Heterogeneous regulation of constitutive thrombomodulin or inducible tissue-factor activities on the surface of human saphenous-vein endothelial cells in culture following stimulation by interleukin-1, tumour necrosis factor, thrombin or phorbol ester

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Thrombomodulin and tissue-factor activities were measured on the surface of confluent human saphenous-vein endothelial cells (HSVEC) cultivated in 96-multiwell plates. Thrombomodulin activity was measured in the presence of purified human thrombin (2.2 nM) and protein C (65 nM). Tissue-factor activity was measured with purified human Factor VII (5 nM) and Factor X (400 nM). Generated activated protein C and Factor Xa released in the supernatant were assayed with chromogenic substrates. Resting cells exhibited significant thrombomodulin activity, but no detectable tissue-factor activity. After 4 h of preincubation with tumour necrosis factor (TNF, 22–2200 pM), interleukin-1 (IL-1, 5.7–570 nM) or phorbol myristate acetate (PMA, 1.61–161 nM) there was an increase in tissue-factor activity and a concomitant decrease in thrombomodulin activity. However, the extent of both responses varied according to the nature of the stimulus. Thrombin (0.44–44 nM) also induced an increase in tissue-factor activity, but had no effect on thrombomodulin activity. Kinetic studies showed that for all stimuli the increase in tissue factor was transient, reaching a maximum after 4–8 h of preincubation with the stimulating agent and returning to normal values after 24 h. IL-1 and TNF induced a time-dependent decrease in thrombomodulin, by respectively 47% and 67% of control values after 24 h. However, PMA induced only a transient down-regulation of thrombomodulin, full activity being recovered after 18 h. Hence this simultaneous assay system, using intact HSVEC and purified human coagulation factors, enabled us to observe that the regulation of thrombin generation could be diversely affected by various substances known to stimulate the endothelium. This suggests that the simultaneous and opposite modulation of these proteins does not represent an unified response of the endothelial cells to procoagulant stimuli. These results also confirm the absence of effect of thrombin on the expression of thrombomodulin on the cell surface.

INTRODUCTION

The generation of thrombin is the culminating event in a series of successive proteolytic reactions, each involving a protein substrate, a proteinase activator, a protein cofactor and an anionic phospholipid surface. The vascular endothelium expresses constitutively a membrane glycoprotein, thrombomodulin, which can bind thrombin and thus exhibit two complementary anticoagulant functions: one is to promote the activation of protein C by thrombin, the other is to alter the procoagulant properties of thrombin (Freyssinet & Cazenave, 1988; Esmon, 1989; Dittman & Majerus, 1990). Protein C is the vitamin-K-dependent precursor of a serine protease, activated protein C (APC), capable of inactivating activated Factors V and VIII, thus exerting a potent negative-feedback control on the generation of thrombin. Tissue factor, a membrane glycoprotein receptor which specifically binds Factor VII or Factor VIIa, is the cellular trigger of the coagulation cascade through activation of Factors X and IX (Nemerson, 1988; Bach, 1988; O'Brien, 1989). Under normal conditions, tissue factor is anatomically sequestered away from the blood; cells in contact with blood (endothelium, leucocytes) do not constitutively express tissue

factor, whereas it is present in the sub-endothelium and other tissues, thus providing an instant trigger for coagulation in the event of a wound and blood loss from the vessel (Drake *et al.*, 1989).

In some pathological situations, when the endothelium is exposed to stimuli such as cytokines, thrombin or endotoxin, it can acquire procoagulant properties (Stern *et al.*, 1988; Drake & Pang, 1989). The effect of such agents on the endothelial expression of thrombomodulin or tissue factor has been described in several experimental systems. An increase in tissue-factor-associated procoagulant activity has been shown after exposure of endothelial cells to interleukin-1 (IL-1) (Bevilacqua *et al.*, 1984, 1985, 1986; Dejana *et al.*, 1987; Carlsen *et al.*, 1988; Zuckerman & Surprenant, 1989; Boeri *et al.*, 1989), thrombin (Brox *et al.*, 1984; Galdal *et al.*, 1985; Almus *et al.*, 1988, 1989; Boeri *et al.*, 1989), tumour necrosis factor (TNF) (Bevilacqua *et al.*, 1986; Carlsen *et al.*, 1988; Zuckerman & Surprenant, 1989; Conway *et al.*, 1989), phorbol ester (Lyberg *et al.*, 1983; Nawroth *et al.*, 1985; Almus *et al.*, 1988; Crossman *et al.*, 1990) or endotoxin (Colucci *et al.*, 1983; Lyberg *et al.*, 1983; Brox *et al.*, 1984; Stern *et al.*, 1985a; Nawroth *et al.*, 1985, 1986a; Noguchi *et al.*, 1989; Surprenant & Zuckerman 1989; Crossman *et al.*,

Abbreviations used: PBS, phosphate-buffered saline (137 mM-NaCl/2.7 mM-KCl/0.9 mM-CaCl₂/0.5 mM-MgCl₂/1.5 mM-KH₂PO₄/6.5 mM-Na₂HPO₄, pH 7.4, 0.295 osM); HSA, human serum albumin; PMA, phorbol 12-myristate 13-acetate; TNF, tumour necrosis factor; IL-1, interleukin-1; HSVEC, human saphenous-vein endothelial cells; APC, activated protein C; S2366, L-pyrroglutamyl-L-prolyl-L-arginine *p*-nitroanilide hydrochloride; S2222, *N*-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine *p*-nitroanilide hydrochloride, with 50% methyl ester; S2765, *N*- α -benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine *p*-nitroanilide hydrochloride.

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1990). Some of these agents have also been shown to decrease the availability and function of thrombomodulin on the endothelium, especially TNF (Conway & Rosenberg, 1988; Moore *et al.*, 1989), phorbol ester [in haemangioma cells] Dittman *et al.*, 1988] or thrombin (Maruyama & Majerus, 1985). In some instances, the fate of both thrombomodulin and tissue factor was followed simultaneously in the same experimental system. Endotoxin (Moore *et al.*, 1987), TNF (Nawroth & Stern, 1986; Brett *et al.*, 1989; Gerlach *et al.*, 1989; Scarpato & Sadler, 1989) or phorbol ester (Scarpato & Sadler, 1989) induced a decrease in thrombomodulin concomitant with an increase in tissue-factor expression by endothelial cells in culture. Infusion of IL-1 in the rabbit had the same effect on the expression of tissue-factor and thrombomodulin activities on native aortic endothelium (Nawroth *et al.*, 1986b). These results have been put forward to suggest that the opposite modulation of these glycoproteins represents a unified response of the endothelium to inflammatory stimuli (Nawroth & Stern, 1986; Moore *et al.*, 1987). Because these results have been obtained in very different experimental situations, it would be hazardous to generalize this concept. Of particular interest is the feedback activity of thrombin, the final product of the coagulation system, in which thrombomodulin and tissue factor play opposite roles, on the expression of these glycoproteins at the cell surface. Thrombin was shown to induce an increase in tissue factor (see above), but there is much discrepancy in the reported effects of thrombin on thrombomodulin expression. Thrombin was found to cause internalization of the thrombin-thrombomodulin complex by human umbilical-cord-vein endothelial cells and A549 lung-cancer cells (Maruyama & Majerus, 1985), but such endocytosis was not observed in human saphenous-vein endothelial cells (HSVEC) and the endothelial cell line EA.hy 926 (Beretz *et al.*, 1989). Moreover, thrombin could even increase thrombomodulin synthesis in mouse haemangioma cells (Dittman *et al.*, 1989). No data are available in the literature on simultaneous determination of the effects of IL-1 on thrombomodulin and tissue-factor activities exposed on the surface of endothelial cells in culture. This prompted us to study in a homogeneous assay system, using cells and reagents of human origin, the simultaneous regulation of thrombomodulin and tissue-factor activities under the influence of thrombin, IL-1, TNF or phorbol ester.

MATERIALS AND METHODS

Reagents

Human protein C, α -thrombin (3000 NIH units/mg) and Factors X and VII were purified from human plasma as previously described (Freyssinet *et al.*, 1988). Purified human fibronectin was prepared by a published method (Ruoslahti *et al.*, 1978). Materials were obtained from the following sources: cell culture media (M199, RPMI 1640, Hepes, L-glutamine, penicillin, streptomycin, fungizone and trypsin-EDTA solution [trypsin (0.5 g/l)/EDTA (0.2 g/l)/NaCl (0.85 g/l)] were from Gibco, Paisley, U.K.; phosphate-buffered saline (PBS) and endotoxin-free human serum albumin (HSA) were from the Centre Régional de Transfusion Sanguine, Strasbourg, France; phorbol 12-myristate 13-acetate (PMA) was from Sigma, St. Louis, MO, U.S.A.; hirudin was from Stago, Asnières, France; chromogenic substrates S2366 (L-pyroglyutamyl-L-prolyl-L-arginine *p*-nitroanilide hydrochloride), S2222 (N-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine *p*-nitroanilide hydrochloride, with 50% methyl ester) and S2765 (N- α -benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine *p*-nitroanilide hydrochloride) were from Kabi Vitrum, Stockholm, Sweden; human recombinant TNF- α (2×10^7 units/mg; M_r 45000) and human recombinant IL-1- β (10^7 units/mg; M_r 17500) were from

Boehringer, Mannheim, Germany. Human serum was prepared from a pool of 100–150 healthy blood donors; samples positive for hepatitis B virus or human immunodeficiency virus (HIV) were discarded before pooling. The serum was inactivated at 56 °C for 30 min.

Cell culture

Human endothelial cells (HSVEC) were collected from fragments of saphenous veins obtained during coronary-bypass surgery (Beretz *et al.*, 1989). The cells were cultivated by a previously described method (Klein-Soyer *et al.*, 1986). The culture medium was M199/RPMI 1640 (1:1, v/v) containing 10 mM-Hepes, 2 mM-L-glutamine, antibiotics (100 units of penicillin/ml and 100 μ g of streptomycin/ml), fungizone (0.25 μ g/ml) and 30% (v/v) human serum. It was verified by a *Limulus* chromogenic assay (Coatest; Kabi Vitrum, Mölndal, Sweden) that all media and buffers had an endotoxin content less than 0.02 ng/ml. The cells were frozen at the second passage and used in experiments from passages 3 to 10. Preliminary experiments were carried out to verify that the number of passages did not alter cell responses. For this purpose, three cell cultures from different donors were assayed for baseline thrombomodulin or tissue-factor activity at confluence at each passage from 3 to 10. There was no significant correlation between either thrombomodulin ($r = 0.198$) or tissue-factor ($r = 0.010$) activity and passage number (results not shown). For all the present experiments, the cells were sub-cultivated in 96-multiwell plates precoated with purified human fibronectin (0.5 mg/ml), at an initial cell density of 10^4 cells/cm².

Assays for thrombomodulin and tissue-factor activities

The assays were performed on confluent cell monolayers (3×10^4 cells/cm²), the culture medium being changed 24 h before each assay.

Dose-action studies. The cells were rinsed $3 \times 200 \mu$ l of RPMI 1640/M199 (1:1, v/v) and then incubated with 200 μ l of RPMI 1640/M199 containing 10 mg of HSA/ml and TNF, IL-1, PMA or thrombin at different concentrations. After incubation for 4 h at 37 °C, the cells were rinsed three times in PBS. Tissue-factor and thrombomodulin activities were then measured. For this purpose, the cell monolayers were covered with 200 μ l of RPMI 1640/M199 without Phenol Red, containing 3 mg of HSA/ml. For tissue-factor assay, purified human Factors VII (5 nM final concn.) and X (400 nM final concn.) were added to each well. At the end of the incubation period (20 min at 37 °C), the supernatants were removed and amidolytic activity of generated Factor Xa was measured in the supernatant at 405 nm, by using a kinetic microplate reader coupled to a microcomputer (Molecular Devices, Palo Alto, CA, U.S.A.), by adding 50 μ l of specific chromogenic substrate S2222 or S2765 to give a final concentration of 0.2 mM. Thrombomodulin activity was evaluated in the same way by addition to the wells of thrombin (0.25 NIH unit/ml, 2.2 nM final concn.) and protein C (65 nM final concn.) for 2 h at 37 °C. At the end of the incubation period, the reaction was blocked by addition of hirudin (1.25 anti-thrombin units/ml). The amidolytic activity of generated APC was measured in the supernatant as described above by addition 50 μ l of substrate S2366 to give a final concentration of 0.1 mM. Each experimental condition was assayed in six different culture wells. The concentration of APC or Factor Xa formed was determined by reference to standard curves constructed with known amounts of Factor Xa or APC. Cells from three wells per experimental condition were detached with trypsin-EDTA and then counted (Coulter Counter Z; Coultertronics, Marnegency, France); the cell number was used to express thrombomodulin

and tissue-factor activities normalized per 10^4 cells. Cells from the other wells were fixed with 2% paraformaldehyde for 20 min at room temperature and then stained with May-Grünwald Giemsa for subsequent study of the morphology of the cells in the monolayer.

Coagulation factors and substrates were added in excess in order to ensure that tissue factor or thrombomodulin was the rate-limiting factor in the assay. It was verified that the activation of protein C or Factor X was linear as a function of time and number of cells present. In any case, less than 20% of total protein substrate, either Factor X or protein C, was converted into its activated form. The activity of controls with only one component added (Factor VII, thrombin or protein C) was always less than 2% of the amidolytic activity measured under the same conditions with the complete assay system. The amidolytic activity generated in culture wells where Factor X was added alone reached more than 10% of that observed when both Factors X and VII were present. However, when a monoclonal anti-(Factor VII) IgG (kindly given by Dr. E. G. D. Tuddenham, Clinical Research Centre, Harrow, Middx., U.K.) was added, activation of Factor X in the absence of added Factor VII was decreased by over 80% in stimulated or resting cells (results not shown). It can thus be concluded that the activation of Factor X in the absence of added Factor VII was due to contamination by residual Factor VII bound to the endothelial-cell surface.

Kinetic studies. The cells were rinsed once with 200 μ l of RPMI 1640/M199 and then incubated with 200 μ l of RPMI 1640/M199 containing 10% human serum and TNF (5 ng/ml, 111 pM), IL-1 (1 unit/ml, 57 nM), PMA (5 ng/ml, 8 nM), thrombin (1 NIH unit/ml, 8.88 nM), or control buffer. In preliminary dose-response experiments, such concentrations were found to

produce sub-maximal responses. The cells were incubated at 37 °C for various times, all incubations were stopped at the same time and the cell monolayers were washed three times with PBS. Thrombomodulin and tissue-factor activities were then measured as described above.

RESULTS

For each experimental condition, simultaneous measurements of tissue-factor and thrombomodulin activities were performed on the same cell population. There was significant thrombomodulin activity on the surface of non-stimulated HSVEC (43.2 ± 2.6 pM-APC generated/min per 10^4 cells; mean $\pm$ S.E.M. of 15 experiments), but only minimal tissue-factor activity (12 ± 1.5 pM-Factor Xa generated/min per 10^4 cells).

Effect of PMA on tissue-factor and thrombomodulin activities

Exposure of cells for 4 h to PMA caused a dose-dependent increase in tissue-factor activity and a dose-dependent decrease in thrombomodulin activity. In both cases, the maximal response was obtained at 16 nM. This concentration of PMA caused a 31-fold increase in the generation of Factor Xa, to 4495 ± 650 pM/min per 10^4 cells, and a 41% decrease in the generation of APC, to 29 ± 2 pM/min per 10^4 cells (Fig. 1).

A concentration of 8 nM-PMA was selected for kinetic experiments. In these assays (Fig. 2), tissue-factor activity increased after as early as 2 h of incubation, reached a maximum between 4 and 8 h (2060 ± 1270 pM-Factor Xa generated) and diminished after 8 h, returning to 440 ± 230 pM-Factor Xa generated after 24 h. Simultaneously, PMA induced a 60% decrease in thrombomodulin activity after 2 h (Fig. 2). Thrombomodulin activity then tended to increase again with time, and the basal activity

Fig. 1. Dose-response of the effect of PMA, thrombin, IL-1 or TNF on the expression of tissue-factor and thrombomodulin activities on the surface of HSVEC

Confluent monolayers of HSVEC were exposed to the indicated concentrations of stimuli for 4 h and then assayed simultaneously for either tissue-factor (●) or thrombomodulin (■) activity as described in the Materials and methods section. Points correspond to means $\pm$ S.E.M. of three to four experiments using cells from different donors; for all experiments, each condition was assayed in sextuplicate. When no error bars are visible, S.E.M. was less than 8% of the mean value.

Fig. 2. Time course of the effect of 8 nM-PMA or 8.8 nM-thrombin on tissue-factor and thrombomodulin activities on the surface of HSVEC

Confluent monolayers of HSVEC were treated for the time indicated with PMA or thrombin (●, ▲, ■, ◆) or control buffer (◇). Tissue factor (top panels) and thrombomodulin (bottom panels) were assayed simultaneously thereafter as described in the Materials and methods section. For tissue-factor activities, three different experiments (●, ▲, ■) performed with cells from different donors are represented. For one experiment with PMA (●), the scale for activated Factor X is different and is shown on the right-hand side of the graph. For thrombomodulin activities, the points (◆) are means of two experiments performed with cells from different donors. For each experiment, each point is the mean $\pm$ S.E.M. of six culture wells. When no error bars are visible, S.E.M. was less than 8% of the mean value.

was recovered after 18 h of incubation with PMA (27 ± 1 pM-APC generated, compared with 32 ± 6 pM-APC generated for basal activity).

Effect of α -thrombin on tissue-factor and thrombomodulin activities

After incubation for 4 h with the cells, thrombin caused a dose-dependent increase in tissue-factor activity, with a maximal response at 8.8 nM (1 NIH unit/ml) (Fig. 1). No significant effect of thrombin on thrombomodulin activity was detected over the whole concentration range (0.88–44 nM). One NIH unit of thrombin/ml (8.8 nM) was used for the kinetic experiments (Fig. 2). The increase in tissue-factor activity was maximal after incubation for 4 h (240 ± 80 pM-Factor Xa generated/min per 10^4 cells), and then decreased rapidly, to return to the vicinity of the basal value after 12 h (15 ± 5 pM-Factor Xa generated/min per 10^4 cells, compared with a basal value of 7 ± 3). Thrombomodulin activity was not influenced upon incubation with 8.8 nM-thrombin for 2–24 h.

Effect of TNF on tissue-factor and thrombomodulin activities

Exposure of cultured endothelium for 4 h to TNF induced a dose-dependent increase in tissue-factor activity (Fig. 1), reaching the generation of 600 ± 120 pM activated Factor X/min per 10^4 cells for 100 ng of TNF/ml (2.2 nM). The kinetic experiments were performed with 5 ng of TNF/ml (111 pM) (Fig. 3). The maximal increase in tissue-factor activity was obtained after incubation for 4 h (1095 ± 250 pM activated Factor X/min per 10^4 cells); the activity then decreased, returning to the basal value after 24 h. Thrombomodulin activity was not significantly altered by preincubation of the cells with TNF (0.02 pM–2 nM) for 4 h. However, 111 pM-TNF induced a time-dependent decrease in thrombomodulin activity, reaching 67% of control values after 24 h.

Fig. 3. Time course of the effect of 57 nM-IL-1 or 111 pM-TNF on tissue-factor and thrombomodulin activities on the surface of HSVEC

Confluent monolayers of HSVEC were treated for the time indicated with IL-1 (●, ▲, ■, ◆) or control buffer (◇). Tissue-factor (top panels) or thrombomodulin (bottom panels) were assayed simultaneously thereafter as described in the Materials and methods section. For tissue-factor activities, three different experiments (●, ▲, ■) performed with cells from different donors are represented. For thrombomodulin activities, the points (◆) are means of three experiments performed with cells from different donors. For each experiment, each point is the mean $\pm$ S.E.M. of six culture wells. When no error bars are visible, S.E.M. was less than 8% of the mean value.

Effect of IL-1 on tissue-factor and thrombomodulin activities

After 4 h of incubation with IL-1, HSVEC expressed a dose-dependent increase in tissue-factor activity, reaching the generation of 1320 ± 320 pM activated Factor X/min per 10^4 cells for 570 nM-IL-1 (10 units/ml) (Fig. 1). Thrombomodulin activity was not significantly altered under the same conditions. A concentration of 1 unit of IL-1/ml (57 nM) was used for kinetic experiments (Fig. 3). The maximal increase in tissue-factor activity was obtained after 4 h of preincubation (1570 ± 470 pM-Factor Xa generated/min per 10^4 cells), the tissue-factor response decreasing thereafter to return to the basal value after 24 h. Thrombomodulin activity was decreased in a time-dependent manner, reaching a 47% decrease after 24 h, from 43 pM (control value) to 23 pM APC generated/min per 10^4 cells.

DISCUSSION

The measurement of thrombomodulin and tissue-factor activities provides an appropriate tool for evaluation of the regulatory role of endothelial cells in the generation of thrombin, especially during inflammation or sepsis, when cells are exposed to various exogenous stimuli. Comparison of results from the literature reporting the study of the various pathways of modulation of thrombomodulin or tissue-factor expression by endothelial cells is sometimes confusing, owing to the variety of cell types, culture methods and experimental protocols which have been used. It should be emphasized that either the culture method, especially with respect to the presence of heparin and growth factors (Almus *et al.*, 1988; Andoh *et al.*, 1990), the origin or passage number of the cells (Dicheck & Quertermous, 1989), or their state of confluence (Gerlach *et al.*, 1989; Andoh *et al.*, 1990) can greatly influence the tissue-factor or thrombomodulin response in endothelial cells. Significant quantitative variations in the expression of tissue factor in human umbilical-cord endothelial cells stimulated by endotoxin (Moore *et al.*, 1987) or thrombin (Galdal *et al.*, 1985; Almus *et al.*, 1988) have been observed when the cells were obtained from different donors. We also observed individual variations in quantitative response (Figs. 2 and 3); this is also illustrated by the S.E.M. values when data obtained with cells from different donors were combined (Fig. 1). The present results were obtained by a two-stage amidolytic assay, which has the following advantages: (i) measurement of thrombomodulin and tissue-factor activities was performed in a homologous system using cells and coagulation proteins of human origin; (ii) the cells were cultivated in a standard medium containing 30% human serum without further addition of heparin or any growth factors, all media and buffers having a low (< 0.02 ng/ml) endotoxin content; (iii) the assay measured thrombomodulin or tissue-factor activity expressed at the luminal cell surface of intact living cells; (iv) the availability of a microtitre-plate assay allowed simultaneous evaluation of several experimental conditions, each one requiring only a limited number of cells (Carson & Archer, 1986; Surprenant & Zuckerman, 1989).

Therefore these homogeneous assays were used to assess the effects of thrombin, IL-1, TNF and PMA on thrombomodulin and tissue-factor activities. According to their respective effects, these agents can be classified within three categories. (1) PMA was the only one to induce at the same concentration a strong increase in tissue-factor activity (at least 31-fold) as well as a 41% decrease in thrombomodulin activity. Both changes were transient; the increase in tissue-factor activity was maximal after 4–8 h and returned to normal values after 24 h of incubation, whereas the decrease in thrombomodulin was maximal after 2 h and returned to normal after 18 h of incubation with PMA.

(2) The cytokines IL-1 and TNF both induced an increase in tissue factor which was maximal after 4–6 h of incubation, and a slow decrease in thrombomodulin activity which was proportional to the time of incubation with the cytokine. Even at the high concentrations of the cytokines that were used, the tissue-factor response did not seem to reach a maximum. It is possible that a further increase in tissue factor could be obtained at higher concentrations of TNF or IL-1. (3) Thrombin induced an increase in tissue-factor activity, but no significant variation in thrombomodulin activity over a 24 h incubation period.

Since the production of thrombin at the endothelial-cell interface is regulated by tissue factor and thrombomodulin, its effect in the present experimental system is of particular interest. Thrombin has been consistently shown to increase the amount of tissue factor present in endothelial cells (Brox *et al.*, 1984; Galdal *et al.*, 1985; Almus *et al.*, 1988, 1989; Boeri *et al.*, 1989), and thus to exert a positive feedback regulation on its own generation. Thrombin is also the specific ligand for thrombomodulin, but its effect on the expression or activity of the endothelial protein is controversial. It was first shown that thrombin could induce endocytosis of the thrombin–thrombomodulin complex in A549 cells and human umbilical-vein endothelial cells, leading to a large decrease in thrombomodulin activity (Maruyama & Majerus, 1985). In another study from our laboratory, the thrombin–thrombomodulin complex was found to remain stable for at least 2 h on HSVEC or the endothelial cell line EA.hy 926 (Beretz *et al.*, 1989). Esmon (1989) also quotes unpublished observations showing that the internalization of thrombin–thrombomodulin complexes can occur on occasion, but is not an obligatory response to receptor occupancy. The present observations confirm that exposure of endothelial cells to concentrations of thrombin reaching 44 nM (5 NIH units/ml) did not significantly modify thrombomodulin activity. Thrombin has been shown to cause the secretion of IL-1 by human umbilical-vein endothelial cells, which could participate in the induction by thrombin of procoagulant activity in these cells (Stern *et al.*, 1985b). In our experiments, dose/action studies, performed in the absence of serum (Fig. 1), show an effect of 8.8 nM-thrombin (1 NIH unit/ml) which is 6-fold higher than the activity observed in the kinetic experiments (Fig. 2), performed in the presence of 10% human serum. This difference can be explained by the partial inactivation of thrombin in the presence of endothelial cells through antithrombin III present in the serum (Lollar *et al.*, 1984).

The differences in the characteristics of the modulation of thrombomodulin and tissue factor are also supported by recent molecular biology data. The induction of tissue factor in endothelial cells by TNF or PMA (Scarpatti & Sadler, 1989; Crossman *et al.*, 1990) can be explained by an increase in transcription and potentially an increase in mRNA stability. On the contrary, the decrease in thrombomodulin induced by TNF would be due to a translational or post-translational mechanism, which could involve internalization and intracellular degradation (Moore *et al.*, 1989). The short half-life of tissue factor mRNA (48 min) and longer half-life of thrombomodulin mRNA (> 8 h) (Scarpatti & Sadler, 1989) or thrombomodulin antigen [11–20 h in mouse haemangioma cells (Dittman *et al.*, 1988)] also support the possibility of different modes of regulation of the amount of protein present at the cell surface. However, it has also been shown in bovine aortic and human umbilical-vein endothelial cells that TNF decreased the amount of cell-surface thrombomodulin, not by internalization but through a 97% inhibition of transcription (Conway & Rosenberg, 1988).

The present data show that, on the surface of endothelial cells in culture, a procoagulant entity, tissue factor, or an anticoagulant one, thrombomodulin, are not simultaneously regu-

lated by stimuli such as TNF, IL-1 or thrombin, which are susceptible to be secreted in pathological situations leading to thrombosis. The expression and availability of functional tissue factor, absent from the surface of the resting cell, can be strongly induced over a 2–8 h period. On the other hand, the constitutive activity of thrombomodulin is much less susceptible to modulation by the same concentrations of substances that induce a maximal response of tissue factor. This is reflected both by the slower onset of the decrease of thrombomodulin and by the fact that the maximal decrease in activity never exceeded 67% after 24 h. Hence, our results do not support the idea that these two opposite activities are simultaneously regulated by symmetrical mechanisms; they suggest that the simultaneous and opposite modulation of the expression of these proteins does not represent a unified response of the endothelium to procoagulant stimuli. Such simultaneous regulation was only observed when the cells were stimulated with PMA, a non-physiological stimulus. This probably indicates that the cytokines do not act by the same mechanism as PMA, and especially that stimulation of protein kinase C might not be the major pathway involved in their mode of action.

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