Identification In Vitro of an Endothelial Cell Surface Cofactor for Antithrombin III

PARALLEL STUDIES WITH ISOLATED PERFUSED RAT HEARTS AND MICROCARRIER CULTURES OF BOVINE ENDOTHELIUM

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ABSTRACT Two in vitro systems were used to identify an antithrombin III cofactor activity on vascular endothelium. Langendorff rat heart preparations or columns packed with endothelium cultured on microcarrier beads were perfused with mixtures of purified thrombin and antithrombin III. With each preparation, accelerated inhibition of thrombin by antithrombin III occurred during passage over endothelium. Platelet factor 4, protamine sulfate and diisopropylphosphoryl thrombin, all antagonists of the antithrombin III cofactor activity of heparin, significantly reduced the capacity of the preparation to inhibit thrombin. It is concluded that a substance with the functional properties of a stationary phase cofactor for antithrombin III is present on the microvascular endothelium and there catalyzes the inactivation of circulating free thrombin.

INTRODUCTION

The finding that inhibition of thrombin by antithrombin III (heparin cofactor) proceeds much more rapidly in vivo than in vitro led us to propose that a substance on the surface of the microvascular endothelium functions as a stationary phase cofactor for antithrombin III (1). Rapid inhibition of thrombin in vivo requires binding of thrombin to the endothelium and occurs predominantly in the microcirculation, where the concentration of cell surface sites is highest. However, because antithrombin III and thrombin concentrations in vivo do not lend themselves to experimental manipulation, the study of thrombin inhibition in vivo has severe limitations. On the other hand, endothelium in monolayer culture presents an insufficient surface to volume ratio for quantitative experiments in vitro. Therefore, to approach structural and functional analysis of endothelium-enhanced thrombin inhibition in vitro, we have used the Langendorff heart preparation (2) and endothelium cultured on microcarrier beads (3) as sources of a large endothelial surface. Our initial findings indicate that the endothelial antithrombin III cofactor has several functional properties in common with the anticoagulant glycosaminoglycans, heparin, and heparan sulfate.

METHODS

Reagents. Published methods were used to prepare thrombin and antithrombin III (4), ^{125}I-thrombin (1) and platelet factor 4 (5). Protamine sulfate was obtained from Eli Lilly & Co., Indianapolis, Ind, and tosyl-glycine-proline-arginine-*p*-nitroanilide was obtained from Boehringer-Mannheim. All experiments were carried out in Hanks' balanced salt solution pH 7.4(6), buffered with 15 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonate in place of $NaHCO_3$, and containing glucose (1 g/liter) and fatty acid-poor bovine albumin (1 mg/ml).

Cell cultures. Monolayer cultures of bovine pulmonary artery endothelium were prepared as described previously (7). Cells from pure monolayers were transferred to microcarriers as described by Ryan et al. (3), except that dextran beads coated with denatured collagen (cytodex 3, Pharmacia Fine Chemicals, Uppsala, Sweden) were used; cells were grown and maintained at confluence in spinner flasks. A representative culture is shown in Fig. 1.

Perfusion experiments. Thrombin (80 ng/ml plus 0.005–0.02 ng/ml of ^{125}I-thrombin) and the experimental reagents, drawn through separate channels of a roller pump, were mixed in a manifold and then perfused through either the coronary microcirculation (2) or columns (0.8 × 0.8 cm) packed with microcarrier cultures. At the beginning of each

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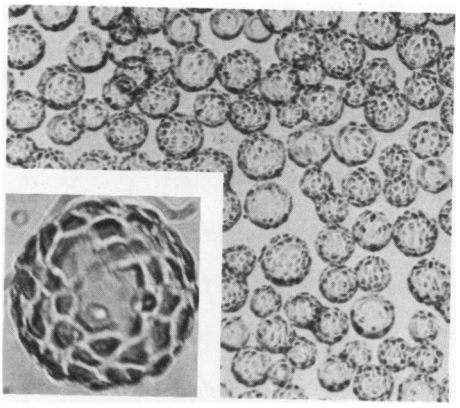

FIGURE 1 Bovine endothelium cultured on microcarrier beads × 60. Just before each experiment, a culture suspension was washed with HBSS, and columns (8 × 8 mm) were packed according to standard chromatographic techniques; at the end of experiments of up to 3 h duration, examination of the cultures by phase contrast microscopy revealed no perturbation of morphology or loss of viability as tested by trypan blue exclusion. Inset: single bead ×240.

experiment, perfusion with thrombin (40 ng/ml final concentration) was continued until ^{125}I counts per minute at the mixing manifold and in the perfusate were equal, then antithrombin III (50 μg/ml)±antagonists were added to the second channel. Approximately 15 s elapsed between mixing of reagents at the manifold and their appearance in the perfusate, a time during which insignificant thrombin was inhibited by bulk-phase antithrombin III (final concentration = 25 μg/ml).

For each determination, perfusate was collected into a test tube for 15 s, and then a 25-μl sample was withdrawn immediately and added to 25 μl of 0.5 mM tosyl-glycine-proline-arginine-*p*-nitroanilide for measurement of thrombin activity; the mixture was incubated at 30°C for 30 min, then 5 vol (250 μl) of 10% acetic acid were added and radioactivity and A_{405} were determined. The thrombin substrate concentration (0.25 mM) was sufficient to arrest further inhibition of thrombin with antithrombin III. With the antithrombin III concentration of 25 μg/ml, inhibition was first-order and slow ($t_{1/2}$ = 4.5 min) relative to the time (30 s) between mixing in the manifold and sampling of the perfusate. Therefore enhanced inhibition of thrombin during passage over endothelium would be reflected by a difference between thrombin specific activity at the manifold and in the perfusate.

RESULTS

Within 2–5 min of initiation of the perfusion of isolated hearts with thrombin, ^{125}I-thrombin in the perfusate reached a steady state equal to that of the manifold and remained constant for up to 3 h and throughout all experimental manipulation. Hydrolase activity reached a steady state in parallel, and in control perfusions was ~90% of the enzyme activity in samples from the manifold (Fig. 2, first bars of each set). Upon addition of antithrombin III to the perfusion, the enzyme specific activity was decreased by about two-thirds (Fig. 2, middle bars).

With individual hearts, a constant degree of inhibition could be maintained for at least 1 h. When the thrombin concentration in the perfusion was increased from 0.1 to 1.0 U/ml, 20% of the enzyme was inhibited. Because of the detection limits of the assay, lower thrombin concentrations (<0.1 U/ml) were not used. Addition to the antithrombin III of diisopropylphospho-thrombin, protamine sulfate, or platelet factor 4 decreased the capacity of the heart to promote inhibition (Fig. 2, third bars of each set). This effect was dose dependent, with the highest response to each antagonist shown in Fig. 2; further increases in antagonist concentrations produced no further effect.

Columns packed with microcarrier cultures of endothelium used in place of the isolated heart also promoted inhibition of thrombin, and diisopropylphospho-thrombin, protamine sulfate, and platelet factor 4 again decreased the capacity of the cultures to promote such inhibition (Fig. 3). However, a much higher concentration of diisopropyl-phospho-thrombin was required for a maximum effect; and a concentration of 1 μg/ml had no effect.

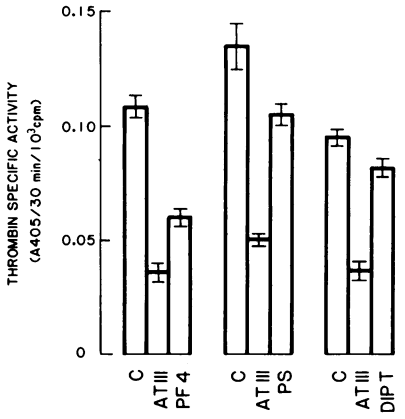

FIGURE 2 Enhanced reaction of thrombin with antithrombin III during perfusion of the myocardium. The first bar of each set shows steady-state controls (C) obtained by perfusing with thrombin alone. At the steady state, the activity of control samples averaged ~0.1 A_{405}/30 min, and had a sp act ~90% of that in samples taken from the manifold, presumably the result of traces of endogenous antithrombin III. Differences among control specific activities reflect differing tracer contents, which were always less than one part in 5,000 (500–1,000 cpm/tube). The middle bars (AT III) show the results obtained after the addition of antithrombin III to the perfusion, and the third bars of each set show the results obtained after the further addition of 10 μg/ml of platelet factor 4 (PF4), 10 μg/ml of protamine sulfate (PS) or 0.4 μg/ml of diisopropyl-phospho-thrombin (DIPT) to the perfusion. Each bar represents the mean±SD of six determinations.

In all experiments with both the hearts and cultures, whether or not an antagonist was present, inhibition of residual thrombin in the perfusates continued to be first-order with the rate ($t_{1/2}$ = 4.5 min) unchanged from that in samples taken from the manifold. When antithrombin III was omitted, no time-dependent inhibition of thrombin in the perfusate was observed.

Analysis of the perfusates by sodium dodecyl sulfate-polyacrylamide gel radioelectrophoresis confirmed that thrombin inhibition resulted from formation of the stable thrombin-antithrombin III complex (Fig. 4).

DISCUSSION

When considered in light of the geometry of cylinders, a substance on the luminal surface of the endothelium cell would be ~1,000 times more concentrated in capillaries than in large vessels. When the velocity of the circulation is factored into its geometry, location of heparin, heparan sulfate or some other cofactor for antithrombin III on the endothelium insures a very brief lifetime for circulating, active thrombin, and perhaps other procoagulants as well. Thus, we envisage every fractional volume of the circulating blood becoming briefly "heparinized" every 5–6 s. In wounds, conversely, where circular perfusion of the microcirculation is interrupted, hemostasis may ensue unimpeded by antithrombin III, which now, separated from its cofactor, is much less potent.

The isolated heart and cultured cell preparations each function in the manner of a stationary phase cofactor for antithrombin III. Like the endothelial cofactor for protein C activation (8, 9), the antithrombin III cofactor appears to saturate with respect to thrombin, as reflected both by the decrease in fractional inhibition as thrombin was increased from 0.1 to 1.0 U/ml (1–10 nM) and by near-maximal inhibition in the heart preparation with 10 nM diisopropylphosphothrombin. These values are of the same order as those obtained in vivo (1), and with antithrombin III and heparin in solution (10). The higher concentration of diisopropylphospho-thrombin required to inhibit the microcarrier cultures suggests a qualitative difference in the product of cell culture.

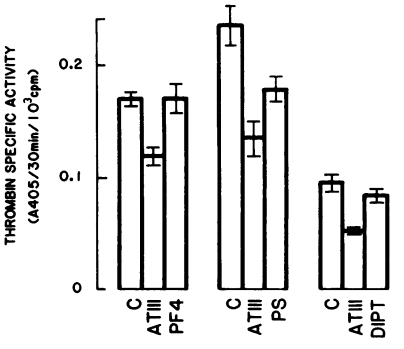

FIGURE 3 Enhanced reaction of thrombin with antithrombin III during perfusion of columns of microcarrier cultures of endothelium. All procedures and conditions were the same as those for Fig. 2, except that the maximum response to diisopropylphospho-thrombin required 40 μg/ml.

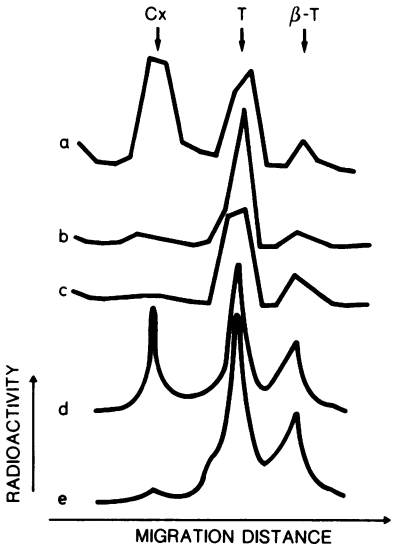

FIGURE 4 Dodecyl sulfate-polyacrylamide gel radioelectrophoresis of heart and microcarrier culture perfusates. Samples of steady-state perfusates were collected directly into 1/10 vol of 10% sodium dodecyl sulfate solution and placed immediately in a boiling water bath for 2 min. The samples were subjected to polyacrylamide gel electrophoresis in sodium dodecyl sulfate, and radioactivity was determined either in slices (2 mm) of gel columns (a–c) or by densitometry of gel slab radioautographs (d–e). The thrombin-antithrombin III complex (Cx) was identified as the reaction product of ^{125}I-thrombin (T), antithrombin III, and heparin (not shown). Differing β-thrombin content reflects differing ^{125}I-thrombin preparations. The radio-electropherograms are: a, perfusate, heart perfused with thrombin and antithrombin III; b, perfusate, heart perfused with thrombin only; c, perfusion buffer containing thrombin and antithrombin III, sampled at the mixing manifold (preperfusion); d, perfusate, microcarrier culture perfused with thrombin and antithrombin III; e, perfusate, microcarrier culture perfused with thrombin only.

Of the substances known to enhance the activity of antithrombin III, extracellular heparan sulfate has been found associated with endothelium in vitro (11) and in vivo (12, 13) and is a candidate for the microvascular antithrombin III cofactor. Recently, Castellot et al. (11) found that heparin also may be associated with endothelium. Results obtained with the heparin antagonists diisopropylphospho-thrombin (1, 11), protamine sulfate and platelet factor 4 (14) are consistent with this view. In addition, interference with the re-

action by platelet factor 4, a platelet secretory product that binds to endothelium-associated heparan sulfate (15), raises the possibility that the normal function of the cofactor might be influenced by pathologic states. With the finding that antithrombin III cofactor activity can be measured accurately and reproducibly in isolated organs and cell cultures, a further characterization of its properties should now be feasible.

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