Increase in Urokinase Plasminogen Activator mRNA Synthesis in Human Carcinoma Cells Is a Primary Effect of the Potent Tumor Promoter, Phorbol Myristate Acetate

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Abstract. The effect of tumor promoters and growth factors on the synthesis of urokinase and urokinase mRNA in human carcinoma cells has been investigated. In urokinase-producing human carcinoma cells (A1251), a 20-40-fold increase in urokinase mRNA level is obtained after treatment with 10 nM phorbol myristate acetate (PMA), a smaller effect (two- to fourfold) with 2 ng/ml platelet-derived growth factor (PDGF) and no effect with epidermal growth factor (EGF) (up to 50 nM). After treatment with PMA, urokinase mRNA level increases already at 30 min peaking 2-4 h thereafter.

Cell line A431, which has an abnormally high number of EGF receptors, shows the same response to PMA, but also responds to EGF (two- to fourfold increase in mRNA). The kinetics are similar to those of A1251. Nuclear transcription experiments show that the PMA-induced increase in urokinase mRNA is due to increased synthesis.

The protein synthesis inhibitor, cycloheximide (10 µg/ml), also increases the level of urokinase mRNA. When both cycloheximide and PMA are used, superinduction is observed. This result may indicate that a short-lived protein negatively regulates the level of urokinase.

The different efficiency of the effectors (PMA and PDGF better than EGF) and their kinetics, as well as the effect of cycloheximide on urokinase mRNA synthesis, (a) are reminiscent of the effect of PDGF and PMA on competence phase genes (Kelly, K., B. H. Cochran, C. D. Stiles, and P. Leder, 1983, Cell, 35:603-610), (b) demonstrate that the synthesis of urokinase is part of the early cellular response to these factors, and (c) provide a preliminary insight in the overproduction of urokinase by primary malignant tumors and transformed cells in culture.

Urokinase is one of the two well-characterized types of plasminogen activator (PA), enzymes that catalyze plasminogen-dependent fibrinolysis. In cell culture and in vivo, modulation of PA level has been shown to occur under a variety of physiological and experimental stimuli, indicating that these enzymes may play a central role in controlling extracellular proteolysis (reviewed in reference 40). The human urokinase gene is transcribed into a 2,500-nucleotides-long mRNA (50), the product of which is a single-chain polypeptide of ~50,000 daltons (44).

Increased production of PA is associated with most spontaneous tumors (reviewed in reference 30). PA increase was shown to be an early, transformation-dependent effect of tumor viruses (16, 34, 41, 47). Recent data indicate that urokinase may be a necessary requirement for the metastatic activity of one type of human tumor (33).

Growth factors and receptors are believed to play an important role in oncogenic transformation. Growth factors have been shown to induce PA synthesis in several cell lines (10, 11, 19, 20, 24, 35). Phorbol esters also induce the synthesis of PA in normal and transformed cell lines (11, 14, 18, 39, 49, 52, 53).

In this context, it seems very important to understand the molecular basis of PA induction by growth factors and tumor promoters as a prerequisite for finding the link between PA production and human neoplasia. In this paper, we have investigated the effect of some growth factors and of a tumor promoter on the expression of the human urokinase gene in human carcinoma cells. Our results indicate that synthesis of urokinase mRNA is increased in transformed cells by factors which in normal cells act at the early competence phase like phorbol myristate acetate (PMA) (21), but not by epidermal growth factor (EGF) which acts later in the cell cycle. Analysis of the kinetics of induction shows this to be primary effect of PMA and not a consequence of its activity on cell growth, revealing a close similarity to the induction of the c-myc oncogene by the same factors (21).
Materials and Methods

Materials

EGF (receptor grade) and 3H-EGF were obtained from Collaborative Research Inc. (Lexington, MA); PMA was obtained by LC Services Co. (Woburn, MA). SDS gel electrophoresis–purified platelet-derived growth factor (PDGF) was a kind gift of Dr. Rick Assoyan (Bethesda, MD). Cycloheximide was purchased from Sigma Chemical Co. (St. Louis, MO); [35S]Methionine, [3H]thymidine, and d-2P-deoxyribofuranosyl and 2P-uridine-triphosphates were from the Radiochemical Center (Amersham Corp., UK). A kit from Bethesda Research Laboratories (Rockville, MD) was used for nick translations.

Cell Culture

Cell lines A1251 (42), A431 (13), and the A431 derivative clone 18 (3) were kept in continuous culture at 37°C in a 5% CO2 atmosphere in Dulbecco’s modified essential medium, supplemented with 10% fetal calf serum and glutamine.

For the analysis of urokinase and urokinase mRNA induction, confluent cells were kept 18 h in serum-free medium before any treatment. When cells were labeled with [35S]methionine, induction was carried out in the labeling medium and the cells were incubated 4 or 18 h. Labeling medium consisted of methionine-free medium supplemented with 0.5% serum and [35S]methionine (0.25 mCi/ml). At the end of the incubation, the medium was harvested and centrifuged to remove cell debris, and the cells were lysed as described (54).

For urokinase mRNA quantitation cells were kept for different times in the presence of the effector, in medium containing 0.5% serum.

PA Assay

Plasminogen activator was assayed in Linbro multi-well plates coated with 3H-fibrin (46). Details of the technique are published elsewhere (14, 54). One unit of PA activity is defined as the amount of enzyme that causes the release of 5% of the total trypsin-solubilized radioactivity in 1 h at 37°C. All activity was dependent on the presence of plasminogen. When present, affinity-purified anti-urokinase IgG was used at a concentration of 0.5 μg/ml.

Protein concentration was measured by the method of Lowry et al. (26) using a standard solution of bovine serum albumin.

Immunoprecipitation

Immunoprecipitation of medium or lysate was carried out in immunoprecipitation buffer (10 mM Tris-HCl [pH 7.5], 0.15 M NaCl, 0.1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM EDTA), as described previously (14). Samples were denatured in β-mercaptoethanol and loaded onto a 10% SDS polyacrylamide gel according to Laemmli (22). At the end of the run, the gel was fixed in 10% acetic acid and fluorography performed as described (23).

RNA Extraction and Analysis

Preparation of RNA, electrophoretic analysis, blotting, and conditions for hybridization have been described elsewhere (50). Dot blot hybridization was carried out as described (28, 34). Nick-translated probes were human urokinase cDNA fragments (50) or β2-microglobulin DNA (21, 36).

Thymidine Incorporation

Thymidine incorporation was quantitated measuring the amount of TCA-precipitable [3H]thymidine incorporated in a 2-h pulse.

Nuclear Transcription Assay

The synthesis of urokinase mRNA was assayed in nuclei of A431 cells as described by Vannice et al. (48). 5 × 10⁶ cells were treated with 30 ng/ml PMA for 3 h, the nuclei isolated and transcribed in the presence of 2P-uridine-triphosphate. 10⁶ cpm of RNA were hybridized with 2.5 μg of either pHUK-8 DNA (a 1.5-kb Pst-I fragment of pHUK-1, 29), or to a cDNA clone of class I HLA-I antigen (1.6 kb) (Boncinelli, E. and A. Simeone, unpublished procedures).

Results

Effect of EGF and PMA on Urokinase Synthesis in A431 and A1251 Cells

Two human carcinoma cell lines, A1251 and A431, have been used. A1251 derives from a kidney carcinoma (42), and A431 from an epidermoid carcinoma of the vulva (13). It has already been reported that EGF increases the fibrinolytic activity of A431 cells (18); we observed that PMA increases urokinase synthesis in A1251 cells (14). We now analyze in more detail the induction of PA in these cells by measuring the enzyme activity (Table I) and urokinase protein synthesis (Fig. 1). Table I shows that PMA (10 nM) increases urokinase level of not only A1251 (14), but also of A431 cells. EGF (50 nM) increases urokinase level in A431 cells only and has no effect on A1251 cells. All fibrinolytic activity is plasminogen dependent (data not shown) and is completely inhibited by monospecific antibodies directed towards human urokinase. This applies to both basal and induced levels of the enzyme.

Table I. Fibrinolytic Activity of A431 and A1251 Cells

|                  | A431 cells | A1251 cells |
|------------------|------------|-------------|
| Basal activity   | 955        | 361         |
| Cell lysate      | 367        | 100         |
| Total            | 1,322      | 461         |
| % inhibition by anti-urokinase IgG |        |
| Fold increase by PMA | 4          | 8           |
| % inhibition by anti-urokinase IgG |        |
| Fold increase by EGF | 2          | 1           |
| % inhibition by anti-urokinase IgG |        |

Fibrinolytic activity was measured in the medium or in the cell lysate by the 3H-fibrin assay (47). Confluent cultures of A431 and A1251 were incubated for 8 h in serum-free medium with or without 10 nM PMA or 50 nM EGF. The activity is expressed in arbitrary units and related to equal amounts of cellular proteins as determined by the Lowry method (26). Fold increase is the ratio of the activity measured in the presence and absence of the effectors. Total activity indicates the sum of medium and cell lysate activities measured separately.

* 0.2 μg of affinity-purified rabbit anti-urokinase immunoglobulins.

Figure 1. Immunoprecipitation of [35S]labeled pro-urokinase from A431 cells. Confluent cells were treated with tetradecanoyl-phorbol-acetate (10 nM), EGF (50 nM) or solvent, for 8 h in methionine-free Eagle’s medium and 0.25 mCi/ml [35S]-methionine. An equal number of trichloroacetic acid–precipitable counts from the cells’ medium was immunoprecipitated in each case and applied to a 10% SDS PAGE after reduction with β-mercaptoethanol. Lane M shows the migration of the markers, the molecular weights of which are indicated to the left.
Thus all fibrinolytic activity in both medium and cells is exclusively due to urokinase.

Urokinase secreted by A431 cells has been labeled with [35S]L-methionine and immunoprecipitated with anti-urokinase IgG. A representative immunoprecipitation of urokinase from the medium of A431 cells incubated for 18 h in the absence and presence of EGF (50 nM) and PMA (10 nM) in serum-free medium is shown in Fig. 1. A single band with an apparent molecular mass of ~48,000 daltons is greatly increased upon both treatments. Under the conditions used in this experiment, most of urokinase is present in the medium (data not shown).

As expected from the data of Table I, EGF has no effect on pro-urokinase synthesis in A1251 cells (data not shown). This finding is not due to the absence of EGF receptors from A1251 cell membranes. We have estimated the number of EGF receptors on A1251 and A431 cell membranes by measuring the binding of [125I]EGF at 0°C and its competition by cold EGF. A1251 cells have approximately three- to sixfold less receptors than A431 cells; i.e., a "normal" number of EGF receptors (data not shown). A431 cells have an abnormally high number of EGF receptors (23). A quantitative radio-immunoprecipitation assay (Corti, A., G. Cassani, M. L. Nolli, and F. Blasi, manuscript in preparation) was used to study the kinetics of PMA and EGF effect on pro-urokinase synthesis in A431 cells. In summary, maximal induction reached threefold with 20 nM EGF and sixfold with 10 nM PMA. Induction becomes evident at early times in the cell lysate (1–2 h) and in the medium (4–6 h) (data not shown). From all the data we conclude that the increase in fibrinolytic activity in A431 and A1251 cells is due to an early increase in pro-urokinase synthesis.

Urokinase mRNA Levels in A431 and A1251 Cells Are Increased by EGF, PMA, or PDGF

Total cytoplasmic RNA isolated from confluent cells, starved of serum for 18 h, was electrophoresed through an agarose–formaldehyde gel, blotted onto a nitrocellulose sheet, and hybridized to a nick-translated cDNA fragment covering most of the urokinase mRNA (50). Northern analysis shows (Fig. 2) that PMA (lanes A and B) or EGF (lanes C and D) increase urokinase mRNA in A431 cells. PDGF has no effect (not shown). The higher molecular weight band, 2,500 nucleotides long, is the mature urokinase mRNA (50). A second band, with a migration slightly faster than 18S, is also regularly observed. Whether it corresponds to a degradation product of urokinase mRNA or to a second mRNA species, remains to be investigated. We have previously reported that A1251 cells also respond to PMA by increasing the level of urokinase mRNA (14). We now show that in these cells PDGF has the same effect (lanes E and F). The extent of induction has been estimated in several experiments by densitometer tracing. In A431 and A1251 cells, PDGF increases the urokinase mRNA level by at least 20-fold, while the effect of EGF on A431 or of PDGF on A1251 is only two- to threefold. The specificity of the effect has been tested by hybridizing parallel filters to a murine β2-microglobulin probe (36). No effect on β2-microglobulin mRNA has been observed (data not shown). As shown in Fig. 3, we have studied the effect of EGF and PMA on urokinase mRNA level of clone 18 variant of A431 cells, a derivative showing a 10-fold reduction in the number of EGF receptors (3). While EGF has no effect (not shown), PMA still causes a ~15–30-fold increase in urokinase mRNA.

The kinetics of urokinase mRNA induction by EGF have been measured by a dot-blot hybridization assay (Fig. 4). An ~40-fold increase can be observed 2 h after the addition of EGF to A1251 cells with an at least fourfold increase already evident within 30 min after addition of EGF. Identical results have been obtained with A431 cells (not shown).

Effect of PMA and EGF on Thymidine Incorporation in A1251 Cells

To correlate the effect of PMA and EGF on urokinase mRNA induction with their effect on DNA synthesis, we have investigated the effect of PMA and EGF on thymidine incorporation in A1251 cells under the same conditions in which urokinase induction was studied. 105 A1251 cells, incubated in medium containing 0.5% fetal calf serum for 18 h in 35-
Figure 4. Dot-blot hybridization analysis of the time course of A1251 urokinase mRNA induction by PMA. Confluent A1251 cells were kept 18 h in serum-free medium and then PMA (10 nM) was added. Cells were harvested and total RNA was prepared at zero, 30, or 120 min after addition of PMA. Two quantities of RNA (0.4 and 4 μg, respectively) were filtered in duplicate through a nitrocellulose filter and hybridized to a nick-translated urokinase cDNA clone (see legend to Fig. 2). Numbers to the left indicate the incubation times, in minutes; those at the bottom indicate the amount of loaded RNA.

Table II. Effect of Serum Starvation, PMA, and EGF on Thymidine Incorporation of A1251 Cells

| % Serum in preincubation medium | Treatment | [3H]Thymidine incorporated |
|---------------------------------|-----------|---------------------------|
| 10                              | None      | 1.22                      |
| 0.5                             | None      | 1.35                      |
| 0.5 10% FCS                     |           | 1.26                      |
| 0.5 10 nM PMA                   |           | 0.67                      |
| 0.5 20 nM EGF                   |           | 0.97                      |

* 5 × 10⁴ trypsinized cells were seeded in 35-mm plates in 4 ml with 10% fetal calf serum. After 6 h the medium was changed and cells preincubated for 14 h in medium containing the indicated amounts of serum.

| % Serum in preincubation medium | Treatment | [3H]Thymidine incorporated |
|---------------------------------|-----------|---------------------------|
| 10                              | None      | 1.22                      |
| 0.5                             | None      | 1.35                      |
| 0.5 10% FCS                     |           | 1.26                      |
| 0.5 10 nM PMA                   |           | 0.67                      |
| 0.5 20 nM EGF                   |           | 0.97                      |

* After preincubation, cells were treated as shown for 3 h, then thoroughly washed with Dulbecco's modified minimal essential medium containing 0.5% serum and incubated in medium containing 0.5% serum for an additional 12 h.

† At the end of this period, cells were incubated in medium containing [3H]-methylthymidine (3 μCi/ml), 20 mM Hapes (pH 7), 1 mg/ml BSA, glutamine, and 0.5% fetal calf serum at 37°C for 2 h. Monolayers were washed, 1 ml of 1 N NaOH was added for 1 h at 37°C, and thymidine incorporation was measured counting the total radioactivity precipitated by 10% trichloroacetic acid. The results are expressed as counts incorporated × 10⁻⁴/plate and are the average of three parallel determinations with <10% deviation.

Effect of Cycloheximide on the Urokinase mRNA

Expression of several genes is rapidly induced by PDGF (5, 21) and PMA (17). Inhibitors of protein synthesis lead to superinduction of the PDGF-induced genes, indicating that their expression may be regulated by some rapidly turning-over protein (18, 21). To determine whether urokinase mRNA is subject to the same kind of superinduction, the effect of cycloheximide on urokinase mRNA level was studied. Confluent A431 and A1251 cells were treated with 10 μg/ml cycloheximide; after 30 min the cells were treated with EGF, PMA, or diluent for an additional 3 h. Fig. 5 shows the results obtained with A431 cells. Cycloheximide increases the level of urokinase mRNA both in control and in EGF- or PMA-treated cells in which a superinduction is observed. Identical results are obtained with A1251 cells and PMA.

PMA Increases the Synthesis of Urokinase mRNAs in A431 Cells

To investigate whether an increase in urokinase mRNA level was due to increased synthesis or to message stabilization, we carried out nuclear transcription assays on control and PMA-treated A431 cells. Nuclei were transcribed in vitro (48) and the [32P]-labeled RNA was hybridized to both a urokinase cDNA probe and to a class I HLA cDNA probe, in a dot-blot assay. The results are shown in Fig. 6. Hybridization to human HLA-1 cDNA was almost identical with PMA-treated RNA. On the contrary, hybridization to the urokinase probe was clearly increased by PMA treatment. Counting of the spots in a scintillation counter shows that hybridization to the HLA-1 probes is increased by 30% by the PMA treatment, while hybridization to urokinase DNA is increased by almost 500%.
Discussion

Transcriptional Induction of the Urokinase Gene

In this paper, we show (a) induction of fibrinolytic activity in two cell lines (A1251 and A431) in which both basal and induced activities are due to the urokinase form of PA; (b) that the increases in PA activity by EGF and/or PMA depends on the increase in urokinase mRNA; (c) that EGF acts only on A431 and not on A1251 cells, even though these are able to bind EGF; (d) that the increases in urokinase mRNA is a fast response of the cells, being approximately 10-fold higher as early as 30 min after the addition of PMA (and EGF in the A431 cells); (e) that protein synthesis is not required for the induction of urokinase mRNA. In fact, treatment of cells with cycloheximide increases urokinase mRNA and enhances the effect of the inducers (superinduction). This raises the interesting possibility that the urokinase gene is controlled by a short-lived protein. (f) Increase of urokinase mRNA depends on the enhancement of transcription of the urokinase gene.

Tumor promoter PMA is a better inducer than EGF in A431 cells; PMA and PDGF also induce urokinase mRNA synthesis in A1251 cells, whereas EGF has no effect. The lack of effect of EGF is not due to inability of these cells to bind the growth factor since A1251 cells have an approximately normal number of EGF binding sites.

The effect of EGF on A431 cell urokinase mRNA may be due to the abnormally high number of EGF receptors in these cells (13). If so, EGF should have no effect on urokinase synthesis in variants of A431 cells with a lower number of EGF receptors. A431 derivative clone 18 (3) does not respond to EGF by increasing urokinase mRNA. The effect of PMA, on the other hand, is identical in A431, A1251, and clone 18 cells. Our conclusion, therefore, is that urokinase can be induced by PMA and not by EGF, at least in the presence of a physiological number of EGF receptors.

The small effect of PDGF on urokinase mRNA synthesis in A1251 is also unexpected. Epithelial cells are in fact not able to bind PDGF (as reviewed in reference 45). On the other hand, many malignant cell lines, including A1251, present nonrandom ectopic protein production (42). The response to PDGF may be due to ectopic synthesis of PDGF receptors.

Induction of Urokinase mRNA Is a Primary Effect of PMA

PMA is known to increase the level of several mRNAs, including urokinase (2). Here we have shown that this induction is due to increase in transcription of the urokinase gene (Fig. 6). In addition, we show that PMA, as EGF, acts early on urokinase gene expression in cell lines where it fails to stimulate DNA synthesis (Table II). This suggests that urokinase synthesis is directly regulated by these effectors, or by events that follow immediately their binding to the cell, and is not a consequence of their effect on cell growth. Phorbol esters may exert both a mitogenic (8, 25) and a differentiating effect (6, 43) on cultured cells. In the former case a stimulation, and in the latter case an inhibition, of thymidine incorporation is observed. In A431 and A1251 carcinoma cells, early induction of urokinase RNA by PMA is followed by an inhibition of thymidine incorporation (Table II). Therefore, there does not seem to be a causal connection between urokinase induction and thymidine incorporation. In fact, in normal mouse bone marrow macrophages, PA induction by PMA is followed by a stimulation of thymidine incorporation (20). Similarly, EGF blocks growth of A431 cells (where it induces urokinase synthesis), but has no effect on the clone 18 variant, in which it fails to induce urokinase. Finally, the induction of urokinase by cycloheximide, which blocks cell growth (Fig. 5), shows that, in agreement with the former interpretation, protein synthesis is not required for urokinase mRNA induction.

Urokinase Has Regulatory Features Similar to Competence Genes

The following features of urokinase gene regulation in transformed cells are similar to those recently reported for several competence-phase genes, including c-myc (5, 21). (a) PDGF and PMA, mitogens active in the early competence phase of cell growth (15, 37), induce the synthesis of urokinase mRNA. EGF, a mitogen acting later in the cell cycle, is a less effective inducer in A431 cells and is inactive in A1251 cells. (b) Induction of urokinase mRNA is an early effect of PMA or PDGF administration. (c) Cycloheximide induces the synthesis of urokinase mRNA and enhances the effect of PMA.

The regulation of c-myc has been studied in growth-arrested normal cells, stimulated to grow by PDGF and PMA (21); our results have been obtained in serum-independent human transformed cells. However, induction of PA synthesis has been reported in normal macrophages stimulated to grow by PMA or colony-stimulating factor (20). It seems, therefore, possible to propose that the increase of urokinase in transformed cells is due to a chronic stimulation of the urokinase gene by growth factors produced by tumor cells, i.e., by the loss of cell-cycle regulation of urokinase gene expression.

Has Urokinase Any Role In Cell Growth and Cancer?

Myra-y-Lopez et al. (29) propose that PA production in malignant tumors is part of the biosynthetic program induced by growth-stimulating hormones. We show that increase of urokinase mRNA is a direct effect of different growth effectors. Recent studies showing identity between oncogenes and...
some growth factors or their receptors (9, 10, 51) and the phenotypic coordinated transforming effect of three growth factor peptides (1) may provide a working hypothesis for this effect. A long-recognized association exists between PA production and human neoplasia (4, 7, 12, 27, 32, 33, 40). The role, if any, of PA in cancer is unknown. The hypothesis that is mostly supported by experimental facts is that PA controls extracellular proteolysis (40). In this respect, it is of the highest interest that anti-urokinase antibodies block the metastatic activity of a human tumor inoculated in the chorionallantoid chamber of the chick embryo (33). Alternative or additional functions are not excluded, particularly those that propose an intracellular role for PA, like down-regulation of membrane receptors (18) or regulation of the structure of cellular cytoskeleton (38).

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