Regulation by Epidermal Growth Factor of Human Squamous Cell Carcinoma Plasminogen Activator-mediated Proteolysis of Extracellular Matrix

Michael J. Niedbala,3 and Alan C. Sartorelli4

Department of Pharmacology and Developmental Therapeutics Program, Comprehensive Cancer Center, Yale University School of Medicine, New Haven, Connecticut 06610

ABSTRACT

The interaction of epidermal growth factor (EGF) with specific cell surface receptors initiates biochemical events in target cells which result in cellular proliferation and differentiation. In this report the regulation of extracellular-associated plasminogen activator (PA) production by EGF in human squamous cell carcinomas and its influence on tumor cell-mediated degradation of extracellular matrix (ECM) is described. The studies utilized the vulvar carcinoma cell line A431, which possesses an unusually large number of EGF receptors (EGF-Rs), and two A431 EGF-R expression variants (A5 and A7), which contain up to 20-fold fewer cell surface EGF-Rs. EGF enhanced the production of urokinase (u) PA activity by two- to threefold in A431 tumor cells, in a concentration-dependent manner, following a 24-h treatment, as determined by substrate hydrolysis assays, while no changes in tissue-type PA occurred. In contrast, A5 and A7 tumor cells failed to demonstrate such a response. Time course studies of the EGF-mediated induction of uPA activity in A431 tumor cells indicated that within 8 h after exposure to EGF, a twofold increase above basal untreated control levels was observed using the substrate hydrolysis assay. EGF increased the steady state levels of uPA mRNA threefold in A431 tumor cells following a 24-h treatment, while in contrast, no such response was observed in EGF-R variant tumor cells. In accord with an EGF enhancement of uPA mRNA levels in A431 tumor cells, a similar increase of two- to threefold in the de novo synthesis of [35S]methionine-radiolabeled uPA was observed by immunoprecipitation following EGF treatment, while no measurable increase was observed in the EGF-R tumor variants. A431 tumor cells progressively degraded 3H-glucosamine-radiolabeled bovine corneal subendothelial ECM in the presence of EGF, resulting in 8.7-, 4.3-, and 1.7-fold increases above untreated control values, after a 48-h exposure to 100, 10, and 1 ng/ml of EGF, respectively. In contrast, A5 and A7 tumor cells did not demonstrate an increase in ECM degradation in the presence of EGF, even though these tumor cells possessed the ability to degrade ECM in the absence of the growth factor. The observed increase in ECM degradation mediated by EGF in A431 tumor cells was dependent upon the presence of plasminogen and could be inhibited by an antianalytic uPA monoclonal antibody. The findings suggest that EGF may be involved in the regulation of the proteolytically active phenotype associated with certain tumor cells and thereby may be a potential chemotherapeutic target for limiting the invasion and metastasis of squamous cell carcinoma.

INTRODUCTION

Pathological evidence indicates that SqCCs are capable of extensive invasion of surrounding normal tissue (1). An initial step in the invasive process is the penetration of the adjacent basement membrane ECM which delineates the epidermal cell compartment from the underlying stromal tissue elements. The transversal of matrix by SqCCs can also occur at distal sites within the metastatic cascade including: invasation/extravasation of either the vascular and/or the lymphatic subendothelial basement membrane, allowing for the systemic dissemination of tumor cells and secondary organ site infiltration. The penetration of matrices by invasive tumor cells is an active multiphenotypic process requiring, along with other necessary tumor cell properties, tumor motility (2) and the elaboration of hydrolytic enzymes, such as collagenases (3), cathepsins (4), and plasminogen activators (5), as well as glycosidases (6, 7).

SqCCs have been shown to overproduce PA in vitro compared to normal epidermal cells (8) and this relationship has also been demonstrated in a variety of malignancies derived from other organ systems (9, 10). The overproduction of PA by SqCCs and other tumor cell types has been observed to parallel their augmented ability to invade and degrade the ECM, while their normal cellular counterparts failed to do so (8, 11). uPA has been shown to correlate with metastasis in some tumor systems (12–14), but this relationship is not absolute (15). Moreover, the PA activity associated with tumor cell invasion is not unique to neoplasia, since it is also indicative of normal physiological forms of invasion and remodeling, such as ovulation (16), trophoblast implantation (17), and neovascularization (18). These findings demonstrate that PA activity is highly regulated under normal physiological conditions and that cellular transformation may disrupt this regulation.

An important link between growth factors and their receptors and oncogene products has recently emerged (19). For example, the proto-oncogene c-erb B-1 encodes for the EGF-R, which is homologous to the v-erb B transforming gene of the avian erythroblastosis virus. Recent studies have demonstrated that in human breast carcinoma overexpression of the EGF-R occurs in a greater proportion of lymph node metastases than in primary tumors (20). In another recent study (21), the presence of EGF-Rs in normal and neoplastic (i.e., transitional, superficial, and invasive carcinoma) human urothelium was investigated by immunohistochemical techniques, with the finding that significantly more invasive tumors (21/24) than superficial (7/24) tumors were positive for the EGF-R. These results suggest that a quantitative increase in EGF-Rs is associated with tumor progression, invasion, and metastasis. The underlying basis for the reported observations is unclear, but it has been shown that the down-regulation of the EGF-R is associated with an elevation of PA activity (22, 23), indicating the potential for the aberrant regulation of PA in tumor cells which over express EGF-R.

In the present report, the effects of EGF on the regulation of PA synthesis, accumulation of extracellular PA activity, and ECM proteolysis by SqCCs have been examined utilizing the A431 epidermoid carcinoma, which possesses an unusually large number (>105) of EGF-Rs (24, 25). In addition, the relationship between the number of cell surface EGF-Rs and...
the molecular regulation of PA activity has been investigated using recently isolated variant sublines (A5 and A7) derived from A431 tumor cells, which have decreased levels of cell surface EGF-R, intrinsic tyrosine kinase activity, constitutive EGF-R mRNA and EGF-R gene copy number. The elucidation of the underlying molecular basis of the regulation of extracellular proteolysis by PA by growth factors such as EGF provides insight into the regulatory mechanisms involved in tumor progression and metastasis.

MATERIALS AND METHODS

Cell Culture. Human epidermoid A431 tumor cells were obtained from the American Type Culture Collection (Rockville, MD) and were routinely maintained in a mixture of DMEM-Ham's F12 medium (1:1, v/v) supplemented with 10% heat-inactivated fetal calf serum, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 2 mM glutamine, 200 µg/ml of streptomycin, and 5 x 10^5 units/liter of penicillin. EGF-R variant cell lines were isolated previously by culturing A431 tumor cells in the presence of EGF (Collaborative Research, Bedford, MA) at a concentration of 600 ng/ml for 2 months (24). These tumor cells were grown in the absence of EGF for four to eight doublings prior to use. The EGF-R levels associated with these tumor cell lines were evaluated by Scatchard analysis; results obtained from these measurements were in agreement with previously published observations (24). Cultures used in experimental studies were maintained in the absence of serum and antibiotics, but medium was supplemented with insulin (5 µg/ml), transferrin (5 µg/ml), and selenium (5 ng/ml). The growth rates of both parental A431 (doubling time, 28 h) and EGF-R variant (doubling times: A5, 36 h; A7, 40 h) cells were different under these conditions, necessitating the expression of data on a per cell basis.

Primary cultures of corneal endothelial cells derived from calf eyes were utilized as a source of ECM and were propagated using methods previously described (7). Culture vessel surfaces coated with radiolabeled ECM were prepared employing D-[6-3H]glucosamine hydrochloride (20 Ci/mmole) as the metabolic precursor for the degradation assays. Immunological verification of the presence of PA in appropriate dilutions of concentrated conditioned medium was conducted using an enzyme-linked immunosorbent assay with monoclonal antibodies directed against uPA and tPA (American Diagnostica, New York, NY). The ELISA assay was employed in an attempt to quantify the relative levels of total PA associated antigen, which includes both enzymatically inactive preforms and enzymatically active forms based upon their immunoreactivity. The MAb used to quantitate the regulation of total extracellular PA in these studies did not discriminate between forms of PA. In these studies, 96-well polystyrene Immunolon II plates (Dynatech Co., Cambridge, MA) were coated with conditioned medium or buffer alone in a 100-µl volume of 50 mM Tris-HCl, 0.1 mM NaCl, and 1 mM EDTA (pH 7.5) and incubated overnight at 4°C in a humidified atmosphere. Nonspecific binding sites were blocked with 5% (w/v) BSA in 100 mM borate acid, 25 mM sodium borate, 75 mM NaCl BS (pH 8.5) for 1 h at 20°C. Antibody wells were washed in PBS and the respective antibodies (1 µg/ml) were added for 4 h at 20°C in BS and 1% BSA. Subsequently, the plates were treated with 5% BSA in BS as described above and incubated overnight at 4°C with 1 µg/ml of rabbit antimesium antikinese phosphatase (Sigma Chemical Co., St. Louis, MO) in BS and 1% BSA. Plates were extensively washed with PBS and the substrate p-nitrophenyl phosphate (1 mg/ml) in 0.1 M glycine, 1 mM MgCl₂, and 1 mM ZnCl₂ (pH 10.4) was added. The absorbance at 405 nm was measured in an automated plate reader (Titertek Multiskan, Finland) and values were compared to standard uPA and tPA curves under conditions of linearity. In addition, the deletion of the primary antibody and substitution of the primary antibody with mouse nonimmune serum was employed as a negative control.

Zymography of PA present in the concentrated conditioned medium was performed on a 10% polyacrylamide gel containing 0.1% SDS polymerized in the presence of plasminogen and casein as previously described (26). Standards of molecular weight (BioRad Lab., Rockville Centre, NY), purified uPA and tPA (American Diagnostica) were applied to each gel.

RNA Isolation, Electrophoresis, and Blot Hybridization. Total cytoplasmic RNA was extracted from exponentially growing cultures by a modification (28) of the guanidium isothiocyanate method (29). Approximately 0.3 to 1 x 10⁶ cells grown in 175-cm² flasks were washed with PBS, solubilized in 4 mM guanidium isothiocyanate and subjected to cesium chloride density centrifugation (38,000 x g for 18 h at 20°C). The RNA pellet was suspended in 10 mM Tris-HCl/1 mM EDTA (pH 7.4), ethanol precipitated, resuspended in the same buffer and quantified by UV absorbance at 260 nm. Protein contamination in RNA preparations was estimated by determining the absorbance ratio of 260/280 nm. RNA (10 µg/ml) samples were denatured in MOPS/formaldehyde at 65°C for 15 min and subjected to electrophoresis in a 1.2% agarose/formaldehyde gel and transferred to nitrocellulose filters (Schleicher and Schuell, Keene, NH) by standard procedures (30). Baked filters were prehybridized for 24 h at 42°C in a solution containing 50% deionized formamide, 1% SDS, 0.2% BSA, 2% polyvinyl pyrrolidone, 0.2% Ficoll, 0.1% sodium pyrophosphate, and 1 mM NaCl in 50 mM Tris-HCl (pH 7.5) supplemented with denatured salmon sperm DNA (100 µg/ml).

Hybridization reactions were carried out for 24 h at 42°C with random primer [³²P]dCTP (3000 Ci/mmol; Dupont-NEN, Boston, MA) radiolabeled DNA (31) derived from the pHUK-8 cDNA fragment of uPA, kindly provided by Dr. Francesco Blasi (Naples, Italy) and the pAI cDNA fragment of β-actin (28). Following hybridization, the filters were washed consecutively with 2 x SSC/0.1% SDS at 20°C, 2 x SSC/0.5% SDS at 60°C and 0.2 x SSC/0.5% SDS at 60°C, each for 30 min. Filters were exposed to Kodak X-OMAT AR film with intensifier screens at −80°C and subsequently analyzed by densitometry.

Metabolic Labeling of Secreted PA with [³⁵S]Methionine and Immunoprecipitation. Cultured tumor cells in exponential growth were preincubated for 18 h in serum-free medium followed by treatment with EGF (0-100 ng/ml) and incubation for 16 h with [³⁵S]methionine (150 µCi/ml; Dupont-NEN) in serum-free methionine-deficient labeling medium. The medium was collected by centrifugation at 800 x g for 10 min to remove cellular elements and concentrated as described above. Equal volumes of culture medium (50 µl) from untreated and EGF-treated cultures were employed for the immunoprecipitation of uPA using a Sepharose-conjugated murine MAb directed against uPA (American Diagnostica). Immunoprecipitation of radiolabeled uPA under these conditions could be prevented by the addition of excess uPA (300 µg) to the reaction mixture. The immunosolubilates were solubilized in Laemmli buffer (32), heated for 5 min at 100°C and subjected to SDS-PAGE using a 10% polyacrylamide gel under nonreducing conditions. Gels were fixed for 1 h in 10% acetic acid/30% methanol, impregnated with En³Hance, dried and exposed to Kodak X-OMAT AR film at −80°C. ³⁵S-radiolabeled molecular weight standards (American Corp., Arlington Heights, IL) were used in parallel lanes for each gel.

Tumor Cell Mediated Degradation of ECM. The ability of A431, A5, and A7 cells to solubilize radiolabeled ECM in the presence of EGF (0-100 ng/ml) was measured as previously described (7). Briefly, tumor cells in exponential growth were harvested by trypsinization, washed in culture medium containing serum and seeded at a density of 2 x 10⁶ cells/10 mm/ml onto [³⁵S]glucosamine-radiolabeled ECM, which was heat treated for 1 h at 80°C as previously described (33). During the course of the assay, tumor cells were incubated in DMEM/Ham’s F12 medium supplemented with 10% ATFCs (34). An analogous series of cultures were maintained in the presence of 10% ATFCs.
depleted of plasminogen by lysine-Sepharose treatment to define the plasminogen dependency of the ECM solubilization. In addition, an anticitcatalytic MAb specific for uPA was used to further discern the role of uPA in tumor cell-mediated degradation of the ECM. Total radioactivity in 50-µl aliquots of the supernatant culture medium was measured at 24-h intervals. Cumulative amounts of radioactivity released by tumor cells were calculated by the subtraction of values obtained from analogous cultures not containing cells (<5% of total radioactivity).

RESULTS

Effect of EGF on Extracellular PA in A431 Parental and EGF-R Variant Tumor Cells. The extracellular levels of uPA present in cell-free conditioned medium from both A431 and EGF-R variant cells were measured following a 24 h exposure to EGF. In these studies, tumor cells were propagated in the serum-free defined medium. Under these conditions, both parental cells and EGF-R variants did not exhibit altered proliferation with EGF treatment during the course of the assay. Differences between these tumor cell lines in the basal levels of uPA activity in the absence of EGF treatment were observed (Fig. 1A). Thus, both A5 and A7 cells exhibited a threefold increase in uPA activity compared to A431 parental cells. In the presence of increasing concentrations of EGF (0–50 ng/ml), however, only A431 cells exhibited a concentration dependent twofold induction in extracellular uPA activity. In contrast, EGF, at analogous concentrations, had little effect on the levels of urokinase activity with both EGF-R variants.

Immunological verification of the presence of total extracellular PA was obtained using a MAb that recognized both the single chain and the active forms of uPA in a Micro-ELISA assay (Fig. 1B). Up to a threefold difference in the basal secreted levels of immunoreactive uPA was observed between parental and EGF-R variant tumor cells. These findings were consistent with the differences in plasminogen-dependent hydrolytic activity described above (Fig. 1A). In addition, EGF treatment of A431 tumor cells resulted in a twofold increase in immunoreactive extracellular uPA, while no significant effect was observed with EGF-R variant tumor cells in the same EGF concentration range.

The extracellular types of PA present in conditioned medium were determined by SDS-PAGE zymography, which allows for a distinction between various molecular weight forms of PA and an identification of PA/inhibitor complexes. A431 cells exposed for 24 h to increasing concentrations of EGF exhibited an induction of a lytic band at M, 55,000 which comigrated with the human uPA standard (Fig. 2A). Interestingly, a parallel induction of M, 100,000 and 33,000 lytic bands were also noted at the highest concentrations of EGF (10–100 ng/ml). A5 tumor cells exposed to EGF under analogous conditions failed to exhibit a detectable change in the M, 55,000 and 33,000 lytic bands, and these tumor cells elaborated a lytic band (M, 100,000) whose presence was not influenced by EGF (Fig. 2B). In addition, neither A431 nor EGF-R cells produced measurable extracellular levels of tPA activity, nor were the levels of tPA influenced by EGF, indicating a specificity for uPA. Further support for these findings stems from the ability of an anticitcatalytic murine MAb (5 µg/ml), which selectively inhibits the activity of uPA when incubated with samples to abolish
EGF-induced Mr 55,000 caseinolytic activity. The caseinolytic activity observed represented true PA activity since, like the uPA and tPA standards and all other lytic bands comigrating with them, these bands failed to demonstrate caseinolytic activity in a replicate gel polymerized in the absence of plasminogen (Fig. 2, C and D).

The effects of a 24-h exposure to EGF on the steady state levels of uPA mRNA expression was assessed in both A431 and EGF-R variant cells. Northern analysis showed that in A431 tumor cells, EGF markedly increased the levels of uPA mRNA in a concentration-dependent fashion (Fig. 3A). The extent of the increase was estimated by densitometric tracings and found to be more than a threefold induction of uPA mRNA following 100 ng/ml of EGF. The apparent size of the uPA mRNA transcript was approximately 2.5 kilobases, which is in agreement with that reported for mature uPA mRNA (5). In addition, a second band migrating slightly faster than 18S was also observed. It is unclear whether this band represented a degradation product of uPA mRNA or was a second uPA mRNA species; further studies will be needed to decide between these alternatives. The A5 EGF-R variant tumor cells exhibited a uPA mRNA with similar migratory characteristics (2.5 kilobases), but these cells failed to exhibit an increase in uPA mRNA following exposure to EGF under similar conditions. These studies indicated that the induction of uPA biosynthesis by EGF in A431 cells occurs at the transcriptional level. Whether this is due to an increase in the rate of mRNA synthesis requires further investigation.

The influence of EGF on the synthesis of uPA protein was investigated utilizing [35S]methionine-labeled A431 and EGF-R variant cells and immunoprecipitating uPA in cell-free medium with an anti-uPA MAb. Analysis of the immunoprecipitates by autoradiography demonstrated that EGF-treated A431 tumor cells secreted progressively higher levels of the Mr 55,000-radiolabeled immunoreactive form of uPA in a concentration-dependent manner when compared to untreated control cultures (Fig. 4A). The increased levels of secreted uPA in EGF treated cultures were shown by densitometric analysis to be twofold higher than those of untreated controls, and is supportive of earlier observations. The A5 EGF-R variant did not exhibit an increase in the synthesis and secretion of the radiolabeled Mr 55,000 immunoreactive form of uPA under similar conditions (Fig. 4B), thereby corroborating the previous findings of the inability of A5 tumor cells to respond to EGF. In addition, a Mr 100,000-radiolabeled uPA cross-reactive protein was immunoprecipitated from A5 tumor cell-conditioned medium, but its level of synthesis was not altered by EGF treatment. This radiolabeled protein was not detected in A431-conditioned medium and may be specific for this EGF-R variant.

The time course of the EGF-mediated induction of uPA in A431 tumor cells was investigated using the chromogenic substrate hydrolysis assay. Tumor cells were treated with 25 ng/ml of EGF and at different incubation times the medium was collected and assayed for uPA activity. By 2 h of EGF treatment, extracellular uPA activity was detected, and within 8 h of EGF treatment, a twofold increase in uPA activity compared to untreated controls was observed (Fig. 5). Similar observations were made by Northern analyses in A431 tumor cells, which demonstrated the presence of uPA mRNA by 30 min and a peaking of the steady state levels of uPA mRNA within 6 h, which remained unchanged for 24 h after exposure to EGF (data not shown).

Regulation of Tumor Cell-mediated Hydrolysis of ECM by EGF. Prior studies have demonstrated the importance of the PA system in the degradation of radiolabeled ECM by A431 tumor cells (8). The regulation of PA activity by EGF may play an important role in the capacity of tumor cells to degrade the ECM, an essential step in the invasion process. For these reasons, we evaluated the ability of A431 and EGF-R variant cells to digest [3H]glucosamine-radiolabeled ECM following exposure to EGF. A431 tumor cells progressively solubilized...
radiolabeled matrix constituents in the absence of EGF over a 96-h period (Fig. 6A). Tumor cell-mediated degradation of the ECM was amplified in a concentration-dependent manner by the presence of EGF. For example, A431 tumor cells degraded the ECM at 8.7-, 4.3-, and 1.7-fold higher levels above that of the untreated control value after 48 h of exposure to 100, 10, and 1 ng/ml of EGF, respectively. These values closely corresponded to the magnitude of extracellular uPA induction by EGF at analogous concentrations in the culture medium. In contrast, A5 (Fig. 6B) and A7 (Fig. 6C) tumor cells failed to exhibit an increase in the degradation of the ECM mediated by EGF, although these tumor cells possessed the capacity to digest the ECM, which is a reflection of their endogenous production of PA.

Additional studies were undertaken to ascertain the role of plasminogen and uPA in the EGF-mediated induction of A431 tumor cell degradation of the ECM. Tumor cells were incubated with either ATFCs or ATFCs depleted of plasminogen, followed by EGF treatment, and the degree of solubilization of radiolabeled ECM was determined. Matrix degradation by A431 tumor cells was decreased 7-fold in cultures containing plasminogen-depleted ATFCs compared to cultures maintained in ATFCs (Fig. 7). Interestingly, EGF treatment failed to augment A431 cell-mediated ECM degradation in plasminogen-depleted ATFCs, indicating the plasminogen dependence of this phenomenon.

A parallel series of experiments was carried out employing an anticatalytic murine MAb which selectively inhibits uPA activity, with a demonstrated IC50 value of 150 ng/ml (8). A431 cells were seeded onto radiolabeled ECM and were subsequently treated with EGF in the absence and continuous presence of 5 μg/ml of MAb. Untreated cells progressively degraded the ECM under these conditions, and MAb treatment effectively inhibited this degradation by 80% after 72 h (data not shown). Treatment of tumor cells with EGF resulted in a threefold increase in the degradation of ECM above that of untreated controls, and this induction was prevented by 90% by a 72-h exposure to MAb. The findings illustrate the importance of the uPA system in the EGF mediated induction of ECM degradation by A431 tumor cells.

DISCUSSION

Increased PA activity has been strongly associated with neoplastic transformation (5), and this proteolytic system has been implicated as an important mediator of tumor cell invasion and metastasis (12). The regulation of the cellular production of PA is believed to have an important role in the control of extracellular proteolysis. A variety of hormones and other effectors has been shown to regulate PA production and activity in both normal and neoplastic tissue (5). Recently, factors which control the growth and the multifunctional changes in the phenotype of various cell types have also been identified as additional regulators of PA (37, 38). In this report, we have analyzed the modulation by EGF of the production and activity of extracellular PA in the A431 human squamous cell carcinoma. In addition, we have investigated the relationship between cell
surface EGF-R number and the regulation of PA using recently isolated and characterized variant sublines derived from A431 cells which contain significantly fewer surface EGF-Rs, as well as less EGF-R mRNA than the parental line (24, 25). The influence of EGF in modulating tumor cell-mediated proteolysis of the ECM, an essential step in the metastatic process, was also measured.

EGF increased extracellular uPA antigenic protein levels and activity twofold in A431 tumor cells, as determined by a micro-ELISA assay and a substrate hydrolysis assay. The EGF-mediated induction of uPA was specific for this PA type, since zymograms and substrate hydrolysis assays, carried out in the presence of soluble fibrin products failed to exhibit any changes in extracellular tPA activity associated with A431 tumor cells. In contrast, the EGF-R variants failed to demonstrate an increase in extracellular-associated uPA following EGF treatment. In addition, relatively high concentrations of EGF (100 ng/ml) led to a decrease in the induction of extracellular uPA activity, which may be due to the internalization of occupied receptors, resulting in a net loss through "down-regulation" of cell surface EGF-R. These findings support the concept that interactions between EGF and its receptor play a significant role in the regulation of uPA. Parental A431 tumor cells contain a very large number of EGF-Rs and high tyrosine-specific protein kinase activity, while the variant cell lines contain fewer EGF-Rs and proportionally reduced kinase activity (24, 25). The tyrosine-specific kinase activity associated with EGF binding to the EGF-R in A431 cells appears to be critical for the induction of uPA, since a reduction in the number of receptor kinase molecules results in a loss of such a response. However, the EGF-R variant cell lines still contain relatively high levels of EGF-R (50-fold more than cultured epidermal cells), and about 40% of the kinase activity of A431 cells (25). The dramatic escape from the EGF-mediated induction of uPA activity in EGF-R variant cell lines is, therefore, not easily explained solely by this modest reduction in the number of EGF-R/kinase molecules. Therefore, more extensive analyses of phosphoproteins of A431 and EGF-R variant cell lines are in progress to determine whether differences in substrate phosphorylations can be detected which may provide further insight into the mechanisms responsible for this differential regulation of uPA. Similar observations have also been reported that describe the relationship between EGF-R number and cell proliferation (36) and differentiation (24).

Supportive evidence for a role for EGF in the regulation of PA activity was described for the human epidermoid HeLa tumor cell line (39), human foreskin fibroblasts (37) and human embryonic lung fibroblasts (38). However, these investigators did not discriminate between the PA types responsible for the observed EGF-dependent increase in fibrinolysis. Moreover, EGF-R down-regulation produced by treatment with super-physiologic concentrations of EGF (200 ng/ml) has recently been reported to correlate with increased cellular uPA activity in A431 cells (22, 23).

In addition to the induction of the $M_\text{r}$ 55,000 form of uPA, EGF also enhanced the secretion of $M_\text{r}$ 100,000 and 33,000 plasminogen-dependent caseinolytic proteins in A431 cells. While the $M_\text{r}$ 100,000 form was identified in A431 EGF-R variants, its level was independent of the presence of EGF. The molecular identity of the $M_\text{r}$ 100,000 lytic band is unclear, but evidence based upon cross-reactivity with a PAI-1 MAb in Western blot analyses exists which suggests that it is a secreted form of PAI-1, which forms SDS-stable complexes (5) with uPA. However, detectable increases in the synthesis and secretion of uncomplexed PAI-1 ($M_\text{r}$ 47,000) mediated by EGF in this tumor cell line were not observed in Western blot analyses and immunoprecipitation studies using MAb specific for PAI-1. We have previously reported that A431 tumor cells secrete an immunologically cross-reactive $M_\text{r}$ 71,000 PAI-1 into the culture medium (8). These findings, together with those described herein, indicate that the $M_\text{r}$ 71,000 PAI-1 may form a complex with the EGF-induced $M_\text{r}$ 33,000 proteolytically active uPA fragment, resulting in the formation of an enzyme/inhibitor complex with an apparent molecular weight of 100,000. These observations correspond to those previously described for human embryonic lung fibroblasts, in which SDS stable complexes between uPA and PAI-1 of a similar 100,000 molecular weight were noted (38). The fact that the uPA/PAI-1 complex shows enzymatic activity is probably due to the proteolysis of the PAI-1 by uPA (40). The $M_\text{r}$ 33,000 lytic band represents an enzymatically active proteolytic fragment of the $M_\text{r}$ 55,000 form of the uPA, based on its comigration and immunologic cross-reactivity with the uPA standard (5).

That the EGF-mediated induction occurred at both the mRNA level and subsequently at the extracellular uPA protein level was demonstrated by Northern analysis and metabolic labeling of tumor cells and immunoprecipitation of uPA, respectively. The enzymatic form of uPA elaborated by these tumor cells in the presence of EGF was further characterized and identified as a $M_\text{r}$ 55,000 single chain form of uPA (scuPA), based upon its unchanged migratory characteristics in SDS-PAGE under reducing and nonreducing conditions, and its resistance to diisopropyl fluorophosphate inactivation. In addition, differences in A431 tumor cell EGF dose-responsiveness which governed uPA activity and antigenicity were observed.  

\* M. J. Niedbala and A. C. Sartorelli, unpublished results.

Fig. 7. The plasminogen dependence of the EGF-mediated induction of the degradation of $[^{14}C]$glucosamine radiolabeled ECM by A431 tumor cells. Tumor cells ($2 \times 10^5$ cells/ml) were plated onto radiolabeled ECM and cultures were maintained in DMEM/Ham's F12 medium (1:1, v/v) supplemented with 10% ATFCS or 10% ATFCS depleted of plasminogen in the absence and presence of 100 ng/ml of EGF. At 24-h intervals, the amount of radioactivity associated with 50-$\mu$l aliquots of the supernatant medium was measured. This level was subtracted from that present in cell-free controls. Values represent the mean ± SD of quadruplicate cultures.
These differences may be accounted for by the ability of EGF to serve as an expression modulator of the proform of uPA and its subsequent activation to enzymatically active uPA. This observation is consistent with a previous report which described the presence of extracellular scuPA in A431 tumor cells (41). Additional studies are currently underway to elucidate the influence of EGF on cellular associated PA activity.

PA-mediated proteolysis of the ECM by normal and malignant cells has been previously described (5, 11, 42, 43). In this paper, we have demonstrated that EGF has a profound effect on the proteolysis of radiolabeled ECM by A431 tumor cells. The observed increase in ECM degradation mediated by EGF in A431 cells was dependent upon the presence of plasminogen and could be inhibited by the use of an anticalytic uPA MAb. These findings are consistent with the observation that EGF increases the level of extracellular uPA. The ability of uPA to mediate the degradation of the ECM may occur via activation of plasminogen to plasmin, which has the capacity to directly degrade matrix constituents (42, 43). Alternatively, plasmin may indirectly activate latent forms of collagenase, which hydrolyzes collagenous elements present in the ECM (44). A431 cells have been reported to only synthesize relatively low levels of latent collagenases, indicating that activation of these enzymes is a possibility, but not a probable mechanism of ECM degradation (45). However, the possibility that plasmin may act in concert with collagenases and/or other proteinases to degrade the ECM is a plausible hypothesis since, based upon the biochemical complexity of the ECM, multiple operative hydrolytic enzymes must be elaborated by invasive tumor cells. EGF can also influence a number of other phenotypic properties, including tumor cell proliferation (36), differentiation (24), and tumor cell motility, as has been recently described in metastatic murine melanoma cells (46). All of these properties may influence the invasive capacity of tumor cells. In summary, the microenvironment and soluble growth factors have the capacity to modulate the invasive and metastatic behavior of cancer cells either directly or indirectly through the regulation of the proteolytically active phenotype. These actions may provide new chemotherapeutic targets for intervention in limiting invasion and ultimately the metastasis of squamous cell carcinomas.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Ivan King for supplying the A431 EGF-R variants and Nancy Salvo for the preparation of the manuscript.

REFERENCES

EGF REGULATION OF PLASMINOGEN ACTIVATOR


Regulation by Epidermal Growth Factor of Human Squamous Cell Carcinoma Plasminogen Activator-mediated Proteolysis of Extracellular Matrix

Michael J. Niedbala and Alan C. Sartorelli


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/49/12/3302

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.