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Expression of Monocyte Chemotactic Protein-1 by Monocytes and Endothelial Cells Exposed to Thrombin

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Thrombin, in addition to being a key enzyme in hemostasis, affects a series of endothelial and leukocyte functions and thus may be involved in the regulation of inflammatory reactions. Because leukocyte recruitment and activation are important events in inflammatory and thrombotic processes, in this study we have examined the possibility that thrombin induces the production of a cytokine chemotactic for mononuclear phagocytes. Human peripheral blood mononuclear cells (PBMC) exposed* in vitro *to thrombin expressed transcripts of monocyte chemotactic protein-1 (MCP-1; alternative acronyms: JE, monocyte chemotactic and activating factor, tumor-derived chemotactic factor). Thrombin was two- to threefold less effective than endotoxin in inducing MCP-1 transcripts in PBMC. Among circulating mononuclear cells, monocytes were identified as the cells expressing MCP-1 in response to thrombin. Monocytes expressed thrombin receptor transcripts. Boiling, hirudin, antithrombin III, and mutation of the catalytic site (serine 205 into alanine) blocked the capacity of thrombin to induce MCP-1 expression. The thrombin receptor-activating peptide mimicked the effect of thrombin in inducing MCP-1 expression. Induction of MCP-1 transcript by thrombin was not reduced by blocking interleukin-1 and tumor necrosis factor, suggesting that these mediators are not involved in thrombin-induced expression of MCP-1. In addition to monocytes, endothelial cells (EC) also expressed MCP-1 in response to thrombin, although at lower levels compared with monocytes. Actinomycin D experiments indicated that induction of MCP-1 by thrombin in PBMC and EC was gene transcription dependent. The inhibition of protein synthesis blocked thrombin-induced MCP-1 expression in PBMC, whereas it superinduced both constitutive and thrombin-inducible expression of MCP-1 in EC, indicating different mechanisms of regulation of this gene in mononuclear phagocytes* versus *endothelial cells. Thrombin stimulated mononuclear cells and EC to release chemotactic activity for monocytes that could be inhibited by absorption with anti-MCP-1 antibodies. Induction of a chemotactic cytokine for monocytes by thrombin points to the importance of this enzyme in regulating inflammatory processes and further indicates that hemostasis, inflammation, and immunity are strictly interconnected processes. (Am J Pathol 1994, 144:975–985)

Endothelial cells (EC) provide a nonthrombogenic surface designed to maintain blood fluidity. On vascular injury, the contact of blood with thrombogenic subendothelial structures stimulates the rapid formation of a hemostatic plug consisting of platelets and fibrin. Thrombin is the final product of the activation of blood coagulation pathways. In addition to playing a central role in hemostasis by converting fibrinogen to fibrin monomers, thrombin has been shown to be involved in the regulation of inflammatory responses. Indeed, thrombin induces in EC a series of functional responses,[1–14] including release of PGI_2[12,13] and

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interleukin-1 (IL-1).[6] In addition, because thrombin is chemotactic for monocytes[15,16] and induces the degradation of various constituents of basement membrane,[17] it may also be involved in the regulation of leukocyte recruitment at inflammatory sites.

Extravasation of leukocytes from blood to tissues during inflammation represents a crucial event. Recently, a novel superfamily of inflammatory mediators called chemokines has been identified[18] and some of these are chemotactic for different leukocyte populations. Monocyte chemotactic protein-1 (MCP-1; alternative designations: JE, monocyte chemotactic and activating factor, tumor-derived chemotactic factor),[18,19] initially identified as a growth factor inducible gene in fibroblasts, has been shown to promote monocyte chemotaxis and functional activation. Various cell types can produce MCP-1, including fibroblasts,[20,21] tumor cells,[22,23] and EC and smooth muscle cells.[21,24–26] We have also demonstrated that monocytes themselves can produce MCP-1,[27] thus suggesting that these cells can autonomously regulate their content in tissues. Stimuli effective in inducing MCP-1 production include proinflammatory cytokines (IL-1 and tumor necrosis factor; TNF) and endotoxin.

Because thrombin appears to participate in the regulation of various aspects of inflammation and induces important functional modifications in cells involved in inflammatory reactions,[1–17] we wondered whether MCP-1 can be induced by thrombin. In this paper we report that human mononuclear phagocytes and EC exposed to thrombin express MCP-1 at the mRNA and protein levels. Inhibition of protein synthesis blocked MCP-1 induction by thrombin in monocytes, whereas it superinduced both constitutive and thrombin-inducible expression in EC. Moreover, we demonstrated that monocytes expressed transcripts for the thrombin receptor and that the 14 residue peptide thrombin receptor-activating peptide (TRAP), which directly activates the thrombin receptor, induced MCP-1 transcript in peripheral blood mononuclear cells (PBMC). The induction of MCP-1 by thrombin required the active catalytic site of thrombin because the mutated thrombin SA205, which has no enzymatic activity, failed to induce MCP-1 transcript. Thus, thrombin can induce the production of a monocyte chemotactic factor by EC, strategically located at the interface between blood and tissues, and by monocytes. These findings point to the importance of thrombin in regulating inflammatory responses and further support the view that hemostasis, inflammation, and immunity closely interact at various levels.

Materials and Methods

Cell Culture Reagents

The following reagents were used for culture and separation of cells: pyrogen-free saline and distilled water (Bieffe, Bergamo, Italy) for clinical use; RPMI 1640 medium (1× concentrated; GIBCO, Glasgow, Scotland, UK); L-glutamine (GIBCO); gentamicin (GIBCO); aseptically collected fetal bovine serum (FBS; lot 1111960, HyClone Laboratories, Logan, UT). The routinely used cell culture medium was RPMI 1640 with 2 mM L-glutamine, 50 mg/ml gentamicin, 10% FBS, hereafter referred to as complete medium. All reagents contained less than 0.125 EU/ml of endotoxin as checked by the Limulus amebocyte lysate assay (Microbiological Associates, Walkersville, MD). Serum was tested after 1/3 dilution and heating at 100 C.

PBMC

Buffy coats from blood donations (courtesy of Centro Trasfusionale, Ospedale Sacco, Milan, Italy) were used in most experiments as a source of PBMC. Alternatively, we obtained heparinized venous blood from healthy laboratory donors. Blood was diluted 1/5 with saline and 40 ml was then placed on 10 ml Ficoll (Seromed, Berlin, Germany) in 50-ml conical tubes (Falcon Becton Dickinson Labware, Lincoln Park, NJ) for centrifugation at 400 × *g* for 20 minutes at room temperature. PBMCs were collected at the interface, then washed with saline and suspended in complete medium at 1–2 × 10^6 cells/ml in 50-ml conical tubes. Stimuli (see below) were then immediately added and cells incubated for various periods of time at 37 C in 5% CO_2 in air. Control cells were incubated for the same period of time without stimuli. After the appropriate treatment, cells were centrifuged at 440 × *g* for 10 minutes at room temperature and subjected to the RNA extraction procedure as detailed below. Each experiment was performed on PBMC from a single individual.

To obtain mononuclear cells depleted of monocytes, PBMC after Ficoll centrifugation were resuspended at 2 × 10^6 cells/ml in complete medium and allowed to adhere in plastic Petri dishes (Falcon) at 37 C for 1 hour; nonadherent cells, usually containing less than 5% nonspecific esterase (NSE)-positive cells, were then recovered, washed in saline, and incubated with stimuli as above. Adherent cells were used as monocyte-enriched populations (≥95% NSE positive).

Cells

EC (2nd to 8th passage) from human umbilical veins was cultured in medium 199 (GIBCO) supplemented with 20% FBS and used in the study as described in previous reports.[24] A human sarcoma cell line (8387) was cultured in minimal essential medium (GIBCO) supplemented with 10% FBS.[22] The lymphoblastoid B cell line Raji was cultured in RPMI 1640 supplemented with 10% FBS.

Reagents

Purified human α-thrombin (specific activity 1665 U/mg) was a kind gift of Dr. J. Fenton II (Albany, NY). Alternatively, thrombin was purified from plasma by barium citrate adsorption and affinity chromatography using antiprothrombin antibodies immobilized on Sepharose 4B (Pharmacia, Uppsala, Sweden). Wild-type recombinant prothrombin was obtained essentially as previously described.[28] Serine 205 (thrombin B chain numbering), one of the residue forming the catalytic triade of the thrombin active site, was modified into alanine by site-directed mutagenesis on the prothrombin cDNA. The modified prothrombin (SA 205) was cloned in the expression vector pMT2. DNA transfection, cell culture, and cell line selection were performed as described.[29] The dihydrofolate reductase-deficient chinese hamster ovary cell line CHO DUCKX-B11[30] was transfected with the expression plasmid by electroporation. Cells were grown in media containing 5 μg/ml vitamin K1. The recombinant prothrombin species were purified by immunoaffinity chromatografy using anti-human prothrombin antibodies immobilized on Sepharose. Human α-thrombin, recombinant wild-type thrombin, and active site-mutated SA205 thrombin were obtained after prothrombin activation with Echis Carinatus venom (Sigma, St. Louis, MO). The procoagulant fraction of the Echis Carinatus venom was purified by ion exchange chromatografy on DEAE Sephadex phast flow (Pharmacia). The purified enzyme was used at a ratio of 1/100 (weight/weight) to activate the prothrombin species for 2 hours at 37 C. Complete activation was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Thrombin was separated from the other activation products by cation exchange chromatography on a mono S column (Pharmacia) developed with a 0.05 to 0.5 M NaCl gradient in 0.05 M MES (4-morpholinethanesulfate, Boerhinger Mannheim, Mannheim, Germany). The thrombin obtained had a specific fibrinogen clotting activity of 2500 NIH U/mg for human thrombin and 2800 NIH U/mg for recombinant thrombin. Human α-thrombin and recombinant wild-type thrombin had 100% enzymatic activity, as measured by the hydrolysis of the chromogenic synthetic substrate Chromozyme TH. Thrombin SA205 had no activity.

Hirudin (specific activity 6.3 U/ng) was from Calbiochem, La Jolla, CA. Antithrombin III was from Behring, Marburg, Germany and was added with 0.3 U/ml heparin as a catalyst. Recombinant human IL-1β (specific activity 10^6 U/mg) was obtained from Dompè, L'Aquila, Italy. Lipopolysaccharide (LPS) (*Escherichia coli* 055: B5) was purchased from Difco Laboratories, Detroit, MA. A monoclonal antibody (mAb) anti-TNF was a kind gift of G. Trinchieri (Wistar Institute, Philadelphia, PA); this antibody, diluted 1:2000, neutralizes 10,000 U of TNF. Recombinant MCP-1 was obtained from Peprotech Inc. (Rocky Hill, NJ); IL-1Ra was from Cetus Corporation (Emeryville, CA). A rabbit antiserum against MCP-1/JE was a kind gift from Dr. B. Rollins (Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA).

JE was identified as a gene expressed in stimulated mouse fibroblasts; human JE was recently shown to be identical to MCP-1.[31] The antibody was used as recently described for vascular cells.[24,25] A Sepharose protein A immobilized anti-MCP-1/JE microcolumn was prepared as described by Leonard and Skeel.[32] The monocyte supernatant was passed through the column and then assayed for the chemotactic activity for monocytes. Normal rabbit serum was used for the preparation of a control column. Recombinant C5a was a generous gift of H. S. Showell, Pfizer Central Research, Groton, CT. FMLP was from Sigma. Actinomycin D (ActD), cycloheximide (CH), emetine, and puromycin used in this study were purchased from Sigma. A 14 residue peptide (SFLLRNPNDKYEPF) (TRAP), corresponding to amino acids 42 to 55 of the human receptor,[33] was used as thrombin agonist because it activates the thrombin receptor.[33] A 13 residue peptide (PESKATNATLDPR), corresponding to amino acids 29 to 41 of the human thrombin receptor, was used as an inactive peptide.

Chemotaxis Assay

PBMC or EC were incubated with thrombin for 4 hours. Then the stimulus was removed and cells added with fresh medium. Supernatants were then harvested after 18 hours of incubation at 37 C in 5% CO_2 in air. Leukocyte chemotaxis was assessed by a microchamber technique.[34] The 25 μl of supernatants diluted in phosphate-buffered saline were seeded in the lower compartment. The two compartments were

separated by a 5-μ pore size polycarbonate filter (Neuroprobe, Cabin John, MD). Chambers were incubated at 37 C for 90 minutes; at the end of incubation filters were removed then fixed and stained with Diff-Quick (Harleco, Gibbstown, NJ), and five oil immersion fields were counted after coding of samples. The statistical significance of migration toward stimulus *versus* medium control was assessed by Dunnet's test.

Northern Blot Analysis

Northern blot analysis was conducted according to standard procedures. Total RNA was isolated by the guanidine isothiocyanate method.[35] The 15 μg of total RNA was analyzed by electrophoresis through 1% agarose formaldehyde gels in the presence of ethidium bromide (Sigma), followed by transfer to Gene Screen Plus membranes (New England Nuclear, Boston, MA). The plasmid containing the human MCP-1 cDNA (0.672 kb *PstI-PstI* fragment)[36] was nick translated with α-^{32}[P]dCTP (5000 Ci/mmol; Amersham, Buckinghamshire, UK). Membranes were pretreated and hybridized in 50% formamide (Merck) with 10% dextran sulfate (Sigma) and washed twice with 2× standard saline citrate (SSC) (1× SSC: 0.15 M sodium chloride, 0.015 sodium citrate) then twice with 2× SSC plus 1% SDS (Merck) at 60 C for 30 minutes and finally twice with 0.1× SSC at room temperature for 30 minutes. The membranes were exposed for 12 to 24 hours at −80 C with intensifying screens. RNA loading and transfer to membranes were checked by examination under ultraviolet light of filters.

Nuclear Runoff Analysis

PBMC and EC were incubated with or without thrombin for 3 and 1.5 hours, respectively. Cells were then washed twice with ice-cold Hanks' balanced salt solution (GIBCO) with Ca^{2+} and Mg^{2+}. To isolate nuclei, cells were resuspended in 0.5 ml of lysis buffer (10 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 10 mM NaCl, 0.5% Nonidet P-40) and incubated for 5 minutes in wet ice. Cells were then centrifuged at 400 × *g* at 4 C and then resuspended in 250 μl of ice-cold freezing buffer (50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, pH 8). Then, 60 μl of 5× runoff buffer (25 mM Tris-HCl, pH 8, 12.5 mM $MgCl_2$, 750 mM KCl, 1.25 mM each of dGTP, dCTP, and dATP) and 100 μCi of α-[^{32}P]UTP (3000 Ci/mmol; Amersham) were added to 230 μl of nuclei suspension and incubated at 30 C for 30 minutes. Elongated transcripts were then isolated using the guanidine-cesium procedure[32] with 50 μg of yeast tRNA as carrier. The RNA pellet was resuspended in 180 μl of ice-cold TNE (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM EDTA) and denaturated by adding 20 μl of 2 N NaOH on ice for 10 minutes. The solution was neutralized by the addition of HEPES, pH 7.2 (0.48 mM final concentration).

The RNA was precipitated by adding 880 μl of ethanol; the pellet was resuspended in 100 μl of hybridization solution (10 mM TES, 2% SDS, 10 mM EDTA, 300 mM NaCl) and radioactivity was checked with a beta counter. Equal amounts of radiolabeled RNAs were used for hybridization (1 to 2 ml for each membrane, 65 C for 48 hours) with DNA immobilized on nitrocellulose filters (Schleicher and Schuell) previously prehybridized at 65 C for 24 hours with the hybridization solution (see above). Membranes were then washed with several changes of 0.2× SSC at 65 C for 30 minutes and incubated at 37 C in 0.2× SSC with 1 μg/ml RNase A for 30 minutes. Membranes were then exposed for autoradiography. For immobilization of DNA on membranes, plasmids (5 μg) were denaturated with 0.3 mM NaOH at 65 C for 60 minutes, neutralized with equal amount of 2 M ammonium acetate, and spotted onto nitrocellulose membranes using a slot blot apparatus. Plasmids used contained MCP-1 and glucose-6-phosphate dehydrogenase (G6PDH) sequences; pBR322 was used as negative control.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

One microgram of total RNA was reverse transcribed and then amplified by RT-PCR. Total RNA (1 μg) was mixed with 2.5 mmol/l $MgCl_2$, 25 mmol/L KCl, 10 mmol/L Tris-HCl, pH 8.3, 1 mmol/L dNTP and 2.5 U/μl Moloney murine leukemia virus reverse transcriptase (Perkin Elmer Cetus, Norwalk, CT) in a final volume of 20 μl. Reverse transcription was conducted at 42 C for 15 minutes. This reaction was then amplified by adding in a final volume of 100 μl 2 mmol/L $MgCl_2$, 50 mmol/L KCl, 10 mmol/L Tris-HCl, pH 8.3, 2.5 U Tac DNA Polymerase, and 0.15 mol/L each specific primers. Primers for amplification by RT-PCR of thrombin receptor sequence[30] were obtained by the phosphoroamidite method with a Beckman 200 A synthetizer and were as follows: forward 5′-TGTGAACTGATCTTTATG-3′ and backward 5′-TTCGTAAGATAAGAGATATGT-3′. Samples were amplified in a Perkin Elmer Cetus thermal cycler using 30 cycles at 95 C (1 minute), 55 C (2 minutes), and 72 C (3 minutes). The 10 μl of each RT-PCR reactions were then elec-

trophoresed through a 1% agarose gel that was stained and photographed.

Results

As shown in Figure 1A, exposure of PBMC, but not of human sarcoma cells, to thrombin resulted in expression of MCP-1 mRNA. Although basal expression of MCP-1 transcript in unfractionated PBMC was usually undetectable or extremely low, MCP-1 transcript was evident after 4 hours of treatment with thrombin and was still high after 48 hours of stimulation. Incubation of cells with or without FBS did not affect MCP-1 expression (data not shown).

In dose-response experiments (Figure 1B) we found that 0.05 U/ml thrombin was sufficient to induce MCP-1 transcripts in PBMC. Induction of MCP-1 transcripts by thrombin was evaluated in five different donors with results similar to those shown in Figure 1. Densitometric analysis showed that when thrombin was tested in parallel with endotoxin at the optimal inducing concentration (5 U/ml and 100 ng/ml, respectively),[21] thrombin was two to three times less effective than endotoxin in inducing MCP-1 transcripts (data not shown). Densitometric analysis was normalized on the expression of G6PDH.

To identify cells expressing MCP-1 in response to thrombin, PBMCs were fractionated by adherence on plastic. Low but detectable expression of MCP-1 was found in untreated adherent monocytes, as expected on the basis of previous reports.[37,38] Expression of MCP-1 was augmented by thrombin in adherent monocytes after 4 hours of stimulation (Figure 1C). Monocyte-deprived lymphoid cells did not express MCP-1 in response to thrombin (Figure 1C). To identify thrombin as actually responsible for the observed effect on MCP-1 expression in circulating monocytes, we used three different approaches. As shown in Figure 2B, boiled thrombin failed to induce MCP-1 in PBMC, thus suggesting that gene induction was not due to undetected endotoxin contaminants. Moreover (Figure 2A) hirudin, which forms a high affinity noncovalent complex with thrombin, prevented MCP-1 induction. Finally, ATIII, a physiological inhibitor of thrombin, abolished induction of MCP-1 by thrombin (Figure 2A).

To obtain evidence that the induction of MCP-1 by thrombin is indeed mediated by the specific receptor for thrombin,[39] we examined the expression of thrombin receptor transcripts for the human monocytes. Having found that monocytes expressed transcript for thrombin receptor (Figure 3), we incubated cells with the 14 residue peptide TRAP, which specifically interacts with the thrombin receptor.[33] TRAP induced expression of MCP-1 in PBMC (Figure 2C). An inactive peptide (corresponding to amino acids 29 to 41 of thrombin receptor) failed to induce MCP-1 mRNA (Figure 2C). Furthermore, the induction of MCP-1 by thrombin required the active catalytic site. Catalytically inactive thrombin (mutant SA205) was tested in two experiments (one shown in Figure 2D). In both inactive SA 205 mutated thrombin had little or no effect on MCP-1 expression.

Because EC are strategically located between blood and tissues, we investigated whether thrombin, which induces a series of functional modifications in

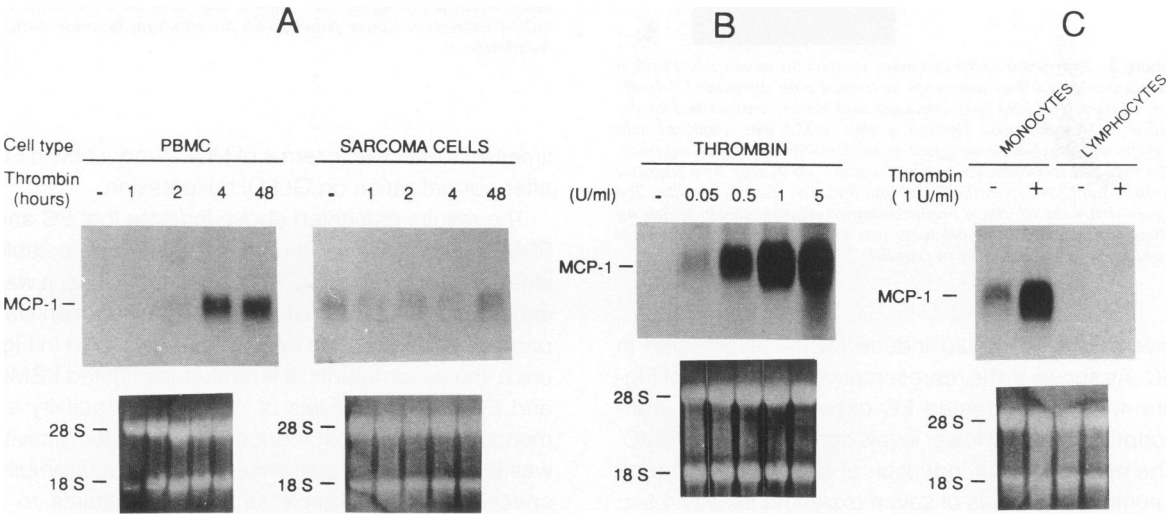

Figure 1. *Induction of MCP-1 transcripts in PBMC and sarcoma cells by thrombin.* A: *Expression of MCP-1 transcripts in PBMC and sarcoma cells exposed to thrombin (1 U/ml) for various periods of time.* B: *Induction of MCP-1 transcripts in PBMC exposed for 4 hours to various dosages of thrombin.* C: *Expression of MCP-1 transcripts in circulating cells enriched for monocytes or lymphocytes and treated with 1 U/ml thrombin for 4 hours. The figure shows four representative experiments out of 11 performed. Lower panels show the ethidium bromide-stained membranes.*

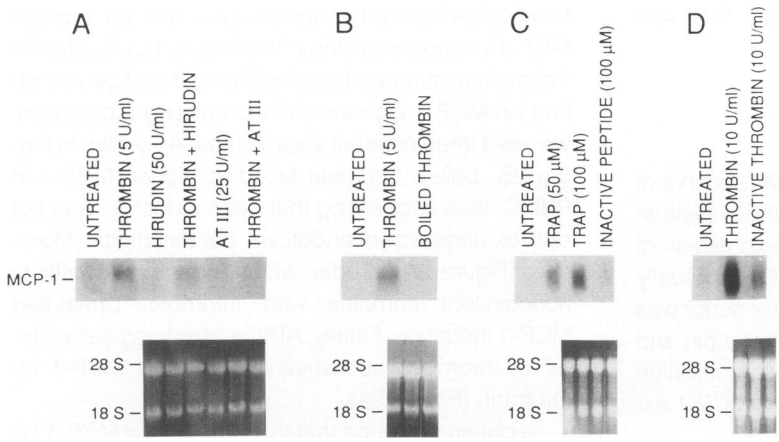

Figure 2. *Induction of MCP-1 transcripts by active and inactive thrombin and by TRAP. Effects of thrombin inhibitors (hirudin and ATIII,* **A**), *heat-inactivated thrombin* (**B**), *TRAP and of an inactive peptide* (**C**), *and catalytically inactive thrombin* (**D**) *on MCP-1 expression in PBMC. Cells were treated with the indicated stimuli for 4 hours. The figure shows four representative experiments out of 12 performed. Lower panels show the ethidium bromide-stained membranes.*

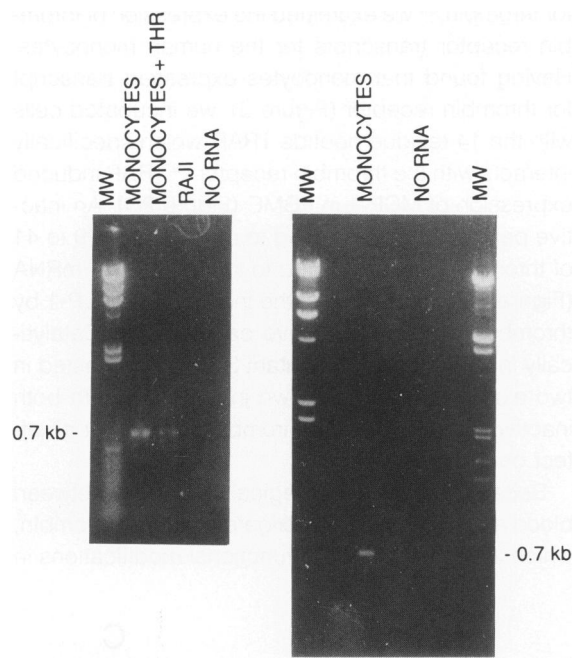

Figure 3. *Expression of the thrombin receptor in monocytes. Purified monocytes were either untreated or treated with thrombin (5 U/ml) for 4 hours. The RNA was extracted and reverse transcribed as detailed in Materials and Methods section. cDNA was amplified with specific oligonucleotide designed to the thrombin receptor sequence. The expected amplification product was 0.7 kb in size. As a negative control, total RNA from the B cell line Raji was used in parallel. The figure shows the ethidium bromide-stained agarose gels in which an aliquot of amplification products was run. DNA molecular weight standards (MW) were run in parallel.*

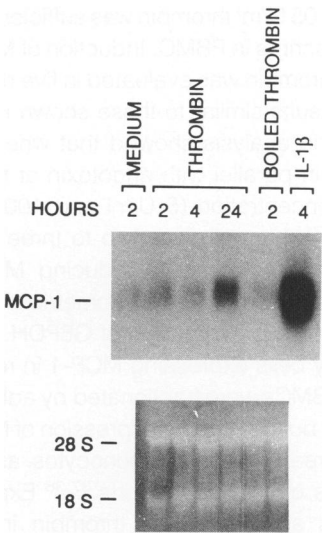

Figure 4. *Expression of MCP-1 transcripts in EC exposed to thrombin for various periods of time. ECs were exposed to thrombin (5 U/ml) for various periods of time or to heat-inactivated thrombin for 2 hours or IL-1β (10 ng/ml) for 4 hours; cells were then examined for MCP-1 expression. Lower panel shows the ethidium bromide-stained membrane.*

these cells,[1–14] could induce MCP-1 expression in EC. As shown in the representative experiment of Figure 4, thrombin-treated EC expressed MCP-1 transcript, although at lower levels compared with PBMC. The mean ± SD of induction of MCP-1 transcript by thrombin in a series of seven experiments was 4.5 ± 2.1 compared with untreated EC. When tested in parallel with optimal concentration of IL-1 (10 U/ml; Figure 4),[24] thrombin (5 U/ml) was found to be two to six times less effective in terms of MCP-1 induction in EC after normalization on G6PDH expression.

The results presented above indicate that EC and PBMC exposed to thrombin have appreciable steady-state levels of MCP-1 transcripts. Thus, it was important to ascertain whether mRNA induction was parallel with translation into protein. As shown in Figure 5, the supernatants of thrombin-stimulated PBMC and EC had high levels of chemotactic activity for monocytes. Thrombin-induced chemotactic activity was blocked by the presence of hirudin, a thrombin-specific inhibitor. Absorption of supernatants to a MCP-1-JE antiserum column significantly reduced, although it did not abolish, the chemotactic activity of thrombin-stimulated PBMC (Table 1).

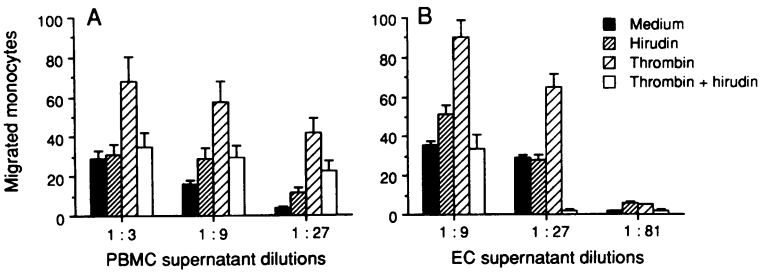

Figure 5. *Induction of monocyte chemotactic activity in supernatants from PBMC and EC exposed to thrombin. PBMC and EC were exposed to thrombin (5 U/ml) with or without hirudin (50 U/ml) for 4 hours. The medium was changed and the incubation was continued for 20 hours. Supernatants were harvested and tested at different dilution for monocyte migration.* **A** *and* **B** *show the monocyte migration in response to supernatants from PBMC and EC, respectively. Bars represent the mean ± SD of triplicates from a representative experiment out of three performed. Migrated monocytes in response to a reference chemoattractant (recombinant C5a) were 135 ± 5 and 110 ± 18 in the experiments of* **A** *and* **B**, *respectively.*

Table 1. *Effect of anti-MCP-1/JE Antibodies on the Chemotactic Activity of Conditioned Medium from Thrombin-Stimulated PBMC*

Stimulus	Migrated Monocytes		
	—	Irrelevant	Anti-MCP-1
FMLP (10^{-8} M)	101 ± 11	95 ± 5	86 ± 7
MCP-1 (50 ng/ml)	75.5 ± 6	80 ± 2	43 ± 4*
Conditioned medium from thrombin-stimulated PBMC	81 ± 4	77 ± 7	42 ± 4*

Results are mean number of migrated monocytes (± SD, 3 to 6 replicates/group) in five oil fields. Thrombin was used at concentration of 5 U/ml. FMLP (formylated peptide) was used as reference chemoattractant.

* $P < 0.05$ *versus* control serum.

In an effort to get more insight into the molecular mechanisms underlying MCP-1 induction by thrombin, we examined the effects of metabolic inhibitors (CH and ActD) along the line of previous studies in which we have examined MCP-1 induction in vascular cells and leukocytes.[24,25,27] As shown in Figure 6, we found that both in EC and PBMC the addition of ActD to thrombin-treated cells blocked MCP-1 induction. The finding that ActD inhibits the thrombin-induced MCP-1 expression suggested that induction required gene transcription. We encountered considerable difficulties in performing runoff analysis on fresh PBMC. In the four experiments performed, only one showed induction, at very low levels, of MCP-1 in thrombin-treated PBMC. No induction was observed in EC (data not shown). In an effort to further elucidate the mechanisms involved in thrombin-mediated induction of MCP-1 transcript, we examined the possibility that this stimulus affected transcript half-life. The half-life of MCP-1 mRNA in untreated cells (PBMC) was approximately 1.5 hours. On exposure to thrombin, transcript half-life was prolonged to 3 hours (Figure 7).

When we used CH, the inhibition of protein synthesis superinduced both constitutive and thrombin-inducible MCP-1 expression in EC. As assessed by the quantitative densitometric analysis of the blot shown in Figure 6, induction of MCP-1 mRNA by CH and thrombin was 30.4-fold with respect to untreated cell, whereas induction of MCP-1 mRNA by thrombin or CH alone was 2.2- and 22.2-fold, respectively. By contrast, CH inhibited MCP-1 induction by thrombin in PBMC (Figure 6). The effect of CH was confirmed by using other protein synthesis inhibitors (puromycin and emetine) (data not shown).

Because MCP-1 is induced by inflammatory cytokine, we investigated whether thrombin-induced mediators could be involved in MCP-1 expression. As shown in Figure 8, blocking of TNF (by anti-TNF antibodies) and IL-1 (by IL-1R antagonist) did not affect the thrombin-induced expression of MCP-1 in PBMC.

Discussion

The results presented here demonstrate that thrombin is an effective stimulus in inducing MCP-1 expression in human circulating mononuclear phagocytes and EC. Induction was found both at the mRNA and protein levels. The presence of MCP-1 in conditioned medium from thrombin-activated PBMC was confirmed with specific blocking antibodies against MCP-1. Induction of MCP-1 by thrombin is likely to be mediated by the thrombin receptor because transcripts coding for this receptor were found in monocytes and the thrombin receptor-activating peptide, which specifically interacts with the thrombin receptor,[33] was able to induce MCP-1 expression in PBMC. Furthermore, because catalytically inactive thrombin failed to induce MCP-1, the response of monocyte to thrombin is dependent on the catalytic activity of thrombin, thus confirming the proteolytic mechanism of thrombin receptor activation.[39]

Thrombin was effective at dosages previously known to induce a series of functional modifications

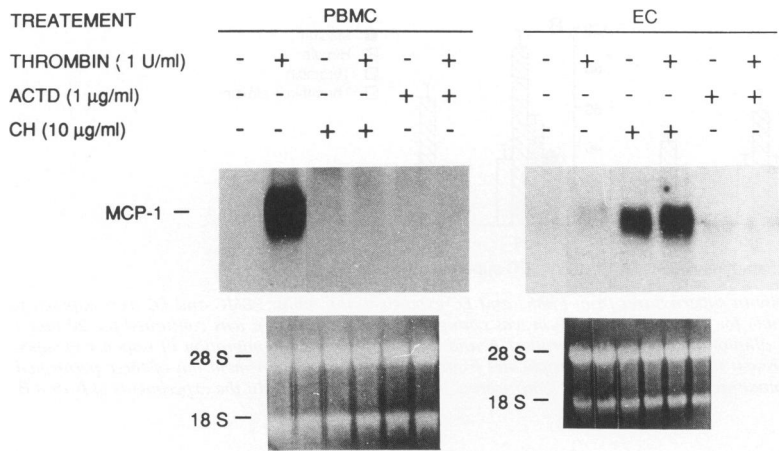

Figure 6. *Effects of metabolic inhibitors on MCP-1 induction in PBMC and EC. Cells were exposed to thrombin (1 U/ml) for 4 hours in the presence or absence of ActD or CH and then examined for MCP-1 expression. Lower panel shows the ethidium bromide-stained membrane.*

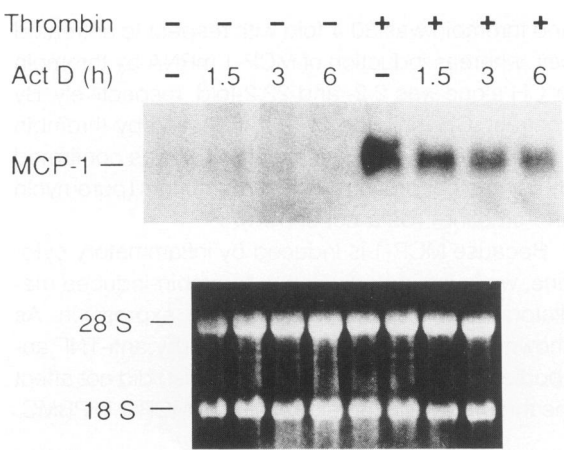

Figure 7. *MCP-1 mRNA half-life in PBMC. Cells were cultured for 4 hours with or without thrombin and then treated with ActD for different times (upper panel). MCP-1 transcript decay was measured by densitometric analysis. Comparable amounts of total RNA were transferred to membranes, as shown by ethidium bromide staining.*

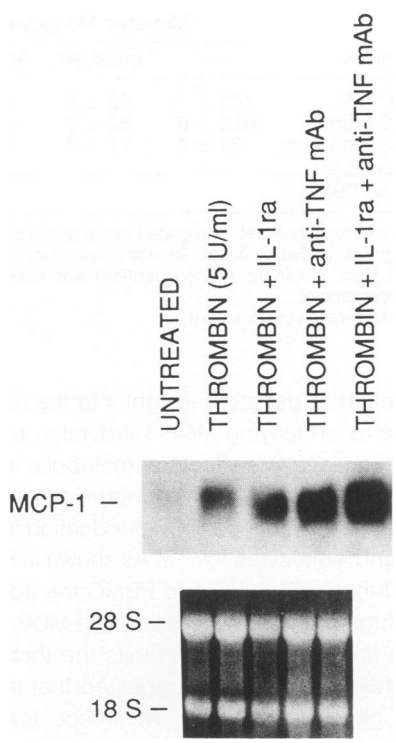

Figure 8. *Effects of anti-TNF monoclonal antibody and IL-1Ra on thrombin-stimulated PBMC. PBMCs were treated with anti-TNF monoclonal antibody (1:2000 diluted) and IL-1Ra (5 µg/ml) for 30 minutes. Then, thrombin was added at the concentration of 5 U/ml and cells were incubated for 4 hours. Cells were then examined for MCP-1 expression. Lower panel shows ethidium bromide-stained membrane.*

in endothelial cells[1–14] and likely to be produced locally *in vivo*.[40] Thrombin was two to three times less effective compared with LPS in PBMC and two to six times less effective compared with IL-1 in EC in inducing MCP-1 transcripts.

The finding that TRAP induced MCP-1 transcript in monocytes points to the role played by the thrombin receptor. Along the same line, recent reports described that thrombin and TRAP induced Ca^{2+} mobilization in monocytes[41] and PGI_2 synthesis, PDGF mRNA expression, and Ca^{2+} mobilization in EC.[42]

Thrombin is a serine esterase enzyme that converts fibrinogen to fibrin monomers and thus plays a central role in hemostasis. In addition, thrombin seems to be also involved in the regulation of certain aspects of inflammatory reactions. This enzyme induces a series of functional modifications in EC, including secretion of PGI_2 and IL-1.[1–14] Moreover, thrombin has been shown to be chemotactic for monocytes,[15,16] and because it also degrades some components of basal membranes,[17] it could facilitate recruitment of leukocytes to inflammatory sites. EC are strategically located at the interface between blood and tissues. Hence, the induction of MCP-1 by thrombin, which is

a product of the activation of blood coagulation pathways, may represent an important mechanism in the recruitment of monocytes at sites of vascular injury. This could be of particular relevance in those situations, eg, atherosclerotic plaques, in which there is infiltration of mononuclear phagocytes at sites of vascular damage. In this context, it is interesting to note that MCP-1 has been identified in macrophage-rich areas of atherosclerotic plaques.[41,42]

As cited above, thrombin elicits a complex series of functional modifications in monocytes and EC. Particularly significant in the context of the present observation is induction of proliferation[10] and associated expression of the c-*fos* proto-oncogene.[14] MCP-1 was first cloned as a growth factor-inducible gene. Moreover, the 5′ region of the MCP-1 gene contains AP-1 sites.[43] The induction of MCP-1 expression reported here may reflect these aspects of the interaction of thrombin with cells.

Cell populations from PBMC enriched for mononuclear phagocytes express MCP-1 gene and protein in response to thrombin. We have recently demonstrated that cytokines and bacterial products can induce MCP-1 expression in human circulating monocytes.[27] The finding that thrombin also induces MCP-1 in human circulating elements of the mononuclear phagocyte system points to the possibility that this cell type can autonomously regulate extravasation and activation of circulating precursors at inflammatory sites.

Thrombin has been reported to cleave the NH_2-terminal part of IL-8,[44] a member of the Cys-X-Cys chemokine family active on neutrophils.[18] On exposure to thrombin, 77aa IL-8 yields a 72aa version of the molecule that is considerably more effective.[44] Thus, thrombin has a complex interplay with the release and action of chemokines.

Inhibition of protein synthesis had opposite effects in EC *versus* PBMC, whereas in EC CH superinduced constitutive and thrombin-inducible MCP-1 gene expression, induction by thrombin in circulating mononuclear cells was abolished. This finding, confirmed with three different inhibitors of protein synthesis, extends to thrombin a previous observation with other stimuli[27] and possibly suggests that the molecular mechanisms regulating the induction of MCP-1 gene expression are different in mononuclear phagocytes compared with cells of other lineages.

The results reported here of regulation of MCP-1 expression by thrombin in PBMC and EC provide further evidence that this enzyme may be important in the regulation of leukocyte recruitment at sites of vascular damage. Because on exposure to inflammatory signals such as LPS or IL-1 monocytes and EC express cell-associated procoagulant activity,[45,46] the results presented here suggest that thrombin, generated at the cell surface, may act on the cells to induce MCP-1, which would in turn amplify monocyte recruitment and participation in inflammatory and thrombotic reactions. These data further suggest that blood coagulation, inflammatory reactions, and immunity interact closely and that recruitment of leukocytes from blood to tissues may represent an important aspect of this interaction.

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