The IL-6-Soluble IL-6Rα Autocrine Loop of Endothelial Activation as an Intermediate Between Acute and Chronic Inflammation: an Experimental Model Involving Thrombin

Valérie Marin,* Felix A. Montero-Julian,† Sandra Grès,* Vera Boulay,* Pierre Bongrand,* Catherine Farnarier,* and Gilles Kaplanski*

Thrombin is a procoagulant and proinflammatory molecule in vivo. In vitro, thrombin has been shown to induce endothelial activation, notably IL-8 secretion and adhesion molecule expression. In this study, we showed that thrombin may induce a new cascade leading from acute to chronic inflammation. Thrombin was able to induce the production of both IL-6 and monocyte chemotactic protein-1 (MCP-1) by HUVEC independently of IL-1$\alpha\beta$ and TNF-α. Addition of physiological concentrations of exogenous soluble IL-6Rα (sIL-6Rα) to thrombin-activated HUVEC was sufficient to increase the amounts of MCP-1 produced, but not those of IL-8. These effects could be blocked by anti-IL-6 or anti-sIL-6Rα blocking mAb, demonstrating the existence of an autocrine loop of MCP-1 secretion, involving the IL-6/IL-6Rα/gp130 complex on HUVEC. In addition, we identified IL-8-activated neutrophils as a potential source of sIL-6Rα because IL-8 induced IL-6Rα shedding from the neutrophil membranes and increased in parallel sIL-6Rα concentrations in neutrophil supernatants. Furthermore, addition of neutrophils to thrombin-activated HUVEC significantly increased MCP-1 secretion, which could be decreased by blocking IL-6. Thus, thrombin-activated endothelium may induce a cascade of events characterized by IL-8 secretion, neutrophil local infiltration, and the release of IL-6Rα from neutrophil membranes. sIL-6Rα may then complex with IL-6 and increase the amount of MCP-1 produced by thrombin-activated endothelium, favoring monocyte infiltration, and the transformation of acute into chronic inflammation. ***The Journal of Immunology,*** **2001, 167: 3435–3442.**

Numerous functional links exist between the coagulation and the inflammatory cascades. Notably, one major procoagulant molecule, thrombin, appears to be a potent proinflammatory agent. Thrombin is a serine esterase that cleaves fibrinogen into fibrin to produce the fibrin clot (1). Thrombin also acts on cells through cleavage of specific receptors, which belong to the family of protease-activated receptors (PARs)[2] (2, 3). Three such receptors, PAR-1, PAR-3, and PAR-4, are cleaved by thrombin on their NH_2 extracellular domain, creating a new NH_2-terminal domain that directly tethered its seven-transmembrane domain part on cells (3–5). Peptides reproducing this N-terminal part, thrombin receptor-activating peptides (TRAPs), can be used to activate directly the PAR-1 receptor, and this method has been used to demonstrate thrombin proinflammatory properties in vivo and in vitro. Thrombin local injection to animals induced inflammation with edema and extravasation, which could be inhibited by hirulog, a thrombin inhibitor, and reproduced by TRAP (6). More recently, a murine model of immune mediated glomerulonephritis (GN) has been described in which hirudin very significantly reduced the severity of leukocyte infiltration and crescent formation (7). Moreover, when the same kind of GN was induced in PAR-1-defective mice, the disease was much less severe than in the wild type (7).

This in vivo evidence correlated with in vitro experiments demonstrating that thrombin exerts proinflammatory activation on various cells, notably on leukocytes and endothelial cells. Thrombin has been shown to be directly chemotactic for both neutrophils and monocytes, and to favor their adhesion to endothelium (8–11). These effects are greatly due to the ability of thrombin to induce type I and type II endothelial activation. Indeed, thrombin has been shown to induce rapid and transient type I endothelial activation, independent of protein synthesis, consisting of P-selectin expression, platelet activating factor, and prostacyclin secretion (11, 12). Thrombin is also able to induce gene transcription and protein synthesis of the major proinflammatory mediators by endothelial cells. Thus, thrombin has been shown to induce in a PAR-1-dependent way, the expression of the leuko-endothelial adhesion molecules, endothelial-leukocyte adhesion molecule-1 (ELAM-1), ICAM-1, and VCAM-1, and to favor neutrophil and monocyte adhesion to endothelium (13, 14). In addition, thrombin induces endothelial production of the chemokines IL-8 and monocyte chemotactic protein-1 (MCP-1), which favor leukocyte activation and migration into inflamed tissues (13, 15, 16, and our unpublished observations). Interestingly, on endothelial cells, thrombin acts independently of the classical proinflammatory cytokines IL-1$\alpha\beta$ or TNF-α and appears to directly activate the transcriptional factor NF-κB (13, 14, 16, 17). Therefore, thrombin appears to be a complete proinflammatory mediator able to induce vasodilatation, vessel permeability, and leukocyte extravasation of both neutrophils during acute inflammation, and mononuclear cells during chronic inflammation.

*Institut National de la Santé et de la Recherche Médicale Unité 387, Hôpital Sainte-Marguerite, and †Immunotech, a Beckman Coulter Company, Marseille, France

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[1] Address correspondence and reprint requests to Dr. Gilles Kaplanski, Institut National de la Santé et de la Recherche Médicale Unité 387-Laboratoire d'Immunologie, Hôpital Sainte-Marguerite, 270 Boulevard Sainte-Marguerite, 13009 Marseille, France. E-mail address: gkaplanski@marseille.inserm.fr

[2] Abbreviations used in this paper: PAR, protease-activated receptor; s, soluble; TRAP, thrombin receptor-activating peptide; MCP-1, monocyte chemotactic protein-1; ELAM-1, endothelial-leukocyte adhesion molecule-1; GRO-α, growth-related oncogene-α; IL-1Ra, IL-1R antagonist; PMN, polymorphonuclear cell; RA, rheumatoid arthritis; GN, glomerulonephritis.

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IL-6 is also an important cytokine in the first steps of inflammation (18, 19). IL-6 is a multifunctional cytokine secreted by various cells, including endothelial cells, and is involved in the acute phase response and in the immune response through T and B cell activation. Interestingly, this cytokine demonstrated both pro- and anti-inflammatory properties in vivo and in vitro (20–24). IL-6 functions in cells are mediated through fixation to a complex receptor, consisting in a transducing protein gp130 and a ligand protein, the IL-6Rα (CD126) (25). Whereas gp130 is present on almost all cell types, IL-6Rα is expressed by a limited number of cells, including hepatocytes, neutrophils, and mononuclear cells (26). However, through membrane shedding or alternative splicing, IL-6Rα can be released by these cells into a soluble form, sIL-6Rα, that can bound IL-6 and protect it against enzyme inactivation (27–29). This complex can then bind to gp130 on cell membranes and activate cells in a mechanism called *trans*-signaling (30). It was recently demonstrated that through *trans*-signaling, the IL-6/sIL-6Rα complex can induce a proinflammatory phenotype in endothelial cells that expresses gp130, but not IL-6Rα (30, 31). In this study, we asked whether thrombin can induce IL-6 secretion by endothelial cells, and we studied the possible mechanisms controlling sIL-6Rα release by neutrophils, which could lead to endothelial *trans*-signaling in an in vitro model linking thrombosis and inflammation.

Materials and Methods

Materials

The following materials were purchased: M199 and RPMI 1640 culture medium, FCS, PBS without Ca^{2+} and Mg^{2+} (BioWhittaker, Fontenay/Bois, France); human α-thrombin (1000 U/mg, controlled for the absence of HIV, hepatitis, plasmin, plasminogen, and fibrin degradation products), FMLP, purified endothelial supplement growth factor from bovine pituitary gland, polymyxin B sulfate (Sigma, Saint-Quentin-Fallavier, France); neutralizing rabbit anti-human TNF-α polyclonal Ab (Genzyme, Le Perray en Yvelines, France); and TRAP-14 (consisting of SFLLRNPNDKYEPF and a control-"scrambled" peptide consisting of NEFSLPKPFRYLNP; Neosystem Laboratories, Strasbourg, France). Recombinant human IL-8 and growth-related oncogene-α (GRO-α) were obtained from R&D Systems (Abingdon, U.K.); IL-1R antagonist (IL-1Ra) was a gift from Dr. C. A. Dinarello (University of Colorado Health Sciences Center, Denver, CO); recombinant human IL-6, Chinese hamster ovary-derived human sIL-6Rα, and the neutralizing anti-sIL-6Rα mAb (clone PM-1) were provided by Dr. K. Yasukawa (Tosoh, Tokyo, Japan) (32, 33). Neutralizing anti-human IL-6 mAb or $F(ab')_2$ (clone AH65) (34), PE-conjugated anti-IL-6Rα mAb (clone M91) were obtained from Immunotech (Marseille, France) and have been previously described (34, 35).

Cell culture

HUVEC were obtained as previously described (13), and used on passage 2 or 3. Cells were grown until confluent in 24-well plates coated with 1% gelatin. The cells were then cultured in M199 containing 20% heat-inactivated FCS, 100 U/ml penicillin G, and 100 μg/ml streptomycin. Forty-eight hours before each experiment, endothelial growth factor was withheld and the cells cultured in the same medium containing 10% FCS for the first 24 h, then 5% for the following 24 h. In some experiments, HUVEC were activated in the absence of FCS. Thrombin, TRAP-14, the control peptide, IL-6, or sIL-6Rα were then added to HUVEC, and after various culture times, the supernatant from each well was collected, centrifuged, and stored at −75°C before assay. In some experiments, IL-1Ra (10 μg/ml), anti-TNF-α polyclonal Ab (10 μg/ml final dilution), neutralizing anti-IL-6 mAb (100 ng/ml), or neutralizing anti-sIL-6Rα mAb (2 μg/ml) was added to the culture. After centrifugation, the supernatants were collected and stored at −75°C for sIL-6Rα assay. All the experiments were performed in the presence of polymyxin B (7 μg/ml) to prevent any biological effect due to endotoxin contamination of the various reagents.

Polymorphonuclear cell (PMN) preparation

PMN were prepared from freshly drawn heparinized blood obtained from healthy donors, as previously described (13). For FACS analysis, cells were adjusted to 4×10^6 cells/ml in RPMI 1640 containing 10% FCS and polymyxin B, then stimulated for 30 min to 24 h with thrombin, IL-8, GRO-α, or control medium, before FACS analysis. At each time, supernatants were also collected and stored at −75°C for sIL-6Rα assay. In some culture experiments, 10^6 fresh PMN were added to either unstimulated or overnight thrombin-activated HUVEC, then after 24 h culture, chemokines were measured in the supernatants.

FACS analysis

For IL-6Rα surface expression, PMN were directly stained with PE-conjugated anti-IL-6Rα mAb (anti-CD126) or with a control isotype (IgG1; Immunotech). Fluorescence was measured on a FACS analyzer (XL; Coultronics, Margency, France). Expression of IL-6Rα was studied by comparing staining with anti-CD126 mAb to that obtained with the control IgG1. Cell surface IL-6Rα expression was expressed as the percentage of mean fluorescence, as follows: [(mean experimental anti-CD126 fluorescence − mean experimental IgG1 fluorescence)/(mean control anti-CD126 fluorescence − mean control IgG1 fluorescence)] × 100.

RNA extraction

Unstimulated and thrombin-activated HUVEC in 25-cm^2 culture flasks were directly solubilized in RNA extraction solution (RNAzol-B; BioProbe Systems, Montreuil, France). Total RNA was isolated using chloroform, and precipitated with isopropanol. RNA was quantified by spectrophotometry at 260 nm.

Synthesis of the cDNA

A 25-μl reverse transcription mixture in strand buffer (25 mM Tris-HCl (pH 8.3), 37.5 mM KCl, 1.5 mM $MgCl_2$) contained 4 μg RNA, 0.1 μg oligo$(dT)_{12–18}$ (Pharmacia LKB Biotechnology, Orsay, France), 0.2 μmol DTT, 13 U of RNase inhibitor (Eurogentec, Angers, France), 400 μM dNTP (BioProbe Systems), 100 U of Moloney murine leukemia virus reverse transcriptase (SuperScript RT; Life Technologies, Eragny, France) and was incubated at 37°C for 60 min.

PCR

PCR amplification of the cDNA using GAPDH primers confirmed that equal amounts of RNA were reverse transcribed. For each condition, three different cDNA concentrations ranging from 32 to 6.4 ng of RNA equivalent concentrations were amplified in 25 μl containing 250 μM dNTP, 2 μM $MgCl_2$, 0.25 U of *Taq* polymerase (Life Technologies), and 1 μM 5′ sense and 5′ antisense specific primers for MCP-1 and IL-6 for 30 cycles as previously described (13). Amplification consisted of 5 min at 94°C followed by 30 sequential cycles consisting of 1 min at 94°C, 1 min at 55°C, and 45 s at 72°C, then a final elongation cycle of 10 min at 72°C in a Crocodile II thermal cycler (Appligen, Illkirch, France). Products of PCR (10 μl) were electrophoresed in a 2% agarose gel (Nusieve; Tebu, Le Perray en Yvelines, France), and then, after ethidium bromide coloration, were quantified using densitometry on a gel imager EASY Herolab (Osi, Elancourt, France). As predicted, the amplification product (amplicon) for IL-6 was 260 bp and for MCP-1 was 274 bp.

RT-PCR specific primers

Specific primers were 5′-TCAATGAGGAGACTTGCCTG-3′ (sense) and 5′-GATGAGTTGTCATGTCCTGC-3′ (antisense) for IL-6; 5′-TCCAG CATGAAAGTCTCTGC-3′ (sense) and 5′-TGGAATCCTGAACCCAC TTC-3′ (antisense) for MCP-1; and 5′-CCACCCATGGCAAATTC CATGGCA-3′ (sense) and 5′-TCTAGACCGCAGGTCAGGTCCACC-3′ (antisense) for GAPDH (Genset, Paris, France).

Cytokine assays

MCP-1 and IL-8 were measured using a specific ELISA (Quantikine, R&D Systems). IL-6, sIL-6Rα, IL-1β, IL-1α, and TNF-α were measured using specific ELISAs from Immunotech.

Statistical analysis

Cytokine levels were expressed as the mean ± SEM of results obtained from at least three individual experiments performed in triplicate. The data were compared using the paired Student's *t* test.

Results

Thrombin induces endothelial IL-6 and MCP-1 secretion in a dose- and time-dependent fashion, through interaction with its specific receptor, independently of IL-1αβ and TNF-α

HUVEC were cultured in medium alone or with increasing concentrations of thrombin for 24 h. As shown in Fig. 1, *A* and *B*,

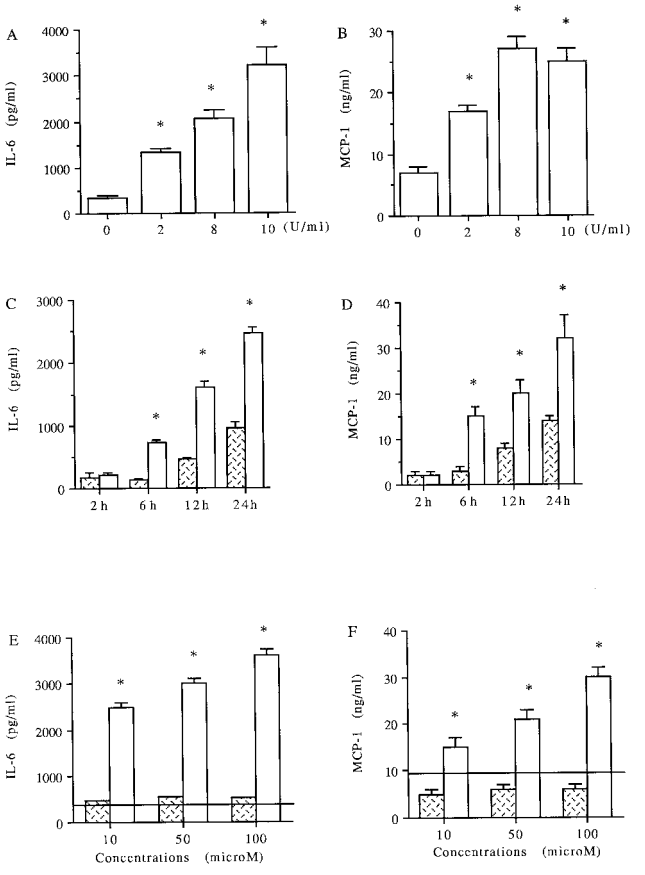

FIGURE 1. Thrombin induces HUVEC production of IL-6 and MCP-1 in a dose- and time-dependent fashion through PAR activation. Increasing concentrations of thrombin were added to HUVEC, and after 24 h, supernatants were collected and IL-6 (*A*, $*$, $p < 0.001$, compared with control medium, $n = 6$) and MCP-1 (*B*, $*$, $p < 0.001$ compared with control medium, $n = 9$) were measured. Thrombin (8 U/ml) was added to HUVEC for different times and both IL-6 (*C*, open bars, $*$, $p < 0.01$, compared with time 2 h, $n = 3$) and MCP-1 (*D*, open bars, $*$, $p < 0.05$, compared with time 2 h, $n = 3$) were measured in the supernatants and compared with HUVEC cultured in control medium (filled bars). Various concentrations of TRAP (open bars), but not of a control peptide (filled bars), induce significant IL-6 (*E*) and MCP-1 (*F*) secretion after 24-h culture ($*$, $p < 0.01$, compared with control peptide at the same concentrations, $n = 3$, horizontal line represents concentrations of each respective chemokine obtained with culture medium alone).

increased concentrations of IL-6 and MCP-1 were obtained with increasing concentrations of thrombin compared with medium alone. A plateau was observed for MCP-1 secretion, in the presence of 8 U/ml thrombin. Thrombin-induced endothelial secretion of IL-6 and MCP-1 was time-dependent, increasing over 24 h (Fig. 1, *C* and *D*). In addition, TRAP-14, a PAR-1/PAR-2 agonist peptide, induced both IL-6 (Fig. 1*E*) and MCP-1 (Fig. 1*F*) secretion in a dose-dependent fashion, whereas a control-scrambled peptide had no significant effect. When HUVEC were cultured with thrombin and saturating concentrations of either IL-1Ra or neutralizing anti-TNF-α Ab, thrombin-induced IL-6 secretion was not modified (data not shown, $n = 4$). Similarly, neither IL-1Ra nor anti-TNF-α significantly decreased thrombin-induced MCP-1 production (thrombin: 24 $\pm$ 2 ng/ml; + IL-1Ra (10 μg/ml): 21 $\pm$ 2 ng/ml; + anti-TNF-α: 28 $\pm$ 3 ng/ml; $n = 4$), whereas in parallel experiments, IL-1Ra significantly inhibited IL-1-induced IL-6 and MCP-1 production by HUVEC (86% inhibition, $p < 0.01$). In agreement with these data, no significant concentration of IL-1$\alpha\beta$ and TNF-α could be detected in thrombin-activated HUVEC supernatants (levels below the detection limits of the assays: 15 pg/ml for IL-1α, 50 pg/ml for IL-1β, and 5 pg/ml for TNF-α, $n = 4$, data not shown).

Thrombin induces increased steady-state levels of IL-6 and MCP-1 mRNA

Using RT-PCR, IL-6 mRNA was not detected in unstimulated HUVEC, but steady-state levels rapidly increased after 1–2 h of thrombin stimulation, reached a maximum after 6 h, and decreased to almost baseline levels after 24 h (Fig. 2). MCP-1 mRNA was not detected in unstimulated HUVEC, and steady-state levels increased after 2 h of thrombin stimulation, with a maximum after 6 h, and maintained sustained levels over 24 h (Fig. 2).

IL-8, but not thrombin or GRO-α, induced IL-6Rα shedding from neutrophils

Because IL-6 in combination with sIL-6Rα has been reported to activate endothelial cells (30), we looked for a possible source of sIL-6Rα production in this model. To define a complete inflammatory cascade, we asked first whether thrombin could induce sIL-6Rα release from PMN. Neither significant shedding of IL-6Rα when PMN were activated with thrombin for 30 min (Fig. 3*A* and Table I) nor significant sIL-6Rα secretion when PMN were activated for 6 h (control medium: 719 $\pm$ 209 pg/ml vs thrombin: 742 $\pm$ 159 pg/ml, $p = 0.7$, $n = 4$) or for 24 h (control medium: 768 $\pm$ 223 pg/ml vs thrombin: 716 $\pm$ 193 ng/ml, $p = 0.5$, $n = 6$) was observed. We have previously reported that thrombin induced an early and sustained secretion of IL-8 by HUVEC (13). We asked whether IL-8 was able to induce IL-6Rα shedding from PMN surface. This experiment was conducted on PMN obtained from 15 healthy volunteers. Using FACS analysis, a significant decrease of IL-6Rα expression on neutrophil membrane was observed, after PMN were stimulated for 30 min with 10 ng/ml IL-8 (Fig. 3*B* and Table I). This loss of IL-6Rα expression on PMN membranes (30–40% loss, $p < 0.01$, Table I) was paralleled by a significant sIL-6Rα increase in IL-8-stimulated PMN supernatants

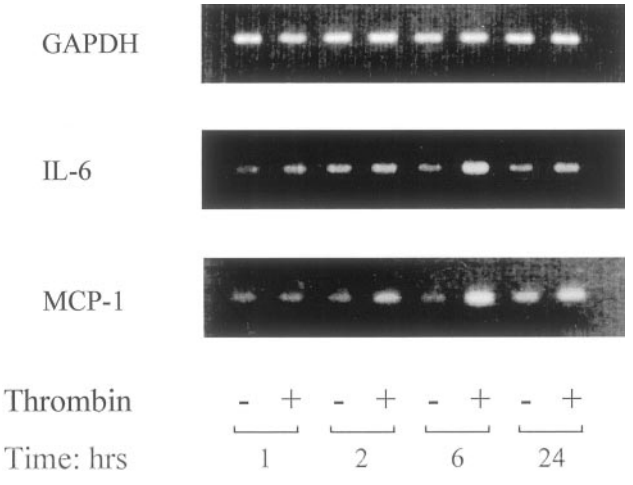

FIGURE 2. Increased IL-6 and MCP-1 steady-state mRNA levels in thrombin-activated HUVEC. RT-PCR products of RNA prepared from HUVEC stimulated by thrombin for different times were deposited on a 2% agarose gel and stained with ethidium bromide. IL-6 mRNA was not detected in unstimulated HUVEC, but was found in thrombin-activated HUVEC with a maximum after a 6-h culture. MCP-1 mRNA was detected in 2-h-stimulated HUVEC, reached a maximum after 6 h, and maintained sustained levels over 24 h.

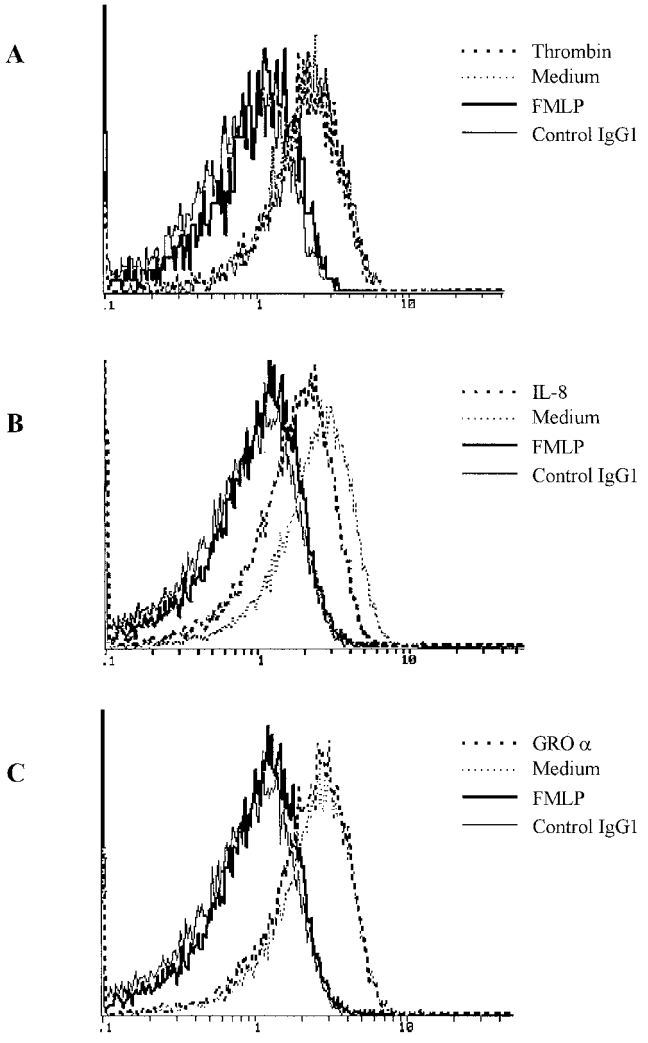

FIGURE 3. IL-8, but not thrombin or GRO-α, induces shedding of IL-6Rα from PMN (FACS analysis). PMN were cultured for 30 min with medium alone, 1 μM FMLP, 8 U/ml thrombin (*A*), 10 ng/ml IL-8 (*B*), or 10 ng/ml GRO-α (*C*), before staining with anti-CD126 mAb or a control isotype and FACS analysis. Fluorescence obtained with thrombin- or GRO-α-stimulated and unstimulated PMN superposed. One experiment representative of 4–9 different experiments is shown.

(160% increase, $p < 0.05$, Table I), consistent with IL-6Rα shedding. Moreover, IL-8 dose-dependently induced IL-6Rα shedding from neutrophil surface, reaching significance for a concentration of 10 ng/ml ($p < 0.05$, Table II). However, IL-8 effects on IL-6Rα shedding were much weaker than those of FMLP, which was used as a positive control in the same experiments (80–90% loss, $p < 0.01$ and 285% increase, $p < 0.05$, Table I). Contrary to IL-8, GRO-α did not appear to induce significant IL-6Rα shedding (Fig. 3*C*) unless very high concentrations (500 ng/ml) were used (Table I).

Exogenous sIL-6Rα increased thrombin-induced MCP-1 but not IL-8 secretion

Addition of various concentrations of sIL-6Rα (10–400 ng/ml) to HUVEC stimulated with thrombin induced a dose-dependent significant increase of endothelial MCP-1 secretion (Fig. 4*A*). MCP-1 secretion was significantly higher (25% increase) with the addition of 200 ng/ml sIL-6Rα, which is a concentration in the range of sIL-6Rα concentrations observed in human fluids (32). The effects of sIL-6Rα on thrombin-induced MCP-1 secretion appeared additive: 8 U/ml thrombin (23 ± 2 ng/ml), 5 ng/ml IL-6 + 200 ng/ml sIL-6Rα (8 ± 1 ng/ml), 8 U/ml thrombin + 200 ng/ml sIL-6Rα (35 ± 1 ng/ml, $n = 4$, data not shown). On the contrary, no significantly increased IL-8 secretion was observed in the same experiments, when HUVEC were stimulated with thrombin in the presence of 400 ng/ml sIL-6Rα (Fig. 4*B*). This was not due to the low concentration of IL-6 present in thrombin-activated HUVEC, because even in the presence of 30 ng/ml IL-6 and 200 ng/ml sIL-6Rα, we observed no increase of IL-8 secretion by HUVEC (data not shown, $n = 3$).

To evaluate the effects of thrombin or FCS concentrations in this phenomenon, we added sIL-6Rα (200 ng/ml) to various concentrations of thrombin-activated HUVEC, in the presence or absence of FCS. In the presence of FCS, 8 U/ml thrombin was necessary to significantly increase MCP-1 secretion, whereas in the absence of FCS, significantly increased MCP-1 secretion was observed with concentrations of thrombin as low as 1 U/ml (Fig. 5, *A* and *B*). These data are consistent with the presence of a thrombin inhibitor in FCS. No increased IL-8 secretion was observed whatever the condition used (Fig. 5, *C* and *D*). Moreover, in the absence of FCS, a significant decrease of IL-8 concentration was observed at 8 U/ml thrombin (Fig. 5*D*).

Autocrine loop of thrombin-induced MCP-1 secretion involving endothelial IL-6 and sIL-6Rα from neutrophils

Addition of 200 ng/ml recombinant sIL-6Rα to thrombin-activated HUVEC significantly increased MCP-1 secretion, and the increased MCP-1 production could be completely reversed by the addition of either neutralizing anti-sIL-6Rα mAb or anti-IL-6 mAb (Fig. 6*A*), demonstrating an autocrine loop of MCP-1 secretion

Table I. *IL-8, but not thrombin or GRO-α, induces neutrophil IL-6Rα shedding*[a]

Conditions	Membrane IL-6Rα Expression (%)	sIL-6Rα Concentrations (pg/ml)
Control	100	488 ± 22
Thrombin (8 U/ml)	106 ± 11	440 ± 50
IL-8 (10 ng/ml)	57 ± 4*	787 ± 33†
GRO-α (10 ng/ml)	92 ± 3	562 ± 28
GRO-α (100 ng/ml)	81 ± 4	589 ± 29
GRO-α (500 ng/ml)	68.5 ± 7†	733 ± 110†
FMLP (1 μM)	1 ± 2*	1397 ± 207†

[a] Membrane expression was determined by FACS analysis, and soluble concentration in neutrophil supernatants was determined by ELISA. *, $p < 0.01$; †, $p < 0.05$, compared to respective controls, $n = 4$.

Table II. *IL-8 induction of neutrophil IL-6Rα shedding (a dose response)*[a]

Conditions	Membrane IL-6Rα Expression (%)	sIL-6Rα Concentrations (pg/ml)
Control	100	338 ± 26
IL-8		
1 ng/ml	97 ± 13	311 ± 41
5 ng/ml	83 ± 10	411 ± 80
10 ng/ml	64 ± 7*	531 ± 69*
100 ng/ml	59 ± 7*	558 ± 80*
FMLP (1 μM)	20 ± 3†	868 ± 144†

[a] Membrane expression was determined by FACS analysis, and soluble concentration in neutrophil supernatants was determined by ELISA. *, $p < 0.05$; †, $p < 0.01$, compared to respective controls, $n = 3$.

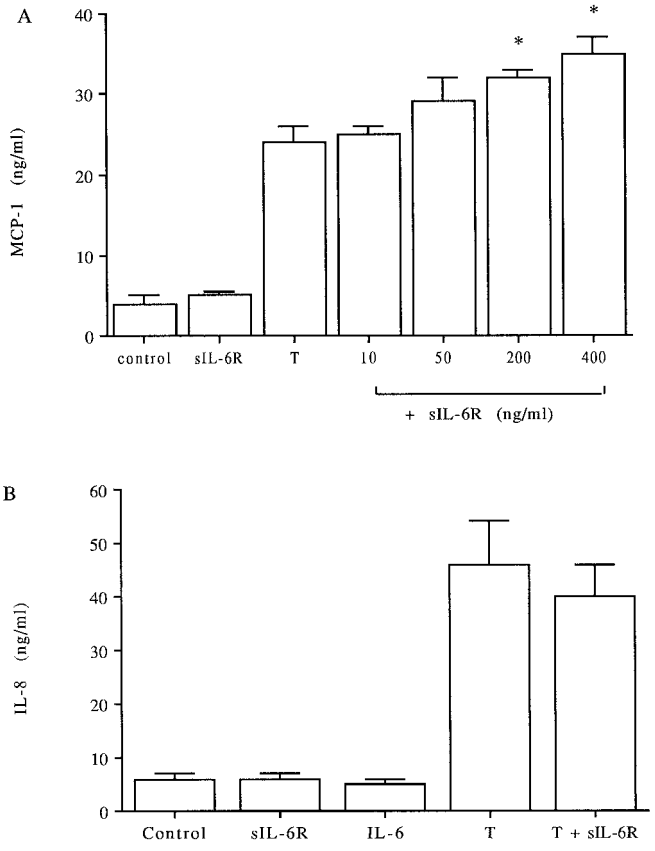

FIGURE 4. Addition of sIL-6Rα to thrombin-activated HUVEC induces MCP-1 (*A*), but not IL-8 production (*B*). Increasing concentrations of exogenous sIL-6Rα were added to thrombin-activated HUVEC, then MCP-1 (*A*) was measured in the supernatant (*, $p < 0.01$, compared with thrombin alone, $n = 3$). High concentrations (400 ng/ml) of sIL-6Rα were added to thrombin-activated HUVEC, and IL-8 secretion was measured in the supernatant after 24-h culture (*B*). Addition of sIL-6Rα (200 ng/ml) alone to unstimulated HUVEC had no effect on MCP-1 or IL-8 secretion compared with medium alone.

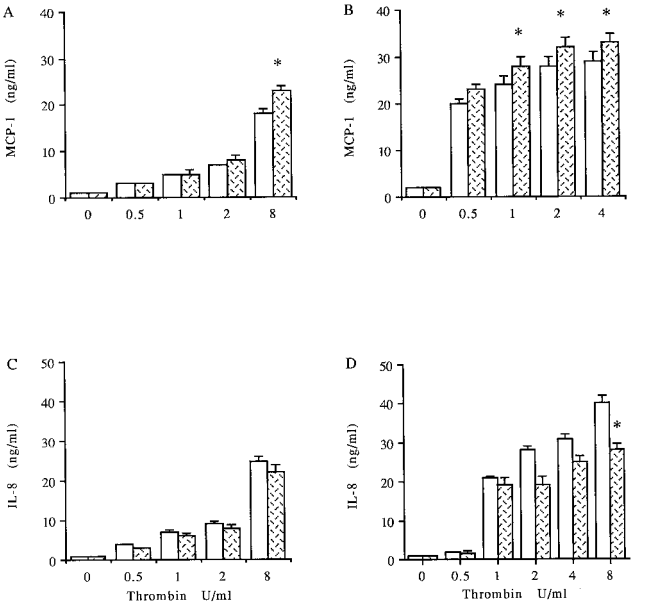

FIGURE 5. Addition of sIL-6Rα to thrombin-activated HUVEC induces MCP-1 (*A* and *B*), but not IL-8 production (*C* and *D*). Thrombin dose-response and influence of FCS. Concentrations of secreted MCP-1 (*A* and *B*) and IL-8 (*C* and *D*) were measured in the supernatants of HUVEC stimulated with increasing concentrations of thrombin with (filled bars) and without (open bars) 200 ng/ml sIL-6Rα in the presence (*A* and *C*) or absence of 5% FCS (*B* and *D*) (*, $p < 0.05$ compared with thrombin alone at the respective concentration, $n = 3$).

involving IL-6 produced by thrombin-stimulated HUVEC and exogenous sIL-6Rα. To evaluate whether such an inflammatory loop involving IL-6 may exist in vivo with PMN as a source of sIL-6Rα, we added freshly prepared PMN to thrombin-activated HUVEC and measured MCP-1 secretion after a 24-h culture. Addition of PMN to thrombin-activated HUVEC significantly increased MCP-1 secretion, which could be partially decreased (25% reduction) by adding anti-IL-6 neutralizing $F(ab')_2$ (Fig. 6*B*). As control experiments, stimulation of fresh PMN with thrombin (8 U/ml), IL-6 (30 ng/ml), or IL-8 (30 ng/ml) did not induce MCP-1 production (levels below the detection limits of the assay, $n = 3$, data not shown).

Discussion

In this study, we observed that thrombin may induce monocyte recruitment through endothelial activation by inducing MCP-1 secretion directly, or indirectly through an autocrine loop involving endothelial IL-6 secretion and IL-8-induced IL-6Rα shedding from neutrophil membranes.

Thrombin is known to induce endothelial MCP-1 and IL-8 secretion by interacting with its PAR-1 receptor, independently of TNF-α and IL-1$\alpha\beta$ (13, 15, 16). Thrombin has also been shown to induce IL-6 secretion by fibroblasts and epidermal cell lines through interaction with the PAR-1 receptor (36). In this study, we observed that thrombin can directly increase mRNA levels and protein synthesis of IL-6 in HUVEC, through interaction with a PAR, likely PAR-1, in a LPS-, IL-1$\alpha\beta$-, and TNF-α-independent way. IL-6 has been reported in one study to directly induce HUVEC activation and leuko-endothelial adhesion, but these data are controversial because HUVEC express the gp130 transducer but not the IL-6Rα (37). Alternatively, IL-6 is able to complex with sIL-6Rα, then associate with gp130 homodimers and activate HUVEC for chemokine production and adhesion molecule expression (30, 31). The same kind of data were observed in our model; addition of physiological concentrations of exogenous sIL-6Rα to thrombin-stimulated HUVEC was sufficient to increase MCP-1 secretion, compared with HUVEC stimulated with thrombin alone, and this can be completely reversed by anti-sIL-6Rα and anti-IL-6 blocking mAbs, demonstrating the existence of an autocrine loop of MCP-1 secretion involving exogenous sIL-6Rα and IL-6 secreted by thrombin-stimulated HUVEC. A similar observation has been made in the case of adhesion molecule expression regulation in a model of TNF-α-activated astrocytes (38). Interestingly, in the same culture condition, we observed no increase in IL-8 secretion, although other authors have found that the IL-6/sIL-6Rα complex induces an autocrine loop of both MCP-1 and IL-8 secretion in endothelial cells (30, 31). These differences are likely due to the different concentrations of sIL-6Rα and FCS present in the different experimental models. In the study by Modur et al. (31), sIL-6Rα concentrations up to 1 μg/ml have been used, and induced both HUVEC chemokine production as well as ELAM, ICAM-1, and VCAM-1 expression, but these concentrations are by far in excess compared with those of sIL-6Rα observed in human fluids, which are rarely higher than 500 ng/ml (32, 39, 40). In the study by Romano et al. (30), the sIL-6Rα concentrations used are comparable to those used in our model and induced both chemokine reduction and ICAM-1 expression, but not ELAM-1 or VCAM-1.

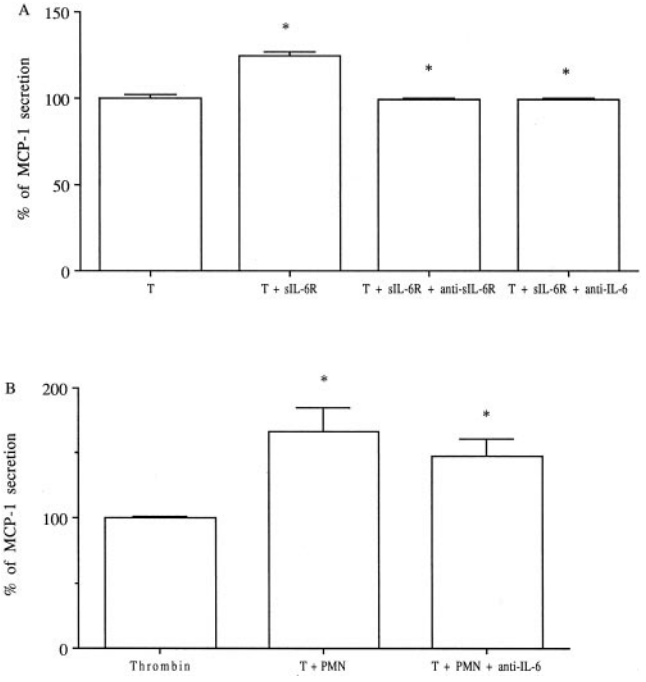

FIGURE 6. Autocrine loop of thrombin-induced MCP-1 secretion involving endogenous IL-6 and exogenous sIL-6Rα (*A*) or fresh PMN (*B*). *A*, Exogenous sIL-6Rα (200 ng/ml) was added to thrombin-activated HUVEC, and MCP-1 concentration was measured in the 24-h culture supernatants (*, $p < 0.01$ compared with thrombin alone, $n = 3$). Addition of blocking mAb against sIL-6Rα or IL-6 in these conditions completely abrogated MCP-1 increase (*, $p < 0.01$ compared with thrombin + sIL-6Rα, $n = 3$). *B*, PMN (10^6/500 μl) freshly isolated from different donors were added to thrombin-activated HUVEC in the absence or presence of anti-IL-6 neutralizing mAb $F(ab')_2$, and MCP-1 was measured in the supernatants after 24 h (*, $p < 0.05$, compared with respective control, $n = 3$).

However, in this study HUVEC were cultured in the presence of 20% FCS and heparin.

However, our results may suggest that HUVEC are more sensitive to stimulation by IL-6/sIL-6Rα for MCP-1 than for IL-8 secretion. A similar observation has recently been made in mesangial cell lines (41). This may be an important point in vivo, because the pro- vs anti-inflammatory functions of IL-6 are still discussed. Notably, studies performed in animals defective for the IL-6 gene have shown that the absence of this cytokine favors on one hand, neutrophilia, high circulating TNF-α concentrations and lethality in response to LPS challenge. In contrast, these animals have decreased IgG and IgA concentrations and a weak ability to develop a Th-1 response against intracellular infectious agents (42). Therefore, IL-6 properties might be rather anti-inflammatory during endotoxemia and acute infection, and proinflammatory during chronic intracellular infection (23, 42). Considering this hypothesis, it is noteworthy that in our study, IL-6/sIL-6Rα is more efficient in inducing MCP-1, a chemokine acting on mononuclear cells rather than IL-8, which is more specific of neutrophils and favors acute inflammation. Thus, the IL-6/sIL-6Rα complex may favor chronic inflammation, activation of APCs, and finally the induction of an immune response with inflammation solving. In agreement with this point of view, the IL-6/sIL-6Rα complex has recently been shown to protect T cells from apoptosis and to favor chronic inflammation (43).

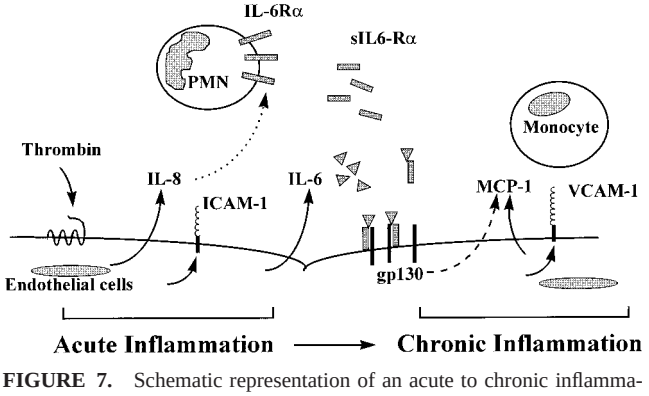

FIGURE 7. Schematic representation of an acute to chronic inflammation shift mediated at the endothelium level by the IL-6/IL-6Rα complex. Thrombin-mediated effects (solid lines), IL-8-mediated effects (dotted lines), IL-6/IL-6Rα-mediated effects (dashed lines).

One important question in such a model in vivo is the potential source of sIL-6Rα in situ. It has been suggested recently that the source of serum sIL-6Rα could be the secretion of an alternatively spliced form by hepatocytes, whereas in situ sIL-6Rα seems to be released by shedding of the receptors expressed on the membranes of inflammatory cells, notably neutrophils and monocytes (26, 27, 40). Therefore, we asked whether thrombin could act as a complete proinflammatory mediator in this model, and induce IL-6Rα release from neutrophils, either through shedding or alternative mRNA splicing. Thrombin did not significantly modify neutrophil membrane IL-6Rα expression or sIL-6Rα concentrations after 30-min stimulation, nor did it induce sIL-6Rα secretion in neutrophil supernatant after 12 h. Because FMLP, a potent neutrophil activator, induces the shedding of IL-6Rα (26, 27, 31), we asked whether IL-8, which is also a well-known neutrophil activator, was able to induce IL-6Rα shedding. In all experiments performed in >15 healthy donors, a significant, although moderate, decrease of IL-6Rα expression on neutrophil membranes and a parallel increase of sIL-6Rα concentrations in the supernatants were observed after 30-min stimulation with IL-8, consistent with receptor shedding from PMN membranes. On the contrary, GRO-α, another chemokine acting on PMN, was not able to induce IL-6Rα shedding unless very high concentrations of this chemokine were used. Because IL-8 acts on PMN through CXCR1 and CXCR2, whereas GRO-α acts through CXCR2 unless high concentrations such as 500 ng/ml are used (44), these data are consistent with the fact that IL-8 induces IL-6Rα shedding through activation of CXCR1. So far, bacterial toxins and C reactive protein are the only physiological molecules known to induce IL-6Rα shedding (45, 46).

This autocrine loop of MCP-1 secretion involving thrombin, IL-8, and IL-6/IL-6Rα is likely to be present in vivo, because addition in vitro of fresh PMN to thrombin-activated HUVEC significantly increased MCP-1 secretion, which was 25% decreased by anti-IL-6 mAb. Thrombin proinflammatory properties and the ability to induce chemokine production may support leukocyte recruitment in tissues in different autoimmune diseases, such as rheumatoid arthritis (RA) or GN, in which both extravascular coagulation and inflammation exist.

Detection of fibrin, fibrinogen, and thrombin/antithrombin III complexes in synovial fluids of RA has clearly established the occurrence of extravascular coagulation in RA synovium (47). Thrombin may play a role in RA pathogenesis through induction of MCP-1 and chronic mononuclear cell infiltration as well as induction of IL-8, which favors neutrophil recruitment during acute flare-up of the disease. In addition to chemokines, RA synovial fluids contain various proinflammatory cytokines, such as TNF-α, IL-1, and IL-6, and have also been shown to contain high concentrations of sIL-6Rα (48). Therefore, all the conditions required for the occurrence of the inflammatory cascade described in this study are present in RA synovial fluids.

Similarly, PAR-1 is constitutively highly expressed on glomerular endothelium, and epithelial and mesangial cells (49). In animal models of immune-mediated GN, thrombin has been shown to play an important pathogenic role consisting in crescent formation, lymphocyte and macrophage infiltration, as well as fibrin deposition (7). MCP-1 is an important cytokine in immune-mediated GN, and it is likely that the ability of thrombin to directly induce chemokine, especially MCP-1, secretion by endothelial and mesangial cells plays an important role in kidney monocyte infiltration (50). In addition, to infiltrate tissues, monocytes interact with adhesion molecules, such as ICAM-1 and VCAM-1, the former playing a critical role in crescent formation (51). Endothelial ICAM-1 and VCAM-1 expression are induced by thrombin through interaction with PAR-1, and allowed mononuclear cell adhesion (14). Moreover, IL-6 has been shown to be secreted by mesangial cells and to be involved in the severity of GN (52), and the IL-6/sIL-6Rα complex increases mesangial MCP-1 production (41). Thus, thrombin may induce IL-6 secretion and the autocrine loop of MCP-1 secretion involving IL-6 and sIL-6Rα described in this study, which may be important in kidney monocyte infiltration.

In conclusion, as shown in Fig. 7, this study suggests that in acutely inflamed tissues containing neutrophils IL-8 and IL-6, IL-8 may induce neutrophil IL-6Rα shedding, which would complex with IL-6 and activate gp130-bearing cells, notably endothelial cells, to induce a stronger MCP-1 secretion, thus mononuclear cell recruitment and a shift toward chronic inflammation, immune response, and possibly inflammation ending.

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