

# Thrombin-induced Expression of Endothelial P-Selectin and Intercellular Adhesion Molecule-1: A Mechanism for Stabilizing Neutrophil Adhesion

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**Abstract.** Thrombin-induced expression of endothelial adhesivity toward neutrophils (PMN) was studied using human umbilical vein endothelial cells (HUVEC). HUVEC were challenged with human  $\alpha$ -thrombin for varying durations up to 120 min, after which the cells were fixed with 1% paraformaldehyde and  $^{51}\text{Cr}$ -labeled human PMN were added to determine PMN adhesion. Endothelial adhesivity increased within 15 min after  $\alpha$ -thrombin exposure, and the response persisted up to 120 min. Expression of endothelial adhesion proteins, P-selectin (GMP-140, PADGEM, CD62), and intercellular adhesion molecule-1 (ICAM-1; CD54) on the endothelial surface was quantitated by increase in the specific binding of anti-P-selectin mAb G1 and anti-ICAM-1 mAb RRI/1 labeled with  $^{125}\text{I}$ . P-selectin expression was maximal at 5–15 min  $\alpha$ -thrombin exposure and decayed to basal levels within 90 min. In contrast, ICAM-1 activity increased at 30 min and remained elevated for 120 min after  $\alpha$ -thrombin challenge. The initial endothelial adhesivity was dependent on P-selectin expression since PMN adhesion occurring within the first 30 min after  $\alpha$ -thrombin challenge was inhibited by mAb G1. The later prolonged PMN adhesion was ICAM-1 dependent since this response

was inhibited by mAb RRI/1 and to the same degree by the anti-CD18 mAb IB4. Anti-ELAM-1 mAb BB11 had no effect on adhesion of PMN to the  $\alpha$ -thrombin-challenged cells. The initial P-selectin expression and PMN adhesion responses were reproduced by the 14-amino peptide (SFLLRNPNDKYEPF) (thrombin-receptor activity peptide; TRP-14) which comprised the  $\text{NH}_2$  terminus created by thrombin's proteolytic action on its receptors. However, TRP-14-induced PMN adhesion was transient, and TRP-14 did not cause ICAM-1 expression. The ICAM-1-dependent PMN adhesion mediated by  $\alpha$ -thrombin was protein synthesis independent since ICAM-1 expression and PMN adhesion were not inhibited by cycloheximide pretreatment of HUVEC. Moreover, Northern blot analysis indicated absence of ICAM-1 mRNA signal up to 180 min after  $\alpha$ -thrombin challenge. In conclusion, thrombin-induced endothelial adhesivity involves early- and late-phase responses. The initial reversible PMN adhesion is mediated by rapid P-selectin expression via TRP-14 generation. Thrombin-induced PMN adhesion is stabilized by a protein synthesis-independent upregulation of the constitutive ICAM-1 activity which enables the interaction of ICAM-1 with the CD18  $\beta_2$  integrin on PMN.

**T**HROMBIN causes polymorphonuclear leukocyte (PMN)<sup>1</sup> adhesion to the endothelial cell membrane by induction of endothelial adhesivity (4, 12, 34, 38). We have shown that a slow intravenous  $\alpha$ -thrombin infusion produces rapid PMN sequestration in pulmonary microvessels secondary to attachment of PMN to vascular endothelial cells (8, 23, 24). Moreover, the PMN sequestration response

typically persisted for several hours (8). The resultant vascular injury and tissue inflammation were critically dependent on PMN sequestration in the microvessels (22). Studies showed that  $\alpha$ -thrombin-induced lung vascular injury was prevented by prior depletion of the circulating PMN count (17, 20, 22, 24).

The basis of increased endothelial adhesivity after  $\alpha$ -thrombin challenge may involve expression of adhesive proteins on the endothelial plasma membrane (14, 26). Thrombin is known to rapidly upregulate P-selectin (GMP-140, PADGEM, CD62), a granular component of the Weibel-Palade body in endothelial cells and azurophilic granules of platelets (5, 13, 26). P-selectin is translocated to the endothelial cell plasma membrane where it rapidly induces PMN ad-

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1. *Abbreviations used in this paper:* HUVEC, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule; PATR, proteolytically activated thrombin receptor; PMN, polymorphonuclear leukocytes; TNF $\alpha$ , tumor necrosis factor  $\alpha$ ; TRP, thrombin receptor activating peptide.

hesion to endothelial cells (14, 15, 26). Another critical endothelial cell adhesion molecule, intercellular adhesion molecule-1 (ICAM-1; CD54), may also be involved in mediating thrombin-induced PMN adhesion (12). ICAM-1 is expressed in response to tumor necrosis factor alpha (TNF $\alpha$ ) and interleukin 1 by a protein synthesis-dependent mechanism (30), but it remains unclear whether thrombin can exert a similar effect on ICAM-1 induction. Previous studies have suggested that thrombin-induced expression of endothelial adhesivity may be dependent on ICAM-1 (12). In this study we examined the time course of expression of endothelial adhesivity using human umbilical vein endothelial cells (HUVEC). These cells were challenged with human  $\alpha$ -thrombin, and the relative contributions of P-selectin and ICAM-1 in mediating increased endothelial adhesivity were examined. The results indicate that the initial transient expression of thrombin-induced endothelial adhesivity is dependent on P-selectin, and that the adhesion response is prolonged and stabilized as the result of ICAM-1 expression. The induction of ICAM-1 expression is a posttranslational event since it did not involve increased ICAM-1 mRNA signal and occurred in the presence of cycloheximide.

## Materials and Methods

### Materials

Ficoll-Paque was obtained from Pharmacia LKB Biotechnology (Piscataway, NJ). Chromium 51 ( $^{51}\text{Cr}$ ) was obtained from New England Nuclear (Boston, MA). DME and HBSS were obtained from Gibco Laboratories (Grand Island, NY). Hepes, EDTA, gelatin (bovine skin type III), BSA, and cycloheximide were obtained from Sigma Chemical Co. (St. Louis, MO). FBS was obtained from Hyclone Laboratories Inc. (Logan, UT). TNF $\alpha$  was obtained from Cetus (Emeryville, CA). Human  $\alpha$ -thrombin was prepared and evaluated as described (11). Anti-CD18 mAb IB4 (37) and mAb OKM-1, a control mAb directed against an irrelevant isotope on CD11b (36, 37), were gifts from Dr. Samuel Wright, Rockefeller University, New York. The anti-ICAM-1 mAb RRI/1 was a gift from Dr. Robert Rothlein, Boehringer-Ingelheim Pharmaceuticals, Ridgefield, CT (30). The anti-endothelial leukocyte adhesion molecule-1 mAb BB11 (1) was a gift from Dr. Roy Lobb, Biogen Inc., Cambridge, MA. The anti-P-selectin mAb G1 and a control nonneutralizing anti-P-selectin mAb S12 (13, 18, 25) were provided by Dr. Rodger McEver, Department of Medicine, University of Oklahoma, OK (13, 18, 25). A control mAb W40 (18) was also provided by Dr. R. McEver. 24- and 96-well culture plates were obtained from Corning Glass Inc. (Corning, NY). The thrombin receptor activating peptide (TRP-14) SFLLRNPNKYEPF was synthesized and confirmed by amino acid analysis and protein sequencing (3, 27, 31, 33). The cDNA clone, CD1.8, for human ICAM-1 was obtained from Dr. Timothy A. Springer, Harvard Medical School, Boston, MA.

### Isolation of Neutrophils

Human PMN were isolated from blood of normal volunteers supplied by the American Red Cross, Albany, NY. The blood was sedimented with the same volume of 4% dextran HBSS without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  with 20 mM EDTA for 40 min at room temperature. After sedimentation, the leukocyte-rich plasma layer was diluted in the same volume of HBSS with EDTA. The Ficoll-Paque (10 ml) layered with diluted leukocyte-rich plasma was centrifuged for 30 min at room temperature at 1,350 rpm (400 g). The pellet containing erythrocytes and PMN was resuspended, and after hypotonic lysis of erythrocyte, PMN suspended in 1 mg/ml BSA, 20 mM EDTA, 25 mM Hepes/HBSS were counted.

### Neutrophil Labeling

The isolated PMN were labeled with  $^{51}\text{Cr}$  by incubating with 1  $\mu\text{Ci}$  of  $^{51}\text{Cr}$  per  $10^6$  cells in a 5%  $\text{CO}_2$  incubator at 37°C for 60 min, washed three times with HBSS, and resuspended in DME ( $2 \times 10^7$  cells/ml).

### Endothelial Cell Culture

HUVEC were cultured as described (9). HUVEC at 17–24 passages were seeded onto gelatin-coated 24- or 96-well plates and grown to confluency.

### Adherence Assay

Confluent HUVEC monolayers in 24-well plates were incubated (37°C; 5%  $\text{CO}_2$ ; 98% humidity) with  $\alpha$ -thrombin or TRP-14 for various times in DME without serum. At the end of each incubation period, HUVEC were fixed with 1% paraformaldehyde/PBS at room temperature for 15 min, and then washed three times with DME without serum. The  $^{51}\text{Cr}$ -labeled human PMN ( $2 \times 10^6$  cells/ml DME) were distributed at 1 ml per well over the HUVEC and cocultured for 60 min at 37°C in 5%  $\text{CO}_2$  and 98% humidity. A comparison was made using live HUVEC to determine the effects of fixation on the expression of endothelial adhesivity in response to  $\alpha$ -thrombin.

To determine the inhibitory effects of mAbs on PMN adherence, HUVEC were treated with mAbs (G1, S12, RRI/1, or BB11) 15 min before  $^{51}\text{Cr}$ -labeled PMN administration at a concentration of 10  $\mu\text{g}/\text{ml}$ , or  $^{51}\text{Cr}$ -labeled PMN were treated with mAb IB4 or mAb OKM-1 30 min before PMN-HUVEC cocultivation. HUVEC monolayers were then gently washed three times with DME without serum to remove the nonadherent PMN. Endothelial monolayers were kept overnight in 1 ml of 1 N NaOH at 4°C. The cell lysates were scraped, collected in polypropylene test tubes, and counted for radioactivity in a Tm Analytical Gamma Counter. Phase contrast microscopy confirmed HUVEC integrity and PMN adherence to HUVEC.

### $^{125}\text{I}$ -labeled Antibody Binding Assay

The time course of expression of endothelial adhesion molecules was determined by the specific binding of  $^{125}\text{I}$ -labeled mAbs to HUVEC after exposure to  $\alpha$ -thrombin or TRP-14 for varying durations. The mAbs were iodinated using the chloramine-T method. The specific activity of the labeled mAbs ranged from 3.7 to 6.6  $\mu\text{Ci}/\mu\text{g}$ . HUVEC grown to confluency in 96-well plates were washed with DME without serum and incubated with either  $\alpha$ -thrombin or TRP-14 for 5, 15, 30, 60, 90, or 120 min. As a positive control, HUVEC were incubated with TNF $\alpha$  (1,000 U/ml). At the end of the treatment periods, the cells were fixed with 1% paraformaldehyde/PBS at room temperature for 15 min and washed three times with DME with 10% FBS, and the  $^{125}\text{I}$ -labeled mAb (10  $\mu\text{g}/\text{ml}$ ) was added with DME with 10% FBS (75  $\mu\text{l}$  per well) and allowed to incubate for 60 min at 4°C. The cells were gently washed three times with DME with FBS to remove nonbinding  $^{125}\text{I}$ -labeled mAbs. The cells were kept overnight in 150  $\mu\text{l}$  of 1 N NaOH at 4°C, after which the cell lysate was scraped, collected in test tubes, and counted for radioactivity in a Tm analytical gamma counter. The specific binding of each mAb was determined by adding 30-fold excess unlabeled mAb. The binding data were normalized to cell protein measured in control cells and after either  $\alpha$ -thrombin or TRP-14 challenge.

### Northern Blot Analysis of ICAM-1 Expression

RNA was isolated according to the method described by Chomczynski and Sacchi (7) with minor modifications. After extraction with acid guanidine thiocyanate phenol-chloroform, RNA was collected by precipitation with isopropanol. The RNA pellet was dissolved in TES buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5% SDS) and extracted once with PCI (phenol-chloroform-isoamyl alcohol, 25:24:1) and once with chloroform/isoamylalcohol (24:1). The aqueous phase was collected by centrifugation at 10,000 rpm at 4°C, and then RNA was precipitated with 2 vol of ethanol in the presence of 0.2 M Na acetate. After standing overnight at  $-20^\circ\text{C}$ , RNA was collected by centrifugation and the RNA pellet was washed with 80% ethanol, dried, and dissolved in a small volume of diethyl pyrocarbamate-treated sterile distilled water.

RNA was quantified by spectrophotometry, and equal amounts of RNA were subjected to electrophoresis on a denaturing agarose-formaldehyde gel and transferred to nitrocellulose using the standard protocol. Human ICAM-1 (0.96 kb Sall to pstI fragment) and glyceraldehyde-3-phosphate dehydrogenase (1.1-kb pstI fragment) were labeled with [ $\alpha$ - $^{32}\text{P}$ ]dCTP using the random primer kit (obtained from 5 Prime to 3 Prime, Inc., Boulder, CO). Hybridization was carried out in the presence of 10% dextran and 40% formamide for 16 h at 42°C. The Northern blots were washed at 42°C twice with  $2\times$  SSC (0.15 M sodium chloride/0.015 M sodium citrate) containing 0.1% SDS at room temperature, and twice with  $0.1\times$  SSC containing

0.1% SDS at 42°C. The washed blots were exposed to Kodak X-OMAT film for development of autoradiogram. The blots were hybridized to ICAM-1, stripped, and rehybridized with glyceraldehyde-3-phosphate dehydrogenase, a constitutively expressed gene.

### Data Analysis

Statistical analysis was performed using the Student's *t* test and  $P < 0.05$  was considered to be significant. The results are shown as mean  $\pm$  SEM.

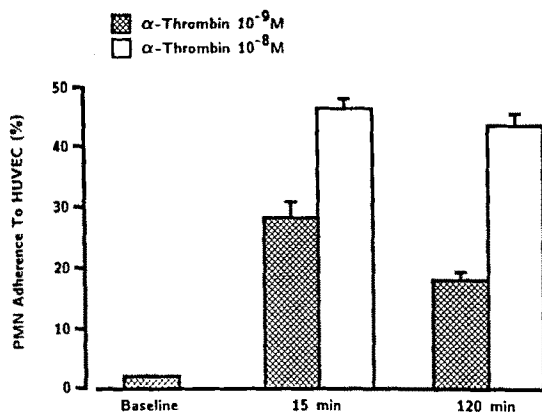
## Results

### Thrombin Induces Rapid Endothelial Adhesiveness

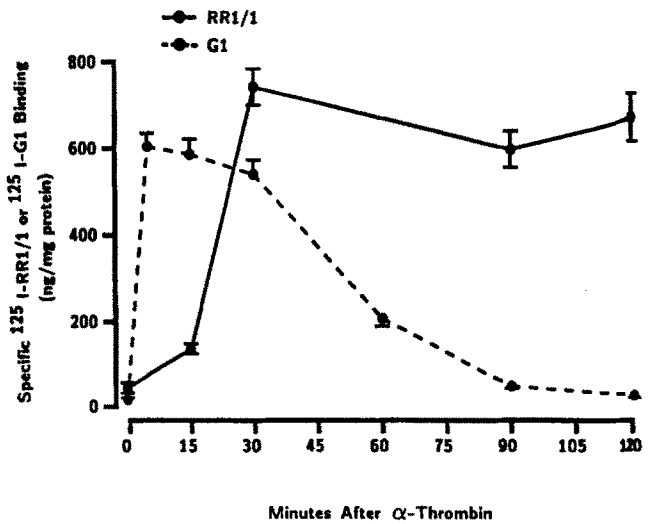
Fig. 1 shows the expression of endothelial adhesiveness at 15 and 120 min after thrombin challenge. Studies were made using thrombin-challenged HUVEC fixed with 1% paraformaldehyde (see Materials and Methods). Endothelial adhesivity increased in a concentration-dependent manner within 15 min after thrombin challenge, and the hyperadhesive response was sustained up to 120 min. The increase in endothelial adhesivity after  $\alpha$ -thrombin ( $10^{-8}$  M) exposure was also evident in unfixed HUVEC; i.e.,  $\alpha$ -thrombin exposure followed by wash (three times) of HUVEC with DME increased PMN adhesion from a basal value of  $5.2 \pm 1.1\%$  to  $45.1 \pm 1.2\%$  at 15 min after  $\alpha$ -thrombin, and to  $52.2 \pm 3.5\%$  at 120 min after  $\alpha$ -thrombin.

### Differential Expression of P-Selectin and ICAM-1

Expression of P-selectin assessed by the specific binding of  $^{125}$ I-labeled mAb G1 to HUVEC peaked within 5 min after thrombin challenge ( $P < 0.05$ ), remained at this value at 15 min, and decreased to near basal level by  $\sim 90$  min (Fig. 2). In contrast, expression of ICAM-1 (assessed by the specific binding of  $^{125}$ I-labeled anti-ICAM-mAb RR1/1) increased at 30 min after thrombin challenge ( $P < 0.05$ ) and remained elevated at approximately maximum value up to the 120-min study period (Fig. 2). The challenge of paraformaldehyde-fixed HUVEC with  $\alpha$ -thrombin did not induce ICAM-1 expression (Table I); ICAM-1 was only expressed in HUVEC



**Figure 1.** Concentration-dependent effects of  $\alpha$ -thrombin on PMN adhesion. The effects of two concentrations of  $\alpha$ -thrombin are shown. HUVEC were treated with  $\alpha$ -thrombin ( $10^{-9}$  or  $10^{-8}$  M) for 15 or 120 min, fixed with 1% paraformaldehyde/PBS, and washed, after which  $^{51}$ Cr-labeled PMN ( $2 \times 10^6$  cells/ml) were layered onto endothelial cells for 1 h and adhesion was determined as described in Materials and Methods. Values are mean  $\pm$  SEM;  $n = 8$  for each condition.



**Figure 2.** Time course of increase in specific binding of  $^{125}$ I-RR1/1 (anti-ICAM-1 mAb) and  $^{125}$ I-G1 (anti-P-selectin mAb) to HUVEC after  $\alpha$ -thrombin ( $10^{-8}$  M) exposure for 5, 15, 30, 60, 90, or 120 min. After each treatment period, HUVEC were fixed and washed, and  $^{125}$ I-labeled monoclonal antibodies were added for 1 h, after which specific binding was determined by adding 30-fold excess unlabeled RR1/1 and G1. Binding data for  $^{125}$ I-RR1/1 and  $^{125}$ I-G1 were normalized to cell protein measured in control cells and after thrombin challenge (see Materials and Methods). Values are shown as mean  $\pm$  SEM;  $n = 6$  for each condition.

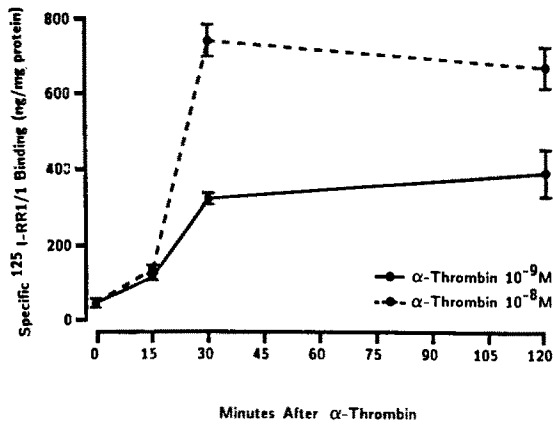
challenged with  $\alpha$ -thrombin, which were then fixed. The binding of control isotype matched IgG,  $^{125}$ I-labeled mAb W40, did not increase after thrombin challenge (i.e., control-specific  $^{125}$ I binding was  $30.6 \pm 1.9$  ng/mg cell protein and values at 15, 30, and 120 min after thrombin were  $24.2 \pm 1.1$ ,  $23.0 \pm 3.1$ , and  $22.5 \pm 1.5$ , respectively).

The expression of ICAM-1 was concentration dependent;  $10^{-8}$  M thrombin resulted in a greater expression than  $10^{-9}$  M thrombin (Fig. 3). Since  $\sim 30$  min was required for the induction of ICAM-1 expression, we examined whether a short thrombin exposure period was sufficient to induce the response. HUVEC were treated with  $10^{-8}$  M thrombin for 1, 5, 10, 30, or 120 min, washed three times, and incubated with DME for 119, 115, 110, 90, or 0 min, respectively, after which they were fixed (as in Materials and Methods), and specific binding of  $^{125}$ I-labeled RR1/1 was determined (Fig. 4). A period of thrombin exposure as short as 1 min resulted in ICAM-1 expression (Fig. 4), indicating that thrombin rap-

**Table I.** Specific Binding of  $^{125}$ I-labeled Anti-ICAM-1 mAb RR1/1 to Paraformaldehyde-fixed HUVEC, Which Were Then Challenged with  $\alpha$ -Thrombin ( $10^{-8}$  M)

Basal	Specific $^{125}$ I-RR1/1 binding to HUVEC (ng/mg total cell protein)			
	30 min		120 min	
	Control	$\alpha$ -Thrombin	Control	$\alpha$ -Thrombin
47.5 $\pm 1.3$	57.3 $\pm 2.6$	63.1 $\pm 1.7$	58.0 $\pm 2.3$	46.1 $\pm 2.1$

Values are shown as mean  $\pm$  1 SEM ( $n = 6$  for each condition). Values are not significantly different from each other. Note: In contrast, the specific binding of  $^{125}$ I-RR1/1 to 120 min  $\alpha$ -thrombin-challenged HUVEC that were then fixed with paraformaldehyde was  $244.1 \pm 14.2$  ng/mg total cell protein.



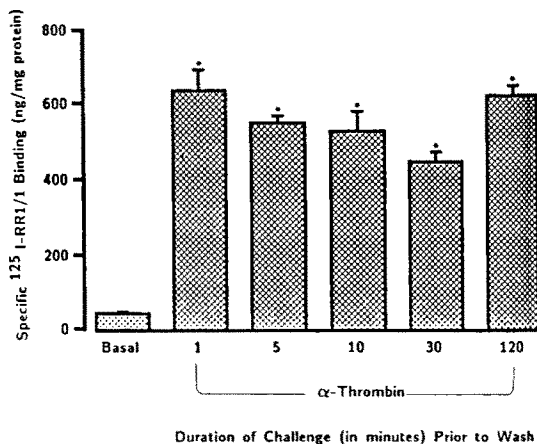
**Figure 3.** Concentration-dependent effects of  $\alpha$ -thrombin on specific binding of  $^{125}\text{I}$ -labeled RR1/1 to HUVEC. HUVEC were treated with  $\alpha$ -thrombin ( $10^{-9}$  and  $10^{-8}$  M) for 15, 30, or 120 min before fixation, after which  $^{125}\text{I}$ -RR1/1 was added for 1 h and specific binding was determined (see Materials and Methods). Values are shown as mean  $\pm$  SEM;  $n = 6$  for each condition.

idly activated the cellular signals responsible for ICAM-1 activation.

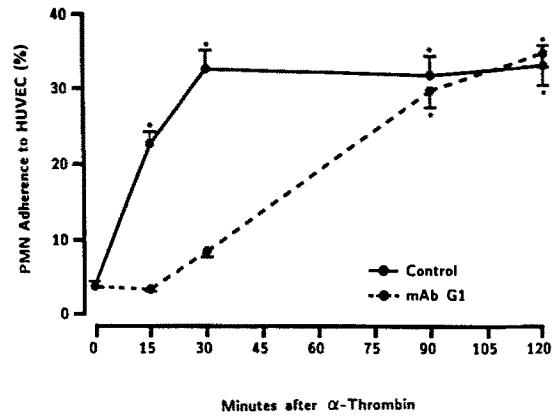
#### Initial PMN Adhesion to Thrombin-activated Endothelial Cells Is P-Selectin Dependent and Prolonged Adhesion Is ICAM-1 Dependent

Fig. 5 indicates that PMN adhesion occurring within the first 30 min of thrombin exposure was P-selectin dependent, since the response was inhibited by treating thrombin-challenged endothelial cells with mAb G1. A nonneutralizing anti-GMP-140 mAb S12 had no effect on this response (Table II).

The sustained PMN adhesion response was inhibited by the anti-ICAM-1 mAb RR1/1 (Fig. 6), indicating the involvement of ICAM-1 in this phase of the response. The magnitude and time course of inhibition with mAb RR1/1 were



**Figure 4.** Effect of varying periods of  $\alpha$ -thrombin exposure on ICAM-1 expression. HUVEC were treated for periods with  $10^{-8}$  M  $\alpha$ -thrombin (i.e., 1, 5, 10, 30, or 120 min), and cells were washed three times and incubated with DME for 119, 115, 110, 90, or 0 min, respectively, after which they were fixed and specific binding of  $^{125}\text{I}$ -RR1/1 was determined (see Materials and Methods). Values are shown as mean  $\pm$  SEM;  $n = 6$  for each condition. \*Different from basal binding ( $P < 0.05$ ).



**Figure 5.** Effect of mAb G1 (anti-P-selectin) of thrombin-induced PMN adhesion to HUVEC. HUVEC were treated with mAb G1 ( $10 \mu\text{g/ml}$ ) 15 min before  $^{51}\text{Cr}$ -labeled PMN administration (see Materials and Methods). PMN adhesion occurring within the first 30 min of thrombin challenge was P-selectin dependent since the responses at 15 and 30 min were inhibited by treatment of thrombin-challenged HUVEC with G1. Values are shown as mean  $\pm$  SEM;  $n = 8$  for each condition. \*Different from basal (0 time) PMN adhesion value ( $P < 0.05$ ).

similar to that observed with the mAb IB4 directed against the CD18  $\beta_2$  integrin on PMN (Fig. 6), supporting the concept that ICAM-1-dependent PMN adhesion is mediated by binding of ICAM-1 on HUVEC to the CD18 integrin on PMN (12, 30). In contrast, control mAb OKM-1 against an irrelevant epitope on CD1b had no inhibitory effect on PMN adhesion to thrombin-challenged HUVEC (Table III). The late-phase response was not affected by the anti-ELAM-1 mAb BB11 (Fig. 7).

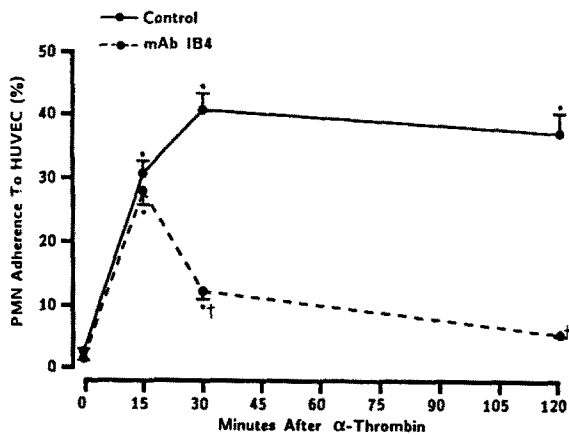
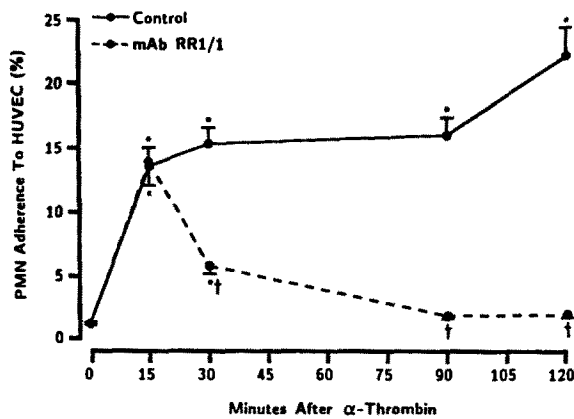
We examined whether the delayed increase in ICAM-1 activity as a result of short-term thrombin incubation (shown in Fig. 4) could mediate ICAM-1-dependent adhesion to endothelial cells. In this study, HUVEC were treated with  $10^{-8}$  M thrombin for 15 min followed by a wash with DME and allowed to incubate in the DME for an additional 165 min, after which the cells were fixed (as described in Materials and Methods) and then treated with either mAb RR1/1 or mAb G1. MAb RR1/1 prevented the PMN adhesion, whereas mAb G1 was ineffective (Fig. 8), indicating that a short-term thrombin exposure (which induced ICAM-1 expression [Fig. 4]) mediated PMN adhesion by an ICAM-1-dependent mechanism.

**Table II.** Effect of Anti-P-Selectin mAb G1 on Thrombin-induced PMN Adhesion

Basal	Percent PMN adhesion after $\alpha$ -thrombin (at 15 min)		
	Control	mAb G1	mAb S12
3.9	36.8*	4.4	38.7*
$\pm 0.7$	$\pm 3.6$	$\pm 0.4$	$\pm 2.8$

HUVEC were challenged with  $10^{-8}$  M  $\alpha$ -thrombin for 15 min before paraformaldehyde fixation. HUVEC were then treated with mAb G1 ( $10 \mu\text{g/ml}$ ) or nonneutralizing control mAb S12 ( $10 \mu\text{g/ml}$ ) directed against P-selectin. Values are shown as mean  $\pm$  1 SEM ( $n = 8$  for each condition).

\* Different from basal and mAb G1 groups ( $P < 0.05$ ).



**Figure 6.** Effect of mAb RR1/1 (anti-ICAM-1) and mAb IB4 (anti-CD18) on thrombin-induced PMN adhesion to HUVEC. (Top) HUVEC were treated with mAb RR1/1 (10  $\mu\text{g/ml}$ ) 15 min before adding  $^{51}\text{Cr}$ -labeled PMN (see Materials and Methods) and (bottom) PMN were treated with mAb IB4 (10  $\mu\text{g/ml}$ ) 30 min before PMN-HUVEC cocubation. The data indicate that the late-phase PMN adhesion was both ICAM-1 and CD18 dependent. Values are shown as mean  $\pm$  SEM;  $n = 8$  for each condition. \*Different from basal (0 time) ( $P < 0.05$ ). †Different from control group values at corresponding times ( $P < 0.05$ ).

### TRP-14 Induces P-Selectin-dependent PMN Adhesion

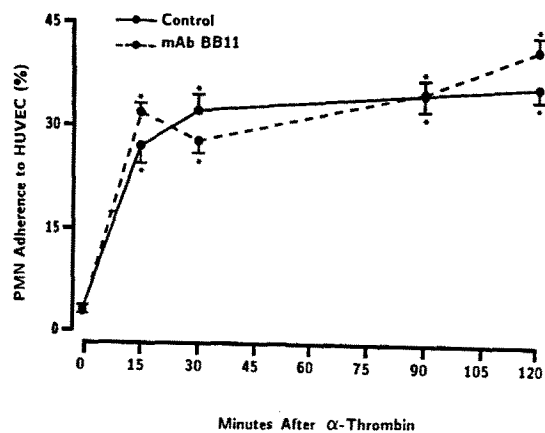
The synthetic peptide (SFLLRNPNDKYEPF), TRP-14, the  $\text{NH}_2$ -terminal portion of the thrombin receptor activated by  $\alpha$ -thrombin (16, 35), caused the rapid expression of P-selectin similar to that observed with  $\alpha$ -thrombin (Fig. 9). A 120-min period of treatment of HUVEC with TRP-14

**Table III.** Effect of Anti-CD18 mAb IB4 on Thrombin-induced PMN Adhesion

Basal	Percent PMN adherence after $\alpha$ -thrombin (at 120 min)		
	Control	mAb IB4	mAb OKM-1
2.1 $\pm 0.1$	30.0* $\pm 1.2$	2.6 $\pm 0.04$	29.1* $\pm 2.1^*$

HUVEC were challenged with  $10^{-8}$  M  $\alpha$ -thrombin for 120 min before paraformaldehyde fixation. PMN were then treated with mAb IB4 (10  $\mu\text{g/ml}$ ) or OKM-1 (10  $\mu\text{g/ml}$ ) (a control mAb directed against an irrelevant epitope on CD11b).

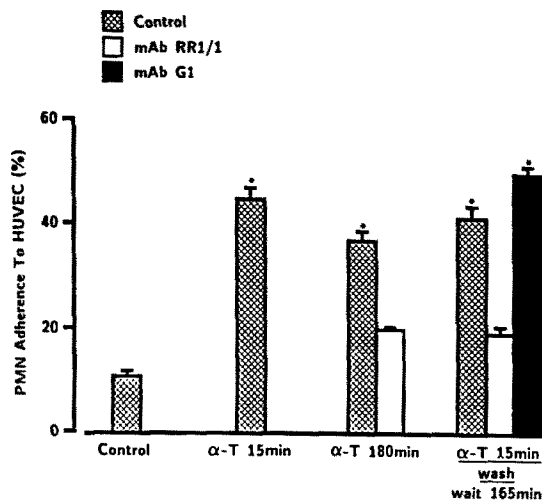
Values are shown as mean  $\pm$  1 SEM ( $n = 8$  for each condition). \* Different from basal and mAb IB4 groups ( $P < 0.05$ ).



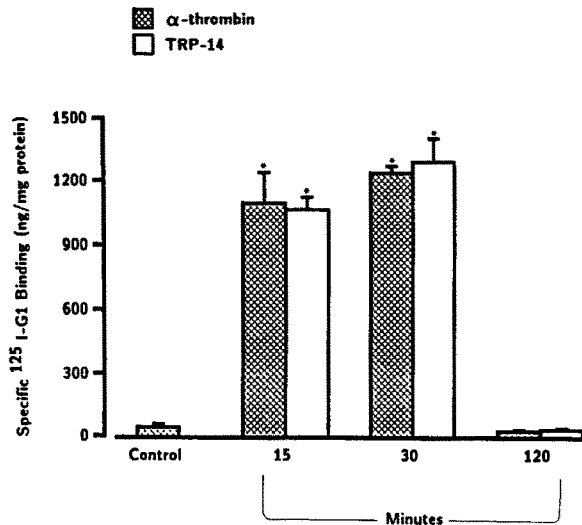
**Figure 7.** Effect of mAb BB11 (anti-ELAM-1) on thrombin-induced PMN adhesion to HUVEC. HUVEC were treated with mAb BB11 (10  $\mu\text{g/ml}$ ) 15 min before addition of PMN (see Materials and Methods); the response was not affected by mAb BB11. Values are shown as mean  $\pm$  SEM;  $n = 8$  for each condition. \*Different from baseline ( $P < 0.05$ ).

or  $\alpha$ -thrombin did not increase the binding of  $^{125}\text{I}$ -labeled mAb G1 (Fig. 9), indicating that the expression of P-selectin mediated by either thrombin or TRP-14 was a transient event.

TRP-14 promoted PMN adhesion within 15 min and the response decreased with time, in contrast to the prolonged  $\alpha$ -thrombin-mediated PMN adhesion response (Table IV). The TRP-14-induced response was absent at 90 min, again in contrast to the thrombin response (Table IV). The TRP-14-mediated PMN response was P-selectin dependent, since it was inhibited by mAb G1 (Fig. 10), whereas adhesion was unaffected by mAb RR1/1 (anti-ICAM-1) or the control mAb S12 (Table V). The rapid time course of the TRP-14-induced



**Figure 8.** Short-term thrombin exposure resulted in ICAM-1-dependent PMN adhesion. HUVEC were treated with  $\alpha$ -thrombin ( $\alpha$ -T;  $10^{-8}$  M) for 15 min, followed by wash with DME, and allowed to incubate in DME for an additional 165 min, after which the cells were fixed and treated with either mAb RR1/1 or mAb G1 (see Materials and Methods). PMN adhesion was prevented by mAb RR1/1 pretreatment of HUVEC. Values are shown as mean  $\pm$  SEM;  $n = 8$  for each condition. \*Different from control ( $P < 0.05$ ).



**Figure 9.** The synthetic peptide (SFLLRNPNDKYEPF) (TRP-14) (i.e., the NH<sub>2</sub>-terminal portion of the thrombin receptor activated by α-thrombin) caused rapid expression of P-selectin similar to that observed with α-thrombin. HUVEC were treated with TRP-14 (10<sup>-4</sup> M) or α-thrombin (10<sup>-8</sup> M) for 15, 30, or 120 min, fixed, and specific binding of <sup>125</sup>I-G1 was quantified (see Materials and Methods). Values are shown as mean ± SEM; n = 6 for each condition. \*Different from control (P < 0.05).

PMN adhesion paralleled P-selectin expression (Figs. 9 and 10). In contrast to the effect of α-thrombin, TRP-14 did not mediate the expression of ICAM-1 (Table VI), indicating that thrombin-induced ICAM-1 activation occurred independently of TRP-14 generation from the proteolytically activated thrombin receptor (PATR).

#### TRP-14 Pretreatment Prevents Thrombin-induced P-Selectin Expression and Delays the Increase in PMN Adhesion

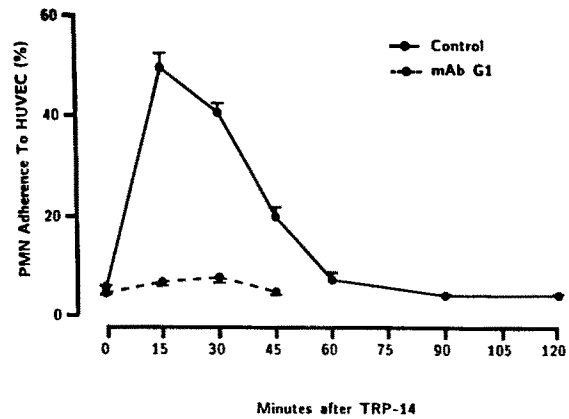
We examined the possibility that TRP-14 acted on the same receptor as α-thrombin to induce the expression of P-selectin. HUVEC were pretreated with either α-thrombin (10<sup>-8</sup> M) or TRP-14 (10<sup>-4</sup> M) for 60 min, after which either TRP-14 or α-thrombin was added to the incubation medium for 30 min. HUVEC were then fixed, and specific binding of <sup>125</sup>I-labeled mAb G1 was determined. The results indicated that pretreatment of the cells with α-thrombin or TRP-14 prevented the TRP-14- or α-thrombin-induced binding of mAb

**Table IV.** Time Course of α-Thrombin- and TRP-14-induced Increases in PMN Adhesion

	Percent PMN Adhesion			
	Basal	15 min	30 min	90 min
α-Thrombin	4.4 ±0.3	45.3 ±2.1	47.8 ±1.1	39.4 ±1.5
TRP-14	6.0 ±1.2	42.3 ±1.7	16.0* ±0.8	6.2* ±0.8

HUVEC were treated with α-thrombin (10<sup>-8</sup> M) or TRP-14 (5 × 10<sup>-5</sup> M) for 15, 30, or 90 min and fixed with paraformaldehyde, and <sup>51</sup>Cr-labeled PMN were added to the monolayer to determine PMN adhesion.

Values are shown as mean ± 1 SEM (n = 8 for each condition). \* Different from α-thrombin values at corresponding times (P < 0.05).



**Figure 10.** The time course of the TRP-14-induced increase in endothelial adhesivity. TRP-14 induced PMN adhesion within 15 min after TRP challenge. HUVEC were treated with TRP-14 (10<sup>-4</sup> M) for 15, 30, 45, 60, 90, or 120 min before fixation (see Materials and Methods). The response was P-selectin dependent since PMN adhesion was inhibited by mAb G1. Values are shown as mean ± SEM; n = 8 for each condition.

(anti-P-selectin) G1 that occurred typically within the 30-min period after TRP-14 or α-thrombin challenge (Fig. 11). Therefore, α-thrombin or TRP-14 pretreatment of HUVEC desensitized endothelial cells to subsequent stimulation with TRP-14 or α-thrombin, indicating that P-selectin upregulation was mediated by action of TRP-14 and α-thrombin on the same receptor site.

Pretreatment of HUVEC with TRP-14 delayed the α-thrombin-induced PMN adhesion; i.e., the adhesion response was not evident within 15 min after α-thrombin challenge, as was the case in control HUVEC that had not been exposed to TRP-14 (Fig. 12). Only the delayed ICAM-1-dependent PMN adhesion response was observed (Fig. 12). This delayed thrombin-induced PMN adhesion response observed after TRP-14 pretreatment was associated with ICAM-1 expression, and the PMN adhesion was inhibited by mAb RRI/1 (Table VII). These findings indicate that TRP-14 pretreatment desensitized the HUVEC to the initial P-selectin-mediated increase in adhesion, but not the subsequent prolonged ICAM-1-mediated PMN adhesion response.

#### Thrombin-induced ICAM-1 Expression Occurs by a Protein Synthesis-independent Mechanism

Treatment of endothelial cells with 6 μg/ml cycloheximide

**Table V.** Effect of Anti-P-Selectin mAb G1 and Anti-ICAM-1 mAb RRI/1 on TRP-14-induced PMN Adhesion

	Percent PMN adhesion after TRP-14 (at 15 min)				
	Basal	Control	mAb G1	mAb RRI/1	mAb S12
	6.1 ±0.2	39.5* ±2.8	7.1 ±0.8	41.2* ±3.3	38.2* ±4.5

HUVEC were treated with 5 × 10<sup>-5</sup> M TRP-14 for 15 min before paraformaldehyde fixation. HUVEC were treated with G1 (10 μg/ml), RRI/1 (10 μg/ml), or S12 (10 μg/ml) (a control nonneutralizing mAb directed against P-selectin).

Values are shown as mean ± 1 SEM (n = 8 for each condition). \* Different from basal and mAb G1 groups (P < 0.05).

**Table VI. Specific Binding of  $^{125}\text{I}$ -labeled Anti-ICAM-1 mAb RR1/1 after TRP-14 ( $5 \times 10^{-5}$  M) Treatment of HUVEC for 120 Min**

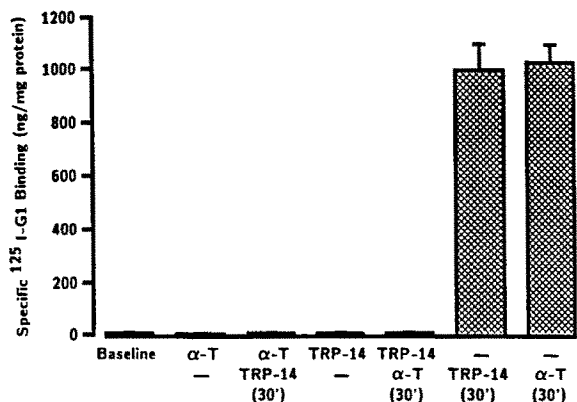
	$^{125}\text{I}$ -RR/1 binding after TRP-14 (at 120 min)	
	Basal	TRP-1
$^{125}\text{I}$ -RR1/1 binding (ng/ml cell protein)	$45.0 \pm 4.2$	$25.4 \pm 0.7$

Values are shown as mean  $\pm$  1 SEM ( $n = 6$  for each group).

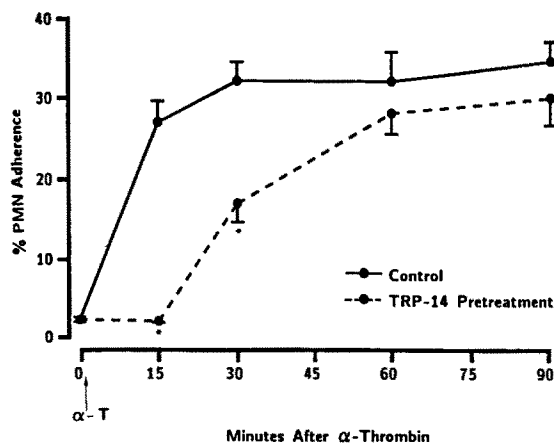
failed to alter the binding of  $^{125}\text{I}$ -labeled mAb RR1/1 induced by  $\alpha$ -thrombin (Fig. 13). Moreover, cycloheximide did not prevent PMN adhesion to the  $\alpha$ -thrombin-challenged endothelial cells (Fig. 13).  $\text{TNF}_\alpha$  treatment of endothelial cells for 120 min induced ICAM-1 expression as well as PMN adhesion, and cycloheximide prevented both ICAM-1 expression and PMN adhesion (Fig. 13). ICAM-1 upregulation induced by  $\alpha$ -thrombin was not the result of increased transcription of the ICAM-1 message since Northern blot analysis did not show an increase in the ICAM-1 mRNA signal up to 180 min after  $\alpha$ -thrombin challenge, whereas in a positive control experiment,  $\text{TNF}_\alpha$  challenge of HUVEC resulted in an increase in ICAM-1 mRNA within 120 min of  $\text{TNF}_\alpha$  exposure (Fig. 14).

## Discussion

Thrombin-induced PMN sequestration in the microvasculature (23, 24) may largely be the result of expression of endothelial adhesivity (4, 12, 34, 38). Studies using  $^{111}\text{In}$ -oxine-labeled PMN indicated that PMN sequestration in the pulmonary microvasculature occurred rapidly after throm-



**Figure 11. Effect of pretreatment with  $\alpha$ -thrombin ( $\alpha$ -T) or TRP-14 on TRP-14- or  $\alpha$ -thrombin-induced specific binding of  $^{125}\text{I}$ -G1 (anti-P-selectin mAb). HUVEC were pretreated with either  $\alpha$ -thrombin ( $10^{-8}$  M) or TRP-14 ( $10^{-4}$  M) for 60 min, after which either TRP-14 or  $\alpha$ -thrombin was added to the incubation medium for 30 min. HUVEC were then fixed and the specific binding of  $^{125}\text{I}$ -G1 was determined. The results also show the effects of  $\alpha$ -thrombin or TRP-14 alone, as well as the effects of TRP-14 and  $\alpha$ -thrombin without the initial treatment with TRP-14 or  $\alpha$ -thrombin. The findings indicate that  $\alpha$ -thrombin or TRP-14 pretreatment prevented the subsequent expression of P-selectin after the second challenge with either TRP-14 or  $\alpha$ -thrombin. Values are shown as mean  $\pm$  SEM;  $n = 4$  for each condition.**



**Figure 12. Effect of TRP-14 pretreatment on  $\alpha$ -thrombin-induced PMN adherence to HUVEC. In the TRP-14 pretreatment group, HUVEC were pretreated with TRP-14 ( $10^{-4}$  M) for 60 min.  $\alpha$ -Thrombin ( $10^{-8}$  M) was then added to the incubation medium for 15, 30, 60, and 90 min. After fixation, PMN adherence was determined (see Materials and Methods). The results indicate that TRP-14 pretreatment significantly reduced the early (i.e., at 15 and 30 min) PMN adherence mediated by  $\alpha$ -thrombin. Values are shown as mean  $\pm$  SEM;  $n = 4$  for each condition. \*Different from control group values at corresponding times ( $P < 0.05$ ).**

bin exposure ( $10^{-8}$  M), and the response persisted for up to 120–180 min (8). The PMN-endothelial interaction may be a critical determinant of vascular endothelial injury since thrombin-induced pulmonary microvascular endothelial injury was prevented after depletion of the circulating PMN count (17, 20). However, the basis of thrombin-induced PMN sequestration in microvessels remains unclear despite the evidence of the involvement of PMN in the pathogenesis of endothelial injury. Previous studies have shown that thrombin promotes PMN adherence to cultured endothelial cells by expression of endothelial adhesiveness (14, 26). This response may involve rapid upregulation of P-selectin (5, 13, 26), which is released from Weibel-Palade bodies in endothelial cells within 15 min after  $\alpha$ -thrombin challenge (14, 15, 26). The translocation of P-selectin to the plasmalemmal membrane may contribute to PMN adhesion to endothelial cells, and thus may be responsible for the rapid intravascular sequestration of PMN (23, 24). However, the transient nature of the P-selectin-dependent response does not explain the persistence of PMN sequestration observed in experimental models of acute lung injury after intravenous  $\alpha$ -thrombin infusion (8).

In this study we examined the relative contributions of P-selectin and another critical adhesion molecule, ICAM-1, in sustaining PMN adhesion to  $\alpha$ -thrombin-activated endothelial cells. The results indicated a rapid expression of endothelial adhesivity occurring within 15 min after  $\alpha$ -thrombin challenge, and this was followed by stabilization of PMN adhesion. The initial response was dependent on the rapid upregulation of P-selectin as evident by increase in the specific binding of  $^{125}\text{I}$ -labeled anti-P-selectin mAb G1 within 15 min after thrombin challenge, and then decrease to basal levels within 60–90 min. The time course of P-selectin expression is consistent with the data of McEver et al. (26) showing that  $\alpha$ -thrombin can rapidly mobilize P-selectin from its endothelial cell stores. The delayed and more sus-



Table VII. Effect of TRP-14 Pretreatment on Thrombin-induced Expression of ICAM-1

	<sup>125</sup> I-RR1/1 binding (ng/mg cell protein)	Percent PMN adherence	Percent adherence + RR1/1
Basal	43.7 ± 5.1	3.4 ± 2.9	-
TRP-14 (5 × 10 <sup>-5</sup> M) Pretreatment for 30 min/α-Thrombin (90 min)	276.1 ± 22.5*	29.8 ± 2.9*	5.5 ± 0.3

HUVEC were pretreated with TRP-14 (5 × 10<sup>-5</sup> M) for 30 min, at which point 10<sup>-8</sup> M α-thrombin was added to the incubation for a 90-min period. The cells were then fixed with paraformaldehyde and binding of <sup>125</sup>I-labeled RR1/1 and ICAM-1 dependence of PMN adhesion response were determined.

Values are shown as mean ± 1 SEM (n = 6 for each condition in binding assay and n = 8 for each condition in adherence assay).

\* Different from basal values (P < 0.05).

tained PMN adhesion after thrombin challenge was ICAM-1 dependent. ICAM-1 expression increased at 30 min after thrombin challenge (i.e., after maximum expression of P-selectin had occurred), and persisted for up to 120 min. This phase of PMN adhesion was ICAM-1 dependent since it was inhibited by the ICAM-1 mAb RR1/1; however, the response was unaffected by the anti-ELAM-1 mAb BB11. These results cannot be explained by the release of secondary medi-

ators from endothelial cells (12) since the cells were fixed with 1% paraformaldehyde after challenge with thrombin. The present results are consistent with the hypothesis that the initial attachment of PMN to α-thrombin-activated endothelial cells is mediated by P-selectin, and the prolonged adhesion response (a characteristic feature of PMN sequestration in the microcirculation [8]) is dependent on ICAM-1 upregulation.

Induction of ICAM-1 by the cytokines (IL-1<sub>α</sub> and TNF<sub>α</sub>) as well as by lipopolysaccharide requires de novo protein synthesis secondary to the transcription of the ICAM-1 mRNA (10). In this study, ICAM-1 activation induced by α-thrombin occurred by a protein synthesis-independent mechanism since neither ICAM-1 expression nor PMN adhesion to thrombin-challenged endothelial cells was affected by cycloheximide pretreatment. Moreover, there was no evidence of increased ICAM-1 transcription; that is, ICAM-1 mRNA signal was not altered within the 2–3-h

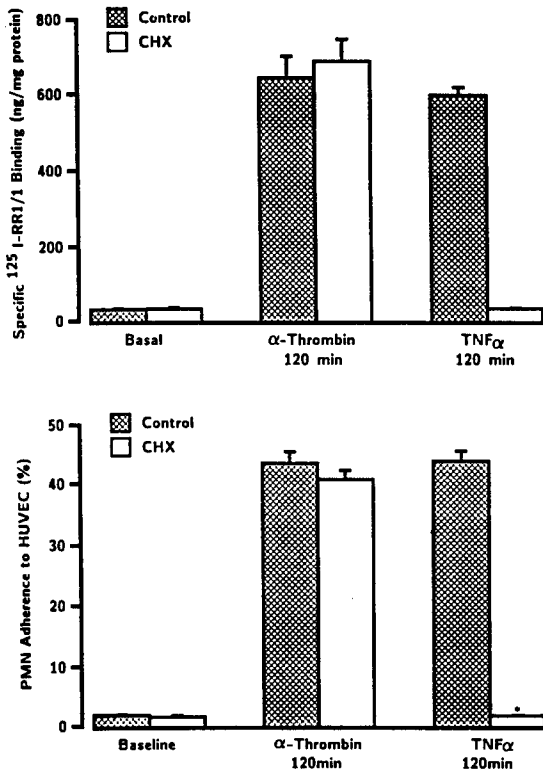


Figure 13. Effect of cycloheximide on thrombin-induced ICAM-1 expression. HUVEC were pretreated with cycloheximide (6 μg/ml) (which we showed to inhibit protein synthesis based on [<sup>3</sup>H]leucine incorporation) 30 min before α-thrombin (10<sup>-8</sup> M) or TNF<sub>α</sub> (1,000 U/ml) challenge. (Top) Treatment of HUVEC with cycloheximide failed to alter the binding of <sup>125</sup>I-RR1/1 induced by α-thrombin challenge. (Bottom) Treatment of HUVEC with cycloheximide also failed to alter the PMN adhesion to HUVEC induced by α-thrombin challenge. In contrast, both specific binding of <sup>125</sup>I-RR1/1 and PMN adherence induced by TNF<sub>α</sub> were significantly reduced by cycloheximide pretreatment. Values are shown as mean ± SEM; n = 6 (binding assay) and n = 8 (adherence assay) for each condition.

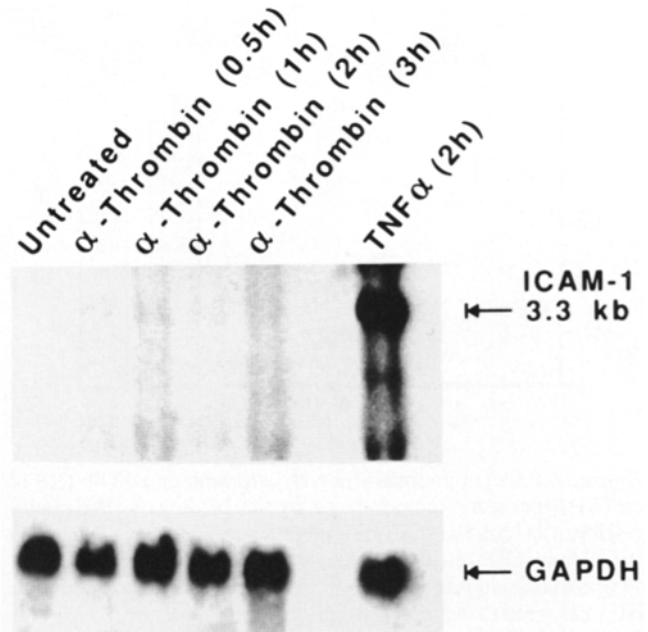
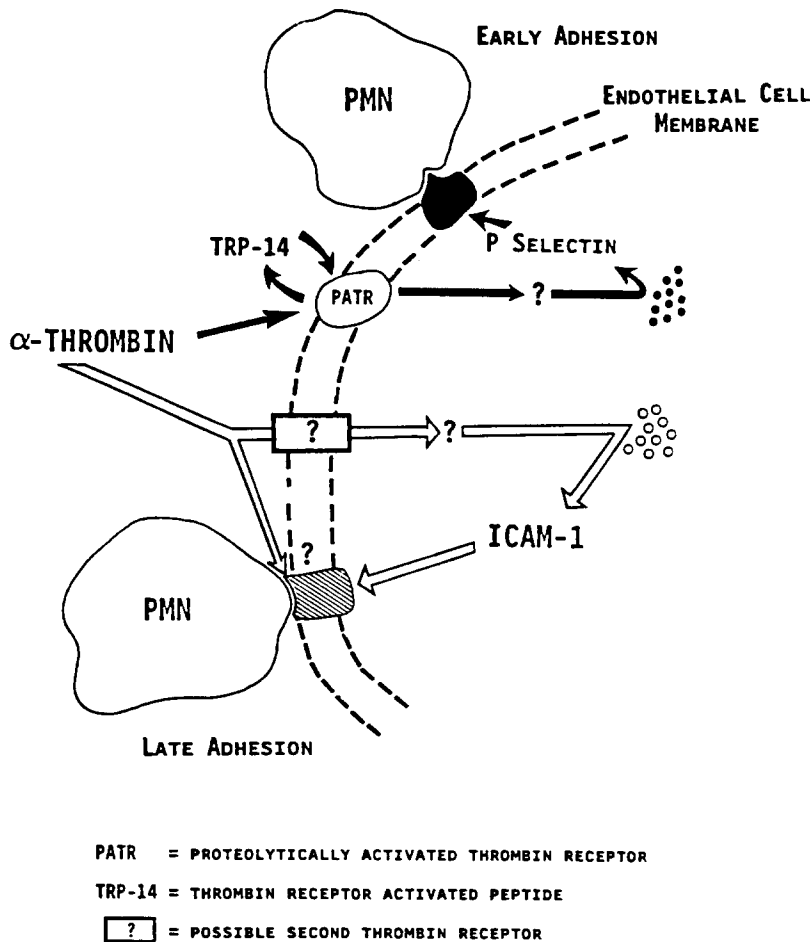


Figure 14. Northern blot analysis of ICAM-1 mRNA signal after α-thrombin challenge up to 180 min. TNF<sub>α</sub> challenge (1,000 U/ml) of HUVEC resulted in an increase of ICAM-1 mRNA within 120 min after TNF<sub>α</sub> exposure, whereas thrombin treatment (10<sup>-8</sup> M) was ineffective. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.





*Figure 15.* The schematic of  $\alpha$ -thrombin-induced endothelial adhesiveness.  $\alpha$ -Thrombin activates the PATR, which has the 14 amino acid new NH<sub>2</sub>-terminal sequence of SFLLRNPNDKYEPF. TRP-14 induces P-selectin expression on the luminal surface of endothelial cells. ICAM-1 expression occurring by a protein synthesis-independent mechanism may be explained by activation of the constitutive cell surface ICAM-1 by thrombin's proteolytic activity or release of cell stores of ICAM-1 by binding of  $\alpha$ -thrombin to another membrane receptor. Another possibility is that  $\alpha$ -thrombin induces a conformational change of PATR, and thereby signals ICAM-1 activation without the involvement of TRP-14. The P-selectin expression is important in early PMN adhesion to endothelial cells, whereas ICAM-1 expression prolongs and stabilizes the PMN adherence response.

period after  $\alpha$ -thrombin challenge. In contrast, TNF $\alpha$  challenge of endothelial cells for 120 min resulted in the typical increase in ICAM-1 mRNA activity as well as ICAM-1 protein expression.

The action of  $\alpha$ -thrombin in mediating ICAM-1 activation may be specific to thrombin since we have shown that another serine protease, plasmin, does not increase endothelial adhesiveness (19). One explanation of thrombin's effect may be a qualitative increase in the activity of the constitutive ICAM-1 on the endothelial cell membrane. A similar phenomenon occurring with CD18 expression on PMN involves clustering of CD18  $\beta_2$  integrin, and is associated with increased amounts of the membrane surface antigen (21, 29). Thrombin-induced ICAM-1 expression does not involve generation of the thrombin receptor activating peptide (SFLLRNPNDKYEPF) (TRP-14) since we showed that the NH<sub>2</sub> terminus peptide generated from the thrombin receptor (35) (which simulates thrombin's effects such as platelet aggregation [16]) failed to promote ICAM-1 expression. This peptide, however, resulted in rapid and reversible P-selectin expression as well as P-selectin-dependent PMN adhesion, consistent with our previous observations (32), suggesting that the signal for P-selectin activation (in contrast to ICAM-1 activation) requires the PATR.

We observed that pretreatment of HUVEC with either  $\alpha$ -thrombin or TRP-14 prevented P-selectin expression in response to a subsequent challenge with either TRP-14 or  $\alpha$ -thrombin. This evidence of cross-desensitization supports the conclusion that P-selectin upregulation was mediated by

activation of PATR (13, 16, 35). The results are in agreement with reports of desensitization of intracellular calcium mobilization by TRP-14 or  $\alpha$ -thrombin (6).

Although the desensitization of PATR by TRP-14 prevented the rapid initial P-selectin-induced PMN adhesion, it did not modify the prolonged ICAM-1-dependent PMN adhesion, indicating that ICAM-1-dependent PMN adhesion was mediated independently of TRP-14. While the induction of P-selectin only requires the presence of TRP-14, ICAM-1 expression may involve activation of another as yet undefined thrombin receptor or direct  $\alpha$ -thrombin-mediated activation of ICAM-1 secondary to a conformational change induced by proteolysis (Fig. 15). It is also possible that a proteolytically induced conformational change of PATR could signal ICAM-1 expression without the involvement of TRP-14.

The dual regulation of thrombin-induced endothelial adhesiveness by both P-selectin- and ICAM-1-dependent mechanisms may be an important process in the development of vascular injury and tissue inflammation. The prolonged intravascular PMN sequestration seen in experimental models of vascular injury after  $\alpha$ -thrombin challenge (8) may be critically dependent on ICAM-1 expression, and thereby may amplify the injury by stabilizing PMN adhesion to endothelial cells. It remains to be determined whether anti-ICAM-1 antibodies prevent the thrombin-induced neutrophil sequestration in microvessels in situ, and also prevent the vascular injury and inflammation attributed to PMN sequestration (8).

Since anti-ELAM-1 mAb BB11 failed to inhibit adhesion

to thrombin-activated endothelial cells, this study excludes the involvement of ELAM-1 in mediation of thrombin-induced adhesiveness. This is in contrast to the described effects of IL-1 $\alpha$ , TNF $\alpha$ , and lipopolysaccharide in inducing ELAM-1-dependent adhesion to endothelial cells (2). Moreover, the ELAM-1-dependent adhesion required induction of ELAM-1 mRNA and de novo protein synthesis (28), which is in contrast to the protein synthesis-independent effect of  $\alpha$ -thrombin in mediating ICAM-1 expression.

In conclusion, we have shown that  $\alpha$ -thrombin induces the expression of endothelial adhesiveness, leading to prolonged PMN adhesion to endothelial cells. The initial PMN adhesion to  $\alpha$ -thrombin-activated endothelial cells is mediated by P-selectin, and the adhesion response is prolonged by ICAM-1 activation. P-selectin expression is rapid and is terminated within 60–90 min, which is the time when ICAM-1 activity peaks. P-selectin expression is the result of activation of PATR and the generation of TRP-14, and is independent of ICAM-1 activation (Fig. 15). ICAM-1 expression does not involve induction of ICAM-1 mRNA, and is probably a post-translational event since ICAM-1 activity and PMN adhesion are unaffected by cycloheximide pretreatment. The increase in ICAM-1 activity may involve proteolytic modification of the surface-bound ICAM-1 or release of preformed ICAM-1 by activation of another thrombin receptor (Fig. 15). Another possibility is that proteolytically induced conformational change of PATR signals ICAM-1 expression. Expression of endothelial ICAM-1 may serve to stabilize PMN adhesion to thrombin-activated endothelial cells, and thus may prolong intravascular PMN sequestration.

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