We report here that human astrocytoma cell line U373-MG is able to express genes of the following components of plasminogen activation system: PAI-1, PN-1, u-PA and t-PA. Treatment of these cells with IL-1β results in accumulation of PAI-1, PN-1 and u-PA mRNAs, whereas t-PA mRNA remains unaffected. IFNγ preferentially enhances PN-1 and PAI-1, EGF enhances PAI-1, u-PA and t-PA expression. Simultaneous addition of anti-inflammatory cytokines IL-4, IL-13 and IL-10 has little effect on the tested components, except induction of u-PA mRNA which was further enhanced by IL-4. We have confirmed interesting time-dependent regulation of plasminogen activation system by EGF/IFNγ. Cells stimulated with EGF/IFNγ show at first increased proteolytic activity but after 24 h inhibition of proteolysis with PAI-1 would prevail. To understand the cooperative effect of EGF and IFNγ in PAI-1 induction the kinetics of activation of STAT1 was studied. It was found that although EGF alone does not activate STAT1, the STAT1 binding activity in the cells treated with the mixture of EGF/IFNγ was considerably prolonged. Our results indicate the importance of inflammatory cytokines and EGF in gene regulation of plasminogen activation system in astrocytoma cells.

**Key words:** IL-1, IFNγ, EGF, PAI-1, u-PA, t-PA, PN-1

**INTRODUCTION**

Plasminogen activation system is a complex and dynamic mixture of zymogens, proteinases and their inhibitors that participate in inflammatory and repair processes occurring in several tissues, including the central nervous system. Plasminogen activators (t-PA - tissue-type plasminogen activator, and u-PA - urokinase-type plasmonogen activator) and inhibitors (PAI-1 - plasminogen activator inhibitor-1, PN-1 - protease nexin-1) are involved in clotting and fibrinolysis, inflammation and tissue remodelling (1)

Plasmin participates in the degradation of extracellular matrix and basement membranes, either directly or indirectly through activation of other zymogens
and metalloproteinases. Plasmin also activates cellular growth factors (1) and enhances secretion of PAI-1 by astrocytes (2). Both u-PA and t-PA activate plasminogen; however, t-PA is primarily implicated in fibrinolysis because of its strong affinity for fibrin, whereas u-PA, bound to its cellular receptor (u-PAR), is associated with tissue remodelling and cell invasion: urokinase-transfected cells show increased ability to promote axon regeneration by changing the matrix components (3). It was also reported that binding of u-PA to its receptor facilitates cell migration not only by plasmin generation but also via a non-proteolytic mechanism with the JAK/STAT signal transduction pathway activated during this process (4).

The components of the plasminogen activator system in astrocytes are modulated by inflammatory cytokines, such as interleukin-1β (IL-1β) and tumour necrosis factor-α (TNFα) (5, 6). We have recently found that also epidermal growth factor (EGF) activates synthesis of u-PA, t-PA and PAI-1 in human astrocytoma cells U373, and its effect on u-PA and PAI-1 is further increased by TNFα and IFNγ (7). Here we demonstrate that cytokine-induced changes in the level of specific mRNAs correlate with changes of the contents of functional proteins, and the synergistic effect of EGF and IFNγ is reflected by the kinetics of activation of STAT-1 signalling pathway.

MATERIALS AND METHODS

Cells culturing and stimulation

Human astrocytoma U373-MG cell line was obtained from American Type Culture Collection (Rockville, MD, USA) and grown in Dulbecco’s modified Eagle medium supplemented with 10% fetal calf serum (FCS). Before the experiments cells were transferred to FCS-free medium and stimulated with 25 ng/ml EGF, 200 U/ml IFNγ, 5 ng/ml IL-1β, 25 ng/ml OSM, 25 ng/ml LIF (all from R&D System Inc., Minneapolis, MN, USA), 100 U/ml IL-4, 100 U/ml IL-13, 100 U/ml IL-10 (all as a gift from Dr. R. de Waal Malefyt, DNAX Research Institute, Palo Alto, CA, USA).

RNA preparation and Northern blot analysis

Total RNA was prepared using the phenol extraction method (8). Samples of RNA (5 μg) were subjected to formaldehyde gel electrophoresis (9) and transferred to Hybond-N membranes (Amersham) according to manufacturer’s instructions. The filters were prehybridized at 68°C for 3 h in 10% dextran sulphate, 1 M sodium chloride and 1% SDS and hybridized in the same solution with cDNAs of PAI-1, u-PA, t-PA and PN-1 labelled by random priming (10). After hybridization, nonspecifically bound radioactivity was removed by washing in 2 x SSC at room temperature followed by 2 washes in 2 x SSC and 1% SDS at 68°C for 20 min. Finally, filters were exposed to X-ray film and obtained picture evaluated by densitometry in a BioRad scanner.
**Gelatin zymography of plasminogen activator activity**

U373 cells were stimulated in FCS-free medium with 25 ng/ml EGF and 200 U/ml IFNγ. Samples of conditioned media were separated in 10% SDS-polyacrylamide gels containing gelatin (0.2%) and plasminogen (12 µg/ml) as substrates for plasmin and plasminogen activator, respectively. The gel was then washed twice for 30 min with 2.5% Triton X-100 to remove SDS and further washed for 10 min with 0.1 M Tris buffer pH 8.0. The buffer was replaced by the reaction buffer containing 0.1 M Tris pH 8.0 with 0.05% Triton X-100 and the gel incubated for 14 h at 37°C to allow proteolysis to occur. Gels were then stained with Blue Amido Black.

**Nuclear extract preparation and EMSA**

Whole cell extracts were prepared as described by Sadowski et al (11). The SIEm67 oligonucleotide interacting with STAT-1 was labelled by filling in 5’ protruding ends with Klenow enzyme and [α-32P]dCTP using standard procedures (9). Gel retardation assays were carried out according to Sawadago et al (12) with 2-5 µg of nuclear extract proteins and approx. 10 fmol (10,000 cpm) of the probe.

**RESULTS**

In agreement with earlier reports (5-7) we found that IL-1 and IFNγ-treated U373 cells show increased levels of mRNAs coding for inhibitors PAI-1 and PN-1 while mRNAs of plasminogen activators (t-PA and u-PA) are much less affected (Fig. 1). In distinction, EGF treatment resulted in enhanced expression of PAI-1, u-PA and t-PA mRNAs, but not of PN-1. Simultaneous stimulation of cells with EGF and IFNγ greatly increased PAI-1 mRNA to the level observed after IL-1 treatment.

Since anti-inflammatory cytokines IL-4, IL-13 and IL-10 are known to influence the expression of several genes stimulated by IL-1 (for references see 13) we tested the preparations of these cytokines in astrocytoma cells. As shown in Fig. 2 IL-4, IL-13 and IL-10 alone or in combination with IL-1 or EGF had no effect on the expression of PAI-1 whereas IL-4 enhanced stimulatory activity of IL-1 on u-PA. Almost identical results were obtained in another experiment when the anti-inflammatory cytokines were added immediately before IL-1 or EGF. It should be added that also cytokines from IL-6 family: leukemia inhibitory factor and oncostatin M did not modulate the expression of the examined components of plasminogen activation system (data not shown).

The synergistic effects of EGF and IFNγ on PAI-1 expression shown in Fig. 1 was examined in detail. Our previous results indicate that the maximum accumulation of PAI-1 mRNA in U373 cells occurs between 12 and 24 h while a similar maximum for u-PA mRNA is observed earlier, between 4 and 12 h (7). This would indicate that in astrocytoma cells stimulated with EGF/IFNγ the initial increase in proteolytic activity
elicited by u-PA is followed by a rise of antiproteases (PAI-1). To test this hypothesis we carried out zymography of conditioned media from U373 cells stimulated with EGF/IFNγ (Fig. 3). We observed a temporary increase in u-PA activity at 6-8 hours that declined to the control level at 18 h (the fast band of approx. 50 kDa). Simultaneously, at 6 h after EGF/IFNγ stimulation, increasing amounts of u-PA/PAI-
1 complexes appeared reaching maximum at 26 h (the slow band of approx. 100 kDa in Fig. 3). The kinetics of formation of these complexes was well correlated with changes in PAI-1 mRNA and increased accumulation of PAI-1 protein as indicated by Western blot (7).

Binding of EGF to its receptor leads to activation of multiple signal transducing components, including Ras-MAPK, PLCγ, JAK-STAT, PI3-K and SHP-2 (14) while IFNγ is known to activate strongly the JAK-STAT pathway, and especially STAT-1 (15). We have studied activation of STAT factors in response to EGF and IFNγ given alone or in a mixture (Fig. 4). Treatment of U373 cells with IFNγ resulted in rapid activation of STAT-1 binding to the SIE probe but after 1 h the level of active STAT-1 declined to almost control values at 6 h. In contrast, stimulation of cells with EGF alone did not activate STAT-1 binding activity. When EGF and IFNγ were added together to U373 culture, rapid activation of STAT-1 was also observed; however, in this case binding to SIE probe was more prolonged than that found after stimulation with IFNγ alone (Fig. 4). We conclude that activation of gene transcription by EGF in U373 astocytoma cells is achieved by a mechanism not involving directly STAT-1, although EGF may prolong activation of STAT-1 by IFNγ. Such mechanism may explain observed in our experiments synergistic enhancement of PAI-1 gene transcription by EGF/IFNγ.
Fig. 3. Time-dependent changes of proteolytic activity corresponding to plasminogen activator system determined by zymography of media from U373 cells stimulated with EGF and IFNγ. Cells were stimulated with 25 ng/ml EGF and 200 U/ml IFNγ as described. Cell culture medium was collected at indicated times (3 - 26 h) and tested for plasminogen activation by zymography in a gel containing gelatin and plasminogen (cf Methods). Bands corresponding to digested gelatin indicate the appearance of active plasmin. The slow band (approx. 100 kDa) most likely represents the complex of plasminogen activators and inhibitors (uPA-PAI-1) while free uPA migrates at approx. 50 kDa. a) Gel containing gelatin and plasminogen; b) Gel containing gelatin only.
DISCUSSION

The results presented in Fig. 1 extend earlier observations of other authors suggesting that components of plasminogen activator system are regulated by inflammatory cytokines, such as IL-1, TNFα and IFNγ (6, 16, 17). We describe here complex and specific regulation of expression of two activators (u-PA and t-PA) and two inhibitors (PAI-1 and PN-1) of plasminogen system by cytokines and EGF in astrocytoma cells. This may be relevant to local changes of cytokine contents in the sites of inflammation, especially in central nervous system (18). Treatment of U373 cells with IL-1 results in more than 10-fold increase in steady-state levels of PAI-1 mRNA (Fig. 1). In agreement with other authors (19) we also observed 2 transcripts of PAI-1 mRNA differing in size (cf. Fig. 2). Since changes in a slowly migrating
component of approx. 3.2 kb were more consistent we used it for densitometric analysis. IL-1 also stimulated expression of another inhibitor - PN-1, whereas the two plasminogen activators, u-PA and t-PA, were mainly regulated by EGF (Fig. 1). However, the final outcome of cytokine stimulation was considerably altered in mixtures of IL-1, EGF and IFNγ.

The conclusions from our experiments based on Northern blot analysis were confirmed by protein determination using Western blot (7) and zymography (Fig. 3). Thus it appears that regulation of gene expression of plasminogen activator system in U373 cells occurs predominantly at the transcriptional level with inflammatory cytokines (IL-1, IFNγ) or cellular growth factors (EGF) as the major players.

Our results provide further support for the anti-proteolytic role of IL-1 in regulation of plasminogen activation system: we show here that IL-1β induces both PAI-1 and PN-1, and down-regulates t-PA synthesis. However, it should be noted that plasminogen generation influences not only degradation of extracellular matrix and cell migration but also several other processes, including NO production. It seems that IL-1 plays the central role in the regulation of NO synthesis within the central nervous system in plasmin-dependent and plasmin-independent manner. The IL-1-induced up-regulation of PAI-1 and PN-1 expression leads to suppression of plasmin-dependent TGFβ generation that is known to inhibit endotoxin-induced NO production (20). Simultaneously, IL-1 has been shown to increase iNOS mRNA expression in astrocytes with IFNγ being the most potent modulator of this process (21). We report here that IFNγ stimulates the expression of PN-1 but inhibits expression of t-PA, which has been reported to be crucial for inhibition of NO production in glial cells. Therefore, we propose a new putative mechanism responsible for the regulation of NO production within the central nervous system.

In distinction to the effects of IL-1 and EGF we found that anti-inflammatory cytokines, IL-4, IL-13 or IL-10, are not so important in modulating basal and induced expression of plasminogen components in U373 cells. The only clear effect of IL-4 was enhancement of IL-1-induced u-PA expression (Fig. 2), in agreement with the results described earlier by Wojta et al. (22) in microvascular human endothelial cells and Paysant et al. (23) in human monocytes. The increased level of u-PA may not necessarily lead to enhanced cell adhesion accompanying inflammation since IL-4 and IL-13 were reported to decrease monocyctic receptors uPAR (23).

The highly complex regulatory mechanisms of the plasminogen activator system point out to necessity of careful extrapolation of the results obtained in vitro with a specific cell line to the situation occurring in tissues in vivo.

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