Tobacco Smoke Induces Urokinase-Type Plasminogen Activator and Cell Invasiveness: Evidence for an Epidermal Growth Factor Receptor–Dependent Mechanism

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Abstract

Multiple tobacco smoke–related premalignant and malignant lesions develop synchronously or metachronously in various organ sites, including the oral cavity. Both field cancerization and clonal migration seem to contribute to the occurrence of multiple tumors. Although the importance of endogenous factors (e.g., oncogenes) in regulating clonal migration is well established, little is known about the role of exogenous factors. Hence, the main objective of this study was to elucidate the mechanism by which tobacco smoke stimulated the migration of cells through extracellular matrix (ECM). Treatment of MSK-Leuk1 cells with a saline extract of tobacco smoke induced the migration of cells through ECM. Tobacco smoke induced the expression of urokinase-type plasminogen activator (uPA), resulting in plasmin-dependent degradation of ECM and increased cell migration. AG1478, a small-molecule inhibitor of the epidermal growth factor receptor (EGFR) tyrosine kinase, a neutralizing antibody to EGFR, or an antibody to amphiregulin, an EGFR ligand, also blocked tobacco smoke–mediated induction of uPA and cell migration through ECM. PD98059, an inhibitor of mitogen-activated protein kinase (MAPK) kinase activity, caused similar inhibitory effects. Taken together, these results suggest that tobacco smoke activated the EGFR→extracellular signal-regulated kinase 1/2 MAPK pathway, causing induction of uPA. This led, in turn, to increased plasmin-dependent degradation of matrix proteins and enhanced cell migration through ECM. These data strongly suggest that chemicals in tobacco smoke can mimic the effects of oncogenes in regulating uPA-dependent cell invasion through ECM. These findings also strengthen the rationale for determining whether inhibitors of EGFR tyrosine kinase reduce the risk of tobacco smoke–related second primary tumors. [Cancer Res 2007;67(18):8966–72]

Introduction

Multiple premalignant or malignant lesions can develop synchronously or metachronously in the oral cavity, urothelial tract, and respiratory epithelium (1–5). One possible explanation for this phenomenon is field cancerization (6). According to this concept, multiple lesions arise because the entire field (e.g., aerodigestive tract) was chronically exposed to carcinogens, such as those in tobacco smoke. In this instance, carcinogens affect the epithelium at multiple sites, leading to numerous mutations and independent growth of multifocal unrelated tumors. However, the independent origin of some second primary tumors (SPT) has been challenged. For example, genetic evidence has suggested a common clonal origin of multiple bladder tumors (1, 2). In addition, studies have suggested that SPT in the head and neck can be a consequence of clonal spreading rather than development of independent lesions (3, 4). In the oral cavity, clonal spreading of both invasive cancer and noninvasive leukoplakic lesions has been described (3, 4). Hence, it seems that both field cancerization and mucosal spreading of clonal cells contribute to the occurrence of multiple tumors. Although the mechanisms underlying field cancerization are well characterized, very little is known about the factors that contribute to clonal migration.

One of the key steps in cell migration is degradation of the extracellular matrix (ECM). Several lines of evidence suggest that the serine proteinase urokinase-type plasminogen activator (uPA), together with its high-affinity receptor, uPAR, is important in initiating a proteolytic cascade that stimulates cell migration (7–12). uPA cleaves the Arg^{560}-Val^{561} bond of plasminogen, thereby generating plasmin, a serine proteinase with wide substrate specificities (13). uPAR helps to localize plasminogen activation to the cell surface and uPA bound to uPAR is relatively resistant to inhibition (14, 15). Plasmin binds to and degrades several components of the ECM, including the adhesive glycoproteins, fibronectin, and laminin (16–18). Moreover, plasmin activates several members of the metalloproteinase family, which degrade collagenous and noncollagenous ECM components (19). Activation of this proteolytic cascade has been implicated in processes requiring cell migration, including inflammation, wound healing, and cancer metastasis (20–24). As detailed above, clonal spreading occurs in smoking-related neoplasia but the effects of tobacco smoke on either cell invasiveness or the uPA-uPAR-plasmin axis are unknown.

In the present study, we first determined that exposure to tobacco smoke extract stimulated the invasiveness of oral leukoplakia cells. Subsequently, tobacco smoke was found to stimulate cell invasion by inducing the expression and release of uPA, leading, in turn, to plasmin-mediated degradation of ECM. Finally, we showed that tobacco smoke–mediated induction of uPA was a consequence of activation of epidermal growth factor receptor (EGFR)→extracellular signal-regulated kinase 1/2 (ERK1/2) mitogen-activated protein kinase (MAPK) signaling. These findings provide new insights into the procarcinogenic effects of tobacco smoke and may help to explain the increased rate of SPT in individuals who continue to smoke (25).

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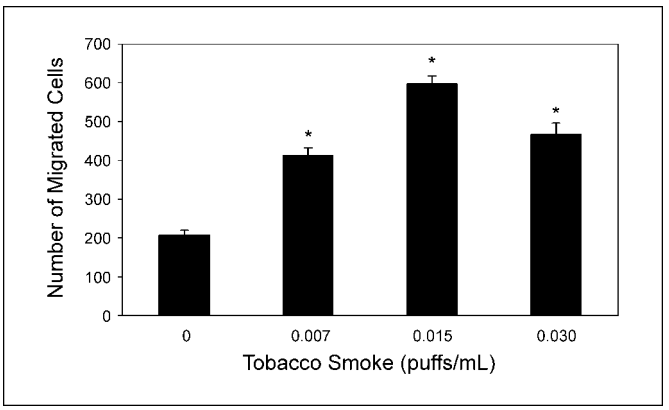

Figure 1. Tobacco smoke stimulates the invasion of MSK-Leuk1 cells. Cells (2×10^4) were aliquoted into an insert containing a porous (8 μm) polycarbonate Matrigel-coated membrane. The cells in the insert were then treated with 0 to 0.03 puffs/mL tobacco smoke in basal medium. The insert was placed into a well containing growth medium and incubated at 37°C for 24 h. Cells that migrated into the lower well were counted after being fixed and stained. *Columns,* mean ($n = 3$); *bars,* SD. *, $P < 0.05$, compared with vehicle-treated control. These data are representative of three independent experiments.

Materials and Methods

Materials. Keratinocyte basal and growth media were supplied by Clonetics Corp. DMEM was from Invitrogen Corp. AG1478 and PD98059 were acquired from Calbiochem. Human Matrigel matrix was from BD Biosciences. Transwell plates with 8-μm pore polycarbonate membrane inserts were purchased from Corning, Inc. [^{3}H]glucosamine was purchased from DuPont/New England Nuclear. Amphiregulin and antiamphiregulin were from R&D Systems, Inc. Anti-EGFR antibody was purchased from Upstate. Antibodies to phosphorylated and unphosphorylated forms of ERK1/2 were from Cell Signaling Technology, Inc. Normal goat IgG and normal mouse IgG were obtained from Santa Cruz Biotechnology, Inc. Plasminogen activator assay substrate (D-Val-Leu-Lys-amino methyl coumarin) was obtained from MP Biomedicals. Human Glu-plasminogen, plasmin, α2 plasmin inhibitor, and antibodies to human uPA and plasmin were obtained from American Diagnostica, Inc. Amiloride and antisera to β-actin were obtained from Sigma Chemical Co. Human dermal fibroblasts were obtained from VEC Technologies, Inc.

Tissue culture. The MSK-Leuk1 cell line was established from a dysplastic leukoplakia lesion adjacent to a squamous cell carcinoma of the tongue (26). Cells were routinely maintained in keratinocyte growth medium supplemented with bovine pituitary extract. Cells were grown in basal medium for 24 h before treatment. Treatment with vehicle (PBS) or a saline extract of tobacco smoke (prepared as described below) was carried out under serum-free conditions. Cellular cytotoxicity was assessed by measurements of cell number, trypan blue exclusion, and release of lactate dehydrogenase. There was no evidence of cytotoxicity in any of our experiments.

Preparation of tobacco smoke. Cigarettes (2R4F, Kentucky Tobacco Research Institute) were smoked in a Borgwaldt piston-controlled apparatus (model RG-1) using the Federal Trade Commission standard protocol. The protocol variables attempt to mimic a standardized human smoking pattern (duration, 2 s/puff; frequency, 1 puff/min; volume, 35 mL/puff). Cigarettes were smoked one at a time in the apparatus and the smoke was drawn under sterile conditions into premeasured amounts of sterile PBS (pH 7.4). This smoke in PBS represents whole trapped mainstream smoke abbreviated as tobacco smoke (27). Quantitation of smoke content is expressed in puffs/mL of PBS with one cigarette yielding about 8 puffs drawn into a 5 mL volume. The final concentration of tobacco smoke in the cell culture medium is expressed as puffs/mL medium. Unless otherwise indicated, all treatments were carried out with either 0.015 or 0.03 puffs/mL tobacco smoke.

Preparation of [^{3}H]glucosamine-labeled ECMs. The preparation of labeled ECM has been described previously (28). Human dermal fibroblasts were plated into 24-well plates (5×10^4 per well) in DMEM supplemented with 10% fetal bovine serum, penicillin (100 units/mL), streptomycin (100 μg/mL), and 4 mmol/L glutamine. On day 3, media were replaced with fresh media containing 1 μCi/well [^{3}H]glucosamine. On day 6, the cell layer was removed by sequential exposure to 0.5% Triton X-100 in PBS (10 min) and 0.20 mmol/L NH_4OH in PBS (3 min). Matrix-coated plates were washed thrice with sterile Dulbecco's PBS (DPBS) and allowed to dry in a laminar flow hood and then stored at 4°C.

ECM invasion assay. Invasion assays were carried out in a 24-well Transwell plates with 8.0-μm pore polycarbonate membrane inserts. Inserts were coated with Matrigel as follows: Matrigel was thawed on ice and diluted with ice-cold sterile water to 75 μg/mL. A 50-μL aliquot of Matrigel was rapidly spread over the surface of the prechilled inserts and allowed to air dry overnight. Before use, coated inserts were rehydrated with serum-free medium for 1 h. MSK-Leuk1 cells were harvested and resuspended to 2×10^5/mL basal medium. After 30 to 60 min of pretreatment with various inhibitors, tobacco smoke was added to the cell suspension. Treated cells (2×10^4 per insert) were added to the matrix-coated insert, and the insert was placed in a 24-well plate containing keratinocyte growth medium. After incubation at 37°C and 5% CO_2 for 24 h, the membrane was fixed for 30 min in chilled 70% ethanol and stained for 25 min with 0.75% hematoxylin. Cells and ECM that were attached to the upper surface of the membrane were removed using a cotton swab. The membrane was then carefully removed from the insert and mounted onto a glass slide. The membranes were examined using a light microscope, and cells that migrated through the matrix-coated insert were counted.

Determination of plasminogen activator activity. MSK-Leuk1 cells were exposed to tobacco smoke and/or other treatments in serum-free medium as indicated in the figure legends. Conditioned media were collected into 1.5 mL microcentrifuge tubes and placed on ice before being cleared by centrifugation (5,000 rpm for 5 min at 4°C). Plasminogen activator activity was quantified using a sensitive functional assay for plasmin as described previously (29). Aliquots of conditioned medium were added to microtiter wells containing 82 μL DPBS plus 0.05% Tween 20, 13 μg of the plasmin substrate D-Val-Leu-Lys-amino methyl coumarin, and

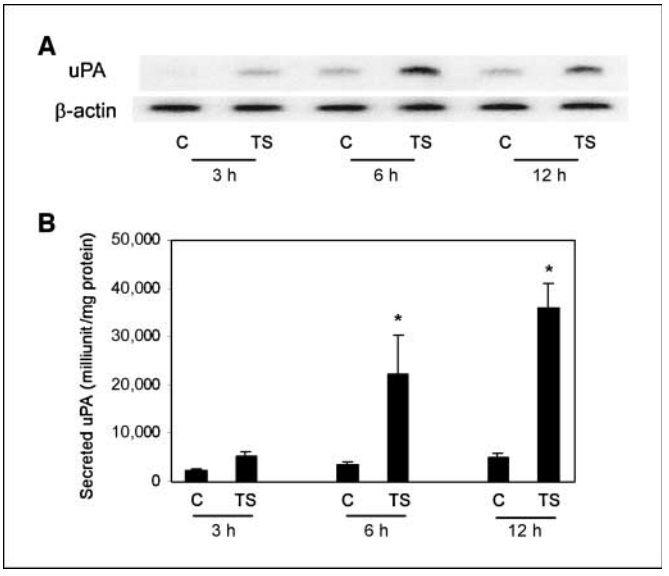

Figure 2. Tobacco smoke stimulates the expression and release of uPA. Cells were treated with vehicle (*C*) or tobacco smoke (*TS*) for 3, 6, or 12 h. *A,* reverse transcription-PCR (RT-PCR) was used to determine the amounts of mRNAs for uPA and β-actin. *B,* amount of uPA activity was measured in medium from vehicle-treated or tobacco smoke–treated cells. *Columns,* mean ($n = 3$); *bars,* SD. *, $P < 0.05$, tobacco smoke compared with control.

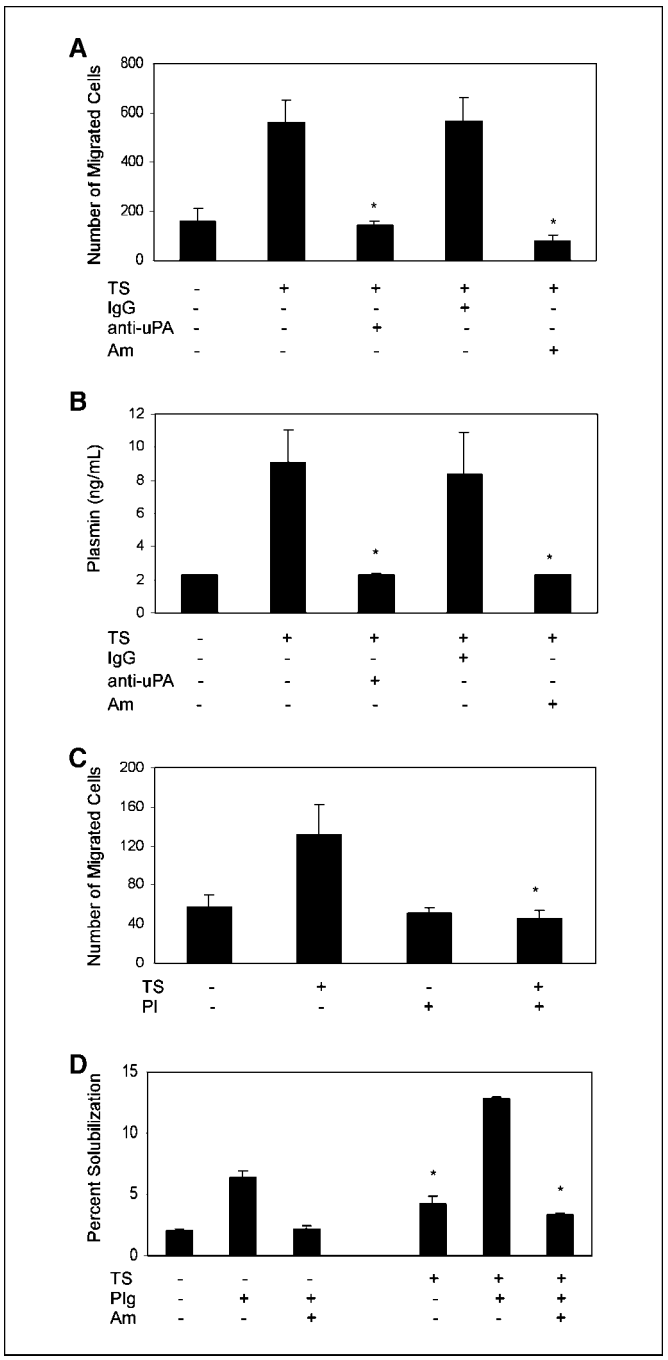

Figure 3. uPA is critical for tobacco smoke–mediated degradation of ECM and increased cell invasion. *A* to *C,* cells were suspended in basal medium and incubated for 1 h with the indicated treatments [antibodies to uPA or IgG at 10 μg/mL, 50 μmol/L amiloride (*Am*), plasminogen inhibitor (*PI*) at 5 μg/mL]. Cells (2×10^4) were then aliquoted into a Matrigel-coated insert. The cells in the insert were then treated with vehicle or tobacco smoke. The insert was placed into a well containing growth medium and incubated at 37°C for 24 h. *A* and *C,* cells that migrated into the lower well were counted after being fixed and stained. *B,* medium was collected and plasmin concentration was determined. *Columns,* means ($n = 4$); *bars,* SD. *, $P < 0.05$, compared with tobacco smoke. *D,* cells were plated on insoluble [^{3}H]glucosamine-labeled ECM in basal medium. Following adherence, medium was replaced with either control or tobacco smoke–conditioned medium. Treatments with 0.5 μg/mL plasminogen (*Plg*) or 50 μmol/L amiloride were as indicated. Twenty-four hours later, incubation media were recovered and solubilized [^{3}H] activity was determined as described in Materials and Methods. *Columns,* mean ($n = 4$); *bars,* SD. *, $P < 0.05$, tobacco smoke compared with untreated control and tobacco smoke + plasminogen + amiloride compared with tobacco smoke + plasminogen.

0.5 μg bovine plasminogen. Samples were mixed and incubated at 37°C for 2.5 h. Cleavage of the substrate was monitored by measuring the increase in fluorescence in a Fluoroscan microplate reader (excitation: 330–380 nm; emission: 430–530 nm). Concentrations of uPA in the test samples were extrapolated from a standard curve prepared with high molecular weight uPA. Plasminogen activator activity in conditioned medium was completely inhibited when preincubated with a polyclonal antihuman uPA IgG.

Reverse transcription-PCR. RNA was prepared using RNeasy Mini kits from Qiagen. RNA (2 μg) was reversed transcribed using murine leukemia virus reverse transcriptase (Roche Applied Science) and oligo $d(T)_{16}$ primer. The resulting cDNA was then used for amplification. The volume of the PCR was 25 μL and contained 5 μL cDNA, 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 2 mmol/L $MgCl_2$, 0.4 mmol/L deoxynucleotide triphosphates, 400 nmol/L forward primer, 400 nmol/L reverse primer, and 2.5 units Taq polymerase (Applied Biosystems). Samples were denatured at 95°C for 2 min and then amplified for 35 cycles in a thermocycler under the following conditions: 95°C for 30 s, 62°C for 30 s, and then 72°C for 45 s. Subsequently, extension was carried out at 72°C for 10 min. Human uPA primers were directed at 1181 to 1200 (forward, 5′-GTCACCACCAAAATG-CTGTG-3′) and 1712 to 1693 (reverse, 5′-ATGCCCTGCCCTTTTTAACT-3′) of Genbank NM-002658. Primers for human β-actin have been described previously (30). PCR products were electrophoresed on a 1% agarose gel with 0.5 μg/mL ethidium bromide and photographed under UV light. The identity of each PCR product was confirmed by DNA sequencing. A computer densitometer (ChemDoc, Bio-Rad) was used to quantify the density of the different bands. Values are expressed in arbitrary units.

Statistics. Mean numbers of transmigrated cells, mean levels of uPA activity, or the mean percent of ECM degradation was compared using single- and two-factor ANOVA where appropriate. Individual comparisons between groups were made using the Newman-Keuls multiple comparison test. A difference between groups of $P < 0.05$ was considered significant.

Results

Tobacco smoke stimulates cell invasiveness by inducing uPA. The effect of tobacco smoke on the ability of MSK-Leuk1 cells to transmigrate through a Matrigel-coated porous (8 μm) insert was determined. The addition of tobacco smoke to inserts led to a 2-fold increase in the number of cells that migrated through the ECM over 24 h (Fig. 1). Given the reported role of uPA in cell migration, we investigated whether tobacco smoke induced uPA expression in MSK-Leuk1 cells. As shown in Fig. 2*A*, levels of uPA mRNA were markedly increased in cells treated with tobacco smoke for 6 to 12 h. Likewise, exposure to tobacco smoke led to a time-dependent increase in the levels of uPA activity in cellular conditioned medium (Fig. 2*B*). We next determined whether tobacco smoke–induced uPA expression was responsible for the enhanced ability of these cells to traverse ECM-coated inserts (Fig. 1). For this purpose, we used neutralizing anti-uPA IgG and amiloride, a selective inhibitor of uPA activity that has no effect on tissue type plasminogen activator or plasmin activities (31). As shown in Fig. 3*A*, the ability of cells to traverse the Matrigel-coated insert was markedly stimulated (2-fold) by exposure to tobacco smoke. The enhanced migration was completely blocked by the addition of anti-uPA IgG, whereas normal IgG had no effect. Likewise, preincubation of cells with amiloride blocked tobacco smoke–induced invasion (Fig. 3*A*). Thus, enhanced uPA expression is causally linked to tobacco smoke–induced migration of MSK-Leuk1 cells through ECM.

Plasminogen activation and subsequent degradation of ECM components is thought to be a principal mechanism by which enhanced uPA expression stimulates cellular invasion. To test whether tobacco smoke–induced uPA expression led to plasminogen activation in the Transwell culture system, 24-h conditioned

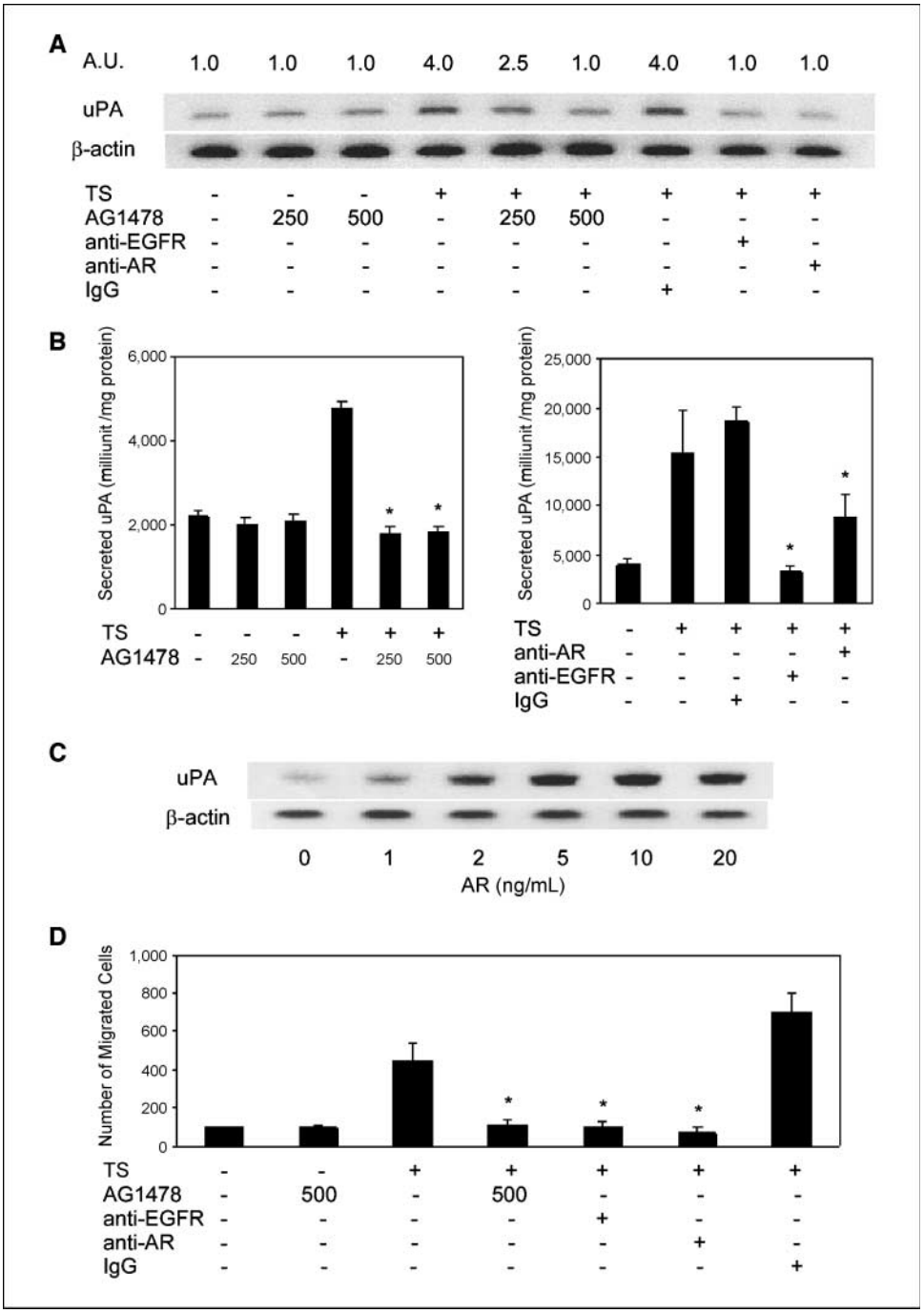

Figure 4. Tobacco smoke–mediated induction of uPA is EGFR dependent. Cells were grown in basal medium for 24 h. *A* and *B,* cells then received the indicated treatments [antibodies to EGFR, amphiregulin (*AR*), or IgG at 10 μg/mL; 250 or 500 nmol/L AG1478] for 2 h before being exposed to tobacco smoke for 6 h. *A,* RT-PCR was used to determine the amount of mRNAs for uPA and β-actin. Results of the data shown are expressed in arbitrary units (*A.U.*). *B,* amount of uPA activity was measured in medium. *Columns,* mean ($n = 3$); *bars,* SD. *, $P < 0.05$, compared with tobacco smoke. *C,* cells were treated with 0 to 20 ng/mL of amphiregulin for 6 h. RT-PCR was used to determine the amounts of mRNAs for uPA and β-actin. *D,* cells were suspended in basal medium and incubated for 1 h with the indicated treatments (antibodies to EGFR, amphiregulin, or IgG at 10 μg/mL; 500 nmol/L AG1478). Cells (2×10^4) were then aliquoted into an insert containing a porous (8 μm) polycarbonate Matrigel-coated insert. The cells in the insert were then treated with vehicle or tobacco smoke. The insert was placed into a well containing growth medium and incubated at 37°C for 24 h. Cells that migrated into the lower well were then counted after being fixed and stained. *Columns,* mean ($n = 4$); *bars,* SD. *, $P < 0.05$, compared with tobacco smoke.

media were collected from the inserts and assayed for plasmin activity. In these studies, cellular incubation medium was not supplemented with exogenous plasminogen. However, Matrigel contains variable amounts of plasminogen (32). As shown in Fig. 3*B*, medium derived from control cells cultured on Matrigel contained low levels of plasmin activity. The level of plasmin activity in the conditioned medium increased dramatically when cells were exposed to tobacco smoke. Furthermore, the tobacco smoke–induced increase in plasmin activity was inhibited by anti-uPA IgG or amiloride. To determine whether tobacco smoke–induced uPA-dependent plasmin activity was necessary for increased cell migration through ECM, migration was monitored in the presence of excess α2 plasmin inhibitor. As shown in Fig. 3*C*, the addition of α2 plasmin inhibitor to cells blocked their migration through Matrigel in response to tobacco smoke.

uPA-dependent plasminogen activation plays an important role in cellular migration via plasmin cleavage of ECM components. Hence, it was next important to determine the effect of tobacco smoke on ECM degradation by MSK-Leuk1 cells. Cell-derived matrices, metabolically labeled with [^{3}H]glucosamine, are useful as a tracer substrate because [^{3}H]glucosamine is incorporated into proteoglycans and adhesive glycoproteins that are susceptible to plasmin cleavage. When cells were plated on labeled matrices in medium alone, a small amount of [^{3}H] activity was solubilized (Fig. 3*D*). The addition of exogenous plasminogen, the substrate for

uPA, led to ~2-fold increase in solubilization of labeled ECM, an effect that was blocked by the uPA inhibitor amiloride. Exposure of cells to tobacco smoke alone led to approximately a doubling of ECM degradation. When cells were incubated with tobacco smoke and plasminogen, ECM degradation was dramatically increased (5-fold), an effect that was blocked by the addition of amiloride (Fig. 3*D*). Taken together, these data indicate that tobacco smoke–mediated induction of uPA leads to increased plasmin production, resulting, in turn, in increased ECM degradation and enhanced ability of MSK-Leuk1 cells to migrate through ECM.

EGFR signaling is responsible for tobacco smoke–mediated induction of uPA and invasiveness. We next investigated the signaling pathway by which tobacco smoke induced uPA and cell invasiveness. Activation of EGFR signaling can induce uPA expression (33). Previously, we found that tobacco smoke stimulated the synthesis and release of amphiregulin, resulting in activation of EGFR signaling (27). It was logical, therefore, to investigate whether tobacco smoke induced uPA expression by an EGFR-dependent mechanism. As shown in Fig. 4*A* and *B*, tobacco smoke–mediated induction of uPA was suppressed by treatment with either a neutralizing antibody to EGFR or AG1478, an inhibitor of EGFR tyrosine kinase. Importantly, the inductive effect of tobacco smoke was also suppressed by the addition of an antibody to amphiregulin. In contrast, control IgG had no effect on the induction of uPA by tobacco smoke. To confirm the significance of amphiregulin as an inducer of uPA, exogenous amphiregulin was used. Treatment with amphiregulin led to dose-dependent induction of uPA (Fig. 4*C*). We next determined whether tobacco smoke–induced transmigration through Matrigel-coated inserts was EGFR dependent. For this purpose, cells were preincubated with AG1478 or neutralizing antibodies to EGFR or amphiregulin. As seen in Fig. 4*D*, tobacco smoke–induced transmigration was blocked by AG1478 or antibodies to EGFR or amphiregulin.

Stimulation of EGFR signaling leads to activation of ERK1/2 MAPK. It was of interest, therefore, to determine whether ERK1/2 was involved in tobacco smoke–mediated induction of uPA and cellular transmigration. As shown in Fig. 5*A*, tobacco smoke caused a rapid increase in ERK1/2 activity. Notably, PD98059, an inhibitor of MAPK kinase, suppressed tobacco smoke–mediated induction of uPA expression and activity (Fig. 5*B*) and the migration of MSK-Leuk1 cells through ECM (Fig. 5*C*).

Discussion

The mechanisms responsible for clonal spreading of noninvasive leukoplakic lesions are unknown (3, 4). Several lines of evidence suggest that uPA, together with its high-affinity receptor, uPAR, is important in initiating a proteolytic cascade that stimulates cell migration (7–12). In this regard, protease activities expressed by buccal cells obtained from smokers and leukoplakia patients were

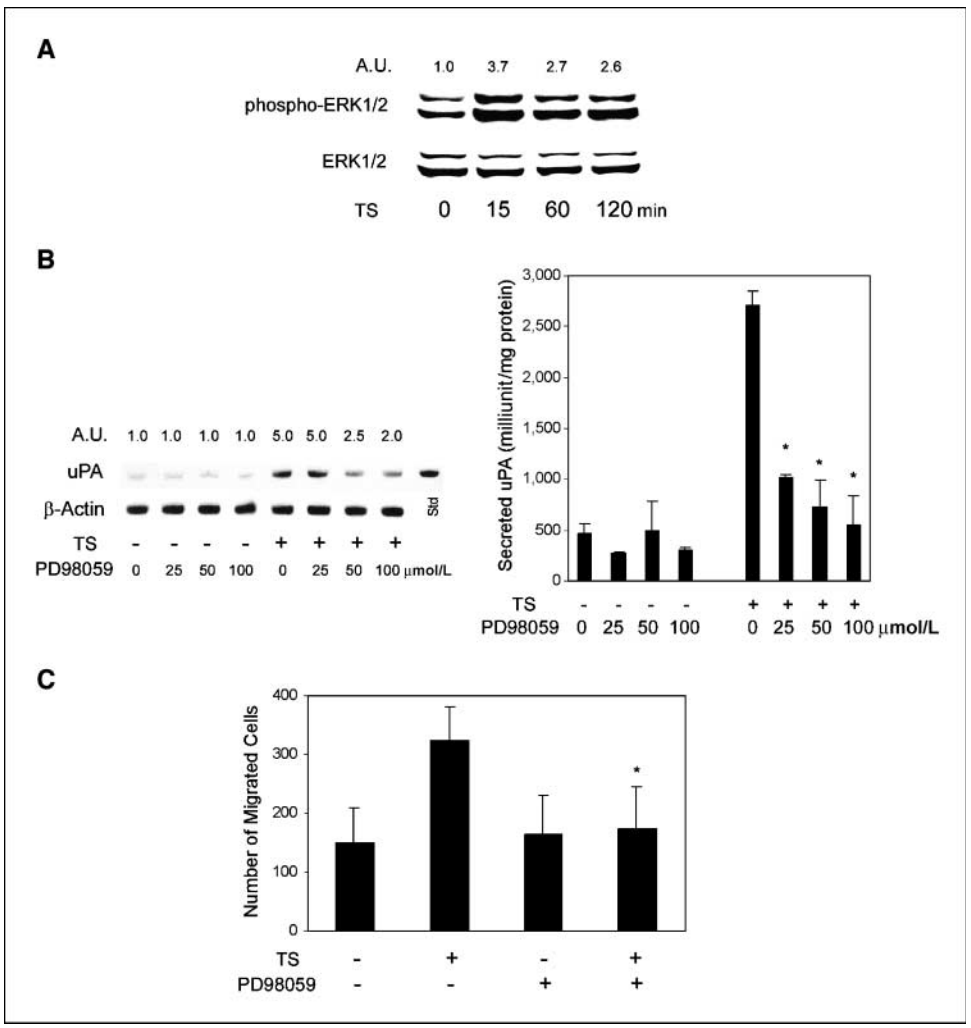

Figure 5. ERK1/2 is critical for tobacco smoke–mediated induction of cell invasion. *A,* cells were treated with tobacco smoke for 0 to 120 min. Cellular protein (100 μg/lane) was loaded onto a 10% SDS-polyacrylamide gel, electrophoresed, and subsequently transferred onto nitrocellulose. The blot was probed with antibodies to the phosphorylated and unphosphorylated forms of ERK1/2. Results of the data shown are expressed in arbitrary units. *B,* cells were treated with vehicle or PD98059 for 2 h. Subsequently, the cells received either vehicle or tobacco smoke as indicated for 6 h. *Left,* RT-PCR was used to determine the amounts of mRNAs for uPA and β-actin. Results of the data shown are expressed in arbitrary units. *Right,* the amount of secreted uPA was measured in the medium. *Columns,* mean ($n = 3$); *bars,* SD. *, $P < 0.05$, compared with tobacco smoke. *C,* cells were suspended in basal medium and incubated for 1 h with vehicle or 25 μmol/L PD98059. Cells (2×10^4) were then aliquoted into an insert containing a porous (8 μm) polycarbonate Matrigel-coated insert. The cells in the insert were then treated with vehicle or tobacco smoke. The insert was placed into a well containing growth medium and incubated at 37°C for 24 h. Cells that migrated into the lower well were then counted after being fixed and stained. *Columns,* mean ($n = 3$); *bars,* SD. *, $P < 0.05$, compared with tobacco smoke.

significantly elevated when compared with a nonsmoking control group (34). In experiments reported here, we have extended these observations by showing that exposure to tobacco smoke led to urokinase-dependent plasminogen activation and increased cell migration of MSK-Leuk1 cells. The importance of uPA in mediating cell invasion was supported by evidence that antibodies to uPA or treatment with amiloride, an inhibitor of uPA, blocked tobacco smoke–mediated induction of cell invasion. The induction of uPA by tobacco smoke stimulated the conversion of plasminogen to plasmin, resulting, in turn, in the degradation of ECM and cell invasion. This mechanism may help to explain why some clinically determined SPT have a common clonal origin in smokers (3, 4). Consistent with this notion, patients who continue to smoke after the successful treatment of their index head and neck malignancy have a significantly increased risk of developing SPT (25). Of course, SPT may also be a consequence of field cancerization (4, 6). Notably, the discovery that tobacco smoke stimulated cell invasion raises the intriguing possibility that clonal migration may occur as a consequence of environmental factors in addition to properties that are intrinsic to the neoplastic cell. Although there is extensive evidence that oncogenes induce uPA and cell invasion (7, 35), this study suggests that tobacco smoke contains chemicals that can mimic these effects of oncogenes. To our knowledge, this is the first study to highlight the potential role of a reversible exogenous factor (i.e., tobacco smoke) in driving cell invasion. Numerous lines of investigation suggest that the uPA-uPAR axis plays a significant role in metastasis (12, 23, 24, 37). Based on the findings in this study, we speculate that tobacco smoke–mediated activation of the uPA-uPAR axis may contribute to the worse prognosis of cancer patients who continue to smoke.

Several observations support a critical role for the EGFR in tobacco smoke–mediated induction of uPA and cell invasiveness. Previously, we showed that tobacco smoke stimulated amphiregulin transcription, resulting in activation of EGFR signaling (30). Here, we found that AG1478, an EGFR tyrosine kinase inhibitor, or antibodies to the ligand-binding site of EGFR or amphiregulin blocked tobacco smoke–mediated induction of uPA and cell invasion. Moreover, treatment of cells with exogenous amphiregulin led to marked induction of uPA. These findings add to growing evidence that disrupting the release or actions of amphiregulin may inhibit carcinogenesis (37, 38). Although this is the first study to show that tobacco smoke induced uPA by an EGFR-dependent mechanism, activation of EGFR is known to regulate the expression of uPA in other systems (33). Previously, growth factor–mediated activation of EGFR was found to stimulate ERK1/2 activity, leading, in turn, to increased uPA expression (33, 39). Similarly, in the current study, several findings suggest an important role for ERK1/2 in tobacco smoke–mediated induction of uPA. Treatment with tobacco smoke activated ERK1/2 MAPK. More importantly, PD98059, an inhibitor of MAPK kinase, blocked tobacco smoke–mediated induction of uPA expression and activity and the migration of MSK-Leuk1 cells through ECM. The activator protein-1 transcription factor complex is likely to contribute to the induction of uPA because it can regulate *uPA* transcription (40) and is activated by ERK1/2. Taking these data together, we conclude that tobacco smoke–mediated activation of EGFR→ERK1/2 MAPK led to induction of uPA and increased cell migration. Previously, binding of uPA to uPAR was reported to stimulate EGFR→ERK1/2 MAPK signaling (41). Hence, it is possible that tobacco smoke will stimulate a positive feedback loop whereby activation of the EGFR→ERK1/2 MAPK pathway causes induction of uPA, which binds to uPAR, causing further activation of EGFR signaling and induction of uPA. Clearly, this type of positive feedback loop could amplify the procarcinogenic effects of tobacco smoke. Inhibitors of EGFR tyrosine kinase are clinically available. This study strengthens the rationale for evaluating whether an inhibitor of EGFR tyrosine kinase can prevent or delay the development of tobacco smoke–related SPT of the aerodigestive tract.

Acknowledgments

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