

Eosinophil Cationic Granule Proteins Impair Thrombomodulin Function

A Potential Mechanism for Thromboembolism in Hypereosinophilic Heart Disease

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Abstract

Thromboembolism is a prominent but poorly understood feature of eosinophilic, or Loeffler's, endocarditis. Eosinophil (EO) specific granule proteins, in particular major basic protein (MBP), accumulate on endocardial surfaces in the course of this disease. We hypothesized that these unusually cationic proteins promote thrombosis by binding to the anionic endothelial protein thrombomodulin (TM) and impairing its anticoagulant activities. We find that MBP potently (IC_{50} of 1–2 μ M) inhibits the capacity of endothelial cell surface TM to generate the natural anticoagulant activated protein C (APC). MBP also inhibits APC generation by purified soluble rabbit TM with an IC_{50} of 100 nM without altering its apparent K_d for thrombin or K_m for protein C. This inhibition is reversed by polyanions such as chondroitin sulfate E and heparin. A TM polypeptide fragment comprising the extracellular domain that includes its naturally occurring anionic glycosaminoglycan (GAG) moiety (TMD-105) is strongly inhibited by MBP, whereas its counterpart lacking the GAG moiety (TMD-75) is not. MBP also curtails the capacity of TMD-105 but not TMD-75 to prolong the thrombin clotting time. Thus, EO cationic proteins potently inhibit anticoagulant activities of the glycosylated form of TM, thereby suggesting a potential mechanism for thromboembolism in hypereosinophilic heart disease. (*J. Clin. Invest.* 1993. 91:1721–1730.) Key words: Eosinophils • thrombomodulin • glycosaminoglycan • cationic protein • major basic protein • eosinophil peroxidase • eosinophil cationic protein

Introduction

Peripheral blood eosinophilia, irrespective of its cause, is frequently complicated by a morbid and potentially lethal form of endocarditis characterized by eosinophils (EOs)¹ adhering to and infiltrating the endocardium, mural thrombosis, endocardial damage, and embolism (1, 2). Such eosinophilic endocarditis may lead rapidly to death from thromboembolic compli-

cations, evolve over months or years to cause progressive endocardial and myocardial damage culminating in congestive heart failure, or resolve over years leaving residual endomyocardial fibrosis (1–3). This unusual form of endocarditis, although rare in temperate climates, causes 10–20% of all cardiac deaths in tropical Africa and Southeast Asia, where chronic hypereosinophilia, caused by endemic parasitic infestations, is common (4).

Mechanisms underlying the pronounced thromboembolic diathesis that characterizes both the acute and chronic phases of eosinophilic endocarditis are poorly understood. However, EO granule proteins have been implicated in the pathogenesis of this disorder. EO-specific granules are comprised almost entirely of four unusually cationic ($pI > 11$) proteins (major basic protein [MBP], eosinophil peroxidase [EPO], eosinophil cationic protein [ECP], and eosinophil-derived neurotoxin [EDN]) that function as potent but nonspecific cytotoxins (reviewed in reference 5). Patients with eosinophilic endocarditis have degranulated circulating EOs (6) as well as high (up to micromolar) serum levels of MBP (7). Moreover, endomyocardial biopsies taken at various stages of this disease uniformly demonstrate dense endocardial and small vessel endothelial surface deposition of MBP (8), ECP (8), and EPO (9).

Endothelial (10) and endocardial (10) cells actively participate in maintaining an anticoagulant surface, in part through their expression of the 105-kD transmembrane protein thrombomodulin (TM) (10–12). Endothelial cell surface TM exerts an anticoagulant effect by avidly (K_d 0.5 nM, [11, 12]) binding circulating thrombin to (a) curb its fibrinogen-cleaving activity, (b) potentiate the interaction of antithrombin III with thrombin, and (c) alter the substrate specificity of thrombin, accelerating greatly its proteolytic activation of circulating protein C to activated protein C (APC). APC, in turn, is a powerful anticoagulant serine protease that, in conjunction with protein S, terminates the procoagulant activity of Factors Va and VIIIa (10–12). Of note, the complete TM molecule is quite anionic ($pI \approx 4$ [13]), in part because of extensive posttranslational glycation of the large extracellular domain of TM with an unusual hypersulfated, chondroitin sulfate E-like moiety (14–20). This bulky polyanionic domain strongly influences all three known anticoagulant functions of TM (14–23).

We hypothesized that eosinophilic cationic granule proteins deposited on endocardial and endothelial surfaces bind electrostatically to the anionic extracellular domain of TM, impair TM anticoagulant function, and thereby contribute to the prominent thromboembolic diathesis that typifies eosinophilic endocarditis. To test this hypothesis, we determined the effect of purified human EO granule proteins upon the ability of endothelial cell-bound TM, isolated full-length TM, and the extracellular domain of TM to generate APC and impair the fibrinogen-cleaving activity of thrombin.

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1. Abbreviations used in this paper: APC, activated protein C; ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin; EO, eosinophil; EPO, eosinophil peroxidase; GAG, glycosaminoglycan; Gla-domainless protein C, γ -carboxyglutamic acid-domainless protein C; MBP, major basic protein; TM, thrombomodulin.

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Methods

Materials

Chromogenic substrates S-2366 and S-2388 were obtained from Kabi Vitrum (Franklin, OH). Benzamidine-free bovine protein C and anti-thrombin III were obtained from Enzyme Research Laboratories, Inc. (South Bend, IN). Bovine plasma thrombin (2,500 NIH units/mg protein), cycloheximide, porcine rib cartilage chondroitin sulfate A, and Hepes were obtained from Sigma Chemical Co. (St. Louis, MO). Hanks' buffered salt solution and Dulbecco's modified DMEM medium were obtained from Gibco BRL Life Technologies Inc. (Grand Island, NY). Squid cartilage chondroitin sulfate E, super special grade, was obtained from Seikagaku America, Inc. (Rockville, MD). Purified, detergent-solubilized rabbit thrombomodulin and Gla (γ -carboxyglutamic acid)-domainless protein C were generously provided by N. L. and C. T. Esmon (Oklahoma Medical Research Foundation, Oklahoma City, OK). The eosinophil granule basic proteins MBP, EPO, ECP, and EDN were purified to physical homogeneity from granule preparations derived from EOs of patients with hypereosinophilic syndrome as previously described (24–26). Human thrombomodulin TMD-105 and TMD-75 were kindly provided by John Parkinson (Eli Lilly and Co., Indianapolis, IN). Human umbilical vein endothelial, porcine aortic endothelial, and human aortic endothelial cells were obtained from collagenase-treated blood vessels as previously described (27) and maintained in DMEM supplemented with penicillin, streptomycin, L-glutamine, and 15% heat-inactivated FCS (Gibco, Grand Island, NY). Cells were grown to confluence and used for experiments \sim 1 wk after initial seeding.

Methods

Immunofluorescent localization of major basic protein in cardiac sections. Formaldehyde-fixed and paraffin-embedded tissue microtome sections were obtained at autopsy from a 70-yr-old male with clinical eosinophilic endocarditis and hypereosinophilia related to the presence of a pulmonary carcinoma that secreted a potent eosinophilopoietic factor, as we have previously described (28). Sections were stained for the presence of MBP using a polyclonal rabbit anti-MBP antibody and visualized by indirect immunofluorescence using a goat anti-rabbit Ig antibody conjugated with FITC as previously described (29). As a control, serial sections were stained with protein A affinity-purified normal rabbit IgG and showed no fluorescence.

Assay of APC generation by endothelial monolayers. Tissue culture medium was aspirated from endothelial monolayers, which were then washed three times with H/H buffer (Hanks' buffered salt solution supplemented with 1 mM magnesium and calcium and 20 mM Hepes buffer, pH 7.4). Monolayers were overlaid with 200 μ l of either H/H buffer or H/H containing increasing concentrations of MBP. Preliminary experiments established that the inhibitory effect of MBP on APC generation by endothelial monolayers was already maximal by 10 min. To ensure that the MBP/TM interaction was complete, however, monolayers were exposed to MBP for 30 min. Supernatant buffer was then aspirated and the monolayers subsequently washed two times in 1 ml of 37°C H/H and overlaid with 500 μ l of H/H supplemented with 3 mM CaCl_2 , 500 nM protein C, and 1.5 nM thrombin. Plates were then incubated at 37°C for 90 min, whereupon APC generation was terminated by the addition of 20 μ l of 6 μ M antithrombin III, vortexed and incubated 5 min further. This 90-min incubation, based on the work of others utilizing endothelial cell monolayers (23, 30, 31), is necessary to obtain optimal sensitivity. APC generation is linear over the 90-min incubation period (not shown). The resulting mixture was then briefly centrifuged to remove cellular debris and transferred to a cuvette containing 0.400 ml of 0.1 molar NaCl, 0.02 molar Tris, pH 7.4, and 0.1% BSA and 50 μ l of either S-2238 or S-2366 (both at 400 nM final). The initial APC generation was then assayed spectrophotometrically by conversion of the chromogenic substrate at 405 nM in a 37°C spectrophotometer cell based on the initial rate of optical density change. Negative controls consisted of empty plastic wells treated in a parallel

fashion. Values obtained from these controls was then subtracted from the rates measured for wells containing monolayers.

Endothelial cell monolayer regeneration of APC-generating capacity after washout of MBP with intact or impaired protein synthetic capacity. 1-cm² monolayers (48-well plate) of porcine aortic endothelial cells were aspirated free of tissue culture medium and washed three times with warmed H/H as described above. Wells were then overlaid with 100 μ l of H/H with or without 10 μ g/ml cycloheximide, then further supplemented with either buffer or 3.3–5 μ M MBP and incubated a further 30 min. At this point, monolayers were either washed free of unbound MBP and buffer and assayed for APC-generating capacity using S-2366 as described above or, alternatively, overlaid with 500 μ l Iscove's DMEM plus 15% FCS with or without the continued presence of cycloheximide, as appropriate. At various time points thereafter (1–16 h), these latter monolayers were then washed free of supernatant medium and assayed for APC-generating capacity. By 16 h, both in the presence and absence of MBP, the cycloheximide-treated monolayers were visibly altered and had low viability as judged by trypan blue exclusion criteria, so no data is shown from this time point.

Effect of intracellular hypokalemia upon MBP-mediated inhibition of APC generation by endothelial monolayers. 1-cm² confluent monolayers of porcine aortic endothelial cells were left normokalemic or rendered intracellularly hypokalemic by treatment with nigericin and exposure to hypokalemic buffers as described by Larkin et al. (32). Tissue culture medium was aspirated from monolayers, which were washed either with buffer B (15 mM Hepes, 100 mM NaCl, 1 mM CaCl_2 , and 1 mM MgSO_4) or buffer B supplemented with 4 mM KCl. Monolayers were then overlaid with 500 μ l of normokalemic buffer or with hypokalemic buffer containing 4 μ M nigericin and incubated 45 min at 37°C before being washed either with normo- or hypokalemic buffer. Monolayers were then assayed for their capacity to generate APC in the presence of 2 nM thrombin, 500 μ M protein C, and 3 mM CaCl_2 in potassium-free H/H buffer for 3 h at 37°C. After quenching of APC generation with antithrombin III, APC was quantitated using the chromogenic substrate S-2366 as previously described.

Inhibition of rabbit TM APC generation by MBP and EPO. To 50 μ l H/H were added 10 μ l 30 mM CaCl_2 and 10 μ l 20 nM rabbit TM and either 10 μ l of H/H buffer or H/H buffer containing various concentrations of MBP or EPO. The solution was vortexed and allowed to incubate at room temperature for 10 min, whereupon 20 μ l of 2.5 μ M bovine protein C and 10 μ l of 20 nM bovine thrombin were added, vortexed, and incubated 10 min at 37°C before addition of 10 μ l of 60 μ M AT-III. After 5 min more of incubation at 37°C, the entire mixture (110 μ l) was transferred to a cuvette and 290 μ l of cuvette buffer containing 400 nM S-2366, mixed, and assayed for initial rate of APC generation spectrophotometrically at 405 nM.

Effect of MBP on thrombin dependence of protein C activation by soluble rabbit TM. 10 μ l of 10 μ M rabbit TM, 10 μ l of 15 mM CaCl_2 , and 10 μ l of 375 nM MBP were added to wells on a 96-well microtiter plate (previously treated with 0.1% Tween 20 detergent and rinsed to render surfaces hydrophilic), mixed, and allowed to incubate 10 min at room temperature. The plate was then allowed to warm to 37°C and 10 μ l of 2.5 μ M protein C was added and mixed. 10 μ l of thrombin at various concentrations was then added, the wells were mixed again, and the plate was incubated 10 min at 37°C. During these 10 min of incubation the rate of APC generation was constant under these conditions (not shown). Therefore, APC generated at 10 min represents accurately an initial rate of APC generation that was used to calculate kinetic parameters, as has been done previously (18, 22). 10 μ l of 30 μ M AT-III was then added, mixed, and incubated 5 min further at 37°C. APC generation was quantitated by adding 150 μ l of cuvette buffer containing S-2366 at a final concentration of 400 μ M and quantitated by initial rate as assayed at 405 nm on a Thermomax microtiter V_{\max} plate reader (Molecular Devices Corp., Menlo Park, CA). Another set of wells was composed as described above with the exception that buffer was substituted for the rabbit TM; values obtained from these wells were subtracted from those obtained from the wells with

rabbit TM at each thrombin concentration to correct for TM-independent cleavage of S-2366 by thrombin.

Determination of apparent K_m of thrombin/rabbit TM complex for protein C. 10 μ l of 450 nM MBP or H/H buffer was combined with 10 μ l of 12 nM rabbit TM and incubated for 10 min at room temperature, whereupon 30 μ l of various concentrations of protein C and 10 μ l of 0.6 nM thrombin were added in a 96-well microtiter plate, mixed, and incubated 10 minutes at 37°C. Final conditions were 3 mM CaCl_2 , 75 nM MBP, and 2 nM rabbit TM. APC generation was terminated by addition of AT-III and assayed using S-2366 as above.

Kinetics of MBP interaction with TM-dependent APC generation. Data from Fig. 5 were analyzed with nonlinear regression analysis (Statistics, Version 5.2, SYSTAT Inc., Evanston, IL) to avoid the hazards of linear analysis related to error distribution (33). Data shown are plus or minus standard deviation.

Polyanion reversal of MBP impairment of rabbit TM. 10 μ l of 10 nM rabbit TM was combined with 10 μ l of 1 μ M MBP in a 96-well plate microtiter well and incubated 10 min at room temperature, subsequent to which 10 μ l of various concentrations of chondroitin sulfate A, chondroitin sulfate E, or heparin or H/H buffer was mixed in and the resulting mixture incubated 45 min further at 37°C. 10 μ l of 2.5 μ M bovine protein C and 10 μ l of 10 nM bovine thrombin were then added, mixed, and incubated 10 min before addition of AT-III and assay of APC generation as described above. The final calcium concentration was 3 mM.

Effect of MBP on APC generation by human TM fragments, TMD-105, and TMD-75. 20 μ l of 2.5 μ M bovine protein C, 40 μ l of H/H buffer, and 10 μ l of 20 nM TMD-75 or TMD-105 were mixed with 10 μ l of either buffer or the indicated concentration of MBP and 10 μ l of 50 mM CaCl_2 , then incubated 5 min at room temperature. The specimen was warmed to 37°C in a water bath and 10 μ l of 20 nM bovine thrombin was added to initiate generation of APC. After 10 min, the reaction was terminated with AT-III, incubated 5 min, and assayed for APC using the chromogenic substrate S-2366 in a final volume of 500 μ l in a 1-cm cuvette. The values obtained from TM-free controls were subtracted from each data point. Final conditions were 500 nM bovine protein C, 2 nM bovine thrombin, 2 nM thrombomodulin, and 5 mM calcium.

MBP interactions with the capacity of TM to prolong the thrombin clotting time. All reagents were made up in H/H plus 0.15% Lubrol PX supplemented with 1.25 mM CaCl_2 . 100 μ l H/H was mixed with 60 μ l of 100 nM TM (either rabbit TM, TMD-105, or TMD-75) and 30 μ l of MBP at 10 times its final concentration (0.5 or 1.0 μ M), mixed, and incubated 10 min at 37°C. 30 μ l of 50 nM bovine thrombin was then added and the mixture incubated 10 min further at 37°C subsequent to mixing with 60 μ l of 10 mg/ml of fibrinogen and initiating the thrombin clotting time. Formation of clot was determined in a fibrometer (Becton Dickinson and Co., Cockeysville, MD). Each determination was made at least in triplicate.

Results

As shown in Fig. 1, endocardial surfaces can accumulate dense deposits of eosinophil cationic granule proteins even at the earliest stage of eosinophilic endocarditis, before morphologically evident damage to endocardium occurs. Fig. 1 *A* shows a hematoxylin-and-eosin-stained section of tissue specimen obtained from the right atrial wall of a 70-yr-old male who developed high grade hypereosinophilia and eosinophilic endocarditis caused by a pulmonary carcinoma that secreted a potent eosinophilopoietic factor, as we have previously described (28). The right atrial endocardial wall appears normal although the lumen is in part obliterated by a cellular clot (labeled *C*), which is composed almost entirely of EOs and has apparently detached from the endocardium (labeled *E*) during the fixation process. Fig. 1 *B* shows a serial tissue section

stained for the presence of MBP by indirect immunofluorescence. Note the bright endocardial MBP deposits, the staining intensity of which rivals that of intact EO granules in the adjacent cellular clot.

To model the endocardial deposition of ECPs seen in eosinophilic endocarditis, we exposed intact endothelial monolayers from three sources to MBP or a buffer control for 30 min, washed away unbound MBP, then assayed their capacity to support generation of APC in the presence of thrombin and protein C. After exposure to MBP, immunofluorescent staining of MBP-treated, but not buffer-treated, monolayers with anti-MBP antibody demonstrated bright cell surface and matrix MBP localization (not shown). As shown in Fig. 2, MBP, the predominant constituent of EO-specific granules (50% of total protein [34]), potentially inhibits TM-dependent APC generation by porcine aortic, human umbilical vein, and human aortic endothelial monolayers. The 50% inhibitory concentration (IC_{50}) is 0.5–2 μ M, within the range of MBP found circulating in the serum individuals with high grade eosinophilia (35). At concentrations > 5 μ M MBP there is no discernible APC generation. This complete abrogation by MBP of the ability of endothelial cells to support thrombin-dependent APC generation is not attributable to a direct cytotoxic effect of MBP on endothelial cells, interference with the assay for APC, or proteolysis of cell surface TM by proteases contaminating our MBP preparation because in experiments not shown we found that: 1) treatment of endothelial cell monolayers with MBP at concentrations up to 10 μ M had no effect on monolayer integrity as assessed morphologically or as quantitated by ^{51}Cr release; 2) virtually identical results were obtained when MBP was not washed out before assay of APC and addition of MBP to APC did not interfere with its detection by chromogenic substrate; and 3) preparations of purified rabbit TM and fibronectin incubated 0.5 h in the presence of high concentrations of MBP showed no evidence of proteolytic digestion by PAGE. In addition to MBP, two other cationic EO granule proteins, EPO and ECP (represented, respectively, by the *open boxes designated EPO and ECP*) also attenuated thrombin-dependent APC generation by porcine endothelial monolayers, in the case of EPO even more potently than MBP. In contrast, BSA at these concentrations had no effect upon APC generation by endothelial monolayers. Thus, the three major cationic proteins comprising EO-specific granules are all potent inhibitors of TM-dependent APC generation by endothelial monolayers.

To assess the durability of MBP inhibition of endothelial monolayer TM function as measured by APC generation and its potential reversibility, we assayed APC generation by monolayers previously treated with nearly 100% inhibitory concentrations of MBP, thoroughly washed, then further incubated in complete tissue culture medium including 10% FCS in either the presence or absence of 10 μ g/ml cycloheximide to inhibit protein synthesis. Fig. 3 shows the results of two such experiments, the first using 3.3 μ M MBP and the second 5 μ M MBP. In the first experiment, MBP-treated endothelial monolayers without cycloheximide (■) regenerate 30% of their capacity to activate protein C 2 h after MBP washout, increasing to 46% by 5 h and 88% after 16 h. By contrast, MBP-treated monolayers incubated in the presence of cycloheximide (▲) recover only 10% of their activity by 5 h. Over this period cycloheximide has no significant effect upon the APC generation capacity of

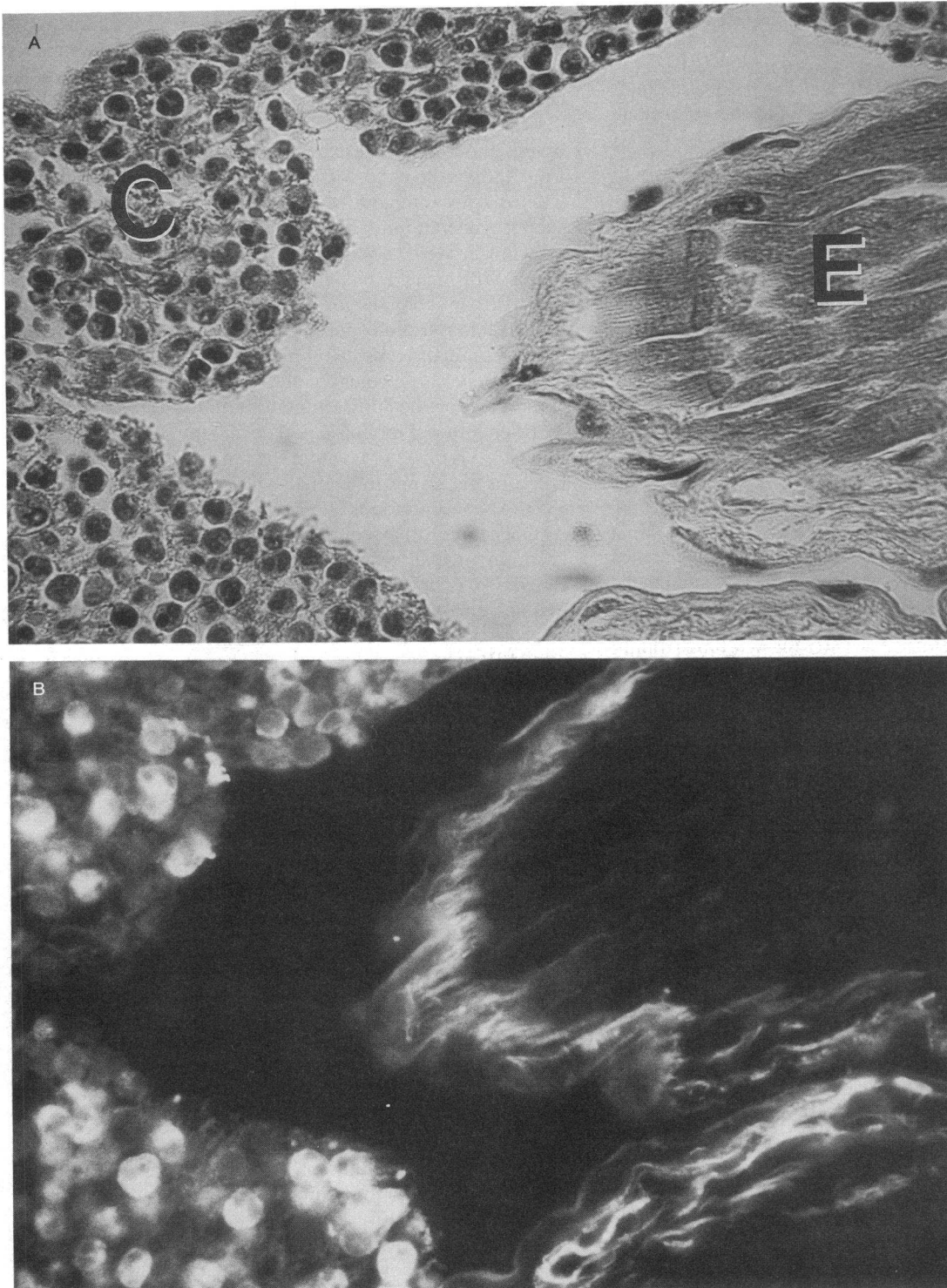


Figure 1. Immunofluorescent localization of MBP in the endocardium of a patient with eosinophilic endocarditis. (A) Hematoxylin-and-eosin section of right atrium: $\times 400$. E, endocardium; C, clot comprised of proteinaceous material and intact EOs. (B) Serial section stained for presence of MBP using indirect immunofluorescence. $\times 400$.

monolayers not treated with MBP. By 16 h, however, the cycloheximide-treated preparations are nonviable, thus making meaningful comparisons impossible. In a second experiment performed using $5 \mu\text{M}$ MBP, monolayers incubated in the absence of cycloheximide (\square) recover more slowly and less com-

pletely than in the first experiment, but cycloheximide-treated monolayers (Δ) remain nearly completely inhibited through 8 h. Thus, inhibition of endothelial surface TM activity resulting from a single exposure to MBP lasts ≥ 8 h in the absence of protein synthesis; however, partial or even complete reexpres-

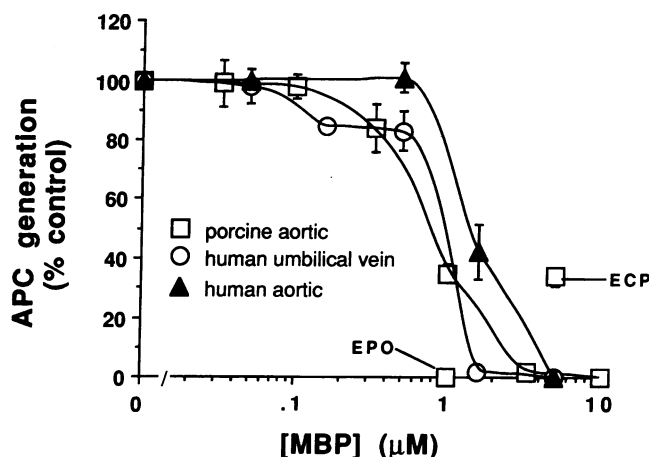


Figure 2. Impaired APC generation by endothelial monolayers exposed to cationic EO granule proteins. 2-cm² endothelial cell monolayers of the indicated derivation were exposed 30 min to 200 μ L of H/H buffer supplemented with the indicated concentrations of MBP, thoroughly washed, and assayed for their capacity to support thrombin-dependent activation of protein C in the presence of 1 mM calcium, 500 nM protein C, and 1.5 nM thrombin over 90 min at 37°C. APC was then quantitated using the chromogenic substrate S-2366, as described in Methods. \square , porcine aortic endothelium; \circ , human umbilical vein endothelium; \blacktriangle , human aortic endothelium. For porcine aortic endothelium only, data are shown for single concentrations of ECP and EPO in the open boxes so labeled. Data are shown \pm standard deviation.

sion of surface TM activity occurs in cells with intact protein synthetic capacity.

These results suggest that de novo synthesis and surface expression of TM is required for MBP-treated endothelial monolayers to recover TM function. Two potential mechanisms underlying the initial inhibition by MBP include durable blockade of surface TM activity or endocytosis of cell surface TM, as has been shown to occur in PMA-treated hemangioma cells (36) and TNF-treated (37, 38) endothelial monolayers. To address the latter possibility, we determined whether intracellular hypokalemia induced by exposure of endothelial cells to nigericin and extracellular hypokalemia, a potent inhibitor of endocytosis (32), also blocks MBP inhibition of endothelial cell surface TM function. As shown in Table I, depletion of intracellular potassium using the nigericin-hypokalemic buffer protocol had no effect on the ability of MBP to inhibit endothelial monolayer generation of APC in the presence of thrombin. This result suggests that MBP inhibits endothelial cell surface TM activity by blocking the function of TM *in situ* rather than by inducing endocytosis.

If EO cationic granule proteins impair APC generation of intact monolayers by interacting directly with TM, then these same proteins should inhibit isolated TM in solution as well. We therefore measured the effect of MBP, EPO, and ECP on APC generation in solutions containing purified full-length rabbit lung TM. As shown in Fig. 4, MBP is a potent inhibitor of APC generation with an IC₅₀ of 100 nM. EPO, the toxicity of which is typically ascribed to its peroxidative catalytic activity, is also an effective inhibitor of APC generation despite the absence of any hydrogen peroxide substrate, with an IC₅₀ of only 10 nM, 10-fold less than that of MBP. Similarly, ECP inhibits

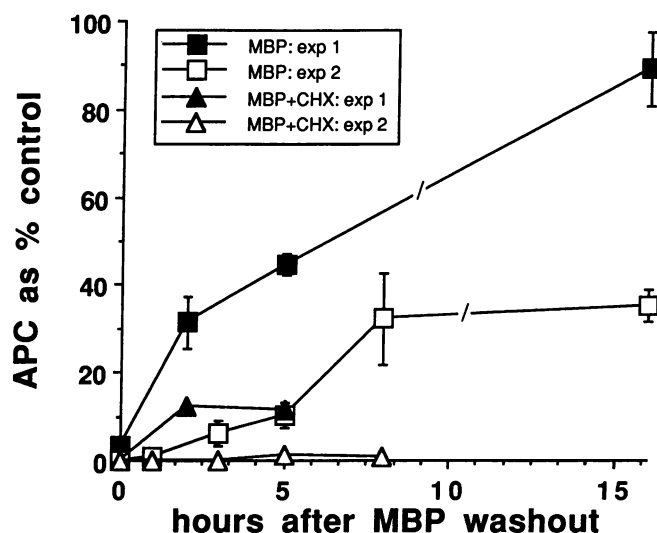


Figure 3. Recovery of APC generation capacity by porcine aortic endothelial cells: time course and requirement for intact protein synthetic capacity. Results of two experiments are shown. In the first, 1-cm² monolayers of porcine aortic endothelial cells were exposed 90 min to 3.3 μ M MBP (\blacksquare) or 3.3 μ M MBP with 10 μ g/mL cycloheximide (\blacktriangle), then thoroughly washed with H/H buffer. One set of monolayers from each treatment group was then assayed immediately for APC generation capacity by incubating 90 min in the presence of 500 nM protein C and 0.25 nM thrombin ($T = 0$ at time of washout). Other sets of monolayers were overlaid with 500 mL of Iscove's DMEM with 15% FCS with (\blacktriangle) or without (\blacksquare) cycloheximide (CHX). These monolayers were washed and assayed for APC generation capacity at various time points hours after washout. APC generation is expressed as a percentage of that of control monolayers first exposed to, then further incubated in MBP-free buffers with or without CHX, as appropriate. At 16 h CHX-treated monolayers were nonviable; therefore, data for these points are not shown. In the second experiment, the protocol was identical except that monolayers were treated with 5 μ M MBP for 30 rather than 90 min in the absence (\square) or presence (Δ) of CHX. Data are shown \pm standard deviation.

APC generation with an IC₅₀ of 5 μ M (not shown). Thus, EO cationic granule proteins inhibit APC generation by soluble rabbit TM as well as by intact endothelial monolayers.

Table I. Effect of Hypokalemia on MBP Inhibition of Endothelial Cell Surface TM Function

Endothelial monolayer preparation	APC generated in absence of MBP exposure	APC generated after exposure to 10 μ M MBP
	<i>mOD₄₀₅/min</i>	
Normokalemic	98 \pm 3	16 \pm 2
Hypokalemic	122 \pm 7	17 \pm 1

1-cm² confluent monolayers of porcine aortic endothelial cells were either left normokalemic or rendered intracellularly hypokalemic by exposure to 4 μ M nigericin and hypokalemic extracellular buffers as described in Methods. Normo- and hypokalemic monolayers were further exposed to, respectively, potassium-containing or -free buffers, each either with or without 10 μ M MBP. Monolayers were then washed and assayed for APC-generating capacity over 3 h in the presence of 500 nM protein C and 2 nM thrombin. Values are means \pm SD.

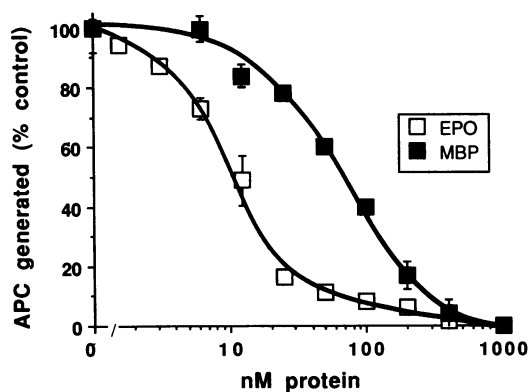


Figure 4. Inhibition of soluble rabbit TM APC generation by MBP and EPO. 2 nM rabbit TM was incubated with the indicated concentrations of cationic protein for 10 min before the addition of 500 nM protein C and 2 nM thrombin at a final calcium concentration of 3 mM, then assayed for APC generation. \square , EPO; \blacksquare , MBP. 100% = 230 mOD/min at 405 nm. Data are shown \pm standard deviation.

To ascertain the mechanism whereby MBP inhibits APC generation by the thrombin/TM complex, we determined the effect of 75 nM MBP (a 65–75% inhibitory dose) on the apparent K_d of rabbit TM for thrombin and the apparent K_m of protein C for the thrombin/TM complex. As shown in Fig. 5 A, this concentration of MBP decreases the V_{max} of APC generation with respect to thrombin to approximately one-third that of untreated rabbit TM. Similarly, as shown in Fig. 5 B, this concentration of MBP also significantly decreases the V_{max} of APC generation with respect to protein C. These impressions were confirmed by nonlinear kinetic analyses of these data,

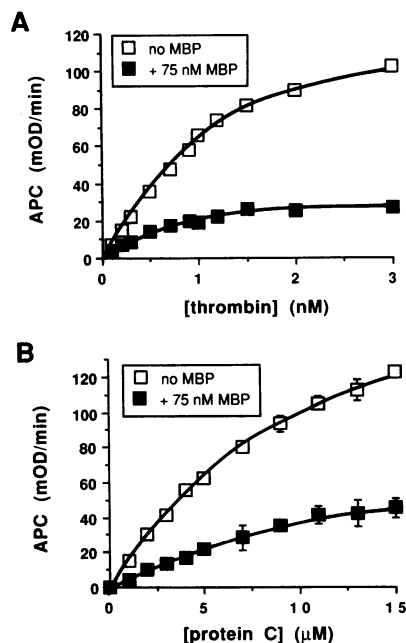


Figure 5. Effect of MBP on thrombin and protein C dependence of protein C activation by rabbit TM. (A) Solutions containing 2 nM rabbit TM were incubated in the absence (\square) or presence (\blacksquare) of 75 nM MBP for 10 min before assaying the APC generation in the presence of 3 mM CaCl_2 and the indicated concentrations of thrombin. Each data point represents the mean of quadruplicate determinations. Data are shown \pm standard deviation. (B) Solutions containing 2 nM (final) rabbit TM were incubated in the presence or absence of 75 nM MBP for 10

min before addition of the indicated concentrations of protein C and 0.1 nM thrombin and assayed for APC generation. Each data point represents the mean of triplicate determinations. Data are shown \pm standard deviation.

Table II. APC Generation Kinetics of MBP Interaction with TM

Sample	K_d for thrombin nM	V_{max} for thrombin mOD ₄₀₅ /min	K_m (apparent) for protein C μ M	V_{max} for protein C mOD ₄₀₅ /min
Rabbit TM	0.76 ± 0.03	116 ± 2	11.0 ± 2.5	209 ± 26
Rabbit TM + 75 nM MBP	$0.60 \pm 0.05^*$	$31 \pm 1^*$	8.7 ± 3.5	$66 \pm 19^*$

Values are means \pm SD.

* P vs. no MBP < 0.05

summarized in Table II. MBP causes a statistically significant but minor decrease in the apparent K_d for thrombin while causing a pronounced decrement of V_{max} to $\sim 25\%$ of its control value. 75 nM MBP does not significantly alter the apparent K_m of the thrombin/TM complex for protein C, though our data do not rule out the possibility of a small change. In contrast, the V_{max} of APC generation with respect to protein C is diminished to 31% of its control value in the presence of this concentration of MBP. These data suggest that MBP inhibits APC generation by the rabbit TM complex without materially affecting apparent thrombin binding to TM or protein C binding to the thrombin/rabbit TM complex. Instead, MBP functions primarily as a noncompetitive inhibitor that impairs the catalytic efficiency of the complete thrombin/TM/protein C complex.

We hypothesized that inhibition by EO granule proteins of TM-dependent APC generation reflects an electrostatic interaction between these extremely cationic proteins and the anionic TM molecule, in particular the large O-linked glycosaminoglycan (GAG) moiety located just external to a hydrophobic transmembrane sequence (10–12, 14–20). This unique GAG is comprised of chondroitin sulfate-like disaccharides, some of which are unusually hypersulfated (and hence more anionic) because they contain two rather than one sulfate per disaccharide unit (14). Because cationic MBP might well bind TM at such a highly anionic site, we predicted that polyanionic substances, and in particular hypersulfated chondroitin sulfates, would reverse MBP-induced inactivation of TM APC generation.

In preliminary experiments not shown, we demonstrated that heparin (3 sulfates/disaccharide), chondroitin sulfate A (1 sulfate/disaccharide), and the hypersulfated chondroitin sulfate E (1.3 sulfates/disaccharide), when present before the addition of MBP, could all effectively block MBP inactivation of rabbit and endothelial cell APC generation. Such apparent blockade might, however, simply reflect polyanion precipitation of cationic MBP, thereby preventing its interaction of TM. We therefore asked instead whether these polyanionic substances could rejuvenate the activity of TM previously inactivated by MBP. As shown in Fig. 6, chondroitin sulfate E and heparin, and, to a lesser extent, chondroitin sulfate A, partially restore the activity of rabbit TM nearly completely inactivated by prior exposure to 200 nM MBP. A hierarchy of efficacy is evident in which heparin > chondroitin sulfate E \gg chondroitin sulfate A, so that hypersulfated chondroitin sulfate E, which closely resembles the GAG moiety of rabbit TM, more effectively reverses MBP blockade of TM function than does “con-

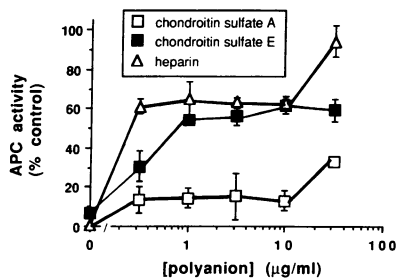


Figure 6. Polyanion reversal of MBP impairment of rabbit TM APC-generating activity. Aliquots of soluble rabbit TM were exposed 10 to 200 nM MBP for 10 min, then incubated further in the presence or absence (buffer control) of the indicated polyanionic substance

for another 30 min before assaying APC in the presence of 2 nM thrombin as previously described. □, chondroitin sulfate A; ■, chondroitin sulfate E; △, heparin.

ventionally” sulfated chondroitin sulfate A. The most anionic polysaccharide, heparin, is also the most effective at reversing MBP inhibition of TM.

To investigate directly the role of the TM GAG domain in MBP inhibition of TM function, we used a pair of recombinant human TM mutant proteins, both containing the entire extracellular domain and differing only in the presence or absence of the chondroitin sulfate-like GAG moiety (19, 20, 22). These proteins migrate on SDS-PAGE with apparent molecular masses 105 kD (TMD-105) and 75 kD (TMD-75). Fig. 7 shows the effect of incubating either TMD 105 (GAG⁺ form) or TMD 75 (GAG⁻ form) with increasing concentrations of MBP before assay of APC generation. In the absence of MBP, APC generation was five times as high with TMD-105 as with TMD-75, in agreement with the original description of these isoforms (22). In the presence of MBP concentrations up to 1 μM, APC generation by TM 105 is progressively inhibited with an IC₅₀ of ~ 100 nM, similar to that of rabbit TM. In striking contrast, TMD-75 APC generation is unaffected over this same range. At 3.3 and 10 μM, MBP TMD-105 and TMD-75 have nearly identical activities and acceleration is evident. For these paired TM proteins, then, the GAG domain is a prerequisite

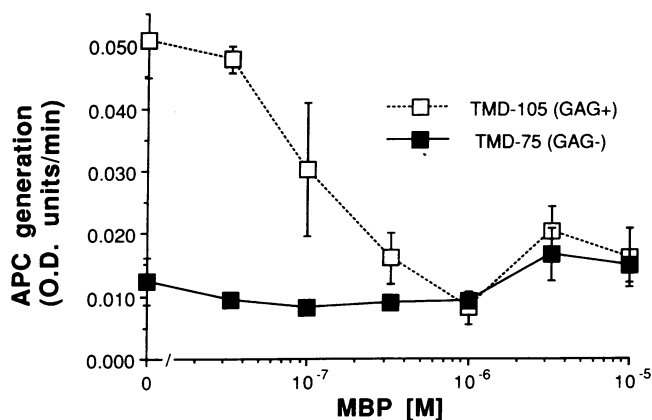


Figure 7. Differential inhibition of APC-generating activity of GAG⁺ TMD-105 and GAG⁻ TMD-75 by MBP. Aliquots of either TMD-105 or TMD-75 (2 nM final) were incubated 5 min in the presence of the indicated concentrations of MBP, then assayed for APC-generating capacity after addition of 2 nM thrombin, as described in Methods. Data shown are ± standard deviation.

for MBP inhibition of APC generation. Moreover, the presence of 1–10 μM MBP alters the APC-generating activity of GAG⁺ TMD-105 to resemble that of GAG⁻ TMD-75, as though the functional influence of the GAG domain on APC generation has been negated.

Because cationic EO granule proteins are potent inhibitors of APC generation by the thrombin/TM complex, we determined whether these same proteins could also abrogate another major anticoagulant action of TM, termination of the fibrinogen-cleaving activity of thrombin. As shown in the top two lines of Table III, addition of 20 nM TM lengthened the thrombin clotting time threefold in the case of rabbit TM and fivefold in the case of TMD-105. As expected, the GAG-domainless variant TMD-75 produced only a slight prolongation of the thrombin clotting time (22). The addition of 0.5 or 1 μM MBP alone had only a minor effect on the clotting time. However, when either rabbit TM or TMD-105 was first incubated with these same concentrations of MBP, its ability to prolong the thrombin clotting time was severely curtailed. In the case of TMD-105, MBP treatment resulted in a slight clotting time prolongation nearly identical to that caused by TMD-75 in the absence of MBP. The minor prolongation of clotting time induced by TMD-75 was also partially reversed by exposure to MBP, but to an extent only slightly greater than that attributable to the effect of MBP itself. Identical results were obtained using 300 nM EPO instead of MBP (not shown).

Discussion

These studies demonstrate that three cationic EO granule proteins known to accumulate on endocardial and endothelial surfaces in eosinophilic endocarditis, MBP, EPO, and ECP, all potentially inhibit the capacity of endothelial cell monolayers to

Table III. MBP Reversal of TMB-mediated Prolongation of Thrombin Clotting Time

Preparation	Clotting time		
	Rabbit TM	TMD-105	TMD-75
	s		
Control	39±1	28±1	28±1.2
+20 nM TM	127±5	142±6	35±1.1
+MBP	48±5	25±1	25±0.6
+TM + MBP	40±2	35±1	29±1.0

Thrombin clotting times were measured using final concentrations of 5 nM bovine thrombin and 2 mg/ml bovine fibrinogen as measured in a fibrometer in H/H buffer supplemented with 0.15% Lubrol PX, pH 7.4. The ability of the designated forms of TM to prolong the thrombin clotting time was assayed by preincubating thrombin 10 min in the presence of 20 nM (final) TM before combining the mixture with fibrinogen to initiate the clotting time. Control clotting times differ between rabbit and the two mutant human TMs because they were assayed in two separate experiments. Where indicated, TM was first incubated 10 min in the presence of MBP (0.5 μM for rabbit TM and 1 μM for TMD-105 and TMD-75) before addition of thrombin, and assay of the thrombin clotting time. Values are means±SD of at least triplicate determinations.

support thrombin-dependent activation of protein C. Such inhibition is not likely a result of internalization or endocytosis of surface TM, as apparently happens in the presence of PMA (36) or tumor necrosis factor (37, 38), because it occurs rapidly, within 10–30 min (Fig. 2) rather than over 12–18 h, and intracellular hypokalemia induced by nigericin and hypokalemic buffers, a known inhibitor of endocytosis, does not block it (Table II). In addition, in experiments not shown we find that 1) after exposure to 10 μ M MBP < 10% of the original APC-generating activity of endothelial cell monolayers is detected in scraped monolayers disrupted by ultrasonication and assayed for total (i.e., surface and intracellular) APC-generating activity and 2) MBP impairs by 70% the total APC generation of endothelial monolayers metabolically inhibited by maintaining them at 4°C. EO granule protein-mediated inhibition of TM activity is not due to an irreversible toxic effect of these proteins since such inhibition occurs at sublytic concentrations (as detected by ^{51}Cr -release assay), can fully reverse > 16 h after washout of the cationic proteins (Fig. 3), and is not accompanied by release of active TM into supernatant fluid (not shown). Immunohistochemical localization of endothelial cell TM after MBP exposure would help establish whether TM is internalized after exposure to MBP. We have attempted such studies using polyclonal goat anti-porcine TM antibodies but have been thwarted by problems related to nonspecific binding of antibodies to MBP-treated monolayers. We therefore conclude that MBP inhibition of endothelial TM function reflects, at least in part, a direct and durable interaction with TM on the cell surface but cannot rule out the possibility that internalization of TM may also play a role in this phenomenon.

This conclusion is further supported by the demonstration that MBP, EPO, and ECP also inhibit APC generation by purified rabbit TM in solution (Fig. 4). EPO and MBP are particularly effective in this regard with IC_{50} s of, respectively, 100 and 10 nM, both roughly 1 μ g/ml, well within the range of concentrations of MBP known (35) to circulate in hypereosinophilic individuals. The differing IC_{50} s of MBP for inhibition of endothelial TM as opposed to soluble rabbit TM may be attributable to such factors as differences in species, glycosylation status, and conformation due to cellular TM being expressed in the context of a complex extracellular matrix and membranous surface environment. In comparing the potency of MBP TM inhibition with that of other cationic substances, Preissner et al. (18) have reported that the IC_{50} with regard to APC generation for the synthetic polycation polybrene was 100 μ g/ml, for poly-L-lysine 3 mg/ml, and for "platelet releasate" 100 μ g/ml. On the other hand, Bourin et al. (17) found little or no effect of the heparin-neutralizing proteins histidine-rich glycoprotein and S-protein in concentrations up to 1,000 nM. This pronounced variance in the ability of cationic proteins to inhibit APC generation suggests factors other than cationicity alone must play a role in this phenomenon. One such factor might be the presence in MBP (39) and EPO (26) of closely juxtaposed cationic and hydrophobic amino acid sequences, a motif common to a variety of membrane-perturbant toxins and venoms (40). In any case, EO granule proteins are the most potent cationic inhibitors of TM APC generation yet described.

MBP inhibits APC generation by rabbit TM not by increasing the apparent K_d for thrombin or the apparent K_m of protein C for the thrombin/TM complex, but rather by acting primarily as a noncompetitive inhibitor. Thus, MBP apparently does

not interfere with the binding of thrombin to TM, known to occur in TM epidermal growth factor-like domains 5, and 6, (41, 42) or with binding of protein C to the thrombin/TM complex, activation of which requires epidermal growth factor-like domain 4 (42, 43). Instead, MBP binds to TM electrostatically, perhaps to the GAG domain (see below), and the presence of TM of this intensely cationic substance may distort the normal conformation of the thrombin/TM/protein C complex so as to diminish its catalytic efficiency. Posttranslational γ -carboxylation of protein C does not appear to be critical in MBP-mediated inhibition of rabbit TM function, because the IC_{50} for MBP inhibition of APC generation using Gla-domainless protein C at 3 mM CaCl_2 is identical to that for native protein C (data not shown).

MBP-mediated inhibition of TM APC generation is due to a reversible, presumably electrostatic interaction with the TM molecule because the activity of MBP-inactivated rabbit TM is substantially regenerated by subsequent exposure to polyanions such as chondroitin sulfate E, heparin, and chondroitin sulfate A. In considering potential binding sites for MBP on TM, we note that Bourin et al. (14) have demonstrated that the large O-linked GAG moiety attached to the extracellular domain of TM is comprised of an unusually hypersulfated, chondroitin sulfate E-like moiety. That chondroitin sulfate E (1.3 sulfates/disaccharide unit) reverses MBP inactivation of rabbit TM much more effectively than does chondroitin sulfate A (1 sulfate/disaccharide unit) (Fig. 6) suggests that MBP binds TM with an affinity roughly equal to that of MBP for chondroitin sulfate E and is thus compatible with MBP binding to the GAG moiety. Alternatively, the greater negative charge of the chondroitin sulfate E and heparin could equally well disengage MBP from the TM molecule no matter where it were bound.

However, our experiments with TMD-105 (GAG^+) and TMD-75 (GAG^-), paired recombinant human TM proteins that differ only in the presence or absence of the GAG moiety, directly implicate the GAG in mediating the interaction of MBP and TM in two ways. First, whereas both intact rabbit TM and TMD-105 are inhibited by MBP with an IC_{50} of ~ 100 nM, TMD-75 is relatively unaffected by the presence of MBP (Fig. 7). Thus, presence of the GAG moiety is a prerequisite for MBP inhibition of the APC-generating capacity of TMD. Moreover, at concentrations of MBP > 10^{-6} M the activities of TMD-105 and TMD-75 are identical, suggesting that at these higher concentrations MBP obliterates any influence of the GAG moiety upon TM activity, and thereby renders it in effect functionally identical to TM-75. Second, in addition to impairing APC generation by TM, EPO and MBP also completely abrogate the capacity of fully glycosylated TM to prolong the thrombin clotting time (Table III), as do other cationic proteins (16–18, 21). Others have previously noted that the ability of TM to prolong the thrombin clotting time is strongly influenced by presence of the GAG moiety, because its removal by chondroitinase ABC severely restricts (18, 22) the capacity of TM to perform this function. Our data confirm the observation (22) that GAG^+ TMD-105 (and rabbit TM) are much more effective than GAG^- TMD-75 in this regard and further demonstrate the differential sensitivity of these related forms of TM to inhibition by MBP. As with APC generation, the presence of MBP converts GAG^+ TMD-105 to function as GAG^- TMD-75. That is, in the presence of MBP, TMD-105, alone very effective, prolonged the thrombin clotting time to

the same minor extent as TMD-75 does in the absence of MBP. In aggregate, these data suggest that functional inhibition of soluble TM by ECPs is primarily mediated through interactions with the anionic GAG moiety, interactions that mitigate its influence on TM anticoagulant actions.

Based on these findings, we propose that in hypereosinophilic states ECPs accumulate in endocardium and endothelial surfaces, bind electrostatically to anionic TM, impair its anticoagulant functions, and thereby promote thrombosis. We recognize, however, that our short-term studies, performed in the absence of plasma, may not accurately reflect in vivo conditions in a person with high grade eosinophilia. MBP, for example, circulates in part as a mixed disulfide linked to serum proteins (35) so that free concentrations are lower than the micromolar concentrations of total MBP actually measured (35) in the serum of such persons. Nonetheless, we find that MBP inhibits endothelial cell and soluble rabbit TM APC generation even in the presence of 33–50% serum, although, as might be expected, the IC_{50} are significantly higher at, respectively, 75 and 25 μ M. Perhaps more directly pertinent to the role of MBP in vivo is the finding that immunofluorescent studies (reference 8 and Fig. 1) uniformly demonstrate high concentrations of MBP (in fact, concentrations approaching those of intact EO granules) on endocardial and endothelial surfaces of patients with eosinophilic endocarditis. Such accumulation of ECPs may occur either as the result of degranulation of attached EOs or, alternatively, from progressive adsorption of circulating free MBP over weeks or months, a situation difficult to replicate in vitro. Of potential relevance in this regard is the finding that MBP inhibition of endothelial monolayer TM function cannot be reversed by subsequent exposure to buffers containing physiological concentrations (40 mg/ml) of BSA (data not shown). Therefore, MBP, once bound to endothelial TM, is tenaciously attached and cannot be dislodged even by the most anionic and abundant serum protein.

The prominent thromboembolic diathesis that characterizes eosinophilic endocarditis is likely a complex phenomenon ascribable to mechanisms in addition to ECP impairment of TM function. For example, tumor necrosis factor- α , a cytokine that exhibits greatly elevated serum levels in parasitic infestations associated with chronic eosinophilia (44) increases procoagulant tissue factor (37) and decreases TM expression in vascular endothelium (37, 38), and, as we have previously shown, renders endothelium more vulnerable to damage by activated EOs (45). ECP accelerates coagulation through a Factor XII-dependent mechanism (46). Moreover, both MBP and EPO evoke nonlytic platelet secretion of serotonin, α -granule, and lysosomal proteins with an EC_{50} of 20–30 μ g/ml (47), a range similar to that in which we demonstrate inhibition of TM-dependent APC generation by intact endothelial monolayers (Fig. 2). Finally, subtle damage to endothelial cell surfaces unrelated to TM and to extracellular matrix wrought by reactive oxygen intermediates or ECPs may render these surfaces procoagulant by a variety of potential mechanisms.

In addition to providing potential insights into the thromboembolic diathesis that attends hypereosinophilic heart disease, our studies further emphasize the importance of the GAG domain in TM function, suggest the potential usefulness of cationic proteins as tools to study TM structure–function relationships, and invite further examination of other potentially relevant cationic proteins that might modulate of TM func-

tion. With regard to the second possibility, we have found in preliminary experiments that MBP appears to unmask a high affinity calcium-binding site on TMD-105, converting its APC-generating calcium concentration optimum to resemble that of TMD-75 (22), chondroitin ABC lyase-cleaved TMD-105 (20), and elastase-cleaved rabbit TM (48), with maximum activity at subphysiologic (0.3 mM) calcium concentrations. Finally, given the relative potency of “platelet releasate” as an inhibitor of TM-dependent APC generation (18) and the pronounced similarities of platelet Factor 4 and MBP with regard to cationicity, heparin binding, and inhibition of angiogenesis (49, 50), it will be of great interest to see if platelet Factor 4 or some other cationic component released by activated platelets also inhibits TM function of endothelial monolayers, a finding of obvious physiologic as well as pathologic relevance.

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