Stimulation of urokinase expression by TNF-α requires the activation of binding sites for the AP-1 and PEA3 transcription factors

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Abstract

The urokinase-type plasminogen activator plays a central role in tissue remodeling by controlling the synthesis of the extracellular matrix-degrading plasmin. Urokinase expression is transcriptionally regulated by a variety of cytokines including TNF-α. The present study was undertaken to identify key transcription factor binding sites in the urokinase promoter necessary for the TNF-α-dependent induction of urokinase expression. TNF-α treatment of a squamous cell carcinoma cell line, UM-SCC-1, which produces no detectable TNF-α, led to a dose-dependent increase in urokinase secretion, thus reflecting a more abundant mRNA. Transient transfections of UM-SCC-1 cells with a CAT reporter driven by 5' deletion fragments of the urokinase promoter indicated that a sequence spanning -2109 to -1870, which contained binding sites for AP-1 and PEA3 was required for the stimulation by TNF-α. Mutation of an AP-1 binding site at -1967 and a PEA3 motif at -1973 completely abrogated the inductive effect of TNF-α on urokinase promoter activity. Mobility shift assays indicated the presence of a jun-containing factor(s) which bound specifically to the AP-1 sequence present in the urokinase promoter. The amount and/or activity of this factor(s) was greatly enhanced by TNF-α treatment. UM-SCC-1 cells transiently transfected with a CAT reporter driven by 3 tandem AP-1 binding sites demonstrated increased CAT activity following TNF-α treatment. Thus, the induction of urokinase expression by TNF-α is likely to involve the altered expression and/or activity of transcription factors which bind to the AP-1 and PEA3 target sequences in the urokinase promoter.

Keywords: TNF-α; Urokinase promoter; AP-1; PEA3

1. Introduction

The urokinase-type plasminogen activator (u-PA) plays a central role in tissue remodeling in a number of physiological and pathological processes including tissue repair, pregnancy, angiogenesis and tumor cell invasion [1,2] by mediating the conversion of the inert zymogen plasminogen into the widely acting serine protease plasmin [3]. Plasmin in turn degrades several extracellular matrix components including laminin and fibronectin [4] and there is some evidence that type IV collagenase may be activated by this serine protease [5].

Urokinase expression is modulated by a variety of growth factors/cytokines including TNF-α [6-8]. TNF-α is a multifunctional Mr 17 kDa cytokine produced primarily by activated macrophages usually in response to inflammatory products and invasive stimuli [9]. This cytokine was initially identified as a mediator of hemorrhagic necrosis of tumors and subsequently found to have a number of biological effects including modulation of growth and differentiation [10,11]. The gene for TNF-α encodes a prohormone that is first inserted into the cell membrane and subsequently cleaved to generate a soluble 17 kDa polypeptide [11]. Trimeric TNF-α binds with equal affinity to two separate and ubiquitously expressed TNF-α receptors (p55 and p75) which are readily distinguished by their divergent cytoplasmic tails [12]. The interaction of TNF-α with its receptor(s) leads to receptor clustering followed by the stimulation of one, or multiple, signal transduction pathways including protein kinase C, protein kinase A and sphingomyelin [9,13,14]. Although the increased production of urokinase by TNF-α has been ascribed to an increased rate of transcription of the plasminogen activator gene, the mechanism by which the promoter is activated in trans has not been investigated. Consequently, we undertook a study to determine the
transcriptional requirements for augmented urokinase promoter activity in response to TNF-α.

2. Materials and methods

2.1. Materials

Recombinant human TNF-α was obtained from Genentech, San Francisco, CA. The urokinase antibody (#389) used in Western blotting was from American Diagnostica, Greenwich, CT. The *jun* Ab (SC 44 × ) antibody, which cross-reacts with *c-jun, jun B* and *jun D*, was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Cell culture

UM-SCC-1 cells were propagated in McCoys 5A medium supplemented with 10% FBS. These cells do not produce any detectable TNF-α as determined by a bioassay on L929 cells [15]. For the collection of conditioned medium, the cells were washed extensively and changed to serum-free medium (McCoys’ 5A supplemented with 4 μg/ml transferrin, 5 μg/ml insulin and 10 ng/ml EGF and 0.1 w/v %BSA) containing TNF-α.

2.3. Western blotting

UM-SCC-1 cells were grown to 70% confluency in 10% FBS and, after extensive washing with serum-free medium, changed to serum-free medium supplemented as needed with TNF-α. The cells were incubated for 24–36 h and the conditioned medium harvested and clarified. The amount of urokinase in the conditioned medium was determined by Western blotting [16]. Briefly, conditioned medium from equal numbers of cells was denatured in the absence of reducing agent and electrophoresed in a 12.5% SDS-PAGE gel. The resolved proteins were transferred to a nitrocellulose filter. The filter was blocked with 3% BSA and incubated sequentially with a polyclonal antibody to urokinase #389 (American Diagnostica, Greenwich, CT) and a horse radish peroxidase conjugated anti-rabbit IgG. Immunoreactive bands were visualized by ECL as described by the manufacturer (Amersham, Arlington Heights, IL).

2.4. Northern blotting

UM-SCC-1 cells at 70% confluency, treated with TNF-α as described above, were extracted for total RNA with 5.0 M guanidinium isothiocyanate. The RNA was purified on a cesium chloride cushion (5.7 M) by ultracentrifugation. The RNA was purified on a cesium chloride cushion (5.7 M) by ultracentrifugation. Purified RNA was electrophoresed in a 1.5% formaldehyde-agarose gel and transferred to Nytran® modified nylon by capillary action. The Northern blot was probed at 42°C with a random-primed, [32P]-labeled 1.5 kb cDNA specific for the urokinase mRNA [17] and subsequently washed at 65°C using 0.5 × SSC in the presence of 1.0% SDS. Loading efficiencies were checked by reprobing the blot with a radioactive cDNA which hybridizes with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

2.5. Chloramphenicol acetyl transferase (CAT) assays

The urokinase promoter CAT constructs used in the present study have been described elsewhere [18–20]. The AP-1 thymidine kinase CAT reporter used was described previously [21]. UM-SCC-1 cells, at 70% confluency, were co-transfected with urokinase promoter-CAT reporter constructs and 5 μg of an expression vector bearing the β-galactosidase gene. Briefly, DNA, in 1 ml of HBS (pH 7.44) buffer (Hepes, 25 mM; MgCl2, 1 μM; CaCl2, 0.1 μM; NaCl, 0.1 M; KCl, 5 μM; and Na2HPO4, 0.7 μM), was mixed with 50 μg hexadimethrine bromide (Aldrich Chemicals, Wisconsin) and added to the cells in 10% FBS. After 5 h, the cells were shocked for 3 min with 33% DMSO and cultured for 12 h after which the transfected cells were treated with TNF-α. After an additional 36 h, the cells were harvested and lysed by repeated freeze-thaw cycles in a buffer containing 0.25 M Tris-Cl (pH 7.8). Transfection efficiencies were determined by assaying for β-galactosidase activity. CAT activity was subsequently measured by incubating cell lysate (normalized for transfection efficiency) at 37°C for 8 h with 4 μM [14C]chloramphenicol and 1 mg/ml acetyl coenzyme A. After 3 h, the acetyl CoA in the reaction mixture was replenished. The mixture was extracted with ethyl acetate and acetylated products subjected to thin layer chromatography using chloroforoform:methanol (95:5) as a mobile phase. The conversion of [14C]chloramphenicol was quantified with a 603 Betascope.

2.6. Mobility shift assays

TNF-α-treated and untreated cells were collected by centrifugation, washed and suspended in a buffer containing 10 mM Hepes (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT and 0.5 mM PMSF. After 15 min on ice, the cells were vortexed in the presence of 0.6% Nonidet NP-40. The nuclear pellet was collected by centrifugation and extracted in a buffer containing 20 mM Hepes (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 1 mM PMSF for 15 min at 4°C. UM-SCC-1 nuclear extract (8 μg) from TNF-α-stimulated and unstimulated cells was preincubated at 4°C for 30 min with or without, 2 μg of a *jun* antibody followed by an incubation at 4°C for 20 min in a buffer (25 mM Hepes buffer (pH 7.9), 0.5 mM EDTA, 0.5 mM DTT, 0.05 M NaCl and 2.5% glycerol) containing 5 fmol (2 × 10^4 cpm) of a Klenow end-labelled (32P-ATP) 30-mer oligonucleotide spanning the AP-1 binding site at −1967 of the
urokinase promoter. Parallel incubations included the wild type (TTCTTTGTCCAGGAGGAAATGAGTCATCTG) and mutated (TTCTTTGTCCAGGAGGAAATCCAGAGTCTG) (lower case letters indicate the mutated nucleotides) AP-1 competitor sequence at a 100-fold excess. To reduce non-specific probe interactions, all incubations were carried out in the presence of 2.0 μg of poly dI.dC. At the end of the incubation period, the reaction mixture was electrophoresed at 4°C in a 6% polyacrylamide gel using a 0.5 × TBE running buffer.

3. Results

3.1. TNF-α increases the amount of urokinase protein / mRNA in UM-SCC-1 cells

A dose-dependent increase in urokinase secretion was evident with UM-SCC-1 cells treated with TNF-α (Fig. 1A). This increase was not secondary to increased growth rate since we could find no evidence of augmented proliferation of UM-SCC-1 cells in the presence of TNF-α.

Northern blotting (Fig. 1B,C) indicated that the increased secretion of urokinase by TNF-α-stimulated UM-SCC-1 cells was a consequence of a larger amount of steady-state mRNA encoding the plasminogen activator.

3.2. Induction of urokinase promoter activity by TNF-α requires 2109 bp of 5′ flanking sequence

We undertook experiments to identify the region of the urokinase promoter required for its stimulation by TNF-α. UM-SCC-1 cells were transiently transfected with a CAT reporter driven by the full length (2345 bp) or 5′ deletion fragments of the urokinase promoter. The transfected cells were subsequently treated with 250 ng/ml of TNF-α for 48 h. After this time, the cells were harvested and assayed for CAT activity. TNF-α treatment of UM-SCC-1 cells transiently transfected with the full length (2345 bp) urokinase promoter-CAT reporter increased [14C]chloramphenicol conversion from 17 to 37% (Fig. 2). A similar augmentation was observed using 2109 bp of the 5′ flank-

![Fig. 1. TNF-α induces urokinase protein and mRNA levels. UM-SCC-1 cells at 70% confluency, were washed extensively and changed to serum-free medium supplemented with, or without, the indicated concentration of TNF-α for 36 h. After this time, the conditioned medium was collected, clarified by centrifugation and the cells counted. Aliquots of the conditioned medium, normalized for cell number, along with authentic urokinase standard (rSC-u-PA) was subjected to Western blotting using a polyclonal antibody (panel A). mRNA was extracted from a parallel flask treated in an identical manner and purified on a CsCl cushion. The purified RNA was electrophoresed in a 1.5% formaldehyde-agarose gel and subsequently transferred to a Nytran-modified nylon filter. The filter was probed with cDNAs specific for the urokinase and GAPDH transcripts (panel B). Radioactivity was quantified using a 603 Betascope and the results are shown in panel C. The experiment was carried out twice.](image-url)
uPA Promoter CAT Constructs

Fig. 2. Stimulation of the urokinase promoter by TNF-α requires 2109 bp of 5’ flanking sequence. UM-SCC-1 cells were transiently transfected with a DNA mixture of a CAT reporter (10 µg) driven by the indicated fragment of the urokinase promoter, 5 µg of a β-galactosidase expression vector and 50 µg of hexadimethrine bromide. After 5 h, the cells were shocked for 3 min with 33% DMSO. The UM-SCC-1 cells were incubated for a further 12 h, after which the cells were treated with 250 ng/ml of TNF-α for a period of 48 h. The cells were harvested, lysed by repeated freeze-thaw cycles and assayed for β-galactosidase activity. Aliquots, adjusted for transfection efficiency, were incubated at 37°C for 8 h with 4 µM [14C]chloramphenicol and 1 mg/ml acetyl coenzyme A. The mixture was subsequently extracted with ethyl acetate and the acetylated products subjected to thin-layer chromatography using chloroform:methanol (95:5) as a mobile phase. The conversion of [14C]chloramphenicol (graph) was quantified using a 603 Betascope. The data are typical of more than 4 separate experiments.

Fig. 3. Stimulation of urokinase promoter activity by TNF-α requires binding sites for AP-1 and PEA3. UM-SCC-1 cells were transiently transfected with a CAT reporter driven by the full-length (2345 bp) wild type urokinase promoter (wt) or the mutated promoter as described in the legend to Fig. 2. The cells were subsequently treated with (+), or without (−), 250 ng/ml TNF-α for 36 h. The cells were harvested, lysed and assayed for CAT activity following normalization of β-galactosidase activities. A schematic of the urokinase promoter spanning −1973 to −1885 is shown at the right with the blocked areas representing mutations. The data are representative of 4 separate experiments.
ing sequence of the urokinase gene. In contrast, \[^{14}\text{C}]\text{chloramphenicol conversions in UM-SCC-1 cells transiently transfected with the CAT reporter driven by 1870 bp of the urokinase promoter was not altered in response to TNF-\(\alpha\). These data suggest that the sequence residing between \(-2109\) and \(-1870\) is critical for the stimulation of urokinase promoter activity by TNF-\(\alpha\). The modest changes in CAT activity seen in TNF-\(\alpha\)-stimulated cells, when compared with those seen by Western and Northern blotting (Fig. 1), may reflect two factors. First, there is a basal activity of the urokinase promoter in the unstimulated UM-SCC-1 cells utilized in the present study. Secondly, a limited supply of transactivators in the TNF-\(\alpha\)-treated cells may be insufficient to stimulate the multiple copies of the exogenous urokinase promoter-CAT reporter introduced into the cells by transfection. Notwithstanding these possibilities, these data suggest that the elevation of urokinase mRNA and protein by TNF-\(\alpha\) in UM-SCC-1 cells reflects, at least in part, increased urokinase promoter activity and requires a region of the promoter residing between \(-2109\) and \(-1870\).

3.3. Binding sites for AP-1 and PEA3 in the urokinase promoter are required for its stimulation by TNF-\(\alpha\)

The expression of c-\text{jun}/c-fos and c-ets, which are involved in transcriptional control via AP-1 and PEA3 binding sites respectively [22,23], is induced by TNF-\(\alpha\) [24–26]. With this in mind, we undertook experiments to determine if target sequences for AP-1 and PEA3 residing between \(-2109\) and \(-1870\) were required for the stimulation of the urokinase promoter by TNF-\(\alpha\). Mutations of the PEA3 (mutant A) and AP-1 (mutant B) motifs at \(-1973\) and \(-1967\) reduced the basal level of the urokinase promoter in UM-SCC-1 cells and abrogated the inductive effect of TNF-\(\alpha\) (Fig. 3). Additionally, a sequence (\(-1947\) to \(-1941\)) in the promoter further downstream was also critical for increased urokinase promoter activity. Thus,

\[\begin{align*}
\text{nuclear extract} & \quad \text{wt AP-1 competitor} \\
\text{- ++ + + + + + +} & \quad \text{- + - - + + + + +} \\
\text{- - - - + - - +} & \quad \text{- - - - + - - +} \\
\text{jun Ab} & \quad \text{mutated AP-1 competitor}
\end{align*}\]

Control +TNF-\(\alpha\)

Fig. 4. TNF-\(\alpha\) stimulates AP-1-binding activity in UM-SCC-1 cells. UM-SCC-1 nuclear extract from TNF-\(\alpha\)-treated, or untreated cells, was preincubated at 4°C for 30 min with, or without 2 \(\mu\)g of a \text{jun} antibody (jun Ab) and then for 20 min with 5 fmol (2 \(\times\) 10⁴ cpm) of an end-labelled (\(^{32}\text{P}\)-ATP) 30-mer oligonucleotide spanning the AP-1 binding site at \(-1967\) of the urokinase promoter. Parallel incubations included a 100-fold excess of an unlabelled oligonucleotide which was either identical to the radioactive probe (wt AP-1 competitor) or which had been point mutated within the AP-1 site (mutated AP-1 competitor). After this time, the reaction mixture was electrophoresed in a 6% polyacrylamide gel using a 0.5 \(\times\) TBE running buffer. The experiment was carried out 3 times.
mutation of this region (mutant F) also led to a reduction in basal activity of the promoter as well as an abolition of the TNF-α-induced response.

3.4. TNF-α stimulates AP-1-binding activity in UM-SCC-1 cells

Since the stimulation of the urokinase promoter by TNF-α could be abrogated by a point mutation of the AP-1 binding site at -1967, we speculated that an increase in AP-1-binding activity brought about by TNF-α could underscore the increased expression of the protease in response to this cytokine. To address this, nuclear extract from TNF-α-treated and untreated cells was incubated with an end-labelled radioactive oligonucleotide probe (Fig. 4) containing the AP-1 sequence (at -1967) present in the urokinase promoter. The reaction mixture was subsequently subjected to gel electrophoresis. An oligonucleotide-containing complex (solid arrow) which could be competed out with an excess of unlabelled oligonucleotide was observed with nuclear extract from untreated UM-SCC-1 cells. The intensity of this band was greatly enhanced with nuclear extracts generated from TNF-α-stimulated cells. The inability of an oligonucleotide sequence, which was mutated in the AP-1-binding region of the probe, to compete with the radioactive oligonucleotide for the binding of the nuclear factor(s) indicated the specificity of this association. A supershifted complex (open arrow) was also evident when nuclear extract was pre-incubated with an antibody which recognizes jun-family members. The intensity of the super-shifted band was substantially higher with nuclear extracts from TNF-α-stimulated UM-SCC-1 cells. These data suggested that TNF-α increases the amount of AP-1-binding activity in UM-SCC-1 cells.

3.5. TNF-α stimulates a reporter construct driven by AP-1 tandem repeats

To determine if the increase in AP-1-binding activity brought about by TNF-α treatment could stimulate promoter activity, UM-SCC-1 cells were transiently transfected with a CAT reporter driven by either a thymidine kinase minimal promoter (PBL CAT) or 3 tandem AP-1 repeats upstream of the minimal promoter (3 X AP-1 PBL CAT) [21]. The cells were subsequently treated with TNF-α and assayed for CAT activity (Fig. 5). A weak activation of the minimal promoter, which was not changed by TNF-α treatment, was observed with UM-SCC-1 cells transiently transfected with the thymidine kinase minimal promoter CAT construct (PBL CAT). In contrast, cultivation of the UM-SCC-1 cells transfected with the AP-1-
driven promoter (3 × AP-1 PBLCAT) with TNF-α elevated CAT activity more than 4-fold over that achieved with untreated cells. These data suggest that the induction of AP-1-binding activity by TNF-α apparent in the mobility shift assays is sufficient to stimulate an AP-1-containing minimal promoter.

4. Discussion

The stimulation of urokinase production in endothelial cells and malignant cell types by TNF-α has been reported previously [6,7,27] and is considered to be a critical factor in tissue remodeling as evident in tumor cell invasion and angiogenesis. The elevated production of the protease by this cytokine in pulmonary epithelial cells has been ascribed to increased transcriptional activity as shown by nuclear run on experiments [28]. We have extended these findings, herein, by showing that the induction of urokinase expression by TNF-α in a squamous cell carcinoma cell line is mediated, at least in part, by binding sites for AP-1 (−1967) and PEA3 (−1973) upstream of the urokinase transcriptional start site.

The AP-1 transcription complex is comprised of heterodimers of jun and fos family members and to a lesser extent homodimers of jun [29,30]. Binding of AP-1 to its cognate sequence leads to altered transcriptional activity. In fact, several observations suggest that modulation of the activity and/or amount of AP-1 is responsible for the increased level of urokinase mRNA/protein evident in TNF-α-stimulated UM-SCC-1 cells. Thus, the binding of the AP-1 complex to its recognition sequence at −1967 has been shown to be critical for the inductive effect of EGF and phorbol ester on urokinase expression [18,20]. Additionally, the induction of c-jun and c-fos expression by TNF-α leads to altered expression of AP-1-containing genes including the chemoattractant JE, elastin and collagenase [31–33]. Indeed, in an earlier investigation, differential screening of cDNA libraries had revealed the c-jun transcription factor to be an overexpressed gene in TNF-α-stimulated human umbilical endothelial cells [25]. These observations were consistent with our gel retardation experiments which showed that the AP-1 activity in UM-SCC-1 cells, which was increased by TNF-α treatment, contained a jun protein(s). Thus, put together, these findings suggest that the effect of TNF-α on urokinase expression is mediated, at least in part, via the modulation of the amount and/or expression of AP-1.

Our study demonstrates that a PEA3 binding site (located at −1973) is also required for the stimulation of urokinase expression by TNF-α. The PEA3 binding site recognizes multiple members of the c-ets family of transcription factors [23] and is required for the basal expression of the plasminogen activator in a number of cell lines [20]. Further, this sequence participates in the induction of urokinase expression in EGF-and phorbol ester-stimulated murine keratinocytes [18,20]. Presumably, TNF-α increases the amount and/or activity of a PEA3-binding protein, this leading to increased urokinase promoter activity. This contention is supported by reports from other laboratories. Firstly, the amount of c-ets mRNA in confluent human umbilical vein endothelial cells was transiently increased by this cytokine [34]. Secondly, and perhaps more importantly, the ability of TNF-α to modulate the expression of member(s) of the c-ets family appeared to be critical for the repression of thrombomodulin expression in TNF-α-stimulated endothelial cells [26].

While the induction of urokinase expression by TNF-α in UM-SCC-1 cells requires transcription factor binding sites for PEA3 and AP-1, the early events in this signal transduction pathway remain to be elucidated. Since the transcription factors, which bind to these promoter sites, have been characterized as effectors of protein kinase C, it is likely that this signal transduction pathway is involved in the TNF-α stimulation of urokinase expression. Indeed, other investigators have demonstrated the requirement for the protein kinase C pathway in the actions of TNF-α. Thus, Hanazawa et al. [32] demonstrated that the induction of the monocyte chemoattractant JE by TNF-α required PKC since this stimulation could be abrogated by a prior desensitization of the PKC pathway. Likewise, the induction of urokinase expression in endothelial cells by TNF-α was blocked with staurosporine and calphostin-C [14], thus testifying to the involvement of protein kinase C.

Although the transcriptional requirements for TNF-α and phorbol ester stimulation of urokinase expression are similar, there are distinct differences which are worth comment. Most prominent is the role of a region of the urokinase promoter which contains the nuclear inhibitor protein (NIP) binding motif, previously characterized as a regulatory component of the human interleukin-3 and stromelysin promoters [35,36]. Mutation of this sequence substantially reduced the stimulation of the urokinase promoter by phorbol ester [18]. In contrast, mutation of this transcription factor binding site (mutant I), did not attenuate the induction of urokinase promoter activity by TNF-α in our cells. In fact, the stimulation appeared to be augmented by this mutation. One possible explanation is that this sequence may contain a repressor motif for TNF-α stimulation which, upon mutation, superinduces the TNF-α response. This key difference in transcriptional requirements for TNF-α and phorbol ester stimulation may be explained in either of two ways. Firstly, it is possible that the stimulation of urokinase expression by TNF-α in UM-SCC-1 cells involves both protein kinase C-dependent and -independent pathways. Alternatively, it may be that the protein kinase C pathways are distinct in the separate cell lines used in the two studies.

In summary, we have demonstrated that the stimulation of urokinase expression by TNF-α in a squamous carcinoma cell line is mediated, at least in part, by members of the PEA3 and AP-1 transcription factor families.
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