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Thrombin causes increased monocytic-cell adhesion to endothelial cells through a protein kinase C-dependent pathway

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The coagulation protein thrombin has been shown to stimulate multiple endothelial-cell (EC) functions, including production of platelet-derived growth factor and of platelet-activating factor (PAF), and neutrophil adhesion. We have found that thrombin causes increased binding of monocytic cells (U937 cells and normal human monocytes) to cultured EC of various species. Maximum adhesion of monocytes to pig aortic EC occurred 6 h after thrombin treatment and remained elevated through 24 h. Stimulation of adherence by bovine α-thrombin was half-maximal at 15 units/ml, and reached a plateau at 50 units/ml. Catalytically inactive thrombin (phenylmethanesulphonyl fluoride-treated) had no effect on monocyte adhesion to EC. Heparin, but not the endotoxin antagonist polymyxin B, suppressed the stimulation of adhesion by thrombin without altering basal adhesion. Two lines of evidence suggested that protein kinase C (PKC) was involved in the intracellular signalling to increase monocyte adhesion to EC. First the PKC activator phorbol 12-myristate 13-acetate (PMA) stimulated monocytic-cell adhesion to EC at a dose consistent with stimulation of PKC (half-maximal response at 1–3 nM) and with a time course similar to that for thrombin stimulation (maximal by 4 h). Diacylglycerol, a physiological activator of PKC, also stimulated U937-cell adhesion to EC. Secondly, H7, a PKC inhibitor, completely blocked stimulation of monocyte adhesion to EC by thrombin or PMA. The structural analogue of H7, HA1004, which preferentially inhibits cyclic-AMP- and cyclic-GMP-dependent protein kinases, had no effect on stimulated monocyte adhesion. The PKC inhibitor also blocked the stimulation of monocyte adhesion to EC by interleukin-1 and endotoxin, but did not alter the basal level of monocyte binding to unstimulated EC. Thrombin stimulation of monocyte adhesion differed from the reported stimulation of neutrophil adhesion by thrombin in that the latter process reached a maximum in minutes rather than hours. In addition, neither PAF itself nor agents known to stimulate PAF production by EC, such as arachidonate and the Ca^{2+} ionophore A23187, had any effect on monocyte adhesion. These results demonstrate a PKC-dependent cytokine-like action of the coagulation protein thrombin in modulating monocytic-cell adhesion to EC, a phenomenon of potential importance in many pathological and physiological processes.

INTRODUCTION

Monocyte adhesion to the vascular endothelium, as an initial event in the emigration of this leucocyte into tissue, may play an integral role in such physiological and pathological processes as wound healing, inflammation and atherogenesis. The binding of monocytes to the endothelium is stimulated by certain leucocyte secretory products, such as interleukin-1 (IL-1) [1] and tumour necrosis factor [2], as well as circulating biological activators such as bacterial lipopolysaccharide (LPS) [3]. The intracellular signalling involved in the induction of monocyte adhesion by these exogenous stimulators remains poorly understood. The stimulation requires several hours and protein synthesis *de novo* [1], and therefore presumably the induction of specific gene products; however, the second-messenger systems participating in this process are completely undefined.

Thrombin is a coagulation-system protease as well as a potent platelet-aggregating substance. Thrombin is known to bind to specific receptor sites on endothelial cells (EC) and to stimulate diverse functions in these cells, examples of which are an increase in prostacyclin production [4,5], the release of von Willebrand factor [6], an increase in expression of the platelet-derived-growth-factor genes [7,8] and an increase in neutrophil binding to the EC surface [9,10]. The ability of thrombin to activate specific second-messenger systems in cultured EC has been the subject of study in recent years. Thrombin causes a transient increase in cytosolic Ca^{2+} in human arterial and umbilical-vein EC [11,12]. The production of specific inositol phosphates is also increased in EC in response to this protease [13,14], and increased levels of diacylglycerol have been observed in thrombin-treated fibroblasts [15,16]. Others have reported an indirect effect of the adenylate cyclase system in the action of thrombin on EC [8].

In the present work we have directly probed a role for α-thrombin in regulating the adhesion of monocytic cells to EC, and we have begun to delineate the intracellular messages involved in this stimulation. We have examined the possible involvement of protein kinase C (PKC) and

Abbreviations used: EC, endothelial cells; PAF, platelet-activating factor (1-alkyl-1-acetyl-*sn*-glycero-3-phosphocholine); PMSF, phenylmethanesulphonyl fluoride; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; LPS, lipopolysaccharide; IL-1, interleukin-1; PBS, phosphate-buffered saline; FBS, fetal-bovine serum; DME/F12, Dulbecco's modified Eagle's medium/Ham's F12 medium (1:1).

platelet-activating factor (PAF), and we have contrasted our results with those published by others on thrombin-induced neutrophil adhesion to vascular EC.

MATERIALS AND METHODS

Reagents

Bovine α-thrombin was purchased from United States Biochemical Corp. (Cleveland, OH, U.S.A.). This protease was characterized by the supplier as chromatographically homogeneous. Phenylmethanesulphonyl fluoride (PMSF)-inactivated thrombin was prepared by incubation of the enzyme with 1 mM-PMSF at pH 7.0 for 1 h, as previously described [17,18]. PAF, PMSF, PMA, phorbol dibutyrate, 1-oleoyl-2-acetyl-*rac*-glycerol, polymyxin B and phorbol were purchased from Sigma (St. Louis, MO, U.S.A.). The protein kinase inhibitors H7 and HA1004 were from Seikagaku America (St. Petersburg, FL, U.S.A.). Arachidonic acid was obtained from Serdary Research Lab (London, Canada). Crude IL-1 was prepared from phytohaemagglutinin-stimulated pig mononuclear cells by the method of Schmidt [19].

Cell culture

Bovine and pig aortic EC were isolated by a modification of the method of Schwartz [20]. Briefly, thoracic aorta segments were split longitudinally, rinsed with serum-free media and the exposed intimal surface was digested with collagenase (2 mg/ml in serum-free media) for 15 min at 37 °C. The detached EC patches were gently collected with a rounded spatula or cotton swab and placed in primary culture. At confluence the cells were subcultured at 1:3 or 1:5 ratios. Human umbilical-vein EC were isolated by a technique similar to that described by Lewis *et al.* [21]. Undamaged segments of 1–3-day-old umbilical cords were drained, and the remaining blood was rinsed from the umbilical vein with phosphate-buffered saline (PBS) via a blunt cannula attached at one end of the vessel. The open end of the cord was sealed, and the cord was distended with 0.06% trypsin/0.2% EDTA and incubated at 37 °C for 15 min. The cord was massaged, cut, and the trypsin digest was collected. The cells were washed and seeded directly into assay plates ($\sim 10^5$ cells/cm^2 area of tissue-culture plastic). All EC were grown in DME/F12 media (Irvine Scientific Co., Irvine, CA, U.S.A.) supplemented with 0.24% $NaHCO_3$, 0.1 mM MEM (minimal essential medium) non-essential amino acids, penicillin (100 units/ml) and streptomycin (100 μg/ml) (supplements from Sigma). The DME/F12 medium was supplemented with 5% (v/v) fetal-bovine serum (FBS) (Gibco) for the pig and bovine cells and 15–20% FBS for the human cells. Cells were subcultured by trypsin/EDTA treatment; pig and bovine EC were used between passages 3 and 20, and human EC in primary culture. U937 cells originally derived from a human histiocytic lymphoma were obtained from the American Type Culture Collection and grown in suspension culture in RPMI-1640 media (MA Bioproducts) containing 5% FBS and routinely subcultured at a 1:5 ratio three times per week [22]. All culture vessels were obtained from Costar.

Separation of human monocytes

Monocytes were separated from peripheral blood (100 units of heparin/ml) by a modification of the method of Recalde [23]. Briefly, total mononuclear cells were collected by Ficoll–Hypaque density-gradient centrifugation [24], washed and resuspended in FBS. The cells were then maintained at 37 °C while 9% NaCl was added at three 10 min intervals (5 μl/ml, then 10 μl/ml, then another 10 μl/ml). After the last 10 min incubation, the cell suspension was mixed with 2 vol. of PBS (with 27 μl of 9% NaCl/ml added) and underlayered with 1 vol. of Ficoll–Hypaque (with 2.8 mg of NaCl/ml added). The gradient was spun at 600 *g* for 20 min, and the monocytes were separated from the Ficoll interface, washed twice with cold PBS and finally resuspended in DME/F12+5% FBS in a Teflon beaker to prevent attachment. Isolated populations were routinely 78–90% monocytes by differential count with buffered Wright–Giemsa stain, and viability was always greater than 90% by Trypan Blue dye exclusion.

Assay for monocytic cell adhesion to endothelial cells

U937-cell adhesion to EC was measured as previously described [22]. Briefly, EC were plated into 24-well plates in their appropriate medium 48–96 h before the assay, and grown to confluence (approx. 2×10^5 cells/well). On the day of the assay, U937 cells or normal human monocytes were labelled for 90 min at 37 °C with 100 μCi of ^{51}Cr as sodium chromate (NEN) in 1 ml of culture medium. The labelled cells were washed by centrifugation and 10^6 viable cells were added per well of EC after removal of the incubation media. This binding phase of the assay was performed at 4 °C for 1 h, then the wells were washed and the cells lysed with 1% Triton X-100, and a sample was removed for γ-radiation counting. The number of U937 cells or monocytes bound per well was calculated from the initial specific radioactivity (c.p.m./cell). All data points represent triplicate determinations, with a S.E.M. less than 10%. Spontaneous release of chromium from the monocytic cells during the assay incubation was less than 5%.

The data of all experiments that were negative are representative of at least two separate experiments with very similar results. All positive results have been confirmed by four or more separate experiments.

RESULTS

Thrombin stimulates monocytic cell adhesion to endothelial cells

Thrombin has been shown to stimulate the adhesion of neutrophils to vascular EC in a rapid process that may involve the generation of PAF [9]. We tested whether α-thrombin was also able to alter adhesion of monocytic cells to cultured EC. Three species of vascular EC, human, bovine and pig, exhibited a 3–13-fold increase in adhesion of U937 cells when treated with bovine α-thrombin at 50 units/ml (Table 1). Binding of normal human monocytes to human umbilical-vein EC was similarly stimulated by thrombin (Table 1). A representative dose–response curve revealed a half-maximal stimulation of U937-cell adhesion to pig aortic EC at 15 units/ml, with maximal response at 50 units/ml (Fig. 1). The thrombin stimulation was as great in the presence of 1% as 5% FBS, and stimulated adhesion was even observed though to a lesser extent (one-third to one-half of the 1%-serum response), when the incubation of EC with thrombin was performed under serum-free conditions after three rinses of the cells (results not shown). These results suggest both that thrombin does not require association with a serum protein to be active

Table 1. Effect of thrombin on monocytic-cell adhesion to various species of endothelial cells

Confluent pig aortic (PAEC), bovine aortic (BAEC) or human umbilical-vein (HUVEC) endothelial cells were treated for 6 h at 37 °C with 50 units of bovine thrombin/ml in DME/F12+5% FBS, or with medium alone. ^{51}Cr-labelled U937 cells or normal human monocytes (10^6 cells/well) were added to triplicate wells of the pretreated EC and incubated for 1 h at 4 °C. Adhesion was quantified as described in the Materials and methods section. Results are means ± S.E.M. ($n = 3$).

Expt. no.	Monocytic cells	EC	EC treatment	Percentage of monocytic cells bound per culture	Increase by thrombin (%)
I	U937	PAEC	Medium	2.1±0.15	–
			Thrombin	29.5±1.40	1350
II	U937	BAEC	Medium	0.5±0.09	–
			Thrombin	6.2±0.53	1140
III	U937	HUVEC	Medium	16.3±0.58	–
			Thrombin	50.2±1.04	208
IV	Normal human monocytes	HUVEC	Medium	8.9±0.96	–
			Thrombin	31.6±2.64	255

and that thrombin is not inhibited by serum proteins in its ability to stimulate monocyte adhesion. Thrombin-induced monocytic-cell binding to EC was minimally inhibited by polymyxin B, an endotoxin antagonist, under conditions where LPS-induced stimulation was nearly completely blocked (results not shown).

The time required for thrombin to induce maximal monocytic-cell adhesion was found to be comparatively long (Fig. 2), 6 h versus the 5–60 min reported by others to be required for thrombin-induced neutrophil binding [9,10]. The stimulated level of monocyte binding to pig aortic EC persisted through 24 h (Fig. 2), whereas thrombin-stimulated neutrophil binding was reported to begin to decay within hours of treatment [9,10]. The time required to attain maximal monocytic-cell adhesion in response to thrombin was also longer than that required by other activators of monocytic-cell adhesion; in our system IL-1, LPS and phorbol esters induced maximal adhesion in 4 h. Because of the relatively long treatment time required, we investigated the possibility that a secondary factor was being produced by EC in response to thrombin, which then acted in an autocrine fashion to induce monocyte adhesion sites. This did not appear to be the case, since conditioned media from thrombin-treated EC did not stimulate control EC which were specifically blocked from thrombin stimulation by prior treatment with heparin (Table 2).

We tested whether the proteolytic activity of thrombin was required for its ability to stimulate monocytic-cell adhesion to EC. The protease was treated with PMSF to destroy more than 80% of the clotting activity of the preparation. The PMSF-treated thrombin also exhibited a greater than 80% decrease in its ability to stimulate monocyte adhesion, suggesting the requirement for a proteolytic cleavage in the process of cell signalling (Table 3). A second line of evidence supported this conclusion in that heparin, which has been reported to inhibit thrombin proteolytic activity in the presence of serum without blocking the enzyme's ability to bind to its cell-surface receptor [25], suppressed thrombin-stimulated monocyte adhesion (Table 3).

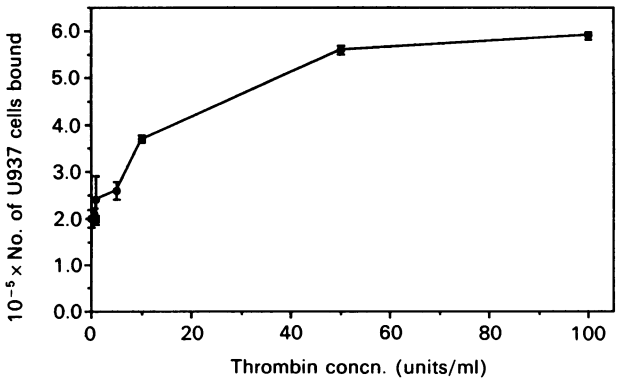

Fig. 1. Thrombin stimulation of U937-cell adhesion to EC

Confluent pig aortic EC were treated with various concentrations of bovine thrombin in DME/F12+5% FBS for 6 h at 37 °C before performing the U937-cell-adhesion assay as described in the Materials and methods section. Results are means ± S.E.M.

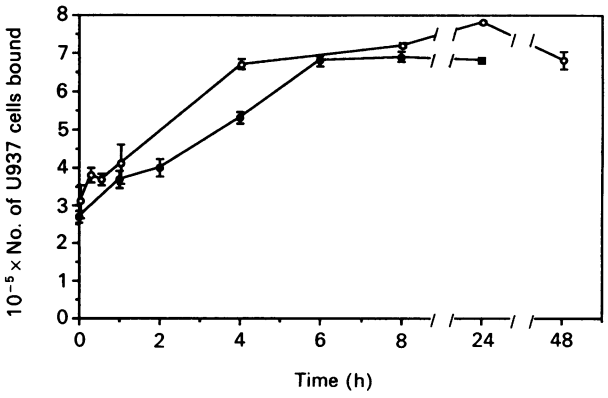

Fig. 2. Time course of thrombin and PMA stimulation of EC to bind U937 cells

Confluent pig aortic EC were incubated with bovine thrombin (●) (25 units/ml) or PMA (○) (10 nM) in DME/F12+5% FBS at staggered times before assay for U937-cell adhesion (see the Materials and methods section). Results are means ± S.E.M.

Table 2. Effect of endothelial-cell conditioning or heparin addition on thrombin's ability to stimulate U937-cell adhesion to endothelial cells

Confluent wells of bovine aortic EC were pre-treated with DME/F12+5% FBS alone or with heparin (200 μg/ml) for 18 h at 37 °C. At 6 h before assay the cells were treated with DME/F12+5% FBS (with or without heparin, 200 μg/ml), bovine thrombin (25 units/ml (with or without heparin), or EC-conditioned media (with or without heparin), and incubated for 6 h at 37 °C. The EC were rinsed, and U937 cells (10^6/well) were incubated with the EC for 1 h at 4 °C. The number bound was determined as described in the Materials and methods section. Conditioned media were prepared by incubating 2 cm^2 wells of pig aortic EC with 0.5 ml of DME/F12+5% FBS alone, or containing bovine thrombin (25 units/ml) for 6 h at 37 °C. The supernatant was collected and stored at 4 °C overnight. Results are means ± S.E.M. ($n = 3$).

Treatment	10^{-5} × No. of U937 cells bound per culture	Increase (%)
Media	0.5±0.02	–
Media + heparin	0.3±0.07	−40
Thrombin (25 units/ml)	4.2±0.22	740
Thrombin + heparin	0.9±0.12	80
6 h-EC-conditioned media	1.2±0.12	140
Conditioned media + heparin	0.5±0.11	0
6 h-EC-conditioned thrombin	3.5±0.24	600
Conditioned thrombin + heparin	0.8±0.12	60

Role of protein kinase C in thrombin-induced monocyte adhesion to endothelial cells

Phorbol esters have been shown to cause increased adhesion of leucocytes to EC [3,26], suggesting a role for PKC in the stimulatory response. We tested the ability of PMA, as well as other known PKC activators, to stimulate monocytic-cell adhesion to EC in our system. PMA, phorbol dibutyrate and 1-oleoyl-2-acetyl-*rac*-glycerol stimulated EC to bind enhanced numbers of U937 cells (Table 4), whereas the non-activating phorbol had no effect in the assay. The concentration range for PMA stimulation (1–3 nM half-maximal and 10 nM maximal response) was indicative of activation of PKC (Fig. 3). In addition, the time required for a response of EC to PMA (Fig. 2) was consistent with a role for PKC in thrombin stimulation of EC. To test further the involvement of PKC in stimulated monocyte adhesion to EC, we investigated the effect of the PKC inhibitor H7, and its structural analogue HA1004, which is a more potent inhibitor of cyclic-AMP- and cyclic-GMP-dependent protein kinases. The inhibitor H7 caused nearly complete inhibition of not only PMA- and thrombin-stimulated U937-cell adhesion to EC, but also adhesion stimulated by IL-1 and LPS (Fig. 4). HA1004, on the other hand, had no effect on the stimulation of monocyte adhesion by any of the activators. Neither drug had any effect on binding of monocytes to sparse unstimulated EC or to EC responding to a 'wound' *in vitro* (results not shown).

Inability of platelet-activating factor to mimic the effect of thrombin

Since PAF production by EC is stimulated by throm-

Table 3. Effect of PMSF inactivation on thrombin's ability to stimulate U937-cell binding to endothelial cells

Confluent pig aortic EC were treated with various concentrations of control bovine thrombin or PMSF-inactivated thrombin in DME/F12+5% FBS for 6 h at 37 °C. The adhesion assay was performed as described in The Materials and methods section. Results are means ± S.E.M. ($n = 3$).

EC treatment	10^{-5} × No. of U937 cells bound per culture	Increase (%)
Medium	1.6±0.37	–
Thrombin		
10 units/ml	3.9±0.06	144
20 units/ml	4.4±0.03	175
50 units/ml	5.4±0.14	238
PMSF-treated thrombin		
10 units/ml	1.5±0.11	−6
20 units/ml	2.5±0.30	56
50 units/ml	3.4±0.07	112

Table 4. Effect of protein kinase C activators on U937-cell adhesion to endothelial cells

Confluent wells of pig aortic EC were treated with DME/F12+5% FBS alone, or containing 40 nM-PMA, 40 nM-phorbol 12,13-dibutyrate (PDBu), 100 μg of 1-oleoyl-2-acetyl-*rac*-glycerol (OAG)/ml or 40 nM-phorbol, and incubated at 37 °C for 4 h (OAG was replaced with a fresh solution midway through the incubation). The EC were rinsed, and ^{51}Cr-labelled U937 cells (10^6 cells/well) were incubated with the EC for 1 h at 4 °C. The number bound was determined as described in the Materials and methods section. Results are means ± S.E.M. ($n = 3$).

Treatment of EC	10^{-5} × No. of U937 cells bound per culture	Increase (%)
Medium	1.8±0.07	–
PMA (40 nM)	5.8±0.13	222
PDBu (40 nM)	4.2±0.17	133
OAG (100 μg/ml)	3.2±0.15	78
Phorbol (40 nM)	2.0±0.10	11

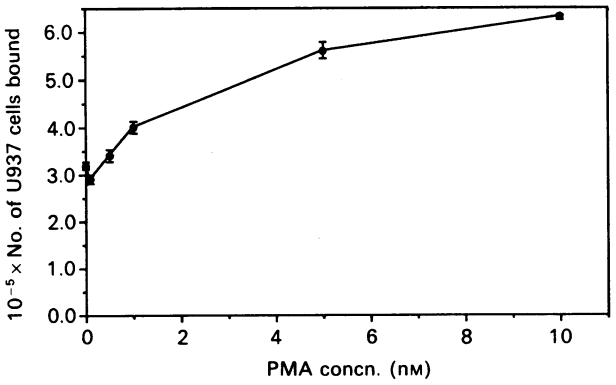

Fig. 3. PMA stimulation of U937-cell adhesion to EC

Confluent pig aortic EC were treated with various concentrations of PMA for 4 h at 37 °C before the U937-cell-adhesion assay. Results are means ± S.E.M.

bin, and since PAF has been implicated in stimulated neutrophil adhesion to EC [9,27], we investigated the effect of PAF on monocytic-cell adhesion to EC. PAF, even at pharmacological concentrations (10 nM–1 μM), had no effect on the ability of EC to bind monocytic cells (Table 5). Thrombin is known as a potent stimulator of prostanoid production by EC [4,5]. Two other agents which stimulate cyclo-oxygenase activity, arachidonate (400 μM) and Ca^{2+} ionophore A23187 (0.6 μM), had no effect on monocyte binding to EC (Table 5). Further evidence that the monocyte-binding ability of stimulated EC was independent of cyclo-oxygenase was the finding that acetylsalicylic acid (100 mg/ml) did not affect the basal or thrombin-stimulated level of adhesion of monocytic cells to EC (Table 5).

DISCUSSION

The binding of blood-borne monocytes to the endothelium has received much attention in recent years as an early, and potentially regulatable, event in such processes as inflammation, wound healing and atherogenesis. Experiments with cultured EC have led to several conclusions about monocyte adhesion that appear to be applicable in multiple species and in multiple vascular beds. First, the expression of monocyte-binding sites on the EC surface is low under 'control' or 'physiological' conditions, i.e. when the cultured EC are confluent, quiescent, and unstimulated by exogenous agents. Secondly, when treated with specific exogenous agents [1–3] or when stimulated to migrate and divide in response to a 'wound' *in vitro* [22], EC exhibit a greatly increased ability to bind monocytes. The increased binding of monocytes that can be induced by such response modifiers as IL-1, tumour necrosis factor and LPS requires protein synthesis *de novo* and is a transient phenomenon. Thirdly, though the above-mentioned stimulators also induce increased neutrophil adhesion to EC, the cell-surface binding site on the EC appears to be distinct for these two leucocytes [28]. Thus monocyte and neutrophil adhesion to EC may be regulated through distinct pathways and induced differentially by certain, as yet undefined, exogenous agents.

We have examined whether α-thrombin, a known stimulator of neutrophil adhesion to EC, is also capable of inducing monocyte-binding sites on the EC surface. Thrombin has been shown to bind specifically to both high- and low-affinity receptors on the EC surface [29]. Catalytic activity of the protease was not necessary for this binding to occur. In addition, Bauer *et al.* [30] have shown that the high-affinity binding of thrombin to EC is inhibitable by heparin. We have demonstrated that

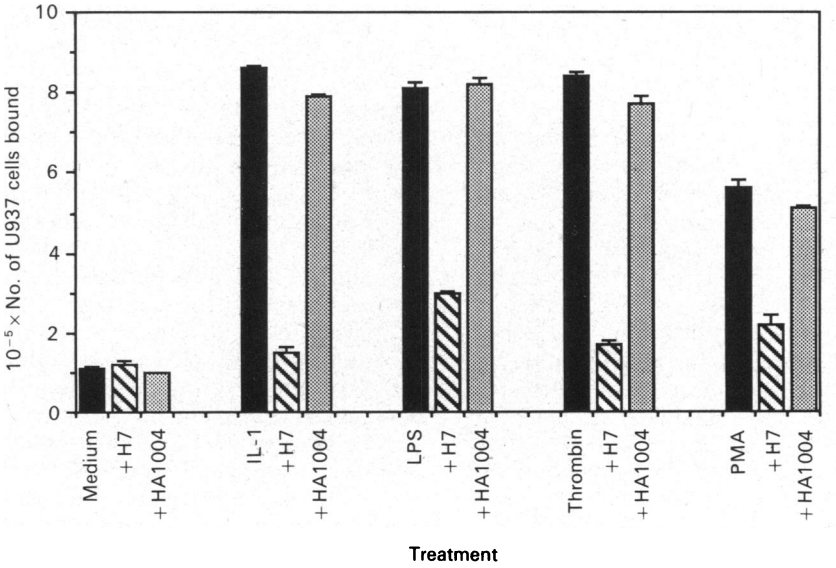

Fig. 4. Effect of protein kinase inhibitors on the stimulation of U937-cell adhesion

Confluent pig aortic EC were pretreated with DME/F12 + 5% FBS media alone, with 50 μM-H7 or with 50 μM-HA1004 for 30 min at 37 °C. Then each stimulator was added in fresh media either alone or with 50 μM-H7 or with 50 μM-HA1004 to the pretreated EC and incubated for another 6 h at 37 °C. Stimulators were used in higher than maximally stimulatory concentrations: crude pig IL-1 (1:10 phytohaemagglutinin-stimulated mononuclear-cell supernatant), LPS (100 ng/ml), bovine thrombin (100 units/ml) and PMA (100 nM). U937-cell adhesion was measured as described in the Materials and methods section. Results are means ± S.E.M.

Table 5. Effect of PAF, Ca^{2+} ionophore, arachidonate and acetylsalicylic acid on U937-cell adhesion to endothelial cells

Confluent bovine (Expt. I) or pig (Expts. II, III, IV) aortic endothelial cells were treated with media (DME/F12 + 5% FBS) or the named agents dissolved in the same media for 6 h at 37 °C when bovine thrombin was the stimulator, or for 4 h at 37 °C when PMA was the stimulator. At the end of the incubation, the media were removed and 10^6 ^{51}Cr-labelled U937 cells were added per 2 cm^2 well. Adhesion was quantified as described in the Materials and methods section. Results are means ± S.E.M. ($n = 3$).

Expt. no.	EC treatment	10^{-5} × No. of U937 cells bound per culture	Increase (%)
I.	Medium	0.05 ± 0.004	–
	Thrombin (25 units/ml)	0.33 ± 0.03	560
	PAF (10 nM)	0.07 ± 0.01	40
	(0.1 μM)	0.07 ± 0.007	40
	(1 μM)	0.07 ± 0.007	40
II.	Medium	0.9 ± 0.05	–
	A23187 (0.6 μM)	1.2 ± 0.04	33
	PMA (10 nM)	4.5 ± 0.14	400
	PMA + A23187	3.6 ± 0.22	300
III.	Medium	1.1 ± 0.10	–
	PMA (10 nM)	5.1 ± 0.21	364
	Arachidonate (400 μM)	1.1 ± 0.25	0
IV.	Medium	1.9 ± 0.25	–
	Thrombin (25 units/ml)	4.3 ± 0.23	126
	Acetylsalicylic acid (100 μg/ml)	2.5 ± 0.19	32
	Acetylsalicylic acid + thrombin	4.4 ± 0.17	132

thrombin stimulates monocyte adhesion to EC, presumably through binding of the protease to its high-affinity EC receptor, since we are able to inhibit completely and specifically only thrombin stimulation by preculturing EC with heparin. PMSF treatment, which greatly decreases the protease activity of thrombin [18], renders the enzyme unable to stimulate monocyte binding to EC. Though thrombin is capable of binding to EC in its catalytically inactivated state, apparently a proteolytic event is necessary for the EC activation sequence to proceed such that monocyte-binding sites are expressed on the EC surface.

Thrombin appears to behave in a similar manner to the cytokines discussed above with respect to stimulated monocyte adhesion; however, we have consistently found that binding-site expression occurs more slowly when EC are treated with this protease compared with treatment with LPS or IL-1 stimulation. The extra time required by thrombin to induce maximal binding of monocytes to EC suggests the possibility that thrombin is acting through an indirect mechanism, for example by inducing the expression of an autocrine stimulator of adhesion, such as IL-1. Though we have failed to detect the generation of such an autocrine activity, this may reflect the limitations in our ability to detect residual stimulator that has not been used by the EC, rather than the non-existence of such a factor. Therefore the possibility remains viable.

A relatively unexplored aspect of the question of regulation of monocyte adhesion to EC is the intracellular steps, including the type and sequence of second-messenger systems, which must occur during the hours that pass between treatment of the EC with an exogenous stimulator and the appearance on the EC surface of monocyte-binding sites. We asked whether the activation of PKC is involved in cytokine- and thrombin-stimulated monocyte adhesion to EC. We found that the increased adhesion caused by all exogenous stimulators tested, including thrombin, LPS, IL-1 and PMA, was fully inhibited by the PKC inhibitor, H7, but not by its structural analogue HA 1004. The latter drug is a more potent inhibitor of cyclic-AMP- and cyclic-GMP-dependent protein kinases than of PKC. PKC activation therefore appears to represent a common step in the induction of monocyte binding sites by very different stimuli. The ability of PMA and diacylglycerol to stimulate monocyte binding to EC also suggests that inositol phosphate production and release of intracellular Ca^{2+} stores, which are induced in EC in response to thrombin, are not involved in the monocyte adhesion pathway. Brock & Capasso [31] have demonstrated in the same EC culture system used in our studies that PKC activators block both the rapid, transient, increase in cytosolic Ca^{2+} and the rise in inositol phosphate levels that are generated in response to thrombin. Our results also indicate that thrombin-induced monocyte adhesion occurs independently of arachidonate metabolism. Neither stimulators of prostanoid production nor inhibition of cyclo-oxygenase altered the level of monocyte adhesion to EC.

The process of EC activation resulting in monocyte adhesion is apparently quite different from that required to stimulate neutrophil adhesion. Neutrophil-binding sites on the EC have decayed by the time monocyte-biding sites are measurably expressed, and monocyte adhesion remains elevated for over 24 h in the pig system. Another major difference is the suspected role of EC PAF production in the adherence of neutrophils. PAF treatment of EC had no effect on monocyte adhesion, and stimulators of arachidonic acid metabolism, which augment PAF production by EC, are inactive in our system.

The state of 'activation' of EC may play a critical role

in early events leading to atherosclerotic lesion formation or wound healing. The hypothetical scenario may progress from bacterial assault (LPS), immune stimulation (IL-1 production by macrophages) or tissue damage (locally high thrombin levels), leading to PKC activation in neighbouring EC. Subsequent increases in the expression of specific genes may then lead to multiple changes in EC function, including the ability to bind blood-borne monocytes. Other cellular events, in addition to PKC activation, that occur as part of the thrombin-induced cascade leading to monocyte adhesion have not yet been identified.

During our final preparation of this manuscript a publication appeared that confirms our observation that thrombin stimulates U937-cell adhesion to human umbilical-vein EC [32]. Our studies extend this initial result by examining adhesion with normal blood monocytes and multiple species of EC and by probing the intracellular events, including PKC activation, that lead to increased monocyte adhesion. In addition, we have contrasted our results with those reported on thrombin-stimulated adhesion of neutrophils and have tested the involvement of PAF in our system.

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