HEPARIN-INDUCED LEUKOCYTE LYSIS IN VITRO

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An investigation on the effect of heparin on leukocytes in vitro was conducted. We have demonstrated that heparin and other mucopolysaccharides destroyed leukocytes as judged by macromolecule leakage. The cytotoxicity of heparin was suppressed by protamine. A medium which mimics intracellular ionic conditions was found to suppress the cytotoxicity of heparin. Macromolecule leakage induced by heparin is thought to be facilitated by passive ion flux. Mepacrine, a phospholipase inhibitor, suppressed the heparin-induced macromolecule leakage from leukocytes. Thus, the activation of phospholipase appears to be involved in the cytotoxicity of heparin. These results suggest that activation of phospholipase and passive movement of ions may be important steps for the heparin-induced leukocyte lysis in vitro.

Keywords — heparin; dextran sulfate; sulfated polysaccharide; leukocyte lysis; protamine sulfate; phospholipase activation

INTRODUCTION

Heparin is well known to have anticoagulant and lipemia clearing activities and is widely used for various therapeutic purposes. The power of heparin to inhibit blood coagulation is thought to be dependent on the activation of antithrombin III, an alpha-2-globulin protease inhibitor, which inactivates progressively both thrombin and factor Xa. The lipemia clearing activity of heparin is mediated by the liberation of the lipoprotein lipase which hydrolyzes triglyceride in chylomicron and low density lipoprotein.

In addition to these activities, heparin forms complexes with various alkaloids, amines, and proteins resulting in possessing different chemical and biological properties from those of the parent substances. Heparin also inhibits delayed-type-hypersensitivity (DTH) reaction and reduces the footpad swelling that results from a local injection of the mitogenic lectin, concanavalin A. Since, there is a dearth of reports that describe the cytotoxicity of heparin on leukocyte we initiated an investigation on the effect of heparin on leukocyte integrity in vitro, and found that heparin caused leukocytes to leak intracellular constituents. The mechanism of heparin-induced cell disruption was also investigated.

MATERIALS AND METHODS

Heparin was obtained from Novo Industry A/S (Denmark), and was purified by Sephadex G-75 column chromatography. After heparin was applied to the column (24 x 780 mm), the column was washed with 0.15 M NaCl solution. Polysaccharide-containing fractions (revealed by a phenol-sulfuric acid method) were pooled and used for the in vitro study. The purified heparin preparation had an anticoagulant activity of 160 USP units/mg. Dextran sulfate (Sigma, USA), deoxyribonuclease (Sigma, type I), protamine sulfate (Sigma, from salmon), heparan sulfate (Seikagaku Kogyo, Co., Japan), carrageenin (Sigma, type I) and chondroitin sulfate (Wako Pure Chemical Industries Ltd., Japan) were used in this study.

Spleen lymphocytes, thymocytes, and macrophages were prepared from ddY mice (male, 6–10 weeks old) by conventional methods. Spleen and thymus were teased, and the splenocytes and thymocytes, respectively, were separated from erythrocytes by ammonium hemolysis solution (10 mM Tris-Cl, pH 7.4 buffer containing 0.84% NH₄Cl). Macrophages were prepared from peritoneal fluids of mice which had been treated 4 d previously with intraperitoneal injection of 2.0 ml of 3.0% thioglycollate. The peritoneal exudate cells thus obtained were incubated in a petri dish for 1.0 h at 37 °C, and the petri dish adherent cells were
recovered with a rubber policeman and used as the macrophage preparation. Each cell suspension was washed 3 times with phosphate-buffered saline (PBS) which consisted of (mM): NaCl, 137; KCl, 2.7; Na₂HPO₄, 8.1; KH₂PO₄, 1.5; pH 7.3, and resuspended in the same buffer.

The reaction mixture containing leukocytes (10⁷ cell), MgCl₂ (1.0 mM) and various concentrations of heparin were incubated in 1.0 ml of PBS at 4°C and then centrifuged for 10 min at 2000 × g. The cells treated with heparin were washed twice with PBS and resuspended in 1.0 ml of PBS containing 10 μg/ml of deoxyribonuclease. After 60 min incubation at 4°C, the cells were pelleted by centrifugation. The absorption at 258 nm of the supernatant fluid was immediately recorded. A positive control of cell lysis was prepared by sonication of cells (intensity 6, Ultrasonic Disruptor model UR-200P, Tomy Seiko Co., Ltd., Japan) before addition of deoxyribonuclease. Cell lysis was measured by obtaining the ratio of the absorption of a supernatant

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**FIG. 1. Effect of Heparin on Splenocytes, Thymocytes, and Macrophages**

Each splenocyte (○), thymocyte (□) and macrophage (△) preparation was added to the culture of 1.0 ml to make a cell density at 1.0 × 10⁷/ml. After heparin, dissolved in saline of 100 μl, was added to the culture to give the indicated concentration shown at the abscissa, cell lysis was determined as described under Materials and Methods. The ordinate indicates cell lysis.

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**FIG. 2. Effect of Sulfated Polysaccharide on Splenocytes**

To splenocyte suspensions (10⁷ cell, 1.0 ml), dextran sulfate (○), chondroitin sulfate (□), carrageenan (●), and heparan sulfate (■) were added. Leakage of macromolecules from splenocytes was measured, and expressed as cell lysis as described in the text. Abscissa indicates the final concentration of each polysaccharide tested.

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**FIG. 3. Effect of Protamine on Splenocyte Lysis Induced by Heparin or Dextran Sulfate**

Protamine sulfate in saline (10 μl) was added to splenocyte cultures (10⁷ cell, 1.0 ml) with heparin (○, 20 units/ml) or dextran sulfate (●, 30 μg/ml). Abscissa indicates the final concentration of protamine sulfate, and the cell lysis was examined by the method described in the text.

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**FIG. 4. Inhibition of Heparin-Induced Splenocyte Lysis by Mepacrine**

Mepacrine in saline (10 μl) was added to splenocyte cultures (10⁷ cell, 1.0 ml) with heparin. Mepacrine concentrations are shown in the abscissa. The insert figure explains the final concentrations of heparin in each column. Cell lysis was examined by the method described in the text.
fluid from a test cell suspension containing a concentration of a sulfated polysaccharide to the absorption of a supernatant fluid from a sonicated cell suspension (control).

RESULTS

Figure 1 shows the experimental results of leukocyte disruption induced by heparin. Splencocytes, thymocytes and macrophages were lysed in a dose-dependent manner to release macromolecules consisting of nucleic acid and protein. Leakage of these cellular components indicates leukocyte disruption and was more intensive with splenocytes and thymocytes than with macrophages. The microscopic examination supported the above findings, that is, heparin caused a progressive cell aggregation and an increase in the number of cells which were stained with trypan blue (deoxyribonuclease was not used and data not shown).

Next, we evaluated the effect of various sulfated polysaccharides which have structural similarity to heparin on splenocytes (Fig. 2). Dextran sulfate was most effective in destroying splenocytes. Carrageenin, which is known for its cytotoxic effect against reticuloendothelial cell\(^9\) also caused splenocyte lysis. Neither chondroitin sulfate nor heparan sulfate was competent in destroying splenocyte.

The effect of protamine, which neutralizes the anticoagulant activity of heparin,\(^9\) was examined to ascertain its effect on the cytotoxicity of heparin. Protamine sulfate was added to the splenocyte cultures with heparin or dextran sulfate. Protamine sulfate progressively suppressed the splenocyte disruption when tested to a concentration of 200 \(\mu g/ml\) (Fig. 3). Protamine sulfate itself had no effect on splenocyte viability under this experimental condition.

Figure 4 shows the suppression of the heparin-induced splenocyte lysis by mepacrine, a phospholipase inhibitor. Mepacrine suppressed the reaction in a dose-dependent manner, and the concentration that caused inhibition was comparable to that used in the inhibition of phospholipases in vitro.

Intracellular ionic conditions in leukocyte are not known exactly, however a buffer system that mimics the intracellular condition, reverse-PBS, which consisted of (mM): NaCl, 2.7; KCl, 137; Na\(_2\)HPO\(_4\), 8.1; KH\(_2\)PO\(_4\), 1.5; MgCl\(_2\), 4.0; pH 7.3, was used.\(^{10}\) We tested the effect of reverse-PBS on the macromolecule leakage from splenocyte induced by heparin. Splenocyte in reverse-PBS was incubated with heparin, and macromolecule leakage was evaluated. This incubation medium inhibited the heparin-induced splenocyte lysis when compared to PBS as shown in Fig. 5.

DISCUSSION

It is unlikely that the complement-required cell lysis or antibody-dependent cellular cytotoxicity participated in the heparin-induced leukocyte lysis since our culture medium contained no serum components and the reaction occurred at 4 °C. Inhibition by reverse-PBS suggests that passive ion flux may play role in cell disruption. Cells treated with heparin might have their surface membrane altered by decreased its barrier capacity against ions. Thus, when an extracellular ionic condition resembling the intracellular condition is used, such as the use of reverse-PBS, heparin-treated cells resisted cell disruption resulting in decreased macromolecule leakage because of decreased passive ion flux.

Excessive activation of phospholipases is known to be one cause of disrupting membrane permeability.\(^{11}\) The results shown in Fig. 5 indicate that the inhibition of phospholipases by mepacrine decreased the heparin-induced macromolecule leakage. Heparin, which influences

*FIG. 5. Effect of Reverse-PBS on the Heparin-Induced Splenocyte Lysis*

Splenocytes were suspended in PBS (●) or reverse-PBS (○) with various concentrations of heparin. Leakage of macromolecules was determined and expressed as cell lysis as described in the test.
various enzymes in vitro,\(^2\) activates endogeneous phospholipases, and the alteration of phospholipase activity changes membrane permeability against ions or perturbs the membrane structure to elicit cell disruption.

Carrageenin, chondroitin sulfate and heparan sulfate, which have little anticoagulant activity, had little effect on splenocyte integrity. On the other hand, heparin and dextran sulfate, anticoagulant sulfated-polysaccharides, induced cell disruption, and their action was blocked by protamine. It is well known that the anticoagulant activity of heparin is inhibited by protamine in vitro as well as in vivo.\(^9\) Therefore, it is used for neutralizing heparin when a hemorrhagic tendency is observed in heparin therapy. The above results (Figs. 2 and 4) show that heparin itself has cytotoxicity on leukocytes and from the view point of hematology it is important to investigate the mechanism of this phenomenon. Experiments to reveal a structure–activity relationship are in progress.

REFERENCES