hyaluronic Acid		Enzymatically depolymerized Hyaluronic Acid	
Ferrous ion Concentration (micromolar	Average MW ($\times 10^6$)	Pooled fractions	Average MW ($\times 10^6$)
0	2.0	HEALON	2.0
50	1.5	11–20	1.5
100	1.0	51–60	1.0
500	0.5	101–110	0.5
1000	0.2		

Enzymatically depolymerized hyaluronic acid was prepared by exposing it to testicular hyaluronidase at room temperature for 10 min. This was then fractionated on a Sephacryl S1000 column and the fractions indicated were pooled (Vo = 10, Vt = 105). Molecular weights for both sets of samples were determined by measuring the Kav for the peak of the elution profile after fractionation on Sepharose 4B-CL and relating this to a standard curve of Kav vs Molecular Weight for polysaccharides.[19]

in vacuo. The average molecular weights of the pooled fractions used were determined by fractionation on a Sepharose 4B-CL column as described above. Characteristics of these preparations are given in the Table. In this assay only one hyaluronic acid concentration of 0.3 mg/nl was used.

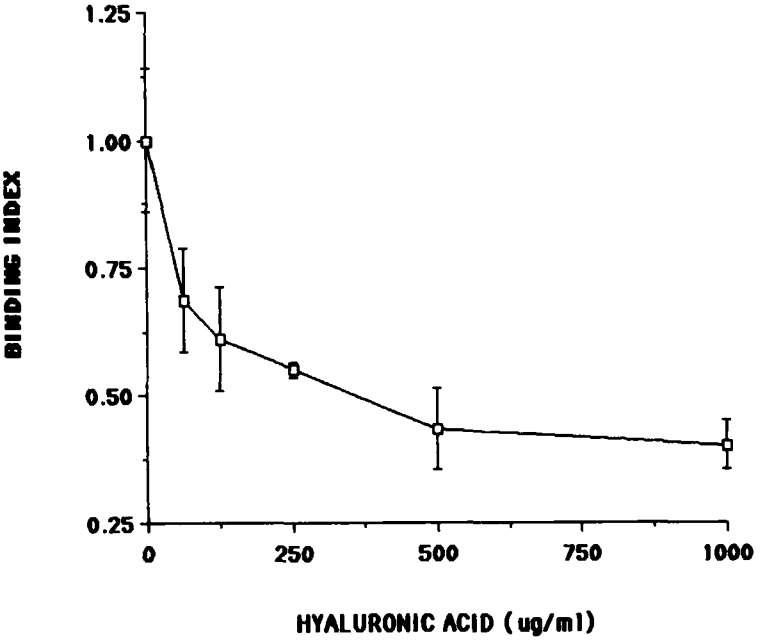

FIGURE 1 Effect of the concentration of a high molecular weight hyaluronic acid preparation (healon-pharmacia) upon Fc receptor binding.

Undergraded hyaluronic acid (Healon) was added to isolated adherent peripheral blood monocytes at the concentrations shown. This had the effect of inhibiting the binding of IgG coated red blood cells expressed as a binding index (see text) in a concentration dependent fashion. Each data point = mean (±S.E.) of triplicate determinations.

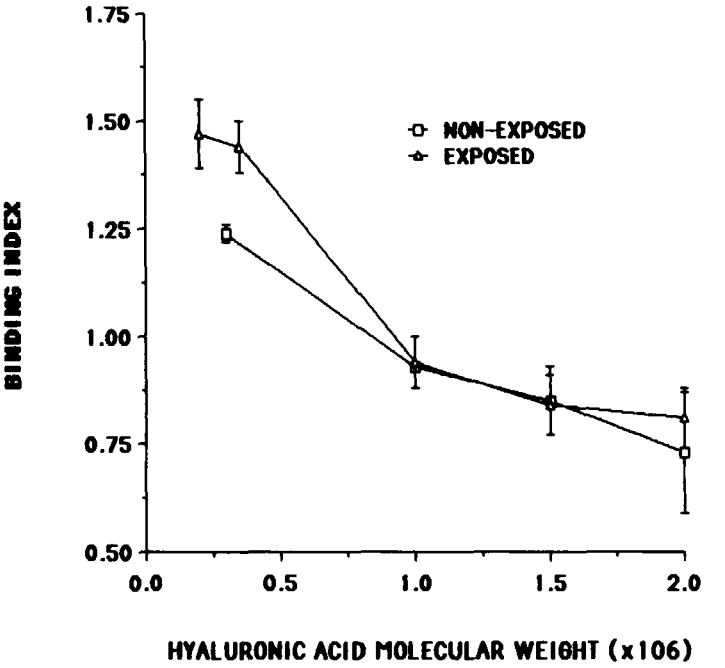

FIGURE 2 Effect of enzymatic and oxygen radical depolymerized hyaluronic acid upon Fc receptor binding.

Preparations of hyaluronic acid exposed to oxygen radicals generated by the ferrous ion autoxidation system and of differing average molecular weights were tested in the macrophage Fc receptor binding assay (—△—). At high molecular weights (1.0, 1.5 and 2.0 $\times$ 10^6) binding was inhibited, whereas at the relatively low molecular weight of 0.5 $\times$ 10^6, stimulation of binding was evident. This phenomenon was also evident using preparations of hyaluronic acid that had been depolymerized by enzymatic digestion (—□—). Each data point = mean $\pm$ S.E. of triplicate determinations.

RESULTS AND DISCUSSION

Using undegraded high molecular weight hyaluronic acid there as a dose dependent inhibition of Fc receptor binding over the concentration range 0.2–1 mg/ml (Figure 1). This extents to human peripheral blood monocyte Fc receptor binding, previous findings at these concentrations and molecular weights, demonstrating inhibition of phagocytosis of urate crystals[16] by neutrophils and inhibition of phagocytosis of latex particles by elicited rat peritoneal macrophages.[15]

At a concentration of 0.3 mg/ml there was a biphasic effect upon Fc receptor binding dependent upon molecular weight (Figure 2). Inhibition of Fc receptor binding was seen with preparations with an average molecular weight of 1 $\times$ 10^6 and above and stimulation of Fc receptor binding was seen in preparations with an average molecular weight of 5 $\times$ 10^5 and below. This effect is seen with both oxygen radical exposed and enzyme digested hyaluronic acid.

The Fc receptor binding assay system used in this study differed from the latex phagocytosis assay studied by Forrester and Balazs[15] because a specific receptor for the uptake of polystyrene latex spheres has not been demonstrated. Latex is considered to be engulfed in a non-specific manner possibly related to its hydrophobic-

ity.[20] The results of our study indicate that this inhibitory effect of high molecular weight hyaluronic acid ($> 1 \times 10^6$) can be extended to those forms of binding that are receptor mediated. However at a low concentration (i.e., 0.3 mg/ml) of low molecular weight hyaluronic acid, a paradoxical stimulation of Fc receptor binding is evident. This is in accord with the findings of Forrester and Balazs for latex phagocytosis, however the molecular weight at which this stimulation first becomes evident is somewhat higher in our system (i.e. 5×10^5 compared with 9×10^4).

Depolymerization of hyaluronic acid by both enzymatic digestion and oxygen radical exposure resulted in hyaluronic acid degradation products that were inhibitory at high hyaluronic acid molecular weights yet caused paradoxical stimulation at lowered molecular weights. Oxygen radical depolymerized hyaluronic acid caused a greater degree of stimulation of binding index but this did not reach statistical significance.

The hyaluronic acid concentration of 0.3 mg/ml was chosen after consideration of Forrester and Balaz's data to give maximal stimulation at the decreased molecular weight and it is pertinent to note that at this concentration a matrix of hyaluronic acid is unlikely to form especially at lowered molecular weights.[21]

Clearly more extensive studies are required to probe the mechanisms involved in both the inhibitory and the stimulatory effects demonstrated. However it is pertinent to consider several recent studies. In considering the inhibitory effect, Forrester and Balazs concluded that electrostatic repulsion (latex, IgG, and hyaluronic acid are electronegative) is unlikely to have an effect since sulphated glycosaminoglycans which are more electronegative are without effect. Similarly an hyaluronic acid induced membrane pertubation is unlikely since other membrane modifying agents were without effect. They favored a steric exclusion of latex from the area of the cell surface thus decreasing the proximity of cell and latex. Alternately they speculated that latex beads and indeed IgG and complement particles are hydrophobic thus making it easier for the cell to effect phagocytosis. Hyaluronic acid, being hydrophilic would decrease this phagocytic advantage. Both of these explanations could apply to our assay. Similarly, steric exclusion effects have been implicated in the inhibitory effect of hyaluronic acid on mitogen induced lymphocyte stimulation.

This biphasic property of hyaluronic acid was chosen to test the postulate that oxygen radical depolymerization could alter the properties of hyaluronic acid independently of the change in molecular weight produced because it was such a subtle change. The fact that we did not find evidence for such an effect of oxygen radical exposure in this case does not necessarily mean that this is true of all the biological properties of hyaluronic acid. Indeed some properties of hyaluronic acid that deserve attention in this regard are the recently described capacity of very small concentrations (i.e. 10 μg/L) of high molecular weight hyaluronic acid, in association with plasma fibronectin, to cause stimulation of phagocyte function.[22]

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