

In vitro studies of the interaction between heparin and eosinophil cationic protein

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Eosinophil cationic protein (ECP) is a protein specific to the granules of human eosinophil granulocytes. ECP is highly cationic and may damage tissue if not inactivated. Heparin is a highly anionic substance present in mast cells and basophil granulocytes. The present *in vitro* study shows that ECP can inactivate the anticoagulant activity of heparin probably by the formation of a complex between the two molecules. This function may be of importance for the microenvironment of allergic diseases where secretion of heparin may promote penetration of mast cell products through tissues. Also this may constitute one mechanism whereby the cytotoxic action of ECP is neutralized.

Key words: eosinophil cationic protein; heparin; *in vitro* test; plasma coagulation.

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Eosinophil cationic protein (ECP) is a highly cationic protein and a major constituent of the human eosinophil granulocyte granules (6). The molecular weight of ECP is 18–21 kD and the molecule contains a large number of arginine residues which probably partly explains the high isoelectric point of the protein. The biological function of ECP is in particular related to its cytotoxic properties, with the capacity to kill a variety of mammalian and non-mammalian cells. Also ECP is able to enhance coagulation *in vitro* by a Factor XII-dependent mechanism (10).

Preliminary experiments suggested that ECP neutralized the anticoagulant activity of heparin. The aim of this study was to clarify the stoichiometric interaction between ECP and heparin.

MATERIAL AND METHODS

ECP with a molecular weight of 18–21,000 was purified from human eosinophil granulocytes. The purity of ECP was evaluated by means of

polyacrylamide-gel electrophoresis in the presence of sodium-dodecyl sulphate (5). Heparin with a molecular weight of $14,000 \pm 1,000$ was purchased from Leo Pharmaceutical, Copenhagen, Denmark. Platelet-poor plasma was prepared by centrifugation of citrated blood $2,000 \times g$ for 20 min. Plasma coagulation time was measured at 37°C in glass tubes by determination of the time from addition of calcium to clot formation. The coefficient of variation of the recalcification assay was less than 4%.

Heparin activity was also determined photometrically by means of Coatest heparin (KABI, Sweden). 200 µl heparin was incubated with antithrombin III (AT III) in excess, to generate heparin-AT III complexes. F X_a in excess was added and incubation continued for 30 s, F X_a not inactivated was allowed to catalyze the splitting of p-nitroaniline from the substrate S-2222 (Bz-Ile-Glu (γ-OR)-Gly-Arg-pNA). After 180 s the reaction was stopped with 50% acetic acid and the absorbance read at 405 nm. The absorbance decreased linearly with increasing heparin concentrations in the concentration range

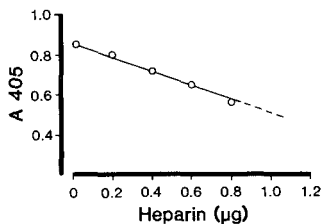


Fig. 1. Heparin activity determined with Coatest-heparin in plasma free medium. A linear relationship was obtained between the absorbance at 405 nm and the amount of added heparin (0–0.8).

studied (0.5–4.0 µg heparin/ml). Incubations were done in 0.05 mol/l Tris buffer, pH 8.4 (Fig. 1).

RESULTS

Increasing amounts of ECP were incubated with 1 µg (0.07 nmol) of heparin in a constant volume. After 90 s 100 µl of citrated plasma was added and incubation continued for 30 s, before addition of 100 µl 0.03 M CaCl_2 . The time until clot formation was recorded, and the results are shown in Fig. 2. The prolonged recalcification time induced by 1 µg of heparin was gradually abolished by preincubation with increasing amounts of ECP, and a complete reversal was seen with 4 µg (0.19 nmol) of ECP. In the next experiment, performed in a plasma-free medium, preincubation of heparin with ECP resulted in neutralization of the heparin activity. A slightly increased neutralization of heparin by ECP was found when the preincubation time was increased from 0 s to 30 s, but further preincubation did not change the degree of neutralization. In Fig. 3 the results are shown when 1.2 µg (0.086 nmol) of heparin and varying amounts of ECP were preincubated for 30 s before the addition of AT III. A complete neutralization of heparin was found by 0.6 µg (0.029 nmol) ECP. No change in the ability of ECP to neutralize heparin was found when

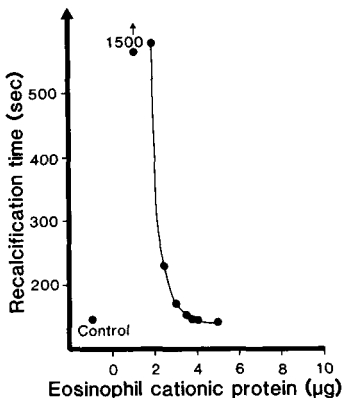


Fig. 2. Neutralization in human plasma of the anticoagulant activity of 1 µg of heparin by increasing amounts of ECP. The test was performed as a recalcification assay, and the time until clot formation was recorded. Control indicates the recalcification time of the plasma without added heparin.

heparin was preincubated with AT III for 30 s before the addition of ECP.

DISCUSSION

Heparin is a highly charged anion and its anticoagulatory effects are neutralized by cationic substances such as protamin. The finding in this study of a neutralization of heparin by ECP is probably related to the fact that ECP also is a highly cationic molecule (9). Thus the neutralization of heparin is likely to be the result of this opposition in charge. Gleich et al. found that major basic protein (MBP), another cationic protein of eosinophil granules, also neutralizes the anticoagulatory effects of heparin (3).

In the plasma-free heparin system the molar relationship for the interaction between ECP and heparin neutralization was 1:3, whereas in the plasma coagulation assay the same relationship for heparin neutralization was 8:3. This means that in the plasma system an excess of ECP was necessary for heparin to be neutralized. This

indicates the occurrence of substances in plasma that bind ECP and is consistent with observations which have shown the binding of ECP to both high and low molecular weight substances (7).

It is possible that ECP and MBP are natural inhibitors of heparin *in vivo*. By inhibition of heparin as well as by enhancing F XII dependent reactions, ECP may enhance blood coagulation (8).

In previous investigations it was found that long-term daily treatment with heparin injections resulted in a rise in blood eosinophil count (4). The number of eosinophils did not increase continuously during treatment as the same number was found after 5 and 10 days. The mechanism for this blood eosinophilia is not known, but it has been found that heparin in a dose-dependent manner inhibits the eosinopenia normally found after administration of ACTH, hydrocortisone, insulin and epinephrin (1).

Heparin is a constituent of tissue mast cells and blood basophils. In immediate type allergic reactions the degranulation of mast cells results in the release of heparin and other bio-active substances (11). The function of heparin in this type of inflammatory reaction is not known, but an inhibition of fibrin deposition might allow for a better penetration of mast cell products through the tissues. Eosinophils are attracted to the site of allergic reactions (2), and neutralization of hepa-

rin could be of value for the localization of the reaction and one of the physiological actions of ECP.

Conversely, ECP and MBP are highly cytotoxic proteins and therefore the binding to heparin could constitute one mechanism by which cells are protected from the deleterious effects of these proteins.

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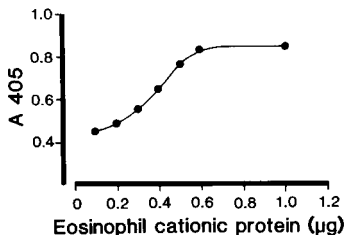


Fig. 3. Neutralization of the heparin activity by ECP in a plasma-free medium as measured by Coatest-heparin. Increasing amounts of ECP was added to 1.2 µg of heparin (0-0).

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