ROLE OF HEPARIN IN RED CELL AGGREGATION

S. M. Bychkov and S. A. Kuz'mina

UDC 612.111.014.46:615.273.53

KEY WORDS: aggregation; red cells; heparin; heparin fractions.

One of the chief functions of hyaluronic acid (HUA) and soluble protein-chrondroitin-keratan-sulfate (PCKS) in the animal organism is to restrict dispersion and concentration of cells, their organelles, and certain other tissue components in the necessary volume [3, 5, 8, 11-14]. Investigations using suspensions of rabbit red cells in saline as the model of isolated cells have shown that heparin (the total preparation) and its two individual fractions, one of which contains three (HP-3) the other four (HP-4) sulfate groups for each repeated dissacharide unit of the macromolecules, weaken or totally suppress this action of HUA and PCKS in a concentration of 10 $mg \cdot ml^{-1}$ [3].

The aim of this investigation was to study dependence of the effect of HP-3 and HP-4 on their concentration on the action of HUA and PCKS, as factors limiting dispersion of rabbit red cells in saline, an essential step in the discovery of the mechanism of action of heparin on aggregation of red cells and other animal cells.

EXPERIMENTAL METHOD

Highly purified preparations of normal potassium salts of HUA, PCKS, HP-3, and HP-4 were obtained by methods developed by the writers previously [1, 2, 4]. A uniform suspension of rabbit red cells, washed to remove plasma, was made up in 0.14 M NaCl, pH 7.2 (phosphate buffer), containing 20% of red cells (by volume). Red cell aggregation was determined quantitatively by measuring the rate of separation of the suspension into phases of solution and red cells at 20°C [3]. The initial rate of separation into phases was determined graphically as the tangent of the angle of slope of the curve to the abscissa.

EXPERIMENTAL RESULTS

Separation of the red cell suspension into phases of solution and cells, induced by HUA, is inhibited by HP-3 and HP-4. The degree of this inhibition, with a constant HUA concentration (1.2 $mg \cdot ml^{-1}$) increased with an increase in the concentration of these heparin fractions; HP-3 was more active than HP-4, moreover, in the same concentrations. With HP-3 and HP-4 in a concentration of 5 $mg \cdot ml^{-1}$ the action of HUA was completely suppressed (Fig. 1a). In the absence of HUA and in the presence of HP-3 and HP-4 in the suspension, no separation into phases took place. Preparation of the red cell suspension induced by PCKS (5 $mg \cdot ml^{-1}$) was activated by HP-3, however, in all concentrations tested (Fig. 1b). HP-4, in a concentration of 0.3 $mg \cdot ml^{-1}$, also activated separation of the red cell suspension into phases in the presence of PCKS, but with higher concentrations of HP-4 the action of PCKS was inhibited and completely suppressed (Fig. 1c). If both HP-3 and HP-4 were present in equal concentrations by weight in the red cell suspension, its separation into phases induced by PCKS took place rather more slowly than in a mixture containing PCKS and HP-3, and a little faster than in a mixture consisting of PCKS and HP-4 (Fig. 1d). It will be evident that HP-3 and HP-4, within the concentration range studied, neutralized each other's action as a factor influencing separation of the red cell suspension in saline into phases, induced by PCKS. The fact that the mutual neutralization of HP-3 and HP-4 was quantitatively not quite complete can be explained by differences in the molecular weights and relative activities of these heparin fractions.

The initial rate of separation of the red cell suspension into phases, induced by HUA, was sharply reduced when HP-3 and HP-4 were added to the suspension even in low concentra-

Biological Structures Research Laboratory, Ministry of Health of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR, S. S. Debov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 102, No. 12, pp. 692-695, December, 1986. Original article submitted June 12, 1986.

0007-4888/86/0012-1681$12.50

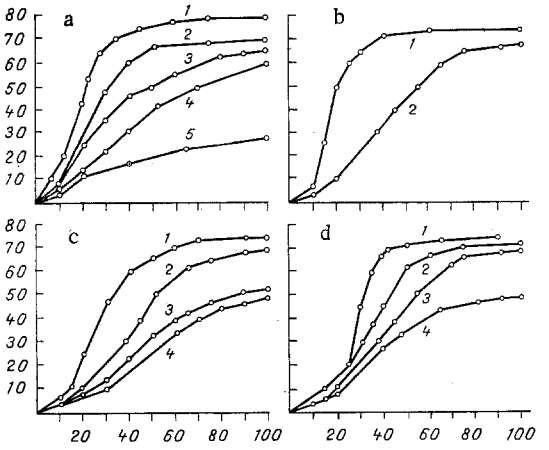

Fig. 1. Effect of HP-3 and HP-4 on kinetics of separation of red cell suspension into phases of solution and cells, induced by HUA and PCKS. Abscissa, time (in min); ordinate, relative volume (in % of phase of solution above aggregated red cells). a: 1) HUA, 2) HUA + HP-4 (0.3 mg•ml^{-1}), 3) HUA + HP-3 (0.3 mg•ml^{-1}), 4) HUA + HP-4 (1.2 mg•ml^{-1}), 5) HUA + HP-3 (1.2 mg•ml^{-1}). HUA concentration in all experiments was 1.2 mg•ml^{-1}; b: 1) PCKS + HP-3 (0.3 mg•ml^{-1}), 2) PCKS; c; 1) PCKS + HP-4 (0.3 mg•ml^{-1}), 2) PCKS, 3) PCKS + HP-4 (0.6 mg•ml^{-1}), 4) PCKS + HP-4 (2.5 mg•ml^{-1}); d: 1) PCKS + HP-3 (1.2 mg•ml^{-1}), 2) PCKS + HP-3 (1.2 mg•ml^{-1}) + HP-4 (1.2 mg•ml^{-1}), 3) PCKS, 4) PCKS + HP-4 (1.2 mg•ml^{-1}). PCKS concentration in all experiments was 5 mg•ml^{-1}. Separation of the red cell suspension into phases did not take place in control experiments in the presence of HP-3 and HB-4 but in the absence of HUA and PCKS.

tions. An increase in their concentrations was accompanied by a very small further decrease in this rate of separation. HP-3 reduced the initial rate of action of HUA in this respect rather more than HP-4 (Fig. 2). A much sharper difference was observed between the effects of HP-3 and HP-4 on the initial rate of separation of the red cell suspension into phases under the influence of PCKS. In this case HP-3 in low concentrations accelerated the action of PCKS on separation of the red cell suspension into phases considerably, whereas in higher concentrations this effect was relatively weaker. Within the concentration range of HP-3 from 0.6 to 5 mg•ml^{-1} the initial rate of this action of PCKS was independent of the HP-3 concentration (Fig. 2). Dependence of the initial rate of phase separation was rather more complex when HP-4 was present in the red cell suspension together with PCKS. With HP-4 in a concentration of 0.3 mg•ml^{-1} the initial rate of phase separation in this case was increased, but if the concentration of this heparin fraction was increased, the initial rate of phase separation fell steadily to values lower than that observed during the action of PCKS in the absence of HP-4 (Fig. 2).

Separation of the red cell suspension into phases as a result of the action of HUA and PCKS, in the absence or in the presence of HP-3 and HP-4, led to the establishment of an equilibrium ratio between the volume of the phase of aggregated red cells and the volume of saline above it, after 80-100 min. The equilibrium volume of aggregated red cells established by the action of HUA (1.2 mg•ml^{-1}) alone was equal to the original volume of red cells (18-20%) used to prepare suspension in saline. This volume of aggregated red cells in the presence of a constant HUA concentration increased with an increase in the concentration of HP-3 and HP-4 as a linear function, but HP-4, which inhibits separation of the red cell suspension into phases by a lesser degree than HP-3, led to a smaller increase in the equilibrium volume of red cells in the presence of equal concentrations of the two heparin fractions. With HP-3 and HP-4 present in concentrations of 5 mg•ml^{-1}, which completely inhibit the action of HUA, the volume of the red cell phase in both cases was close to that in the control test (Fig. 3a, b). In all concentrations of HP-3 that activate the action of PCKS (5 mg•ml^{-1}) on separation of the suspension into phases, the equilibrium volume of aggregated red cells was less than that formed in the presence of PCKS alone in the same concentration (Fig. 3c). HP-4, in a concentration of 0.3 mg•ml^{-1}, which potentiates the action of PCKS (5 mg•ml^{-1}) on separation of

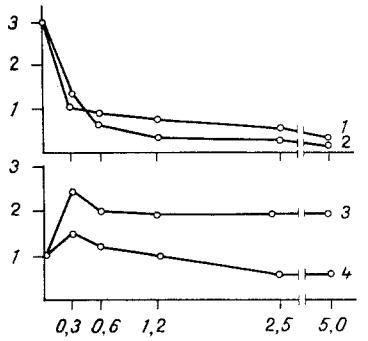

Fig. 2. Dependence of initial rate of separation of red cell suspension into phases of solution and cells on HP-3 and HP-4 concentration with a constant concentration of HUA and PCKS. Abscissa, concentration of HP-3 and HP-4 (in $mg \cdot ml^{-1}$); ordinate, relative volume of solution above red cell aggregates (in %/min). 1) HUA + HP-4, 2) HUA + HP-3, 3) PCKS + HP-3, 4) PCKS + HP-4. HUA concentration 1.2 $mg \cdot ml^{-1}$, PCKS concentration 5 $mg \cdot ml^{-1}$.

the red cell suspension into phases, reduced the equilibrium volume of the phase of aggregated red cells, but in the presence of higher concentrations of HP-4, which inhibit this action of PCKS, the equilibrium volume of these red cells became greater than that in the absence of HP-4. Under these circumstances, this volume was independent of the HP-4 concentrations within the range from 0.6 to 5 $mg \cdot ml^{-1}$ (Fig. 3d). In a red cell suspension containing, besides PCKS, equal concentrations of HP-3 and HP-4, the equilibrium volume of aggregated red cells was equal to that found in the presence of PCKS alone (Fig. 3d).

The effect of HP-3 and HP-4 on aggregation and steric exclusion of red cells from their suspension in saline, induced by HUA and PCKS, is thus determined by the structure of HP-3 and HP-4 and also by the nature of the HUA and PCKS macromolecules. Both heparin fractions inhibit this function of HUA, but the degree of this inhibition differs from each of them. HP-3, in all concentrations tested, increases the steric exclusion of red cells due to PCKS, but HP-4 has this effect only in low concentrations, and in higher concentrations it inhibits this action of PCKS. The factors described above also determine the equilibrium volume of the phase of aggregated red cells. As the result of the action of HUA, PCKS, HP-3, and HP-4 this volume is greater in cases when HP-3 and HP-4 inhibit steric exclusion of the red cells due to HUA and PCKS than when they potentiate this action of these proteoglycans. In turn, the macrostructure of the phases consisting of aggregated erythrocytes themselves is determined by the same relations between the components.

Aggregation of red cells and of others induced by HUA and PCKS is due to the ability of these proteoglycans to create flexible three-dimensional structures in solutions which expel these cells from the space which they occupy and concentrate them in the smallest possible volume [3, 5, 8, 11-14]. Only a few comments are possible on the mechanism of the effect of HP-3 and HP-4 on this action of HUA and PCKS. HP-3 and HP-4, which themselves create a uniform distribution of small red cell aggregates, cannot expel these structures into a limited phase by a steric mechanism [3]. HP-3 and HP-4 macromolecules carry an exceptionally high negative charge, and this factor undoubtedly plays an important role in the effect of HP-3 and HP-4 on the action of HUA and PCKS in this respect. However, there are no grounds for denying the participation of nonelectrovalency interactions of HP-3 and HP-4 with red cell surface components, similar to interaction of heparin with fibronectins [7, 9]. Consequently, under certain conditions, one or other of the interactions mentioned above may predominate. HP-3 and HP-4 differ from one another in the values of their charges, in anticoagulating activity (both are greater in the case of HP-4), and the conformation of their macromolecules [4, 6], as is shown by differences in the degree and character of the influence of these heparin fractions on the action of HUA and PCKS, as factors concerned in aggregation and steric exclusion of red cells. HP-3 and HP-4 may also have an influence on structures formed in solutions by HUA and PCKS macromolecules. Under these complex physicochemical conditions, as a result of interaction between the above-mentioned biopolymers and the surface of red cells, and also, probably, with one another in relation to aggregation of red cells and separation of their suspension into phases, phenomena such as inhibition, activation, synergism, and com-

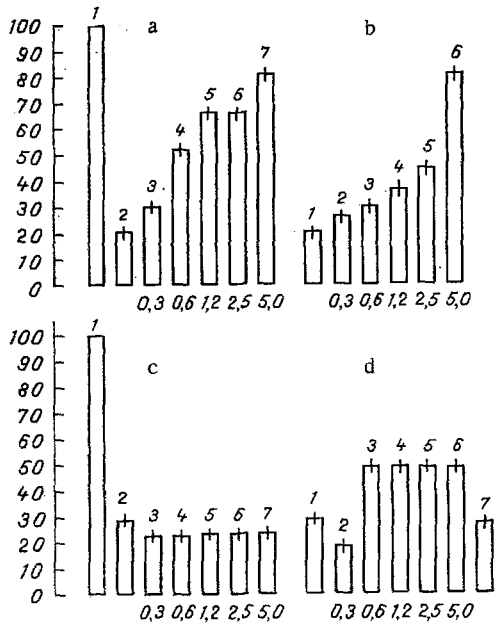

Fig. 3. Effect of HP-3 and HP-4 on relative volume of phase of aggregated red cells established after action of HUA and PCKS. Horizontal axis — concentration (in mg•ml^{-1} of HP-3 and HP-4. Vertical axis — relative volume of phase of aggregated red cells (in %) after separation of suspensions into phases of solution and aggregated red cells, with establishment of the steady state after 80-100 min. a) HUA + HP-3: 1) control (red cell suspension without proteoglycans), 2) HUA, 3-7) HUA + HP-3; b) HUA + HP-4: 1) HUA, 2-6) HUA + HP-4; c) PCKS + HP-3: 1) control, 2) PCKS, 3-7) PCKS + HP-3; d) PCKS + HP-4: 1) PCKS, 2-6) PCKS + HP-4, 7) PCKS + HP-3 + HP-4 (1.2 mg•ml^{-1}). HUA concentration 1.2 mg•ml^{-1} and PCKS concentration 5 mg•ml^{-1} in all cases.

petition may arise between the individual components. Special investigations are necessary to shed light on these problems.

According to the results of direct counting of aggregated red cells, HP-3 and HP-4 in a concentration of 10 mg•ml^{-1}, when concentrations of HUA and PCKS are relatively low, prevent the formation of large, distinctly formed aggregates [3, 5]. This does not contradict the results of studies of separation of red cell suspensions into phases of solution and cells. Instead, these results are mutually complementary. Because of the opposite nature of the effects of HP-3 and HP-4 (in concentrations of between 0.6 and 5 mg•ml^{-1}) on the action of PCKS as a factor in steric exclusion, the mutual neutralization of their effects, the complex dependence of the initial rate of separation into phases, and of the size of the equilibrium volume of the aggregated red cell phase, on the concentrations of these heparin fractions in the presence of a constant PCKS concentration, and also because of the quantitative differences in the effects of HP-3 and HP-4 on steric exclusion of red cells induced by HUA, it can be concluded that HP-3 and HP-4 can facilitate or prevent both aggregation and steric exclusion of red cells from a suspension in saline, induced by HUA and PCKS, depending on the ratio between the concentrations of all components of the system.

The effect of heparin [3] and its fractions on red cell aggregation found in [3] can hardly be limited to those cells. Very probably these biopolymers regulate the action of HUA and PCKS, mentioned above, on many cells in the animal organism. This is in agreement with the fact that steric exclusion from solutions containing HUA and PCKS, discovered first in experiments on erythrocytes, was later confirmed for other cells also [12-14]. HUA and PCKS are always present together in particular quantitative or structural relationships in the tissues of the body [8], and this greatly complicates the dependence of aggregation and steric exclusion on the concentrations of all components involved in these phenomena. The facts discovered in the present investigation are in full agreement with the unique assortment of varied func-

tions of heparin in the animal organism, which depend on the physical and chemical properties of its macromolecules [6, 10].

LITERATURE CITED

1. S. M. Bychkov and V. N. Kharlamova, Biokhimiya, 33, 840 (1968).
2. S. M. Bychkov and M. F. Kolesnikova, Biokhimiya, 34, 204 (1969).
3. S. M. Bychkov and S. A. Kuz'mina, Byull. Éksp. Biol. Med., No. 6, 40 (1973).
4. S. M. Bychkov and V. N. Kharlamova, Byull. Éksp. Biol. Med., No. 3, 289 (1976).
5. S. M. Bychkov and S. A. Kuz'mina, Byull. Éksp. Biol. Med., No. 9, 284 (1977).
6. S. M. Bychkov, Vopr. Med. Khim., No. 6, 725 (1981).
7. S. M. Bychkov, Vopr. Med. Khim., No. 6, 2 (1983).
8. S. M. Bychkov and S. A. Kuz'mina, Vopr. Med. Khim., No. 1, 19 (1986).
9. D. A. Zlatopol'skii and V. I. Mazurov, Vopr. Med. Khim., No. 6, 2 (1985).
10. A. M. Ul'yanov and L. A. Lyapina, Usp. Sovrem. Biol., 83, 69 (1977).
11. P. A. Edwards, Nature, 271, 248 (1978).
12. K. M. Jon and S. Chin, Microvasc. Res., 11, 121 (1976).
13. J. E. Morris, J. Cell Biol., 79, 157A (1978).
14. J. E. Morris, Exp. Cell Res., 120, 141 (1979).

EFFECT OF THIOL-SPECIFIC REAGENT ON *Pseudomonas aeruginosa* PAEI AND PAEII RESTRICTION ENDONUCLEASE ACTIVITY

N. N. Sokolov, A. B. Fitsner, N. V. Anikeicheva, É. B. Khoroshutina, and O. T. Samko

UDC 579.841.11:579.222:577.152].088.1

KEY WORDS: restriction endonucleases PaeI and PaeII; thiol-specific reagents; SH-groups.

Because of their unique properties class II restriction endonucleases are widely used in research in molecular biology and genetic engineering [2]. By now more than 400 restriction endonucleases have been described, their physicochemical and catalytic properties and the structure of many of these enzymes have been reasonably well studied [7] and techniques have been developed for seeking them in microorganisms and isolating them [3, 4, 6]. However, there is hardly any information in the literature on sulfhydryl groups of restriction endonucleases and their role in interaction with the DNA substrate. The only exception is an investigation [8] of the sensitivity of 11 restriction endonucleases to the action of alkylating and mercury compounds.

The aim of this investigation was to study the importance of SH-groups for manifestation of activity of new restriction endonucleases PaeI and PaeII isolated by the writers from *Pseudomonas aeruginosa*.

EXPERIMENTAL METHOD

Restriction endonucleases PaeI and PaeII were purified from strains of *Ps. aeruginosa* by the method developed by the writers previously [5]. DNA was isolated from phage λ as described previously [1]. The incubation mixture (30-60 µl for determination of the activity of restriction endonucleases PaeI and PaeII contained 10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1 µg DNA of phage λ, and 1-5 µl of the enzyme preparation. KCl was added to the samples for determination of restriction endonuclease PaeII activity in a final concentration of 20 mM, the samples were incubated at 37°C for 1-2 h, and the reaction was stopped by addition of a mixture containing Na_2-EDTA (pH 8.0), bromphenol blue, and glycerol to final concentrations of 20 mM, 0.02%, and

Research Institute of Medical Enzymology, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR S. S. Debov.)

Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 102, No. 12, pp. 695-697, December, 1986. Original article submitted November 22, 1985.