# Urokinase expression in mononuclear phagocytes: cytokine-specific modulation by interferon-γ and tumor necrosis factor-α

Margaret R. Gyetko, Susan B. Shollenberger, and Robert G. Sitrin

*Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, University of Michigan Medical Center, Ann Arbor*

**Abstract: This study delineates the regulatory effects of inflammatory cytokines on mononuclear phagocyte plasminogen activator (PA) activity. The mechanisms by which mononuclear phagocytes modulate PA activity are described. Mononuclear phagocytes regulate net PA activity by the balanced expression of urokinase-type PA (uPA), in either secreted or membrane-associated forms, and a specific plasminogen activator inhibitor, PAI-2. Therefore, understanding how immunomodulators regulate macrophage PA activity requires that the comparative effects of uPA and PAI-2 be elucidated. We determined how recombinant interferon-γ (IFN) and tumor necrosis factor-α (TNF) regulate plasminogen activation in monoblast-like U937 cells and normal human monocytes. In U937 cells, both IFN and TNF induced concurrent increases in secreted PA and PA inhibitor activities. These effects were accompanied by increased immunoreactive uPA and PAI-2 in conditioned media (enzyme-linked immunosorbent assay) and steady-state levels of cellular uPA and PAI-2 mRNA (Northern analysis). To determine the relative abilities of IFN and TNF to either promote or inhibit plasmin generation, we directly compared the effects IFN and TNF, using optimal stimulating concentrations. IFN induced PA activity to 180% of the level achieved by TNF. In contrast, IFN elicited only 78% of the PA inhibitor produced by TNF stimulation. These differences in secreted activity can be explained by the shift in balance between uPA and PAI-2 proteins. Immunoreactive uPA was induced equally by IFN and TNF, but TNF generated higher levels of PAI-2. The same overall pattern of results was seen in normal human monocytes. IFN and TNF differ greatly in the ability to augment receptor-bound PA activity in U937 cells, as IFN induced a twofold increase but TNF had no effect. We conclude that IFN and TNF modulate mononuclear phagocyte proteolytic activity through coordinate regulation of secreted and receptor-bound uPA, balanced against concurrent expression of PAI-2. These effects are cytokine specific, as IFN is superior to TNF in stimulating expression of both secreted and receptor-associated PA activities. These properties suggest mechanisms by which mononuclear phagocytes control proteolysis in cytokine-rich inflammatory foci. *J. Leukoc. Biol.* 51: 256-263; 1992.**

***Key Words:*** *macrophage · fibrinolysis · plasminogen activator inhibitor (PAI-2) · protease · inflammation*

## INTRODUCTION

Substantial evidence indicates that plasmin formation through the action of plasminogen activators (PAs) is crucial for the orderly generation and resolution of inflammation [13]. Mononuclear phagocytes are the dominant source of PA activity among leukocytes [13]. During recruitment, mononuclear phagocytes utilize urokinase-type plasminogen activator (uPA) to focus plasmin activity in the immediate pericellular environment, thereby effecting sufficient extracellular matrix degradation to permit migration across tissue planes [16, 26]. Plasmin is believed to be important in inflammatory tissue remodeling through its degradation of provisional fibrin-fibronectin matrices and many of the glycoprotein constituents of basement membranes [13]. Some immunomodulatory effects of uPA are indirect, as uPA has been shown to be a chemotactic factor for polymorphonuclear leukocytes and a lymphocyte mitogen [5, 7]. Further, uPA activates some cytokines, such as tumor necrosis factor (TNF), transforming growth factor β, and interleukin-1, but inactivates others, such as interferon-γ (IFN) [24, 25, 31, 37]. Thus, the expression of PA activity is intimately involved in the course of the inflammatory response by directing inflammatory cell trafficking, matric remodeling at the inflammatory site, and cytokine-mediated cell-to-cell signaling.

Because expression of macrophage PA activity affects so many aspects of the inflammatory response, it is necessary to understand how this system is regulated. These mechanisms are undoubtedly complex, as mononuclear phagocytes express not only uPA and a plasma membrane receptor for uPA (uPAR) but also a PA inhibitor (PAI-2) [4, 41]. It appears that mononuclear phagocytes have the capacity to modulate expression of each component of the PA system independently. We have shown, for example, that uPA and PAI-2 synthesis can be modulated in either parallel or divergent fashion depending on the agonist used [19, 28]. Agonist-specific effects on uPA binding sites have also been shown [27]. The net expression of mononuclear phagocyte PA activity, however, is a reflection a dynamic balance of these highly interactive proteins. Therefore, examining one of these proteins in isolation is insufficient. Instead, it is essential to adopt a more integrated approach in which changes in PA activity are assessed in the context of concomitant changes in PA inhibitor activity.

The factors in the inflammatory milieu responsible for regulating macrophage PA activity remain poorly characterized. Cytokines likely provide important regulatory signals

Abbreviations: BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; IFN-γ, interferon-γ; PA, plasminogen activator; PAI, PA inhibitor; PMA, phorbol myristate acetate; TNF, tumor necrosis factor; uPA, urokinase-type PA; uPAR, uPA receptor; UV, ultraviolet.

Reprint requests: Margaret R. Gyetko, Pulmonary and Critical Care Medicine Division, University of Michigan Medical Center, 3916 Taubman Center, Ann Arbor, MI 48109-0360.

Received June 29, 1991; accepted August 12, 1991.

for macrophage PA activity. PA activity is expressed abnormally in vivo in inflammatory lung disease, such as sarcoidosis, in which cytokines are pathogenetically important [3, 20]. In vitro, stimulated lymphocytes stimulate net macrophage PA activity, but the mechanisms are unclear because individual effects on uPA, PAI-2, and uPAR were not described [18, 40]. Conversely, inflammatory cytokines and hematopoietic growth factors have been shown to induce changes in either PA, PA inhibitor, or uPAR in isolation, but the effects of these mediators on the relative balance between these components has not been delineated [9, 27, 32, 36]. Accordingly, it is not known whether individual cytokines affect net PA activity or the components of the PA system in an agonist-specific manner. To determine whether there is cytokine specificity in the regulation of the mononuclear phagocyte PA system, we compared the effects of IFN and TNF on the relative expression of PA and PA inhibitor activities in human mononuclear phagocytes. We specifically examined the regulatory effects of these inflammatory cytokines on uPA and PAI-2 synthesis, as well as on surface receptor-associated PA activity.

## MATERIALS AND METHODS

### Reagents

Plasminogen was prepared from outdated human plasma by lysine-Sepharose affinity chromatography (Pharmacia Chemicals, Piscataway, NJ) [14]. Plasminogen preparations were rendered free of active plasmin by treatment with phenylmethylsulfonyl fluoride (1 mM) for 16 h at 25°C, followed by extensive dialysis in 0.05 M phosphate buffer, pH 7.5. The human PAI-2 cDNA, generously provided by Andrew C. Webb (Department of Biological Sciences, Wellesley College, Wellesley, MA), was the subcloned internal *Pst*I-*Dra*I fragment of the pcD-1214 clone in pGEM-2 (Promega, Madison, WI), inserted between the *PSt*I and *Sma*I sites in the polylinker [42]. Human urokinase cDNA was obtained from the Japanese Center Resources Bank-Gene, National Institute of Health, Tokyo [34]. This is a nearly full-length clone inserted in the *Pst*I-*Pvu*II site of pcD. Culture medium and additives were found to contain less than 0.1 endotoxin unit/ml, as determined by a *Limulus* amebocyte lysate assay (Whittaker Bioproducts, Walkersville, MD).

### Isolation of Monocytes

Human peripheral blood monocytes were purified from buffy coats provided by the American Red Cross, Detroit, MI. Buffy coats were diluted 1:1 with 5 mM EDTA–normal saline and purified by density gradient centrifugation through Lymphoprep (Nycomed AS, Oslo, Norway). After washing the RPMI-1640 (Gibco, Grand Island, NY), cell number was determined by counting in a hemocytometer and viability was assessed by trypan blue exclusion. Differential cell counts were determined by examining Giemsa-stained cytocentrifuge samples. The mononuclear cells were resuspended at $2 \times 10^6$ cells/ml in complete medium consisting of RPMI-1640 supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), gentamicin (100 μg/ml), L-glutamine (2 mM), and 5% fetal bovine serum (Hyclone, Logan, UT), and the monocytes were purified by adherence in 16-mm plastic dishes (Corning, Corning, NY) at $5 \times 10^6$ cells/well (approximately $2 \times 10^6$ monocytes) for 2 h in humidified air containing 5% $CO_2$ at 37°C. Nonadherent cells were removed by washing with RPMI-1640 at 37°C, and adherent cells were cultured in complete medium or serum-free medium as indicated below.

### U937 Cell Culture

U937 cells (American Type Culture Collection, Rockville, MD) were maintained in complete medium in humidified air containing 5% $CO_2$ at 37°C. Cells were routinely passaged into 50 ml of fresh medium by seeding $5 \times 10^6$ cells into 75-$cm^2$ tissue culture flasks (Corning) at 3- to 4-day intervals. Cells were always passaged 24 h before stimulation with cytokines.

### Preparation of Cells for Determination of PA and PA Inhibitor Activities

U937 cells were washed extensively in RPMI-1640 and suspended in serum-free medium supplemented with 0.1% (w/v) bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO), dispensed ($10^6$ in 1 ml) in sterile 12 × 75 mm polypropylene tubes (Falcon, Lincoln Park, NJ), and incubated with varying concentrations of recombinant IFN and TNF (Genzyme, Boston, MA). After 24 h, cells and conditioned media were separated by centrifugation. The PA and PA inhibitor activities of the conditioned media were then determined by esterolytic assays. The cells were washed extensively with RPMI, counted, and used immediately to measure receptor-bound uPA activity (see below).

Adherent monocytes were cultured in serum-free medium supplemented with 0.1% human serum albumin (American Red Cross) for 24–48 h with varying doses of IFN and TNF. The conditioned media were then removed and the PA and PA inhibitor activities detemrined by esterolytic assays. As an internal quality control for each experiment, monocytes were cultured in parallel wells with 10 ng/ml phorbol myristate acetate (PMA) to ensure that there was the expected increase in PA inhibitor activity [35]. On occasion, cells were refractory to PMA and were considered to be either endogenously activated or injured during purification. Data derived from these cell preparations were not used.

### Determination of Receptor-Bound PA

To quantitate the uPA activity associated with the plasma membrane uPAR, U937 cells were washed extensively with RPMI-1640. To dissociate the uPA from its receptor, cells were then treated with 50 mM glycine, 100 mM NaCl, pH 3, for 3 min, 0°C, at $3 \times 10^6$ cells/ml [4, 39]. A 40% volume of neutralizing buffer (0.5 M HEPES, 100 mM NaCl, pH 7.5) was then added. As a control, cells were treated with a neutral buffer consisting of 50 mM glycine, 100 mM NaCl, pH 7.4. The neutral buffer did not displace detectable PA activity (data not shown). Samples were centrifuged, and the supernatant was assayed for PA activity as detailed below.

### Esterolytic Assays for PA and PA Inhibitor Activities

Plasminogen activator activity was measured with the esterolytic assay of Coleman and Green, with minor adaptations for use in 96-well plates [8]. Test samples (10 μl) were mixed with an optimal amount of plasminogen in 0.67 M glycine, 0.17% BSA, 1.7 μM Tris, 0.02% triton X-100 (50 μl) and incubated at 37°C for 30 min. The plasmin generated by this step was then quantified by addition of the synthetic plasmin substrate thiobenzyl benzyloxycarbonyl-L-lysinate (0.2 mM; Calbiochem. La Jolla, CA) and a color reagent, 5,5'-dithiobis 2-nitrobenzoic acid (2.2 mM; Calbiochem) in 200 μl of 0.2 M phosphate, 0.2 M NaCl, 1% Triton X-100. After 30 min at 37°C, optical absorbance was read at 414 nm with a multichannel spectrophotometer (Flow Laboratories, McLean, VA). After subtracting control values of wells lacking test samples, PA activity was determined from a standard

curve generated with commercially prepared urokinase (Calbiochem) and expressed in milliPloug units (mPU).

Because uPA is secreted as a proenzyme, stable complexes with PAI-2 do not form in conditioned media until exogenous plasminogen is introduced in the first phase of the esterolytic assay [41]. Therefore, by modifying the esterolytic assay, we can measure either PA or PA inhibitor activity in conditioned media. To measure PA inhibitor activity, serial dilutions of the test samples were coincubated with 2 mPU of urokinase and the residual PA activity was measured in standard fashion. PA inhibitor activity was calculated from a plot of the sample concentration (reciprocal of the dilution factor) versus residual PA activity and expressed as PA inhibitor units/ml (1 PAI unit = 1 mPU PA inhibited). Endogenous PA activity was included with the exogenous 2 mPU when calculating PA inhibition.

### uPA and PAI-2 Antigens

Levels of immunoreactive uPA and PAI-2 in 24-h conditioned media were measured using Tint-Elize-uPA and Tint-Elize-PAI-2 enzyme-linked immunosorbent assay (ELISA) kits according to manufacturer's directions (American Diagnostica, Greenwich, CT).

### mRNA Analysis

For these experiments, U937 cells were cultured in serum-containing medium ($2.2 \times 10^7$ in 50 ml) in 75-cm$^2$ flasks in the presence or absence of IFN (0–1000 U/ml) and TNF (0–500 U/ml) for periods ranging from 0.5 to 12 h. Cells were washed with RPMI-1640 and flash frozen at −70°C. To extract cellular RNA, cell pellets were sonicated on ice in urea (5 M), LiCl (3 M), and heparin (14 U/ml), according to the method of Auffray and Rougeon [1]. RNA was pelleted after overnight precipitation at −20°C and extracted repeatedly with phenol-chloroform. The aqueous layer was precipitated with ethanol-Na acetate at −20°C. The RNA pellet was suspended in RNAase-free water and concentration determined by spectroscopy at 260 nm.

Human monocytes were purified from mononuclear cells by adherence to 60-mm plastic tissue culture dishes (Corning). Mononuclear cells ($2 \times 10^7$) were plated, routinely yielding $8 \times 10^6$ monocytes/dish. The adherent monocytes were incubated for 4 h in serum-containing medium in the presence and absence of IFN (1000 U/ml) and TNF (500 U/ml). The medium was then removed and the monocytes lysed directly in the culture dish by repeated pipet aspiration in 4.23 M guanidine isothiocyanate (IBI, New Haven, CT), 0.5% sarcosyl, 25 mM citric acid, and 0.72% 2-mercaptoethanol. The RNA was purified by phenol-chloroform extraction and precipitation at −70°C in isopropanol-Na acetate [6] and quantitated as above.

The RNA was size fractionated electrophoretically on 1% agarose gels containing 3.5 $\mu$M formaldehyde and 20 pg/ml ethidium bromide [30]. Visualization of ribosomal bands under ultraviolet (UV) light provided internal size markers for each lane and also confirmed that the RNA content was equal in corresponding lanes. The RNA was transferred to Hybond nylon filters (Amersham, Arlington Heights, IL) according to the method of Southern and fixed by exposure to UV light [30]. The cDNA of interest was labeled with [$^{32}$P]dCTP (Amersham) by random priming, achieving specific activities of approximately $5 \times 10^8$ cpm/$\mu$g DNA [17]. The nylon filters were then hybridized with $2 \times 10^7$ cpm of [$^{32}$P]cDNA for 18 h at 65°C, followed by serial washes of increasing stringency, the final wash consisting of 0.1× standard saline citrate (0.15 M NaCl/0.15 M Na citrate), 0.1% sodium dodecyl sulfate at 68°C [30]. The filters were then developed by autoradiography, using Kodak XAR-5 X-Omat AR film at −70°C (Eastman Kodak, Rochester, NY).

### Statistics

For experiments utilizing U937 cells, comparisons between groups were performed using an unpaired Student's *t*-test [45]. Where appropriate, data were log transformed to ensure equivalent variances between groups. For experiments utilizing peripheral blood monocytes, a paired Student's *t*-test was used to compensate for interdonor variability in the levels of PA and PA inhibitor activities expressed under control conditions.

## RESULTS

### Cell Culture

U937 cells were always >95% viable at the onset of cytokine stimulation. After incubation in serum-free medium for 24 h, cells were routinely >90% viable, even at the highest concentrations of cytokines (1000 U/ml IFN and 500 U/ml

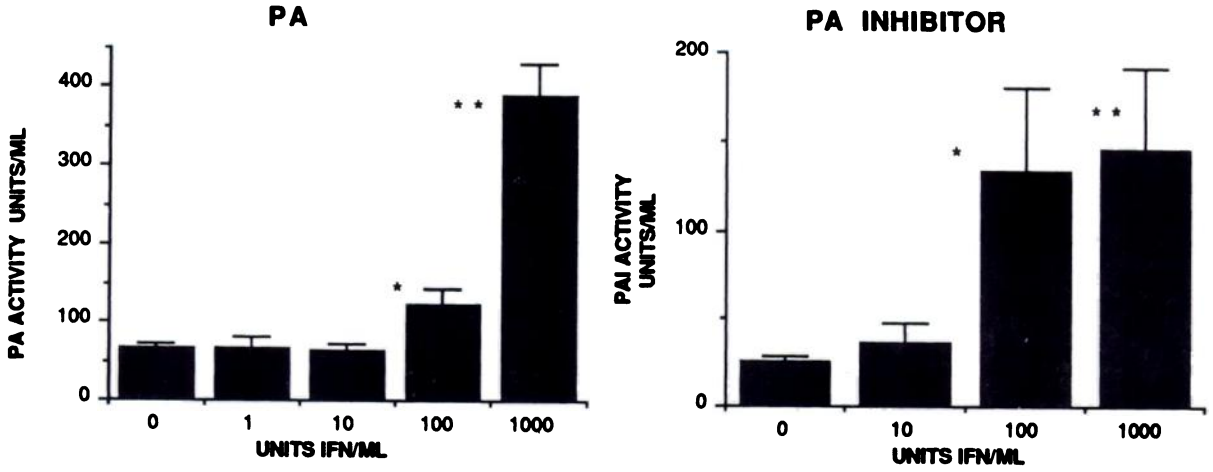


**Fig. 1.** PA and PA inhibitor activities of IFN-stimulated U937 cells. IFN induced a dose-related increase in both PA (left) and PA inhibitor (right) activities. PA activity is expressed in milliPloug units (mPU) and PA inhibitor in PAI units (1 PAI unit = 1 mPU inhibited). PA: $^*P < .05$; $^{**}P < .001$. PA inhibitor: $^*P < .01$; $^{**}P < .005$.

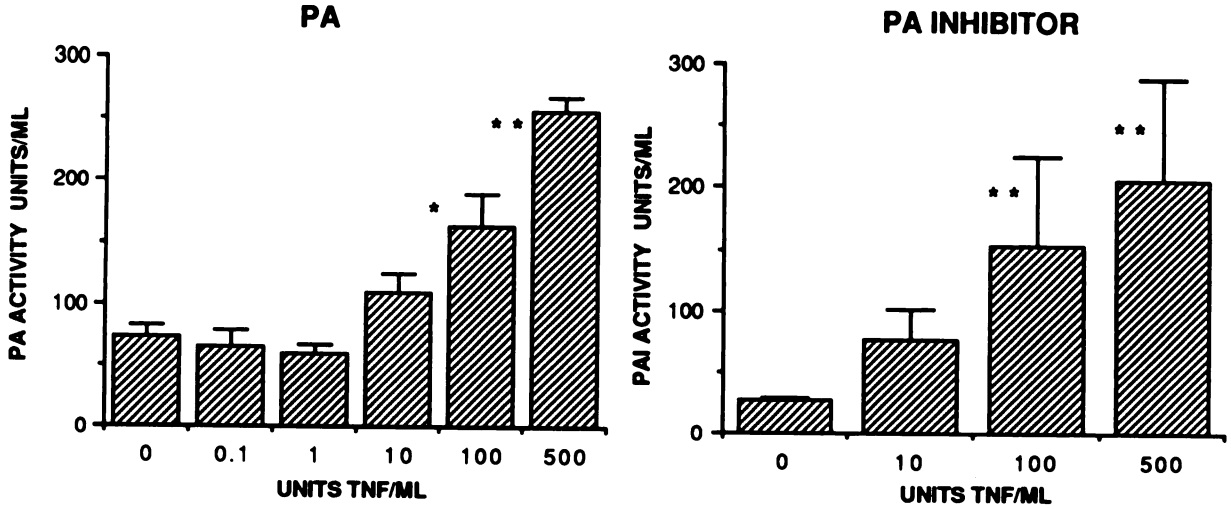


Fig. 2. PA and PA inhibitor activities of TNF-stimulated U937 cells. TNF induced a dose-related increase in both PA and PA inhibitor activities. Data are expressed as in Fig. 1. PA: $*P < .01$; $**P < .001$. PA inhibitor: $**P < .005$.

TNF). Higher concentrations significantly reduced cell viability in 24-h cultures. Mononuclear cells consisted of approximately 40% monocytes, and adherent cell preparations were routinely >94% monocytes and >95% viable. The effects of cytokines and serum-free medium on monocyte viability were identical to those observed in U937 cells.

## Effects of IFN and TNF on PA and PA Inhibitor Activities of U937 Cells

To determine the effects of IFN on PA and PA inhibitor expression, conditioned media were assayed after incubation for 24 h with 0–1000 U/ml IFN. Conditioned media of control cells consistently contained low levels of PA activity (54.7 ± 8.1 mPU/ml). IFN induced a marked increase in PA activity in a dose-dependent manner, reaching a maximum at 1000 U/ml (391 ± 40.2 mPU/ml; $P < .001$) (Fig. 1). Conditioned media of control cells also showed activity when assayed for PA inhibitor (25.8 ± 2.4 PAI units/ml). In parallel with the increase in PA activity, IFN induced a dose-dependent increase in PA inhibitor activity that reached a maximum at 1000 U/ml (146.0 ± 46.2 PAI units/ml; $P < .005$) (Fig. 1).

Cells were stimulated with 0–500 U/ml TNF for 24 h to determine the effects on secreted PA and PA inhibitor activities. Conditioned media from control cells contained PA activity (71.8 ± 10.0 mPU/ml). TNF increased PA activity in a dose-dependent manner, reaching a maximum at 500 U/ml (255.9 ± 11.4 mPU/ml; $P < .001$) (Fig. 2). TNF induced a parallel increase in PA inhibitor activity, from control levels of 25.8 ± 2.4 PAI units/ml to a maximum of 206 ± 83.4 ($P < .005$) in response to 500 U/ml (Fig. 2). The maximal increase in PA activity was significantly less than that achieved by the highest concentration of IFN ($P < .02$). However, the difference between the cytokines in maximally inducing PA inhibitor activity was not statistically significant.

To compare directly the effects of IFN and TNF on secreted PA and PA inhibitor activities, cells were stimulated in parallel using a maximal concentration of either IFN (1000 U/ml) or TNF (500 U/ml). IFN stimulation increased PA activity to 180 ± 21.3% of the level produced by TNF (Fig. 3; $P < .01$). By contrast, IFN-induced PA inhibitor activity was only 78 ± 11.7% of the activity induced by TNF ($P = .15$). Thus, the two cytokines differed markedly in altering the balance between secreted PA and PA inhibitor activities ($P = .008$; Fig. 3), with IFN producing a secretory profile more amenable to fluid-phase plasminogen activation.

## Measurement of uPA and PAI-2 Antigens

Macrophages secrete uPA in a zymogen form that is not inhibited by PAI-2 until it is activated by plasmin [41]. This permits measurement of both PA and PA inhibitor activities in conditioned media. When plasminogen is added in the initial phase of the esterolytic assays, however, there is a potential for forming uPA–PAI-2 complexes that do not contribute to either net PA or PA inhibitor activities. We therefore used ELISA assays for uPA and PAI-2 to determine how the results of the activity assays reflected changes in the relative balance between secreted uPA and PAI-2 proteins. We stimulated U937 cells with maximal concentrations of IFN and TNF (1000 and 500 U/ml, respectively) and assayed conditioned media for uPA and PAI-2 antigens. In keeping with

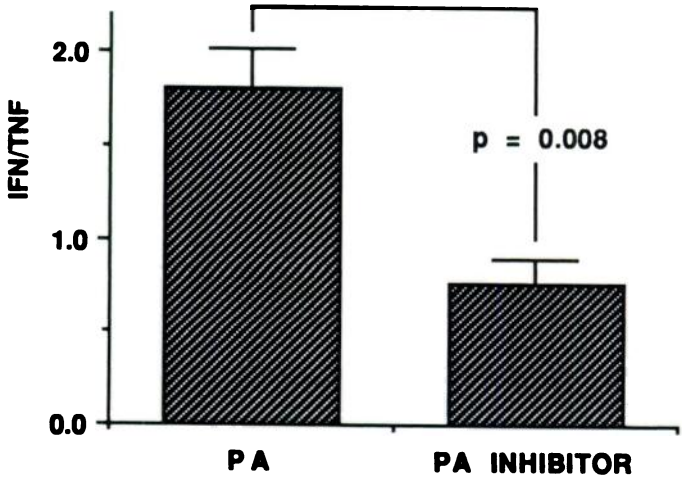


Fig. 3. Comparative effects of IFN and TNF on secreted PA and PA inhibitor activities in U937 cells. Activities induced in parallel cultures by IFN (1000 U/ml) and TNF (500 U/ml) are expressed as a ratio (IFN/TNF). IFN induced 180% of the PA activity produced by TNF (IFN/TNF >1; $P < .01$). IFN elicited only 78% of the PA inhibitor activity produced by TNF (IFN/TNF <1; $P = .15$). IFN/TNF (PA) ≠ IFN/TNF (PA inhibitor); $P = .008$.

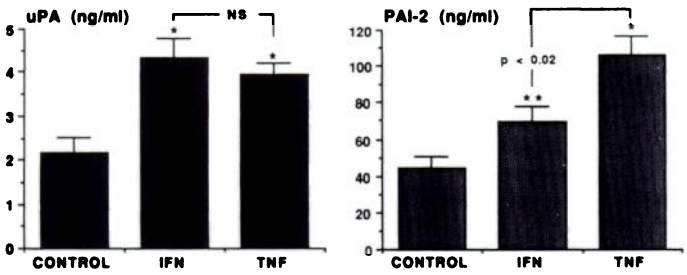


**Fig. 4.** Effects of IFN and TNF on secretion of uPA and PAI-2 antigens by U937 cells. IFN (1000 U/ml) and TNF (500 U/ml) induced significant increases in uPA and PAI-2 antigens over unstimulated controls. IFN- and TNF-induced uPA antigen levels were virtually identical, whereas TNF induced significantly higher levels of PAI-2 antigen than did IFN ($P < .02$). *$P < .002$; **$P < .05$.

the expression of functional activity, IFN and TNF induced significant increases in both uPA and PAI-2 antigens (Fig. 4). IFN and TNF produced equal levels of uPA antigen. By contrast, the level of PAI-2 antigen secreted by IFN-stimulated cells was significantly lower than that induced by TNF ($P < .02$). Therefore, IFN produces higher levels of net PA activity than TNF because there is less offsetting inhibition by PAI-2, not because uPA secretion is relatively enhanced. This demonstrates that although IFN and TNF are alike in increasing both uPA and PAI-2 levels, they differ significantly in their modulation of the relative balance of enzyme and inhibitor and thus in their induction of net PA activity.

## Effect of IFN and TNF on mRNA for uPA and PAI-2

We next determined whether IFN- and TNF-induced increases in uPA and PAI-2 protein levels could be explained by proportionate increases in steady-state mRNA levels. Cells were incubated with either IFN (1000 U/ml) or TNF (500 U/ml) for varying time intervals prior to lysis and RNA extraction, as detailed in Materials and Methods. The same filters, stripped and reprobed, were used for determination of both uPA and PAI-2 mRNA. IFN induced substantial increases in mRNA for both uPA and PAI-2 (Fig. 5A). Both message levels peaked after stimulation for 4–6 h. The message levels declined at similar rates, returning to near control levels within 12 h. TNF also increased mRNA for both uPA and PAI-2 over a similar time period (Fig. 5B). The increase in uPA mRNA levels over controls was consistently smaller in response to IFN than TNF, whereas the two cytokines induced similar increases in PAI-2 mRNA. The same pattern was seen with monocytes (Fig. 6; see below).

## Effects of IFN and TNF on PA and PA Inhibitor Expression by Monocytes

We sought to determine whether the results of experiments with U937 cells accurately reflect the effects of IFN and TNF on authentic human mononuclear phagocytes. Like U937 cells, monocytes expressed secreted PA inhibitor activity that increased from 63 ± 19 PAI units/ml in controls to 106 ± 33 ($P < .05$) in response to IFN (1000 U/ml) and to 167 ± 50 ($P < .05$) in response to TNF (500 U/ml) (Fig. 6). In contrast to U937 cells, PA activity was not detectable in any of the monocyte supernatants. Extending the incubation times to 48 h did not appreciably alter these results (data not shown).

Monocytes were incubated with either IFN (1000 U/ml) or TNF (500 U/ml) for 4 h prior to lysis and RNA extraction. In parallel with the PA inhibitor activity data, both IFN and TNF increased mRNA for PAI-2 (Fig. 6). Interestingly, uPA mRNA was consistently detectable under control conditions. IFN and TNF induced marked increases in uPA mRNA that were quite similar to the results obtained with U937 cells. Thus, the apparent absence of PA activity in monocyte-conditioned media is likely due to a lower level of uPA synthesis and a predominance of PAI-2 secretion, rather than a fundamental difference in uPA regulation between monocytes and U937 cells.

## Determination of Receptor-Bound uPA

We further determined whether stimulation with IFN or TNF for 24 h also modulated the uPA activity bound to macrophage plasma membrane receptors. In parallel with the effects of IFN and TNF on secreted PA activity, we found that the cytokines had markedly different effects on receptor-bound uPA of U937 cells. Even relatively low doses of IFN (100 U/ml) increased receptor-bound uPA above control

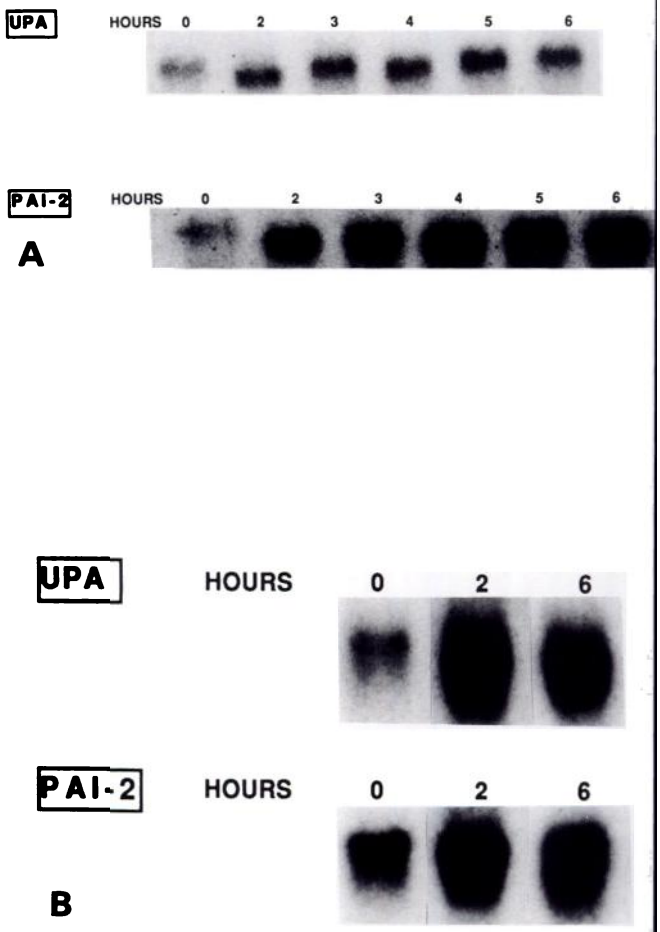


**Fig. 5.** (A) Northern blot analysis of U937 cells incubated with IFN (1000 U/ml) for 0–6 h. Filters were hybridized with a cDNA probe for uPA (2.4 kb; top) and PAI-2 (2.0 kb; bottom). IFN increases mRNA for both uPA and PAI-2, with the maximal increase occurring at approximately 6 h. (B) Northern blot analysis of U937 cells incubated with TNF (500 U/ml) for 0–6. Filters were hybridized with a cDNA probe for uPA (top) and PAI-2 (bottom). TNF increases mRNA for both uPA and PAI-2, peaking at approximately 6 h.

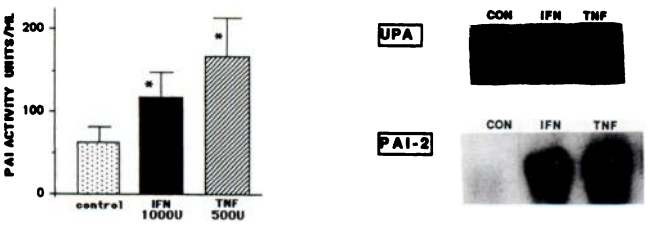


**Fig. 6.** Effects of IFN and TNF on normal human monocytes. Stimulation of monocytes with IFN increased secreted PA inhibitor activity. Stimulation of monocytes with TNF also increased secreted PA inhibitor activity (left; $^*P < .05$). Data are expressed as in Fig. 1. Northern blot analysis (right) shows that stimulation of monocytes with either IFN or TNF increases mRNA for both uPA and PAI-2.

values (from 61.8 ± 15.3 mPU/1 × $10^6$ cells to 117 ± 21.1; $P < .02$) (Fig. 7). By contrast, TNF had no effect on receptor-bound uPA activity, even at the highest doses used (500 U/ml). These experiments were limited to U937 cells as the levels of PA activity that could be extracted from monocytes by acid washing were not consistently above the detection limit of the esterolytic assay.

## DISCUSSION

Mononuclear phagocytes control plasminogen activation by combined expression of uPA, PAI-2, and the uPA receptor. We have shown that macrophages can be induced to regulate the uPA and PAI-2 genes in tandem or divergent fashion [19, 28]. The ability to regulate the components of the PA system independently enables macrophages to express a broader range of PA activity than would be possible if modulation of uPA synthesis were inextricably linked to parallel changes in PAI-2 synthesis. This also suggests that equivalent changes in PA activity can be achieved mechanistically by a spectrum of concomitant changes in uPA and PAI-2 expression. Clearly, then, the overall regulation of macrophage PA activity cannot be understood by isolated studies of uPA, PAI-2, or the uPA receptor. In the present study, we investigated the regulatory effects of IFN and TNF on the relative balance of uPA and PAI-2 expression by mononuclear phagocytes.

Both IFN and TNF increased concurrent secretion of PA and PA inhibitor activities (Figs. 1 and 2). Increased expression of uPA and PAI-2 adequately explains these functional changes, as increased levels of uPA and PAI-2 antigens were present in conditioned media (Fig. 4). These findings extend previous observations that IFN can augment expression of uPA mRNA in macrophages [9] and that in other cell types, such as H1080 fibrosarcoma cells, TNF can augment PAI-2 synthesis [32]. This cytokine responsiveness is consistent with the genetic structure of the uPA and PAI-2 genes, which both contain AU-rich sequences in the 3' untranslated regions that are typically associated with rapid modulation by inflammatory cytokines [2, 44].

It is important to note that the responses to IFN and TNF were similar only to the extent that both cytokines caused up-regulation of uPA and PAI-2. When we compared the increases elicited by IFN and TNF, substantial differences in the magnitude of responses were revealed. IFN caused a significantly greater increase in secreted PA activity than did TNF (Figs. 1–3). However, PA inhibitor activity did not follow this pattern, as the PA inhibitor actively elicited by IFN was approximately 75% of the response to TNF (Figs. 1–3). Clearly, modulation of the balance between PA and PA inhibitor activity is cytokine specific.

By examining synthesis of uPA and PAI-2 proteins, we determined the likely basis for the difference between IFN and TNF in augmenting PA activity, IFN and TNF produced identical increases in uPA secretion, but PAI-2 secretion was significantly greater in response to TNF than IFN (Fig. 4). Thus, the cytokine-specific effects on PA activity can be attributed not to a direct effect on uPA expression, but rather to differences in the counterregulatory effects of PA inhibitor.

The up-regulation of uPA and PAI-2 mRNA suggests that both cytokines augmented neosynthesis of these proteins (Figs. 5 and 6). However, in contrast to protein levels, the increase in uPA mRNA was consistently greater in response to TNF than IFN. The discrepancy between steady-state mRNA levels and protein expression suggests that other regulatory mechanisms, such as translational or posttranslational steps in uPA synthesis, are also differentially affected by these cytokines. This does not appear to be the case for PAI-2 regulation, as we did not observe notable discrepancies between stimulated levels of PAI-2 mRNA, antigen, and activity.

We also observed that PA activity associated with plasma membrane uPAR was differentially affected by IFN and TNF. IFN doubled the acid-dissociable PA activity, whereas TNF had no effect (Fig. 7). By directly measuring receptor-associated enzymatic activity, we extended previous work in which uPAR was enumerated by antibody binding alone [26]. In that study, the number of uPAR was increased by IFN and to a lesser extent by TNF. However, here we show that the number of uPAR occupied by endogenously generated uPA was up-regulated only by IFN. Our results could be explained in several ways, possibly by up-regulation of uPAR expression, with IFN inducing a larger response than TNF. Alternatively, the lower levels of PAI-2 secretion induced by IFN may have permitted greater expression of receptor-associated PA activity. This could occur either by less inhibition of the enzyme on the cell surface or by reduction of inhibitor-mediated internalization of receptor-bound enzyme [11]. To resolve these issues, it will be necessary to perform studies of receptor-associated PA enzymatic activity combined with formal analysis of uPA receptor expression. This work will be particularly important because receptor-associated uPA may have unique functional properties that create a propitious site for proteolysis in the immediate pericellular environment [10, 26, 33].

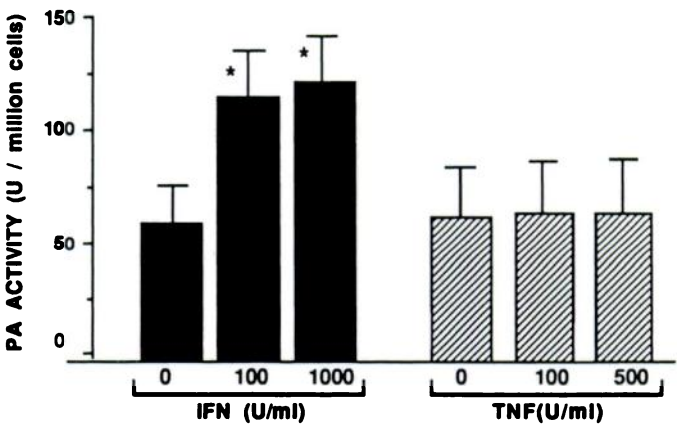


**Fig. 7.** Effects of IFN and TNF on the level of receptor-associated PA activity of U937 cells. IFN doubled receptor-associated PA activity ($^*P < .05$), while TNF had no effect. Data are expressed in mPU per million cells.

It appears that the U937 cell is a valid model for uPA and PAI-2 synthesis by authentic mononuclear phagocytes, as the responses of peripheral blood monocytes to IFN and TNF effectively duplicated those of U937 cells (Fig. 6). The absence of detectable PA activity in monocyte supernatants was the only distinction between the cell types. Although we cannot state conclusively that a posttranscriptional processing step prevented expression of functional PA activity, it is more likely that small amounts of secreted uPA were effectively quenched by PAI-2. U937 cells, being a monoblast-like malignant line, could be expected to produce higher levels of uPA than the more differentiated monocyte [13]. Furthermore, previous studies have suggested that down-regulation of uPA production and enhanced production of PAI-2 are features of greater mononuclear phagocyte differentiation [29, 43].

There is strong evidence that factors regulating immune and inflammatory responses interact extensively with the proteins controlling plasmin-mediated fibrinolysis. For example, fibrin degradation products are known to be chemotaxic for leukocytes, suppressive of lymphocyte function, and directly injurious to endothelial cells [12, 15, 38]. As discussed previously, macrophage PA activity has also been implicated in the activation as well as in the degradation of growth factors and cytokines [24, 25, 31, 37]. In vitro, products of stimulated lymphocytes have been shown to regulate macrophage PA and PA inhibitor activities [18, 40]. In chronic immune reactions in vivo, there is abnormal regulation and functional expression of local PA and PA inhibitor proteins [20, 22]. In several murine models of granulomatous inflammation, plasmin-mediated fibrinolysis is sequentially linked to the inflammatory course of the disease; local PA inhibitor activity is enhanced as the granulomatous lesion evolves, and PA activity appears later as granulomas regress. This sequential expression was shown to be modulated by T cells [21–23]. Collectively, these observations strongly suggest that cytokines exert a major influence on macrophage-derived PA activity in immune reactions and inflammatory foci. Because we have shown that individual cytokines can produce distinct profiles of macrophage PA activity, it is possible that local plasmin activation is controlled by phasic changes in cytokine activity during the course of an inflammatory response. In reciprocal fashion, changes in plasmin generation could influence the release and function of the same cytokines.

In summary, we have demonstrated that IFN and TNF stimulate both PA and PA inhibitor activities in mononuclear phagocytes. There are potentially important distinctions between the effect of these cytokines, as IFN elicits a profile of secreted and receptor-associated activities that surpasses TNF in promoting plasminogen activation. By further characterizing the factors regulating PA expression, we hope to elucidate the complex mechanisms by which fibrinolysis is integrated with the pathogenesis of specific immunity and inflammation.

## ACKNOWLEDGMENTS

This work was supported by a Veterans Administration Career Development Award, by a research grant from the American Lung Association, and by NHLBI grant HL-39672.

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