

A Novel Biologic Activity of Thrombin: Stimulation of Monocyte Chemotactic Protein Production

By Giuseppe Grandaliano, Anthony J. Valente,
and Hanna E. Abboud

*From the Department of Medicine, Division of Nephrology, and the Department of Pathology,
The University of Texas Health Science Center at San Antonio, San Antonio, Texas
78284-7882*

Summary

Thrombin is a serine protease that is released at sites of vascular injury and exerts a variety of biologic effects on different cell types. Thrombin is postulated to play a role in the pathogenesis of a number of diseases including atherosclerosis, since it activates vascular smooth muscle and endothelial cells. Thrombin mediates these effects through a specific receptor that is upregulated in vascular cells in atherosclerosis. Atherosclerosis and glomerulosclerosis are characterized by the presence of monocyte-macrophages in the lesions. Monocyte chemotactic protein (MCP-1) is believed to be an important mediator of monocyte recruitment to the tissue and can be induced in a broad variety of cells including mesangial cells. We studied the effect of thrombin on MCP-1 production and gene expression in well-characterized human mesangial cells, vascular pericytes that play a central role in fibrosis of the glomerular microvascular bed. α thrombin stimulates MCP-1 production and gene expression in mesangial cells in a dose- and time-dependent manner. Experiments with diisopropylfluorophosphate thrombin and γ thrombin demonstrate that this thrombin effect requires both receptor binding as well as catalytic activity, features consistent with the known properties of the recently characterized and cloned thrombin receptor. Moreover, a human thrombin receptor activating peptide (TRAP₁₋₇) also stimulates MCP-1 production. Northern blot analysis demonstrated that mesangial cells express an mRNA transcript that hybridizes with labeled human thrombin receptor cDNA. These data describe a novel biologic activity of thrombin and suggest an additional mechanism by which this coagulation factor may participate in the progression of glomerulosclerosis, and by analogy, atherosclerosis.

Thrombin is a serine protease released at sites of vascular injury during activation of both intrinsic or extrinsic coagulation pathways. In addition to its central role in clotting, thrombin exerts a variety of cell-activating functions (1). It is mitogenic for fibroblasts, vascular smooth muscle cells and glomerular mesangial cells (2-4). Thrombin also stimulates platelet-derived growth factor production and monocyte adhesion in endothelial cells (5-7). These findings indicate a potential role for thrombin in the development and progression of atherosclerosis. The observation (8) that the recently cloned thrombin receptor is widely expressed within human atherosclerotic lesions *in vivo* supports this contention. Mesangial cells are vascular pericytes within the glomerular microvascular bed. Proliferation of these cells and mesangial matrix expansion are characteristic of glomerulosclerosis and resemble the intimal thickening and smooth muscle cell proliferation present in atherosclerosis (9). Another essential feature of both pathological processes is the presence of monocytes in the lesions (9). Once in the arterial wall or in the mesangial area, monocytes represent a reservoir and a source of cytokines or growth factors promoting and/or maintaining the activation of resident cells (10). The mechanisms by which monocytes are recruited to the blood

vessel wall and the mesangial area, to participate in the development of atherosclerotic or glomerulosclerotic lesions, remain to be defined. Monocyte chemotactic protein 1 (MCP-1) is a potent and specific chemotactic factor for monocytes (11). MCP-1 expression has recently been identified in human and experimental atherosclerotic lesions, indicating its potential role in the recruitment of monocytes into the lesions (12-14). Mesangial cells in culture produce MCP-1 constitutively and in response to different cytokines (15, 16). In this study, we examined the effect of thrombin on MCP-1 production and gene expression in well-characterized human mesangial cells. The results demonstrate a stimulatory effect of thrombin on MCP-1 protein and gene expression that is dependent on both intact binding site and catalytic activity. This novel biologic effect of thrombin may be of fundamental importance in the recruitment of monocytes and therefore in the development and progression of focal or other forms of glomerulosclerosis and, perhaps in analogy, atherosclerosis.

Materials and Methods

Materials. Highly purified human α thrombin, (2,750 U/mg) diisopropylfluorophosphate (DIP) thrombin and γ thrombin (sp

act, 16.7 U/mg) were kindly provided by Dr. J. Fenton (New York State Department of Health, Wadsworth Center for Laboratories and Research, Albany, NY). Human α thrombin (sp act 4,000 U/mg) was purchased from Sigma Chemical Co. (St. Louis, MO). Thrombin solutions were tested for endotoxin content by the limulus amoebocyte lysate assay (Association of Cape Cod, Inc., Woods Hole, MA) and found to contain <0.1 ng/ml LPS. The human thrombin receptor activating peptide NH₂-Ser-Phe-Leu-Leu-Arg-Asn-Pro-COOH (TRAP₁₋₇) was synthesized using F-moc chemistry on a peptide synthesizer (model 430A; Applied Biosystems, Inc., Foster City, CA) in the University of Texas Health Science Center Institutional Protein Core Facility and purified using reverse phase-high pressure liquid chromatography. The purified peptide was verified using amino acid compositional and mass spectrometry. Tissue culture materials were obtained from GIBCO BRL (Gaithersburg, MD). ³²P-CTP (3,000 Ci/mmol) was obtained from New England Nuclear (Boston, MA) and ¹²⁵I-Bolton-Hunter Reagent from ICN Radiochemicals (Irvine, CA).

Mesangial Cell Culture. Human mesangial cells were cultured from glomeruli isolated from normal human kidneys judged unsuitable for transplantation or normal areas of surgical nephrectomy samples. The cells have been extensively characterized and their identity was confirmed by immunohistochemical staining and by electron microscopy as detailed previously (17). The cells were grown in Waymouth's medium containing 17% FCS. For all the experiments, the cells were used between passages 6 and 10.

Measurement of MCP-1 by Radioimmunoassay. MCP-1 levels were measured by a specific and sensitive RIA using specific anti-MCP-1 antiserum as described previously (18). Highly purified human MCP-1 (15,000 mol wt form) was isolated from serum free-conditioned medium of a human U105 tumor cell line using a combination of affinity chromatography on orange A-Sepharose, ion exchange, and reverse phase HPLC. 2–5 μ g of the purified protein was labeled with ¹²⁵I using the Bolton-Hunter reaction as described previously (18). Specific activities of 100–300 cpm/pg of protein were routinely obtained. Protein-associated counts were $>90\%$ precipitable in 10% TCA. In a preliminary study, it was determined that sodium sulfate at a final concentration of 15% wt/vol would precipitate $<10\%$ of ¹²⁵I-MCP-1 in the presence of nonimmune serum, whereas $>90\%$ could be salt precipitated after reaction with 1:100 dilution of the specific rabbit antiserum to baboon MCP-1. Based on these observations, the following standard protocol was adopted for a RIA. To a 150- μ l volume of the test solution was added 50 μ l of ¹²⁵I-MCP-1 (5×10^5 cpm/ml) in assay buffer (150 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 0.5% human serum albumin) and 100 μ l of the rabbit antibody to MCP-1 (diluted 1:300 in assay buffer). The samples were incubated at 37°C for 2 h and then 100 μ l of 60% calf serum as carrier and 1 ml of 19.6% sodium sulfate were added and incubation continued at room temperature. The tubes were centrifuged at 1,500 g for 20 min, the supernatant was discarded, and the activity in the precipitate was counted. All determinations were made in triplicate; replicate values varied by $<10\%$. Under these conditions, $\sim 60\%$ of the total added counts were bound maximally (Bo). Pooled serum-free medium conditioned by cultured human U105 tumor cells was used as a working standard. Dilutions of the standard were made in assay buffer and determinations made in triplicates as with the test samples. A plot of the logit transformation of the proportion of the tracer bound (B/Bo) against the logarithm of the concentration of the standard was linear ($r^2 > 0.95$) over the standard range of 5–70 ng/ml MCP-1. Each working standard pool (60–80 ng/ml MCP-1) was standardized with freshly prepared highly purified MCP-1 quantitated as before. Where necessary, test samples

were diluted in assay buffer to bring them into the linear range of the curve. To determine the effect of α , γ or DIP thrombin and TRAP₁₋₇ on MCP-1 protein production, confluent human mesangial cells in 12-well dishes were rinsed twice and incubated in serum-free medium with or without the indicated doses of highly purified human α , DIP, or γ thrombin. At the indicated time points, the medium was collected and assayed for MCP-1 using the RIA described above. Each experimental point was determined in duplicate or triplicate.

RNA Isolation and Northern Blotting. Total RNA was prepared from human mesangial cells by lysis in guanidine isothiocyanate and centrifugation on cesium chloride gradients as previously described. Poly(A)⁺RNA was selected by oligo (dT) cellulose chromatography. For Northern blotting, total RNA or poly(A)⁺RNA was electrophoresed through 1% agarose-formaldehyde gel, transferred to GeneScreen (New England Nuclear), and prehybridized at 42°C for 2 h in 50% formamide, 0.5% SDS, 2 \times Pipes-NaCl-EDTA buffer, and 0.1 mg/ml salmon sperm DNA. Baboon MCP-1 cDNA or human thrombin receptor cDNA (19) was labeled by random priming using a commercial kit (Amersham Corp., Arlington Heights, IL) and [³²P]dCTP (3,000 Ci/mmol). The probes (2×10^6 cpm/ml) were added to 10 ml of prehybridization solution, and the blots were hybridized for 16 h at 42°C. Blots were then washed and autoradiography was performed with Kodak x-ray film and intensifying screens at -70°C . The MCP-1 probe was then removed by boiling, and the same blot was rehybridized to a cDNA encoding the ribosomal protein 36B4 (20).

Statistical Analysis. Comparison of MCP-1 secretion in the presence or absence of thrombin was performed by Student's *t* test for paired data.

Results

We first examined the effect of α thrombin on MCP-1 secretion using a specific and sensitive RIA. In response to different concentrations of α thrombin, there was a dose-dependent increase of MCP-1 released into the media (Fig. 1). A significant increase over basal values was observed at 1 U/ml thrombin. Peak effect occurred at 5 U/ml; concentrations of thrombin as high as 20 U/ml had no additional

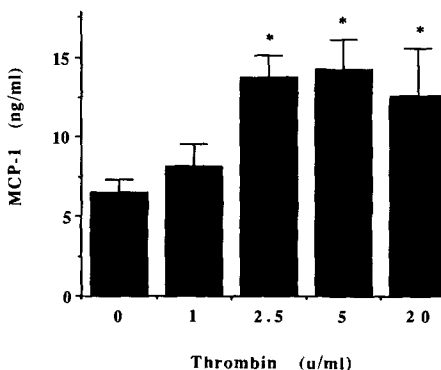


Figure 1. Dose-response for the effect of α thrombin on MCP-1 production. Human mesangial cells were grown to confluence in 12-well plates. The cells were rinsed and incubated in serum-free medium with different concentrations of α thrombin. After 24 h, the medium was collected and assayed for MCP-1 by RIA as described in Materials and Methods. Data are expressed as mean \pm SEM from four to seven separate experiments. (*) $p < 0.05$ compared with control.

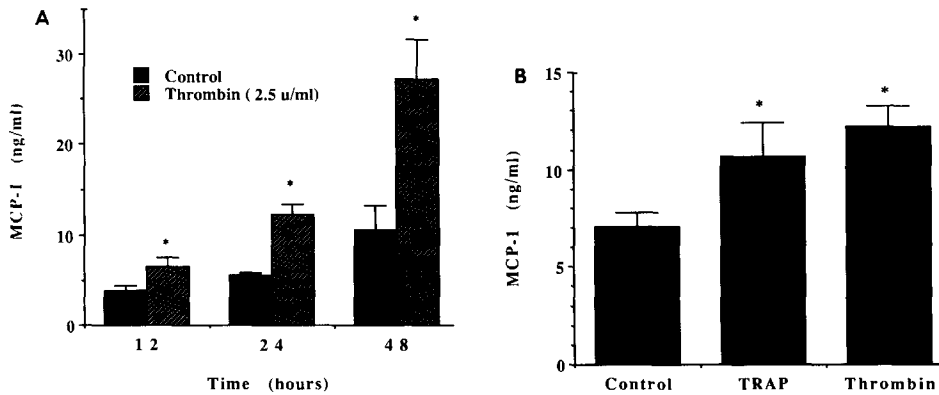


Figure 2. (A) Time course for the effect of thrombin on MCP-1 production. Human mesangial cells were grown in 12-well plates, rinsed, and incubated in serum-free medium with 2.5 U/ml of thrombin. The medium was collected at 12, 24, and 48 h and assayed for MCP-1 by RIA as described in Materials and Methods. Data are expressed as mean \pm SEM from three or four separate experiments. (*) $p < 0.05$ compared with control. (B) Effect of TRAP₁₋₇ on MCP-1 production. Experiments were carried out as in A. Thrombin (2.5 U/ml) or TRAP₁₋₇ (50 μ M) was added and medium collected at 24 h for MCP-1 assay. Data are expressed as mean \pm SEM from three separate experiments. (*) $p < 0.05$.

effect on MCP-1 production. As shown in Fig. 2 A, a significant increase in MCP-1 production in response to α thrombin was detectable at 12 h and continued to increase for at least 48 h after its addition. To determine if thrombin-induced MCP-1 release is due to de novo protein synthesis, we examined the effect of thrombin on MCP-1 mRNA levels. Fig. 3 shows that thrombin induced MCP-1 gene expression. The stimulatory effect occurred as early as 2 h (twofold), reached a maximum at 8 h (10-fold), and was still present at 24 h (fivefold) after the addition of thrombin. To investigate if α thrombin-induced MCP-1 gene expression and protein secretion were dependent on its enzymatic activity, derivatives of thrombin modified at the catalytic or binding sites were tested. DIP-treated α thrombin is blocked at the catalytic site, but can still bind the receptor via the anion binding exo-

site. γ thrombin on the other hand has the binding site disrupted but still has an active catalytic site (3, 19). Neither DIP nor γ thrombin treatment of mesangial cells stimulated MCP-1 release (Fig. 4) or influenced MCP-1 mRNA levels (Fig. 5) when used in equimolar concentrations to α thrombin (1.7×10^{-8} or 0.625 μ g/ml). These data indicate that both proteolytic activity and intact binding exosites are required for thrombin stimulation of MCP-1 production and gene expression. These data also suggest that the effect of thrombin on MCP-1 production by mesangial cells may be mediated by a specific cell surface thrombin receptor similar to that recently characterized by Vu et al. (19). Evidence that this receptor is involved in mediating thrombin's effect is provided by the experiment demonstrating that TRAP₁₋₇ (SFLLRNP), at concentrations of 50 μ M also stimulated MCP-1 production in cultured human mesangial cells (Fig. 2 B). Northern blot hybridization of poly(A)⁺RNA from mesangial cells with a labeled human thrombin receptor cDNA, detected a single mRNA species of 3.5 kb, in agreement with the size of thrombin receptor mRNA detected in other cell types (Fig. 6).

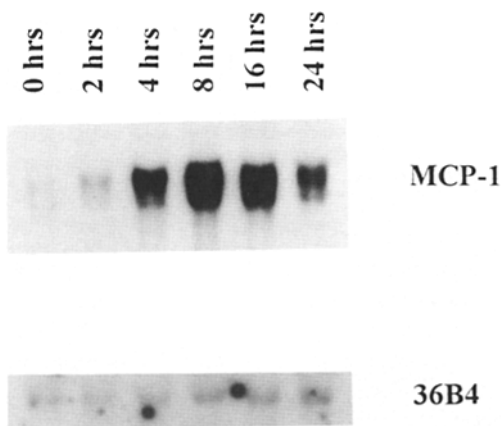


Figure 3. Time course for the effect of thrombin on MCP-1 gene expression. Confluent mesangial cells were washed and incubated in serum-deprived medium for 48 h before exposure to thrombin (2.5 U/ml). At the indicated time points, the cells were harvested and RNA was isolated. Control represents RNA isolated at time zero. Each lane contains 15 μ g of total RNA. After hybridization with MCP-1 cDNA, the probe was removed by boiling and the blot was rehybridized to a 36B4 cDNA. Blots were exposed to Kodak Xomat film with intensifying screens overnight at -70°C for the MCP-1 cDNA and for 10 d for 36B4 cDNA. Compared with control (time zero), thrombin elicited two-, seven-, ten-, nine-, and fivefold increase at 2, 4, 8, 16 and 24 h, respectively.

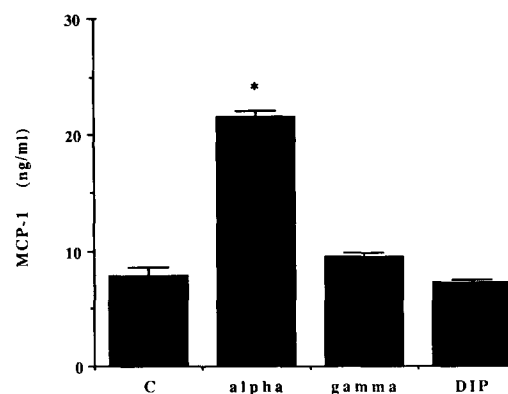


Figure 4. Effect of γ thrombin and DIP thrombin on MCP-1 production. Confluent mesangial cells were rinsed and incubated in serum-free medium in the presence of α , γ or DIP-thrombin. After 24 h, the medium was collected and assayed for MCP-1 by RIA. Data represent mean \pm SEM from three separate experiments. (*) $p < 0.05$ compared with control.

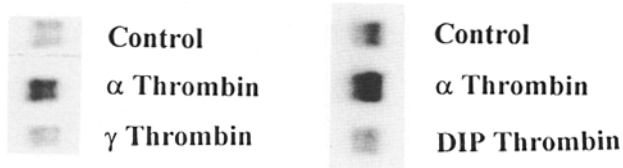


Figure 5. Effect of DIP thrombin (*bottom right*) and γ thrombin (*bottom left*) on MCP-1 gene expression. Confluent mesangial cells were incubated in serum-free medium for 48 h before the addition of α (2.5 U/ml), γ (0.625 μ g/ml), or DIP thrombin (0.625 μ g/ml). After 8 h the cells were harvested and RNA was isolated. Each lane contains 15 μ g of total RNA. Blots were hybridized with MCP-1 cDNA, washed and exposed to Kodak Xomat films with intensifying screens overnight at -70°C .

Discussion

In this study, we describe a novel biologic effect of thrombin: stimulation of MCP-1 production and gene expression. This effect of thrombin is demonstrated in cultured human glomerular mesangial cells, vascular pericytes that acquire effector functions during glomerular injury. The rapid increase in mRNA levels in response to thrombin suggest that increased transcription is an important mechanism of increased MCP-1 production. However, changes in mRNA stability and/or regulation of MCP-1 protein translation cannot be excluded. Stimulation of MCP-1 expression by thrombin requires both an intact receptor binding site on the molecule and enzymatic activity, consistent with the properties required for stimulation of the recently cloned thrombin receptor (19). Moreover, we demonstrate that the synthetic peptide human TRAP₁₋₇ with sequences similar to the amino acid terminus of the cleaved human thrombin receptor also stimulate MCP-1 production, and that human mesangial cells predominantly express a single mRNA transcript upon hybridization with labeled cDNA of the recently cloned thrombin receptor (3, 19). Therefore, this biologic activity of thrombin, at least in part, appears to be mediated by a specific thrombin receptor. These data establish an important link between the clotting cascade and monocyte recruitment to this microvascular bed. Monocyte infiltration is a prominent feature of both human and experimental forms of glomerular injury (21, 22). Histological and ultrastructural studies in human kidney biopsies have identified monocytes within the mesangium and sometimes in direct contact with mesangial cells in a variety of renal diseases, including focal glomerulosclerosis acute proliferative glomerulonephritis and crescentic forms of glomerulonephritis (21, 22). The generation of thrombin in the glomerular microenvironment is suggested by the presence of fibrin deposition and microthrombi within the glomer-

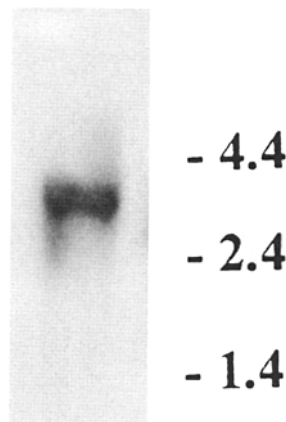


Figure 6. Human glomerular mesangial cells express thrombin receptor mRNA. 5 μ g of poly(A)⁺ RNA from mesangial cells were size fractionated through a 1% agarose-formaldehyde and transferred onto a nylon membrane. The blot was hybridized with a ³²P-labeled human thrombin receptor cDNA, washed, and exposed to Kodak Xomat film with intensifying screens overnight at 70°C . Numbers on the right represent sizes in kilobases of RNA standards run in the same gel and stained with ethidium bromide.

ular capillaries in severe inflammatory and vascular diseases of the kidney, including transplant rejection (23, 24). In addition, a positive correlation between fibrin deposition and monocyte infiltration was observed in a large series of human renal biopsies (25). In the rat remnant kidney model, characterized by progressive renal injury, monocytes are detected in glomeruli as early as 2 wk after renal ablation (26). In this model, Floege et al. (26) reported that activation of the coagulation system precedes the influx of monocytes. These observations suggest a role for certain factor(s) of the coagulation cascade in monocyte recruitment. Whereas fibrin itself or fibrin degradation product(s) have been implicated as mediators of monocyte influx to the glomerular vascular bed, in rabbits with nephrotoxic nephritis, defibrination reduced glomerular fibrin deposition but had no effect on monocyte accumulation within the mesangial area (27). Thrombin is not the only inducer of MCP-1 production by mesangial cells. Mesangial cell production of MCP-1 is stimulated in response to proinflammatory cytokines and lipoproteins (14, 15, 28). It is interesting to note that thrombin itself is chemotactic for monocytes (29), hence activation of mesangial cells by thrombin to release MCP-1 would result in further recruitment and activation of monocytes with subsequent amplification of the inflammatory response. To our knowledge, this is the first report to demonstrate the stimulation of MCP-1 production by thrombin in any cell type. Our finding may have major implications as to the role of thrombin in monocyte recruitment to other micro or macro vascular beds. MCP-1 gene expression is also upregulated by thrombin in glomerular vascular endothelial cells (Fouqueray, B., G. Grandaliano and H. E. Abboud, unpublished observation).

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Address correspondence to Dr. Hanna E. Abboud, Department of Medicine, Division of Nephrology, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78284-7882.

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