Activation of Endogenous Factor V by a Homocysteine-induced Vascular Endothelial Cell Activator

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Abstract

Vascular endothelium possesses multiple procoagulant properties, including synthesis and expression of Factor V. We studied the effects of homocysteine on the regulation of endothelial cell Factor V activity. Elevated levels of homocysteine are associated with the congenital thrombotic disorder homocystinuria. Treatment of cultured endothelial cells with 0.5–10 mM homocysteine had no effect on cell morphology, but did increase Factor V activity and prothrombin activation by Factor X_a. A radioimmunoassay for endothelial cell Factor V demonstrated that homocysteine treatment did not increase Factor V antigen levels. ^{125}I-prothrombin was activated by treated endothelial cells and Factor X_a in the presence of thrombin inhibitors. Exogenous ^{125}I–Factor V was cleaved by homocysteine-treated but not control endothelial cells. ^{125}I–Factor V cleavage products distinct from those generated by thrombin and Factor X_a were identified. These data provide evidence for regulation of endothelial cell Factor V activity, and indicate that increased Factor V activity associated with homocysteine-treated vascular endothelium results primarily from induction of an activator of Factor V.

Introduction

Vascular endothelium normally presents a nonthrombogenic surface to flowing blood (1, 2). Endothelial cell surface glycosaminoglycans (3) and thrombomodulin (4) both serve as potent inhibitors of coagulation, while vessel wall generation of prostacyclin (5) and plasminogen activators (6) limit platelet plug formation and fibrin deposition, respectively. In contrast to these mechanisms of thrombo-resistance, several procoagulant properties of endothelial cells have been described. With injury, procoagulant (tissue factor) activity is expressed by vascular tissue to rapidly initiate coagulation (7). In addition, disrupted endothelial cells also possess a serine protease activator of Factor XII (8). On the other hand, intact endothelium possesses receptors for Factors IX_a (9) and X_a (10, 11) that may allow for intrinsic pathway activation of Factor X and prothrombin on endothelial cells, respectively. Furthermore, another procoagulant property has been described in association with the surface of undisturbed aortic endothelial cells—Factor V (10, 12). Although the biosynthesis of Factor V by endothelial cells has been demonstrated (13), the factors that regulate its activity have not been described. Enhanced coagulant activity of Factor V would be important because the resulting increased thrombin formation would amplify coagulation by thrombin feedback activation of the pro–co-factor plasma proteins, Factors V and VIII (14).

Homocystinuria is a congenital thrombotic disorder in which affected patients have elevated plasma levels of homocysteine, a sulfur-containing amino acid (15). Homocystinuria results most commonly from deficiency of cystathionine β-synthase, an enzyme necessary for transsulfuration (15). Untreated patients with homocystinuria usually have fasting homocysteine levels of 0.2 mM; their nonfasting levels may be higher (15). Homocysteine levels are undetectable in fasting normal human plasma. Clinical manifestations of patients with this disorder include dislocation of the optic lens, osteoporosis, and mental retardation; however, the life-threatening complications of homocystinuria are accelerated vascular disease and thromboembolic events (15). The mechanisms by which thrombotic events occur in these patients are unknown. Some investigators have reported increased platelet turnover in affected patients (16), while other investigators have not confirmed this observation (17, 18). Additionally, abnormalities in platelet adhesion and aggregation have been described in some homocystinuric patients (for review see reference 15). Other mechanisms that may contribute to thrombosis in homocystinuria include endothelial cytotoxicity (19) and activation of Factor XII by homocystine (20). Although progressive vascular disease occurs in homocystinuria, no vascular endothelial cell procoagulant properties have been identified that might contribute to thrombosis in this disorder. In the present report, it is demonstrated that cultured bovine aortic and human umbilical vein endothelial (HUVE)[1] cells exhibit enhanced Factor V activity and increased prothrombin activation after treatment with homocysteine. The data suggest that homocysteine-induced Factor V activity results from activation of Factor V by an endothelial cell activator. These observations may account, in part, for the increased incidence of thrombosis in patients with homocystinuria.

Methods

Homocysteine, homocysteine thiolactone, methionine, homocystine, cysteine, cystine, lipopolysaccharide (endotoxin, trichloroacetic acid, or

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1. *Abbreviations used in this paper:* ABAE, adult bovine aortic endothelial; APC, activated protein C; BCE, bovine corneal endothelial; DAPA, dansyl arginine-4-ethylpiperidine amide; DFP, diisopropylfluorophosphate; HUVE, human umbilical vein endothelial; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBS, Tris-buffered saline.

phenol extract from *Escherichia coli*, serotype 055:B5), hirudin, Trizma base, rabbit brain cephalin, SP-Sephadex C-50, porcine heparin, bovine serum albumin (BSA, radioimmunoassay grade), diisopropylfluorophosphate (DFP), and Factor V–deficient plasma were obtained from Sigma Chemical Co. (St. Louis, MO). Dansyl-L-glutamyl-glycyl-L-arginine-chloromethylketone was provided by Calbiochem-Behring Corp. (San Diego, CA). Interleukin 1 was supplied by Genzyme (Boston, MA). Sodium [^{51}Cr]chromate (specific activity 200 Ci/g) and sodium [^{125}I]iodide (specific activity 15 Ci/g) were obtained from New England Nuclear (Boston, MA). Iodogen was obtained from Pierce Chemical Co. (Rockford, IL). A protein A suspension (IgGsorb) was supplied by The Enzyme Center (Malden, MA). Medium for cell culture was obtained from the University of California, San Francisco Cell Culture Facility and had <10 pg/ml endotoxin. Calf serum was provided by HyClone (Logan, UT) and had <13 pg/ml endotoxin.

Cell culture techniques. Adult bovine aortic endothelial (ABAE), bovine corneal endothelial (BCE), and human foreskin fibroblasts were cultured as previously described (2). The bovine aortic cells were used between passages 3 and 10. HUVE cells were obtained as described (21) and were used between passages 2 and 3. Both fibroblast growth factor and porcine heparin were added to cultured HUVE cells. Assays were performed on cells plated in 35-mm petri dishes.

Cell viability was determined using a ^{51}Cr-release technique. Cells were incubated in serum-containing medium with 1 μCi/ml ^{51}Cr for 16 h, washed three times with medium (without serum), and incubated in serum-containing medium with or without homocysteine. After an additional 16 h, the medium was removed from each dish and centrifuged, and the supernatant was counted to determine ^{51}Cr release. The specific activity of the labeled cells was ~1,200 cpm/μg cell protein. Cytotoxicity was calculated using the formula $(A - B)/(C - B)$, where A represents ^{51}Cr cpm in the test sample, B represents ^{51}Cr cpm in the control sample, and C represents maximal ^{51}Cr cpm released by treating radiolabeled monolayers with Tris-buffered saline (TBS)-1% Triton X-100. Experimental data were statistically analyzed using a significance level of $P < 0.05$ (22).

Coagulation proteins. Factor X and prothrombin were purified from human plasma as described (23). Human Factor V was purified according to the methods of Kane and Majerus (24). Bovine protein C and bovine thrombin were kindly provided by Dr. Walter Kisiel (University of New Mexico, Albuquerque, NM). Protein C was activated and assayed for anticoagulant activity as described (25). A burro anti-bovine Factor V IgG was supplied by Dr. Paula Tracy and Dr. Kenneth Mann (University of Vermont, Burlington, VT); this antibody detects both bovine Factor V and Factor V_a (13). A rabbit anti–human Factor V antiserum was provided by Dr. Philip Majerus (Washington University, St. Louis, MO). Protein concentrations were assayed using the technique of Bradford (26). Prothrombin and Factor V were iodinated using the Iodogen technique (24). Factor V and Factor X activities were measured using standard clotting assays (27, 28). Procoagulant activity of cultured cells was assayed by mixing 0.1 ml cell lysate, 0.1 ml normal pooled plasma, and 0.1 ml 25 mM $CaCl_2$ at 37°C. Procoagulant activity was considered to be tissue factor if Factor VII was required in the assay.

Homocysteine treatment of cultured cells. Homocysteine was dissolved in medium at the desired concentration (0.5–10 mM), sterile filtered, and added to cell monolayers 3–4 d postconfluence. Most incubations were performed for 16 h in the tissue culture incubator. For determination of total Factor V activity, cell monolayers were washed three times with 20 mM Tris containing 150 mM NaCl (TBS), pH 7.4, and 0.1 ml TBS was added to each dish. The cells were scraped from the dish and transferred to plastic tubes that were rapidly frozen in a dry-ice bath, then stored at −70°C for several hours. At the time of assay, the samples were thawed at 37°C, and a 0.1-ml aliquot was used in a clotting assay to quantitate Factor V activity. Activity was expressed as milliunits per microgram protein, with 1 U representing the activity in 1 ml pooled human plasma. Intact ABAE cell monolayers that had been washed with TBS were used in a previously described assay with purified prothrombin (90 μg/ml), Factor X_a (20 ng/ml), and 10 mM $CaCl_2$ (12) to measure prothrombin activation.

Activation of Factor V in homocysteine-treated endothelial cells. For these experiments, control or homocysteine-treated cells were washed three times with medium (without serum), then incubated with activated protein C (APC, 6 μg/ml) for 15 min at 37°C. After the APC incubation, DFP (final concentration, 0.2 mM) was added to inactivate APC. The monolayers were then washed 10 times with TBS. For measurement of cellular Factor V activity, cell lysates were obtained as described above, frozen, thawed, and assayed using a clotting method (27). For measurement of prothrombin activation before and after APC treatment, washed monolayers were assayed as described above.

Cleavage of ^{125}I-prothrombin by Factor X_a in the presence of control or homocysteine-treated cells was monitored. The thrombin inhibitors dansyl arginine-4-ethylpiperidine amide (DAPA, 100 μM) or hirudin (20 U/ml) were added to the incubation medium (12). Aliquots of the incubation mixture were removed at intervals, subjected to nonreduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), dried, and processed for autoradiography (12).

Radioimmunoassay was used to quantitate the amount of Factor V (V_a) in detergent-solubilized control or homocysteine-treated HUVE cells (29). Human umbilical vein endothelial cells grown in 35-mm petri dishes were treated with TBS or 10 mM homocysteine for 16 h. The monolayers were then washed 10 times with TBS, and lysed with 0.1 ml of a buffer containing TBS, 10 mM benzamidine, 5 mM $CaCl_2$, 0.5 mg/ml BSA, and 1% Triton X-100. The lysates were then frozen at −70°C. Endothelial cell lysates were thawed and centrifuged at 15,000 *g* for 30 min to remove insoluble material prior to assay. A Factor V standard curve was constructed using purified human Factor V (14–896 ng/ml) diluted in TBS containing 10 mM benzamidine, 5 mM $CaCl_2$, 0.5 mg/ml BSA, and 0.2% Triton X-100. Iodinated Factor V was prepared (24) and had specific activities of 2,000–6,000 cpm/ng; ~10,000 cpm was added to each assay tube. Incubations were initiated with the addition of rabbit anti–human Factor V antiserum (diluted 1:400) and conducted at 4°C for 16 h. Control incubations included those omitting the antiserum or using normal rabbit serum instead of the anti–Factor V antiserum. After the overnight incubation, rabbit IgG (10 μg) was added to each tube. Protein A (0.015 ml of a 10% suspension) was then added to the tubes, and a 1-h incubation was performed with mixing. Next, the samples were centrifuged (15,000 *g* for 5 min), and the protein A precipitates were washed twice with buffer. The immunoprecipitate was then counted with a gamma counter.

Cleavage of ^{125}I–Factor V by homocysteine-treated cells. Freshly prepared ^{125}I–Factor V (100 ng) was added to ABAE cells in control medium or medium containing 10 mM homocysteine. The effects of serum-containing medium on cleavage of ^{125}I–Factor V was studied by comparing serum-free medium with serum-containing medium. After 16 h of incubation, the medium was removed, and the cell monolayers were lysed in gel buffer (30). The cell lysates were analyzed for ^{125}I–Factor V cleavage by SDS-PAGE and autoradiography.

Cleavage of ^{125}I–Factor V by Factor X_a. Because Factor X_a has been demonstrated to activate Factor V (31), it was of interest to determine whether the ^{125}I–Factor V cleavage profile induced by homocysteine-treated endothelial cells resembled that following cleavage by Factor X_a. These experiments were a modification of the studies of Foster et al. (31). In our activation assay, ^{125}I–Factor V (1 μg/ml) was incubated in 0.5 ml total volume containing TBS-5 mM $CaCl_2$ (0.4 ml), 0.045 ml cephalin, 100 μM DAPA, and Factor X_a (500 ng/ml). This mixture was incubated at 37°C; at intervals from 30 s to 30 min, aliquots were removed, added to reduced gel buffer, and processed for SDS-PAGE (5%). Autoradiography of the dried gel was then performed.

Results

Endothelial cell cytotoxicity and Factor V activity after homocysteine treatment. In preliminary experiments, confluent cultures of human umbilical and bovine aortic endothelial cells were incubated with homocysteine thiolactone or homocysteine to determine the cytotoxicity of these two compounds. Homocysteine thiolactone (10 mM) was found to induce gross changes

in endothelial cell morphology and to induce lysis of 42% of the cells during a 16-h incubation. In contrast, exposure of ABAE cells to homocysteine (0.5–10 mM) caused no changes in morphology as observed by phase microscopy (retraction, detachment). ^{51}Cr cytotoxicity experiments in which 10 mM homocysteine was incubated for 16 h resulted in 2% more ^{51}Cr being released compared with that of untreated cells. This increase was statistically significant ($P < 0.05$). All subsequent experiments were performed with the less cytotoxic compound, homocysteine.

A dose-response curve of homocysteine-induced Factor V activity of ABAE cells after 16 h of incubation is shown in Fig. 1. Time-course studies using 10 mM homocysteine indicated that a period of ~8 h was required from initial exposure of cultured endothelial cells to homocysteine before an increase in Factor V activity was detected. Thereafter, Factor V activity in ABAE cells increased steadily through 16–24 h, then slowly declined (data not shown). For convenience, a 16-h incubation time was used in most experiments. Homocysteine concentrations of 0.5–1 mM required 24–30 h of incubation for expression of increased Factor V activity. Concentrations >10 mM were not tested because of the insolubility of this compound.

Purified Factor V (125 μg/ml) was also incubated with 10 mM homocysteine in TBS containing 5 mM $CaCl_2$ at 37°C, and aliquots were analyzed for activation of Factor V by the functional clotting assay. No increase in Factor V coagulant activity occurred with homocysteine incubation; however, thrombin treatment (1 U/ml) of Factor V resulted in a 25-fold increase in coagulant activity after a 5-min incubation at 37°C (data not shown). These data indicate that homocysteine does not directly activate Factor V.

Effect of other sulfur-containing amino acids on endothelial cell Factor V activity. To determine the selectivity of the homocysteine-induced increase in endothelial cell Factor V activity, other sulfur-containing amino acids were assayed in a similar manner. A comparison of endothelial cell Factor V activity after treatment of ABAE cells with homocysteine, homocystine, methionine, cysteine, and cystine is shown in Table I. Homocystine and cystine were only tested at the 2-mM concentration because of their insolubility in culture medium. Homocysteine treatment (10 mM) resulted in a fourfold increase in Factor V activity, whereas cysteine (10 mM) resulted in a twofold increase. Cystine-, homocystine-, and methionine-treated cells exhibited minimal or no increase in Factor V coagulant activity.

Effect of other stimuli on endothelial cell Factor V activity. Other stimuli that have been demonstrated to induce procoagulant (tissue factor) activity in vascular endothelial cells were tested for their ability to increase endogenous Factor V clotting activity. One stimulus was mechanical wounding of the cell monolayer using a sterile pipette tip to remove approximately one-third of the original cell population. A subsequent incubation for 12–16 h showed no increase in Factor V activity (control cells, 0.24 mU/μg protein vs. wounded cells, 0.20 mU/μg protein). Another stimulus was interleukin 1, which we found to induce a 15-fold increase in HUVE tissue factor activity, similar to results of previously published studies (32). However, treatment of vascular endothelial cells with interleukin 1 (7 U/ml) for 5–16 h did not result in increased Factor V activity. In contrast, treatment of either ABAE or HUVE cells with endotoxin (250 ng/ml to 8 μg/ml for 6–8 h), which causes expression of tissue factor activity, did result in a three- to fourfold increase in Factor V activity.

Homocysteine-induced prothrombin activation. Factor V activity expressed on the aortic endothelial cell surface results in Factor X_a–catalyzed prothrombin activation (10, 12). Fig. 2 in-

Table I. Effect of Sulfur-containing Amino Acids on ABAE Cell Factor V Activity

Amino acid	Concentration	Factor V activity (% of control)
	mM	
No addition	—	100
Homocysteine	2	145
	10	405
Homocystine	2	97
Methionine	5	103
	10	162
Cysteine	5	209
	10	233
Cystine	2	75

ABAE cells were incubated in control or treatment medium for 16 h. The monolayers were then washed and assayed for Factor V activity as described in Methods. The data are expressed in terms of percent Factor V activity expressed by control ABAE cells (cells incubated in medium alone). Each value represents the average of two experiments.

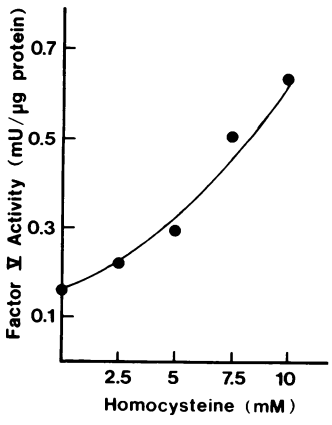

Figure 1. Dose-response curve of homocysteine-induced Factor V activity. Confluent ABAE cells were incubated with increasing concentrations of homocysteine for 16 h. The monolayers were then washed and processed for Factor V assay as described in Methods. The data are expressed as Factor V activity (milliunits per microgram protein). Each value represents the mean of three experiments.

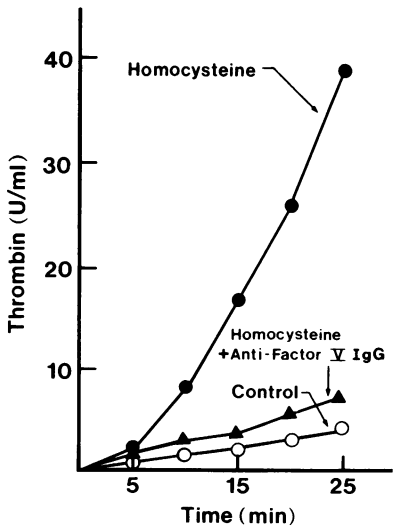

Figure 2. Activation of prothrombin by control or homocysteine-treated ABAE cells and Factor X_a. After a 16-h incubation, cell monolayers were washed and incubated with prothrombin, calcium, and Factor X_a as outlined in Methods. Anti-bovine Factor V IgG (100 μg/ml) was added to some homocysteine-treated ABAE cell dishes 10 min before initiation of prothrombin activation. Each value represents the mean of two experiments.

dicates that intact homocysteine-treated ABAE cells enhanced prothrombin activation ninefold compared with that of untreated ABAE cells. This increase in prothrombin activation was inhibited ~80% by 100 μg anti-bovine Factor V IgG, but not by a control antibody (goat anti-rabbit IgG). Homocysteine-treated endothelial cells were unable to activate prothrombin in the absence of Factor X_a.

Effect of activated protein C. APC has been previously demonstrated to inactivate purified Factor V_a in the presence of phospholipid and calcium (33) or when bound to the platelet surface (34). Incubation of homocysteine-treated ABAE cell monolayers with APC (6 μg/ml) for 15 min, followed by washing, lysis, and freeze/thawing, resulted in inhibition of the increased Factor V activity (Table II). In related experiments, homocysteine-treated ABAE cell monolayers subsequently incubated with APC (6 μg/ml) and washed exhibited markedly reduced prothrombin activation compared with cells not exposed to APC (Fig. 3). These data support the hypothesis that increased ABAE cell Factor V activity and prothrombin activation induced by homocysteine result from increased endothelial cell Factor V_a.

Comparison of Factor V activities of vascular and nonvascular cells. A comparison of endogenous Factor V activities of vascular and nonvascular cells and their responses to homocysteine is shown in Fig. 4. Of the cell types studied, only vascular endothelial cells (ABAE and HUVE) exhibited increased Factor V activity after homocysteine treatment. For example, as depicted in Fig. 4, homocysteine treatment resulted in an approximately sixfold increase in Factor V activity in HUVE cells compared with an approximately fourfold increase observed with ABAE cells. Nonvascular cells (BCE, fibroblasts) had little or no basal Factor V activity, and homocysteine treatment failed to induce an increase.

The ability of homocysteine to enhance secretion of Factor V (V_a) into conditioned medium was also studied. Control HUVE cell medium contained 900±33 mU of Factor V (V_a) activity per microliter as determined by the clotting assay (n = 3). Homocysteine-treated (16 h) HUVE cell medium contained 817±90 mU of Factor V (V_a) activity per microliter. In contrast, control ABAE cell medium contained 310±30 mU/μl, which increased to 1,120±90 mU of Factor V (V_a) activity per microliter after homocysteine treatment.

Demonstration of an endothelial cell activator of Factor V. The increase in Factor V activity and prothrombin activation associated with homocysteine-treated endothelial cells could result either from increased synthesis of Factor V by the stimulated cells and/or activation of cell-associated Factor V. Two methods were used to distinguish between these possibilities. In an initial study, ^{125}I-prothrombin cleavage by Factor X_a in the presence of control or homocysteine-treated cells was monitored in an assay containing either DAPA or hirudin. The rationale for these experiments is based on our previous observation that prothrombin activation by ABAE cells and Factor X_a requires thrombin as an activator of Factor V (12). In the presence of thrombin inhibitors, ^{125}I-prothrombin is not cleaved by control cells and Factor X_a. Therefore, if homocysteine-treated cells contained Factor V_a, the presence of thrombin inhibitors would not inhibit cleavage of ^{125}I-prothrombin. Fig. 5 is an autoradiogram demonstrating that untreated cells and Factor X_a activate ^{125}I-prothrombin in the absence of DAPA (lane *2*), but not in its presence (lane *3*). This suggests that thrombin formation is required for ^{125}I-prothrombin cleavage, most likely as an activator

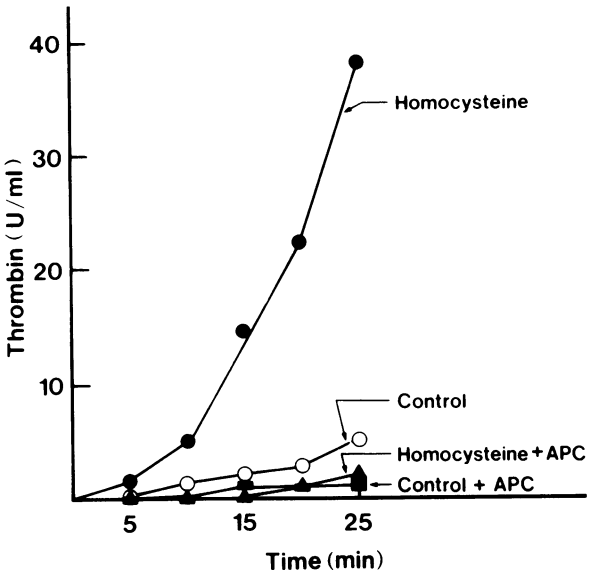

Figure 3. Inhibition of homocysteine-induced prothrombin activation by APC. After incubation with homocysteine for 16 h, cell monolayers were washed and incubated with APC (6 μg/ml) for 15 min. They were washed again and assayed for prothrombin activation as outlined in Methods. Each value represents the mean of two experiments.

Table II. Inhibition of Homocysteine-induced ABAE Cell Factor V Coagulant Activity by Bovine APC

Medium	Factor V activity
	mU/μg protein
Control	0.2
Control + APC	0.1
Homocysteine	2.2
Homocysteine + APC	0.2

After incubation of ABAE cells in control or homocysteine-containing medium for 16 h, the monolayers were washed, incubated with APC (6 μg/ml) for 15 min, washed again, and then harvested for Factor V assay as described in Methods. Each value represents the mean of three experiments.

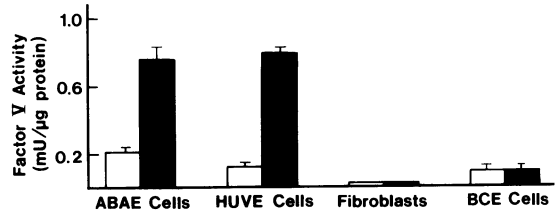

Figure 4. Comparison of Factor V activities of vascular and nonvascular cells. Vascular (ABAE, HUVE) and nonvascular (fibroblasts, BCE) cells were incubated with buffer or with 10 mM homocysteine for 16 h, then washed and processed for Factor V clotting assay as described in Methods. Approximately 1×10^6 cells were used in each experiment. Open bars represent control treatment; closed bars represent homocysteine treatment. The data are expressed as Factor V activity (milliunits per microgram protein). Each value represents the mean±SEM of four experiments.

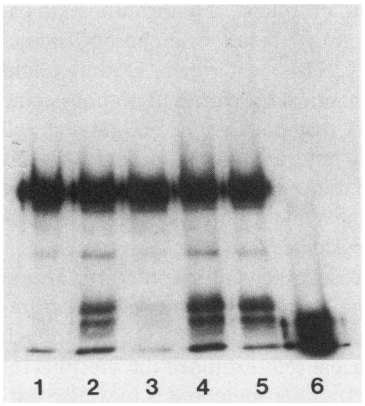

Figure 5. Cleavage of ^{125}I-prothrombin by Factor X_a in the presence of control or homocysteine-treated ABAE cells. Confluent ABAE cells were incubated with ^{125}I-prothrombin (25 μg/ml) and Factor X_a (20 ng/ml) with or without the thrombin inhibitor DAPA (100 μM) in TBS containing 10 mM $CaCl_2$. Aliquots of the incubation mixture were obtained after 30 min and subjected to nonreduced SDS-PAGE (7.5%) followed by autoradiography. Lane *1:* purified ^{125}I-prothrombin (no cells); lane *2:* control cells with ^{125}I-prothrombin and Factor X_a, without DAPA; lane *3:* control cells with ^{125}I-prothrombin and Factor X_a, with DAPA; lane *4:* homocysteine-treated cells with ^{125}I-prothrombin and Factor X_a, without DAPA; lane *5:* homocysteine-treated cells with ^{125}I-prothrombin and Factor X_a, with DAPA; and lane *6:* purified ^{125}I-thrombin (no cells). Equivalent cpm of the incubation mixture were applied to each gel lane.

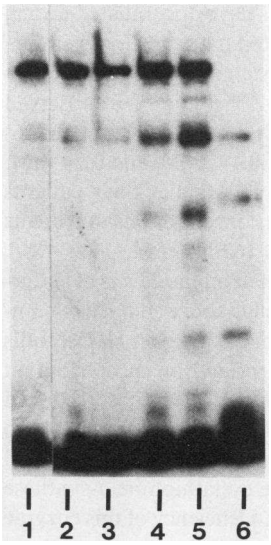

Figure 6. Autoradiogram of ABAE cell–associated exogenous ^{125}I–Factor V (V_a). ^{125}I–Factor V (100 ng) was added to ABAE cells incubated in serum-free medium (either control medium or homocysteine medium) for 6 or 16 h. At each interval, the medium was removed, and the cells were lysed and processed for SDS-PAGE and autoradiography. Lane *1:* purified ^{125}I–Factor V; lane *2:* control cells (6 h); lane *3:* control cells (16 h); lane *4:* homocysteine-treated cells (6 h); lane *5:* homocysteine-treated cells (16 h); and lane *6:* thrombin-treated ^{125}I–Factor V (0.5 U/ml, 15 min, 37°C). Equivalent cpm were applied to each gel lane.

of Factor V (12). Homocysteine-treated cells (in the absence of DAPA) activated more ^{125}I-prothrombin than control cells did (lane *4*), a finding consistent with functional measurements of prothrombin activation (Fig. 2). However, unlike control cells in the presence of DAPA, homocysteine-treated cells and Factor X_a activated ^{125}I-prothrombin in the presence of DAPA (lane *5*), indicating a thrombin-independent mechanism for prothrombin activation by Factor X_a and treated cells. Similar data were obtained when another thrombin inhibitor, hirudin, was substituted for DAPA.

To determine whether homocysteine affected the total Factor V content of HUVE cells, Factor V antigen was quantitated by radioimmunoassay of control or homocysteine-treated cells. The limit of detection in our assay was 50 ng of added Factor V per milliliter. Postconfluent control HUVE cells ($\sim 1 \times 10^6$ cells, 50 μg protein) contained 103±4 (SEM, $n = 7$) ng of Factor V antigen as determined by radioimmunoassay. Treatment of HUVE cells with 10 mM homocysteine for 16 h resulted in a Factor V antigen level of 90±3 ng. Thus, homocysteine treatment does not increase the quantity of Factor V.

In other experiments, direct demonstration of a Factor V activator in homocysteine-treated cells was attempted. Fig. 6 depicts an autoradiogram of ABAE cell-associated ^{125}I–Factor V from cells incubated for 6 or 16 h in serum-free medium containing buffer or 10 mM homocysteine. It can be seen that intact ^{125}I–Factor V was associated with both control and treated cells after 6 and 16 h. However, homocysteine-treated cell lysates also contained cleavage products of ^{125}I–Factor V. The most prominent fragment had an apparent molecular weight of 250,000. Less prominent Factor V fragments associated with homocysteine-treated endothelial cells had apparent molecular weights of 180,000, 110,000, 86,000, and 82,000. All cleavage products were more prominent after a 16-h incubation. When serum-containing medium was used, the exogenous ^{125}I–Factor V was cleaved by both control and homocysteine-treated cells (data not shown). Inclusion of 100 μM DAPA in the ^{125}I-Factor V–homocysteine-treated endothelial cell incubation did not prevent ^{125}I–Factor V cleavage.

A comparison of the ^{125}I–Factor V cleavage fragments generated by homocysteine-treated endothelial cells with those resulting from thrombin activation (0.5 U/ml, 15 min, 37°C) is also shown in Fig. 6 (lane *6*). The apparent molecular weights of the thrombin-treated Factor V_a fragments were 185,000, 114,000, 79,000, and 76,000. Whereas the cleavage patterns produced by thrombin and homocysteine-treated endothelial cells were similar, the low molecular weight Factor V_a species generated by thrombin consistently had a lower molecular weight than those associated with homocysteine-treated cells.

To determine whether Factor X_a activation of Factor V resulted in cleavage products similar to those produced by homocysteine-treated cells, the ^{125}I–Factor V cleavage products generated by these two treatments were compared using 5% SDS-PAGE and autoradiography. Factor X_a cleaved ^{125}I–Factor V, resulting in cleavage fragments with apparent molecular weights of 185,000, 155,000, 130,000, 76,000, 72,000, 70,000, and 58,000. Partial cleavage was noted as soon as 30 s; after 15 min, cleavage appeared complete. When lysates of homocysteine-treated cells previously incubated with ^{125}I–Factor V were compared with the Factor X_a–induced cleavage products of ^{125}I–Factor V, the cleavage patterns were distinct.

Additionally, co-incubation of homocysteine-treated ABAE cells with the specific Factor X_a inhibitor dansyl-glutamyl-glycyl-arginine-chloromethylketone (35), using concentrations of 10^{-6}–10^{-5} M, did not prevent increased Factor V activity as measured by clotting assay. Finally, incubation of homocysteine-treated cells with the Factor X_a inhibitor did not prevent cleavage of ^{125}I–Factor V. In data not shown, we demonstrated that this peptide inhibitor was a potent Factor X_a antagonist, as quantitated by a Factor X_a clotting assay. These data indicate that the endothelial cell activator of Factor V is not similar to Factor X_a, and that the activator described in this study appears to be distinct from previously described activators of Factor V.

Discussion

The data in the present report show that endothelial cell Factor V activity is increased by homocysteine treatment. The minimum concentration of homocysteine required for significant enhancement of procoagulant activity in cultured endothelial

cells was 0.5 mM. Several investigators have documented fasting blood levels of homocysteine in affected patients. For example, Uhlemann et al. (17) found homocysteine levels ranging from 0.01 to 0.09 mM in their study. Hill-Zobel et al. (18) observed fasting levels of 0.03–0.11 mM in their report. Harker et al. (16) described three patients with levels of 0.17, 0.20, and 0.24 mM; however, no comment was made as to whether their patients were fasting. Therefore, homocysteine concentrations in patients with homocystinuria range from 0.01 to 0.24 mM.

De Groot et al. (36) recently reported on the effects of sulfur-containing amino acids on HUVE cell cytotoxicity. They compared the ^{51}Cr cytotoxicity of these metabolites on HUVE cells obtained from a normal female subject and on those from an obligate heterozygote for homocystinuria. They observed that cultured cells from the heterozygote were more susceptible to injury induced by sulfur amino acids than normal endothelial cells were, suggesting that the enzyme cystathionine β-synthase was expressed in normal endothelium. Deficiency of this enzyme in either the heterozygote or homozygote for homocystinuria would be expected to result in intracellular accumulation of homocysteine. The absence of endothelial cell cystathionine β-synthase in these patients might lower the plasma concentration of homocysteine required for expression of procoagulant activity, and may explain why the normal endothelial cells in our studies required higher concentrations of homocysteine than those usually observed in homocystinuric patients to see enhanced procoagulant activity. Therefore, a homocysteine-induced increase in vascular endothelial cell procoagulant activity may contribute to the thrombotic tendency in certain homocystinuric patients and may explain why these patients develop vascular disease in the absence of a consistent platelet abnormality.

Factor V is a critical regulatory co-factor in the coagulation mechanism. Modulation of Factor V activity by platelets or endothelial cells may be an important determinant of prothrombin activation by these cells. Activation of Factor V by thrombin results in an approximate 30-fold increase in activity (24). Although Factor X_a can activate Factor V, thrombin is considered to be the primary activator of this co-factor (31). Additionally, a platelet protease has been identified that potentially might serve as an activator (37). With regard to endothelial cell–associated Factor V, exogenously added thrombin is unable to activate the Factor V (12). However, thrombin generated on the endothelial cell surface is capable of feedback activation of Factor V (12). The data in this report suggest that, in addition to thrombin, ABAE cell Factor V activity can be regulated by an endogenous endothelial cell activator in response to homocysteine. This effect of homocysteine does not require cellular disruption or gross injury. Both activation of exogenous ^{125}I–Factor V and prothrombin activation on the surface of intact endothelial cells treated with homocysteine were documented in the present study. This suggests that the endothelial cell activator of Factor V induced by homocysteine has access to surface-bound Factor V.

In related studies, an endogenous activator of Factor V in monocytes has been suggested in a preliminary report by Tracy and Rohrbach (38). Cleavage of Factor V by disrupted monocytes or their conditioned medium was observed; two proteolytic activation patterns of Factor V could be identified (38). Additionally, a platelet-associated protease has been observed to cleave Factor V_a bound to intact platelets (39).

We used homocysteine instead of the thiolactone derivative because of marked endothelial cell cytotoxicity of the latter compound. Wall et al. (19) previously noted the cytotoxicity of homocysteine thiolactone and postulated that thrombosis in patients with homocystinuria might result from homocysteine-mediated endothelial injury. However, when Dudman and Wilcken (40) analyzed the chemical properties of homocysteine thiolactone, they concluded that changes in endothelial cell morphology and cytotoxicity reported after treatment with this analogue were probably due to the thiolactone moiety, and not necessarily from homocysteine alone. Consequently, our studies were conducted with homocysteine.

In addition to homocysteine, other sulfur-containing amino acids, such as methionine, are observed in increased concentrations in the blood of homocystinuric patients (15). Although homocysteine-treated endothelial cells exhibited the most Factor V coagulant activity (Table I), other metabolites may also participate in the generation of increased endothelial cell Factor V activity in these patients.

The possibility that endotoxin present in tissue culture medium or serum might contribute to the effects on endothelial cell Factor V activity was considered. Our reagents contained <15 pg/ml endotoxin. When experiments using this quantity of commercial endotoxin were performed, endothelial cell Factor V activity remained at the same level as in untreated cells.

The levels of HUVE cell Factor V antigen obtained by our radioimmunoassay ($\sim$100 ng/10^6 cells) are comparable with that obtained using a competitive enzyme-linked immunosorbent assay, $\sim$1.2 μg/10^7 cells (41). If it is assumed that Factor V has a specific activity of 82 U/mg (24), the radioimmunoassay data would suggest that a 35-mm petri dish of HUVE cells has 8 mU of Factor V coagulant activity. This is comparable with our data, which show that HUVE cell lysates possess 6–8 mU of Factor V activity.

In addition to increasing ABAE Factor V activity, homocysteine treatment resulted in a three- to fourfold increase in secretion of this activity into the conditioned medium. This is consistent with previous observations that [^{35}S]methionine-labeled ABAE cells synthesize Factor V and secrete it into conditioned medium (13), and also indicates that aortic endothelial cell perturbation with homocysteine enhances secretion of Factor V (V_a). No such increase in Factor V (V_a) secretion occurred with HUVE cells treated with homocysteine. Possible explanations for this finding include the use of heterologous serum to culture HUVE cells or a true inability of this endothelial cell line to alter secretion of Factor V (V_a) in response to homocysteine treatment.

The inhibition of both the increased Factor V activity and enhanced prothrombin activation of homocysteine-treated cells by APC suggests that endogenous Factor V_a is present in treated cells. Stern et al. (42) have reported that the inactivation of exogenous Factor V_a by APC is enhanced by the presence of endothelial cells. Thus, vascular endothelial cells not only enhance the activation of protein C by thrombomodulin (4), but they also can promote APC-mediated inactivation of both endogenous and exogenous Factor V_a. These data also indicate that APC can inactivate the unique Factor V_a species generated by homocysteine-treated cells.

The data in Fig. 6 indicate that homocysteine-treated ABAE cells can activate ^{125}I–Factor V; Factor V fragments were observed that were similar, but not identical, to those associated with increased Factor V activity after activation by thrombin or Russell's Viper venom (24). Although the degree of activation of Factor V by homocysteine-treated cells is much less than that seen with thrombin (Fig. 6), even the minimal cleavage observed

resulted in a ninefold enhancement of prothrombin activation (Fig. 2). The observation that Factor V_a possesses ~400-fold more coagulant activity than the pro-co-factor (43) means that even a low degree of activation will result in significant enhancement of coagulation.

Although Factor X_a can activate Factor V, the cleavage products generated were distinct from those produced by homocysteine-treated cells. Foster et al. (31) observed that of the Factor X_a–induced ^{125}I–Factor V cleavage products generated after 30 min of incubation, the most prominent cleavage products had apparent molecular weights of 94,000 and 56,000. In contrast, the prominent cleavage products that we found after 30 min of incubation had apparent molecular weights of 185,000, 70,000, and 58,000. The explanation for this difference may be related to the origin of the proteins in each study: Foster et al. (31) used bovine Factor V and Factor X_a, whereas the Factor V and Factor X_a that we used were prepared from human plasma. Differences in molecular weight between thrombin-activated fragments of bovine (31) and human (44) Factor V have been previously noted. In any event, homocysteine-treated endothelial cell activation of ^{125}I–Factor V resulted in cleavage products distinct from those generated by thrombin or Factor X_a.

Our data show that serum-free medium is required for the activation of exogenous Factor V; it is thought that this requirement is due to the presence of serum proteases, which degrade the added Factor V. In the absence of cells, ^{125}I–Factor V was not cleaved by serum-containing medium. The following data suggest that there is an enzyme that activates Factor V in endothelial cells: (*a*) inactivation of homocysteine-induced Factor V activity by activated protein C (Table II and Fig. 3); (*b*) ^{125}I-prothrombin activation by homocysteine-treated ABAE cells and Factor X_a in the presence of the thrombin inhibitor DAPA (Fig. 5); and (*c*) cleavage of exogenous ^{125}I–Factor V by homocysteine-treated, but not control endothelial cells (Fig. 6). The inability of DAPA to inhibit homocysteine-treated endothelial cell prothrombin activation and ^{125}I–Factor V cleavage suggests that this endothelial activator is not thrombinlike. Additionally, the radioimmunoassay results indicate that increased amounts of Factor V (V_a) antigen are not present in homocysteine-treated endothelial cells.

Our data do not address other potential endothelial cell hemostatic mechanisms that might be affected by exposure to homocysteine, such as tissue factor expression and fibrinolysis. However, with regard to endothelial cell Factor V activity, homocysteine exerts its procoagulant effect primarily by inducing an activator of Factor V. Expression of this activator may be one mechanism by which perturbed vascular endothelium promotes coagulation in the absence of thrombin.

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