Biochem. J. (1991) 276, 739–743 (Printed in Great Britain)

Up-regulation of thrombomodulin by activation of histamine H_1-receptors in human umbilical-vein endothelial cells *in vitro*

Kazunori HIROKAWA and Nobuo AOKI*
The First Department of Medicine, Tokyo Medical and Dental University, Yushima, Bunkyo-ku, Tokyo 113, Japan

Previous reports demonstrated that the expression of thrombomodulin (TM) in endothelial cells was modulated by various agents. Although TM was down-regulated by endotoxin or cytokines, up-regulation of TM was accomplished when endothelial cells were stimulated with unphysiologically high concentrations of cyclic AMP derivatives or tumour-promoting phorbol esters. We investigated the expression of TM in human umbilical-vein endothelial cells (HUVECs) by physiological substances that can be released into the bloodstream. Histamine (0.1–10 μM, 1–48 h) increased TM activity, TM antigen in cell lysates and TM mRNA levels, but 5-hydroxytryptamine and bradykinin had no effect. Enhancement of TM activity by histamine was completely blocked by the H_1-selective antagonist pyrilamine, whereas the H_2-antagonist cimetidine had no effect, showing that histamine up-regulates TM activity via H_1-receptors on HUVECs. Enhanced TM activity by histamine and the resultant increase in protein C activation might play a role in a feedback regulation for prevention of vascular thrombosis.

INTRODUCTION

Thrombomodulin (TM), expressed on endothelial cells, is a physiologically important modulator of blood coagulation [1,2]. TM accelerates thrombin-catalysed protein C activation [3]. Activated protein C is a protease, and with its cofactor protein S [4] acts as an anti-coagulant by inactivating the coagulation factors Va [5] and VIIIa [6]. Recently, TM structure [7,8], location [9], regulation of expression [10–13] and implication as a therapeutic agent [14,15] have been described. Among these, the regulatory mechanisms for TM expression are important to investigate the modulation of coagulation by endothelial cells. Down-regulation of TM and the accompanying expression of tissue factor occurred on incubation with endotoxin [16], tumour necrosis factor [17] and interleukin-1β [18] in cultured endothelial cells. These results are believed to be analogous to hyper-coagulable states frequently resulting in thrombosis and/or disseminated intravascular coagulation in various patients. In contrast, up-regulation of TM activity was observed when the cells were stimulated with tumour-promoting phorbol esters, unphysiologically high concentration of cyclic AMP derivatives, and the adenylate cyclase activator forskolin [13]. Although thrombin and cycloheximide increase TM mRNA levels, they do not increase TM activity in endothelial cells [19].

In the present studies, we investigate regulation of TM activity by mediators of inflammation. Although several inflammatory cytokines inhibit surface TM activity and induce procoagulant activity of endothelial cells [16–18], there is a possibility that inflammation by some mediators provokes an increase in the anticoagulant activity of endothelial cells. Such increased TM activity and resultant increase in protein C activation might play a role as a feedback regulation to circumvent or prevent vascular thrombosis in pathological states associated with various diseases. Histamine is an important mediator of inflammation, and antagonists of histamine are effective in moderating allergic and acute inflammatory responses. In this study, we measured the effects of histamine on surface TM activity, total TM antigen in cell lysates, and TM mRNA levels in human umbilical-vein endothelial cells (HUVECs). Our results demonstrate that histamine up-regulates TM expression in HUVECs by activation of H_1-receptors on the cells.

EXPERIMENTAL

Materials

Acetylsalicylic acid (aspirin), indomethacin, histamine dihydrochloride, 5-hydroxytryptamine (serotonin) creatinine sulphate complex, bradykinin (acetate salt), cimetidine, pyrilamine (maleate salt), *o*-phenylenediamine, pig mucosal heparin, human α-thrombin (4000 units/mg of protein), and BSA were purchased from Sigma (St. Louis, MO, U.S.A.). Iscove's modified Dulbecco's medium, fetal-calf serum, penicillin/streptomycin and 0.05% trypsin/0.02% EDTA in Dulbecco's phosphate-buffered saline (137 mM-NaCl, 3.67 mM-KCl, 9.66 mM-$NaHPO_4$, 1.72 mM-KH_2PO_4) were purchased from GIBCO (Grand Island, NY, U.S.A.). Endothelial-cell growth supplement was purchased from Collaborative Research (Belford, MA, U.S.A.). Human protein C was purchased from American Diagnostica (New York, NY, U.S.A.). Human antithrombin III was purchased from Green Cross (Osaka, Japan). The chromogenic substrate S-2366 was purchased from Kabi Vitrum (Stockholm, Sweden). Staurosporine was purchased from Kyowa Medex (Tokyo, Japan). The nick-translation kit was purchased from Boehringer (Mannheim, Germany). The total RNA isolation kit was purchased from Invitrogen (San Diego, CA, U.S.A.). [α-^{32}P]dCTP (6000 Ci/mmol) was purchased from New Research Products (Boston, MA, U.S.A.). A *Tth111*I–*Nhe*I fragment in the coding region of human TM was kindly provided by Dr. K. Nawa (Research Institute, Daiichi Pharmaceutical Co., Tokyo, Japan) [20] and used as a hybridization probe. cDNA for human β-actin was purchased from Wako Pure Chemicals (Osaka, Japan). All other chemicals were reagent-grade products.

Methods

Cell culture. HUVECs were harvested from umbilical-cord veins within 12 h after delivery, by the method of Jaffe *et al.* [21]. The cells were cultured and characterized by the methods

Abbreviations used: HUVECs, human umbilical-vein endothelial cells; PGI_2, prostaglandin I_2; TM, thrombomodulin.

* To whom correspondence and reprint requests should be addressed.

previously reported [13]. All experiments were performed on cells within three passages.

Assay of surface TM activity. Surface TM cofactor activity was determined by using HUVEC monolayers [$(1.0–1.5)\times10^5$ cells/well] in 48-well tissue-culture plates, human thrombin and human protein C by previously reported methods [13,22].

Endothelial cells in each well were counted by the method originally described by Drysdale *et al.* [23]. Surface TM activity per 10^5 cells was expressed as the percentage of the control, ±S.D. of four determinations. Data were analysed by Student's *t* test.

E.l.i.s.a. for TM antigen in cell lysates. At the indicated times, medium was removed from the 48-well culture plates and quadruplicate cell monolayers were extracted with 200 μl of 0.5% (w/v) Triton X-100 in Dulbecco's phosphate-buffered saline, pH 7.4. After 30 min, the cell monolayers were suspended with Pasteur pipettes. Cell lysates, after removal of cell debris, were stored at −20 °C until assayed. Cell numbers were counted in another matched 48-well culture plate that had been identically treated with agents. Cell supernatants from the experimental wells were also stored for measurement. KA-2 or KA-4 anti-(human TM) monoclonal antibodies, whose characteristics were previously reported [24], were used to determine total TM antigen in cell lysates.

Microtitre plates coated with KA-2 and horseradish-peroxidase-conjugated KA-4 were used for e.l.i.s.a. assays. Horseradish peroxidase was conjugated to KA-4 by the method of Nakane & Kawaoi [25]. E.l.i.s.a. assays were performed as previously reported [24].

Northern blotting. Total cytoplasmic RNA was prepared from confluent HUVECs by methods previously reported [13]. Isolated RNA was analysed by Northern blotting by standard techniques [26]. The probe employed for hybridization was a ^{32}P-labelled 450 bp DNA fragment corresponding to the human TM coding region, nick-translated to a specific radioactivity of > 10^8 d.p.m./μg. Autoradiograms of Northern blots were prepared as described previously [13]. After washing with distilled water at 65 °C for 30 min, the filter was re-hybridized with ^{32}P-labelled cDNA probe for human β-actin. After densitometric analysis of autoradiographs. TM mRNA levels were normalized to the concentration of β-actin mRNA.

RESULTS

Effects of vasoactive amines on surface TM activity in HUVECs

The effects of a 4 h exposure to vasoactive amines on surface TM activity was determined (Fig. 1). Histamine (0.1, 1, 10 μM) enhanced surface TM activity (121±11.3, 152±6.89, 134±10.0% of control respectively), and up to 10 μM-bradykinin or 5-hydroxytryptamine had no effect on surface TM activity.

Time course of surface TM activity and antigen in HUVECs incubated with histamine

HUVECs, isolated and cultured from three different cords, were incubated with 10 μM-histamine for the indicated times, washed, and surface TM activity was determined. Surface TM activity increased significantly between 4 and 8 h of incubation and declined by 16 h, as shown in Fig. 2.

Total TM antigen in lysates of HUVECs, derived from another cord, incubated with 10 μM-histamine increased after 6 h incubation and declined slightly by 24 h (Fig. 3*b*), almost parallel to increased surface TM activity (Fig. 3*a*).

Expression of endothelial-cell TM mRNA

Northern blotting of TM mRNA demonstrated that 10 μM-histamine increased endothelial-cell TM mRNA levels, as shown in Fig. 4. Increased TM mRNA levels occurred after 2 h of incubation (230% of control), reached a maximum at 8 h (350% of control) and declined between 16 and 24 h, but remained slightly elevated relative to control levels (140–150% of control).

Effect of selective H_1 and H_2 antagonists on enhancement of TM activity in HUVECs by stimulation with histamine

To determine which type of histamine receptor is involved in enhancement of TM activity, HUVECs were co-incubated for 6 h with 10 μM-histamine and two types of histamine-receptor

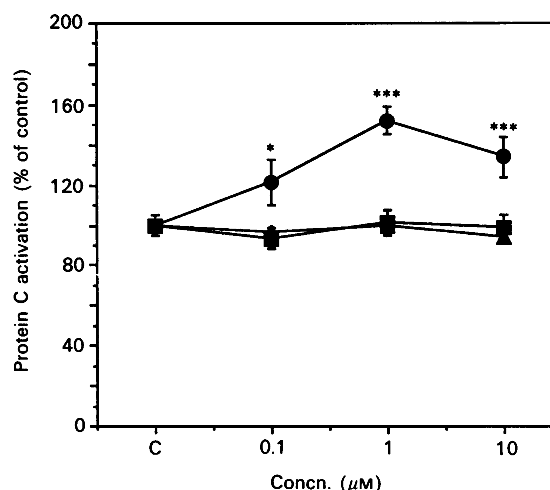

Fig. 1. Effects of vasoactive amines on surface TM activity in HUVECs

After incubation with histamine (●), bradykinin (▲) or 5-hydroxytryptamine (■), surface TM activity was measured as described in the Experimental section. Surface TM activity per 10^5 cells is expressed as a percentage of the control (C), as means±S.D. of four determinations. Asterisks represent significant differences from the control (*$P < 0.05$, ***$P < 0.001$).

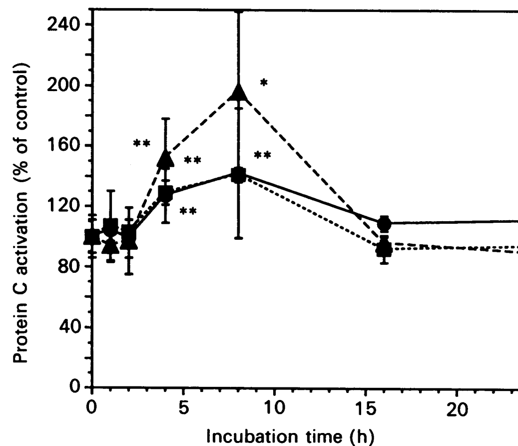

Fig. 2. Time course of surface TM activity in HUVECs incubated with histamine

HUVECs grown in 48-well plates were incubated without or with 10 μM-histamine at 37 °C in 5% CO_2. At the indicated times, surface TM activity was measured as described in the Experimental section. Each symbol (●, ▲, ■) represents the result measured on HUVECs derived from different cords. Asterisks represent significant differences from the unstimulated control cells (*$P < 0.05$, **$P < 0.01$).

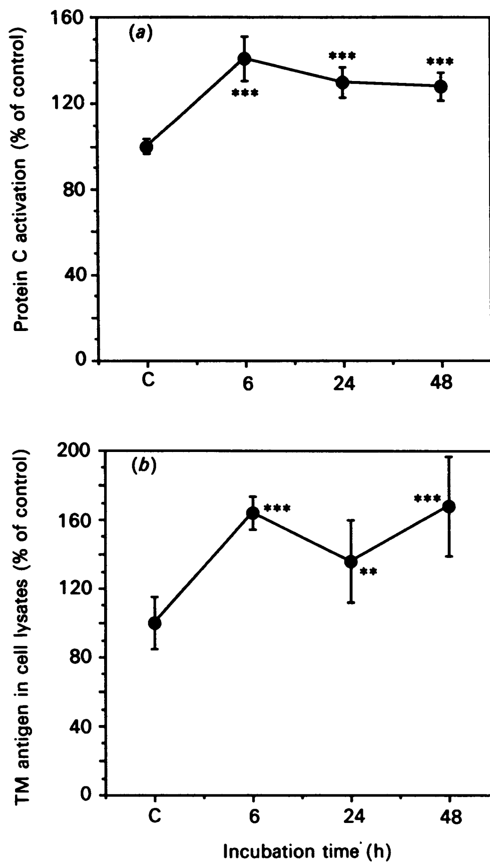

Fig. 3. Time course of surface TM activity and total TM antigen in HUVECs incubated with histamine

HUVECs grown in 48-well plates were incubated without or with 10 μM-histamine at 37 °C in 5 % CO_2. At the indicated times, surface TM activity was measured as described in the Experimental section. In separate matched 48-well plates that had been treated identically with agents, medium was removed and quadruplicate cell monolayers were extracted with 200 μl of 0.5 % (w/v) Triton X-100 in phosphate-buffered saline. Total TM antigen in cell lysates was measured by e.l.i.s.a. methods as described in the Experimental section. Surface TM activity (*a*) and TM antigen per 10^5 cells (*b*) are expressed as percentages of the control (C), as means ± S.D. of four determinations. Asterisks represent significant differences from the unstimulated control cells (** $P < 0.01$, *** $P < 0.001$).

antagonists, i.e. cimetidine (H_2-antagonist) or pyrilamine (H_1-antagonist), and surface TM activity was determined (Figs. 5*a* and 5*b*). Neither antagonist affected surface TM activity in HUVECs incubated without histamine. Although cimetidine (0.1–100 μM) did not affect the histamine-induced enhancement of TM activity, pyrilamine (0.1–100 μM) abrogated the enhancement of TM activity in a dose-dependent manner. Almost the same decrease in enhancement of TM activity was observed in HUVECs co-incubated with histamine and another H_1-receptor antagonist, promethazine (results not shown). These results suggested that enhancement of TM activity by histamine results from activation of H_1-receptors in HUVECs.

Effect of histamine on TM activity in HUVECs pretreated with aspirin or indomethacin

Since histamine increases the synthesis and release of prostaglandin I_2 (PGI_2) in endothelial cells [27,28], the question arose as to whether the enhancement of TM activity by histamine may be due to the actions of PGI_2 or other prostanoids. To exclude these possibilities, the effects of histamine on TM activity in HUVECs

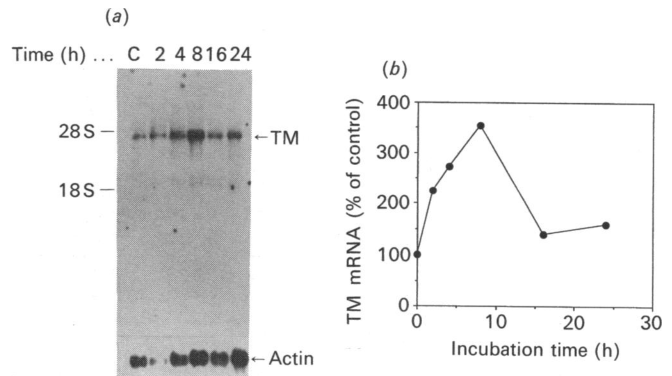

Fig. 4. Effects of histamine on the expression of endothelial-cell TM mRNA

(*a*) HUVECs were incubated with 10 μM-histamine for the indicated times (c, control). Total RNA was isolated, electrophoresed, and transferred to a Hybond-N filter as described in the Experimental section. The filter was hybridized with a ^{32}P-labelled DNA probe for human TM and re-hybridized with a probe for β-actin. The positions of the rRNA size markers are indicated in the left margin. (*b*) Autoradiographs were analysed by densitometry, and values for TM were normalized to human β-actin. Normalized data are expressed as the percentages of TM mRNA relative to unstimulated control cells.

pretreated with aspirin or indomethacin, which inhibit cyclo-oxygenase, were investigated. Aspirin (0.1–1 mM) or indomethacin (1–10 μM) did not abrogate the enhancement of TM activity by histamine, and 1 mM-aspirin even slightly further enhanced the increased TM activity in HUVECs incubated with 100 μM-histamine (Fig. 6). These results suggested that the enhanced TM activity by histamine is not due to PGI_2 or other prostanoids produced by cyclo-oxygenase.

DISCUSSION

Among the vasoactive amines tested, only histamine increased surface TM activity (Fig. 1) resulting from increased TM mRNA levels (Fig. 4). Histamine released from mast cells in response to various stimuli is known to increase vasopermeability *in vivo*, and this action of histamine induced oedema, which represents a symptom of inflammation. Killackey *et al.* [29] reported that histamine increased permeability of microcarrier-cultured endothelial monolayers, and that neither bradykinin nor 5-hydroxytryptamine had any effect. It is possible that bradykinin does not increase permeability by interacting directly with the endothelium, although bradykinin can increase vasopermeability *in vivo* and has been shown to mediate mast-cell histamine release. Our results suggest that vasoactive amines which directly increase the permeability of endothelial cells may increase surface TM activity.

As shown in Figs. 2 and 3(*a*), surface TM activity in HUVECs incubated with 10 μM-histamine increased with time. Total TM antigen in the cell lysates of HUVECs incubated with histamine also increased almost parallel to surface TM activity (Fig. 3). As we reported previously [13], dibutyryl cyclic AMP (4–8 h), forskolin (16–48 h) and phorbol myristate acetate (24–48 h) increased TM activity in HUVECs. Similarly to those agents, up-regulation of TM by histamine is thought to be due to increased synthesis *de novo* of TM protein resulting from increased TM mRNA levels.

The time course of changes in endothelial TM mRNA levels by histamine (increase after 2–8 h incubation and then return to control levels) was similar to those obtained from HUVECs

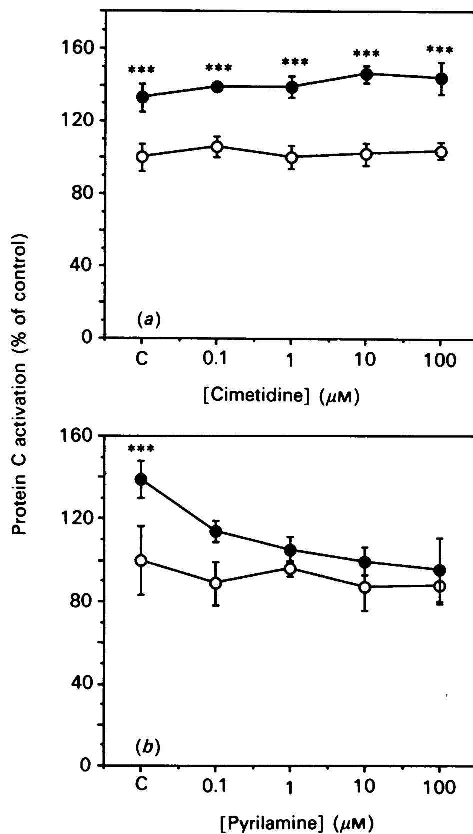

Fig. 5. Effects of selective H_1- and H_2-receptor antagonists on enhanced TM activity in HUVECs stimulated with histamine

HUVECs grown in 48-well plates were incubated without (○) or with (●) 10 μM-histamine at 37 °C in 5 % CO_2. After 6 h incubation, surface TM activity was measured as described in the Experimental section. (*a*) HUVECs were cultured with cimetidine for 30 min before addition of histamine, then co-cultured with cimetidine and 10 μM-histamine for 6 h at 37 °C. (*b*) HUVECs were cultured with pyrilamine for 30 min before addition of histamine, then co-cultured with pyrilamine and 10 μM-histamine for 6 h at 37 °C. Asterisks represent significant differences from unstimulated controls (C) (***$P < 0.001$).

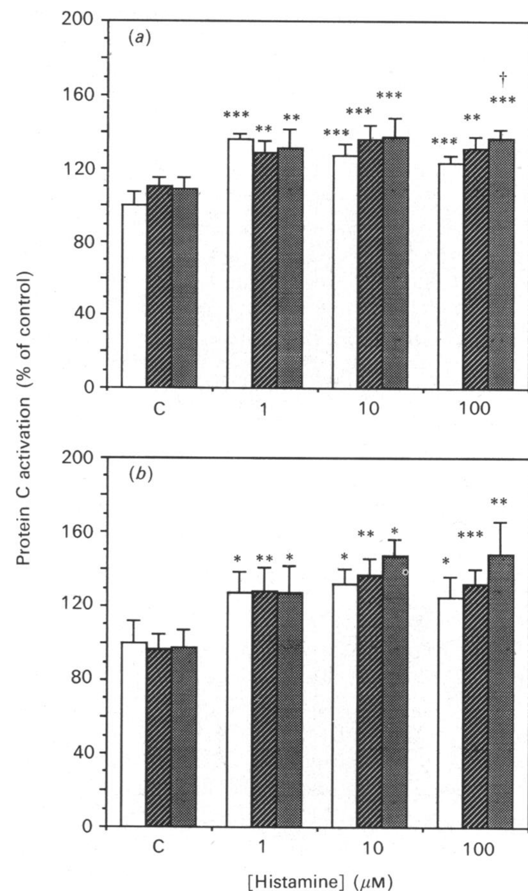

Fig. 6. Effect of histamine on surface TM activity in HUVECs pretreated with aspirin or indomethacin

(*a*) HUVECs were cultured with aspirin (■, 0.1 mM; ■, 1 mM; □, control) for 30 min before addition of histamine, then co-cultured with aspirin and histamine for 6 h at 37 °C. (*b*) HUVECs were cultured with indomethacin (■, 1 μM; ■, 10 μM; □, control) for 30 min before addition of histamine, then co-cultured with indomethacin and histamine for 6 h at 37 °C. HUVECs were then washed and surface TM activity was measured as described in the Experimental section. Asterisks represent significant differences from unstimulated control cells (C) (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Significant differences from surface TM activity of cells incubated with only histamine at the same concentration are indicated by † ($P < 0.05$).

treated with dibutyryl cyclic AMP [13] or thrombin [19]. Although the enhancement of TM activity by dibutyryl cyclic AMP is transient (2–8 h) and thrombin decreases surface TM activity in HUVECs, histamine increased both surface TM activity and total TM antigen (Fig. 3). The increase lasted for various lengths of time, 8–48 h depending on the HUVEC's origin. This is similar to the effect of forskolin [13]. Although forskolin, an activator of adenylate cyclase, increased intracellular cyclic AMP in HUVECs, histamine did not do this (results not shown).

As shown in Fig. 5, enhancement of TM activity by histamine was blocked by the H_1-selective antagonist pyrilamine, whereas the H_2-antagonist cimetidine had no effect, showing that histamine is acting through H_1-receptors. Rotrosen & Gallin [30] reported that H_1-receptor occupancy increased endothelial cytosolic Ca^{2+}, decreased F-actin (which indicates endothelial-cell shape change), and promoted albumin diffusion across cultured endothelial monolayers. Activation of H_1-receptors on HUVECs is also involved in stimulation of synthesis and release of PGI_2 [27], synthesis of platelet-activating factor [28], inositol phosphate accumulation [31] and increase in intracellular Ca^{2+} [32]. Our results further suggest that histamine is a physiological agonist for endothelial anticoagulant function, although the precise molecular mechanism for increased TM activity is not known.

Since PGI_2 release has been associated with increased release of cyclic AMP [33], and derivatives of cyclic AMP up-regulate TM in HUVECs [13], mouse embryonal carcinoma cells [34,35], and megakaryoblastic leukaemia cells (K. Ohashi, K. Hirokawa, N. Komatsu & N. Aoki, unpublished work), PGI_2 released by histamine and the subsequent increase in intracellular cyclic AMP may be a causal factor in up-regulation of TM. However, pretreatment of HUVECs with aspirin or indomethacin did not abrogate enhancement of TM activity by histamine, as shown in Fig. 6, although those pretreatments were reported to inhibit PGI_2 release from HUVECs [33]. In addition, histamine did not increase intracellular cyclic AMP in HUVECs, whereas forskolin increased the levels (results not shown). Hence we exclude the possibility that the enhancement of TM activity by histamine was due to increased intracellular cyclic AMP levels induced by PGI_2 released from endothelial cells. Johnson *et al.* [36] reported that histamine increased the release of not only prostaglandins (PGI_2,

PGE_2, $PGF_{2\alpha}$) but also hydroxyeicosatetraenoic acids from endothelial cells. Thus it is possible that increased lipoxygenase-derived products released from HUVECs incubated with histamine may up-regulate TM, since aspirin or indomethacin cannot inhibit lipoxygenase activity.

Histamine has been shown to promote hydrolysis of phosphatidylinositol 4,5-bisphosphate and to increase cytosolic Ca^{2+} in populations of endothelial cells [32]. Hydrolysis of phosphatidylinositol 4,5-bisphosphate generates inositol phosphate, which increases cytosolic Ca^{2+}, and a natural activator of protein kinase C, diacylglycerol [37]. As reported previously [13], a potent activator of protein kinase C, phorbol myristate acetate, increased TM activity (24–48 h) and TM mRNA levels (4–40 h; maximum at 24 h) in HUVECs after a transient decrease in TM activity (0.5–16 h). Up-regulation of TM by histamine occurred at earlier time points than that by phorbol esters, and was not preceded by a transient decrease in TM activity (Figs. 2, 3*a*, 4). In addition, enhancement of TM activity by histamine was not abrogated by an inhibitor of protein kinase C, staurosporine, and the intracellular Ca^{2+} antagonist TMB-8 did not affect the increase in TM activity by histamine, whereas the Ca^{2+} ionophore A23187 decreased TM activity (results not shown). Hence we could not attribute up-regulation of TM exclusively to activation of protein kinase C or increased cytosolic Ca^{2+}.

Although it was reported that histamine augmented endothelial-cell proliferation [38,39], histamine-increased TM mRNA levels was not a result of a general activation of the cells, since all values were normalized to β-actin mRNA. The concentration of histamine used in the present study is not unphysiologically high, since plasma histamine at the time of an episode of ventricular fibrillation in a patient after the induction of anaesthesia was observed to be 110 ng/ml (approx. 0.6 μM), although normal histamine levels are less than 1 ng/ml (5 nM) [40]. At sites of inflammation, the local concentration of histamine may be higher than those values and might reach the levels used in the present experiments. Thus we conclude that histamine may have a role in regulation of coagulation via the modulation of endothelial functions.

Histamine increases endothelial anticoagulant activities by increasing TM activity, synthesis and release of PGI_2 [27,28] or endothelial-cell-derived relaxing factor ('EDRF') [41], and simultaneously increases endothelial procoagulant activities by increasing synthesis of platelet-activating factor [28], synthesis of tissue factor [42], and the release of von Willebrand factor [43]. Thus histamine is a bidirectional agonist for regulation of coagulation through its effects on endothelial cells.

We thank the nursery staff of the Department of Obstetrics, Japan Red Cross Medical Center (Tokyo), for collecting umbilical cords and placentae, Ms. M. Tanaka and Ms. M. Hayashi-Wada for their skilful technical assistance, and Dr. K. Nawa for providing us with the gene fragment corresponding to human TM coding region. We also gratefully thank Dr. D. J. Stearns-Kurosawa for discussions and critical reading of the manuscript.

REFERENCES

1. Esmon, C. T. (1989) J. Biol. Chem. **264**, 4743–4746
2. Dittman, W. A. & Majerus, P. W. (1990) Blood **75**, 329–336
3. Esmon, C. T. (1987) Science **235**, 1348–1352
4. Walker, F. J. (1980) J. Biol. Chem. **255**, 5521–5524
5. Walker, F. J., Sexton, P. W. & Esmon, C. T. (1979) Biochim. Biophys. Acta **571**, 333–342
6. Fulcher, C. A., Gardiner, J. E., Griffin, J. H. & Zimmerman, T. S. (1984) Blood **63**, 486–489
7. Suzuki, K., Kusumoto, H., Deyashiki, Y., Nishioka, J., Maruyama, I., Zushi, M., Kawahara, S., Honda, G., Yamamoto, S. & Horiguchi, S. (1987) EMBO J. **6**, 1891–1897
8. Wen, D., Dittman, W. A., Ye, R. D., Deaven, L. L., Majerus, P. W. & Sadler, J. E. (1987) Biochemistry **26**, 4350–4357
9. Maruyama, I., Bell, C. E. & Majerus, P. W. (1985) J. Cell Biol. **101**, 363–371
10. Dittman, W. A., Kumada, T., Sadler, J. E. & Majerus, P. W. (1988) J. Biol. Chem. **263**, 15815–15822
11. Conway, E. M. & Rosenberg, R. D. (1988) Mol. Cell. Biol. **8**, 5588–5592
12. Moore, K. L., Esmon, C. T. & Esmon, N. L. (1989) Blood **73**, 159–165
13. Hirokawa, K. & Aoki, N. (1990) J. Biochem. (Tokyo) **108**, 839–845
14. Kumada, T., Dittman, W. A. & Majerus, P. W. (1987) Blood **71**, 728–733
15. Gomi, K., Zushi, M., Honda, G., Kawahara, S., Matsuzaki, O., Kanabayashi, T., Yamamoto, S., Maruyama, I. & Suzuki, K. (1990) Blood **75**, 1396–1399
16. Moore, K. L., Andreoli, S. P., Esmon, N. L., Esmon, C. T. & Bang, N. U. (1987) J. Clin. Invest. **79**, 124–130
17. Nawroth, P. P. & Stern, D. M. (1986) J. Exp. Med. **163**, 740–745
18. Nawroth, P. P., Handley, D. A., Esmon, C. T. & Stern, D. M. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 3460–3464
19. Dittman, W. A., Kumada, T. & Majerus, P. W. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 7179–7182
20. Nawa, K., Sakano, K., Fujiwara, H., Sato, Y., Sugiyama, N., Teruuchi, T., Iwamoto, M. & Marumoto, Y. (1990) Biochem. Biophys. Res. Commun. **171**, 729–737
21. Jaffe, E. A., Nachman, R. L., Becker, C. G. & Minick, C. R. (1973) J. Clin. Invest. **52**, 2745–2756
22. Sala, N., Owen, W. G. & Collen, D. (1984) Blood **63**, 671–675
23. Drysdale, B.-E., Zacharchuk, C. M. & Shin, H. S. (1983) J. Immunol. **131**, 2362–2367
24. Kimura, S., Nagoya, T. & Aoki, N. (1989) J. Biochem. (Tokyo) **105**, 478–483
25. Nakane, P. K. & Kawaoi, A. (1974) J. Histochem. Cytochem. **22**, 1084–1091
26. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, pp. 188–206, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
27. Baenziger, N. L., Force, L. E. & Becherer, P. R. (1980) Biochem. Biophys. Res. Commun. **92**, 1435–1440
28. McIntyre, T. M., Zimmerman, G. A., Satoh, K. & Prescott, S. M. (1985) J. Clin. Invest. **76**, 271–280
29. Killackey, J. J., Johnston, M. G. & Movat, H. Z. (1986) Am. J. Pathol. **122**, 50–61
30. Rotrosen, D. & Gallin, J. I. (1986) J. Cell Biol. **103**, 2379–2387
31. Lo, W. W. Y. & Fan, T.-P. D. (1987) Biochem. Biophys. Res. Commun. **148**, 47–53
32. Jacob, R., Merritt, J. E., Hallam, T. J. & Rink, T. J. (1988) Nature (London) **335**, 40–45
33. Bull, H. A., Rustin, M. H. A. & Dowd, P. M. (1989) Br. J. Dermatol. **120**, 757–765
34. Imada, S., Yamaguchi, H., Nagumo, M., Katayanagi, S., Iwasaki, H. & Imada, M. (1990) Dev. Biol. **140**, 113–122
35. Imada, M., Imada, S., Iwasaki, H., Kume, A., Yamaguchi, H. & Moore, E. E. (1987) Dev. Biol. **122**, 483–491
36. Johnson, A. R., Revtyak, G. & Campbell, W. B. (1985) Fed. Proc. Fed. Am. Soc. Exp. Biol. **44**, 19–24
37. Nishizuka, Y. (1988) Nature (London) **334**, 661–665
38. D'Amore, P. & Shepiro, D. (1977) J. Cell. Physiol. **92**, 177–184
39. Tharp, M. D. (1989) J. Invest. Dermatol. **93**, 107S–112S
40. Wolf, A. A. & Levi, R. (1986) Circ. Res. **58**, 1–16
41. Weinheimer, G. & Osswald, H. (1989) Naunyn-Schmiedeberg's Arch. Pharmacol. **339**, 14–18
42. Galdal, K. S. (1984) Haemostasis **14**, 378–385
43. Hamilton, K. K. & Sims, P. J. (1987) J. Clin. Invest. **79**, 600–608

Received 31 August 1990/16 January 1991; accepted 13 February 1991