Endogenously produced urokinase-type plasminogen activator is a major determinant of the basal level of activated ERK/MAP kinase and prevents apoptosis in MDA-MB-231 breast cancer cells

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SUMMARY

Urokinase-type plasminogen activator (uPA) binds to the uPA receptor (uPAR) and activates the Ras-extracellular signal-regulated kinase (ERK) signaling pathway in many different cell types. In this study, we demonstrated that endogenously produced uPA functions as a major determinant of the basal level of activated ERK in MDA-MB-231 breast cancer cells. When these cells were cultured in the presence of antibodies that block the binding of uPA to uPAR, the level of phosphorylated ERK decreased substantially. Furthermore, conditioned medium from MDA-MB-231 cells activated ERK in MCF-7 cells and this response was blocked by uPA-specific antibody. The mitogen-activated protein kinase kinase inhibitor, PD098059, decreased expression of uPA and uPAR in MDA-MB-231 cells. Thus, uPA and the uPAR-ERK signaling pathway form a positive feedback loop in these cells. When this feedback loop was disrupted with uPA- or uPAR-specific antibody, uPA mRNA-specific antisense oligodeoxynucleotides or PD098059, cell growth was inhibited and apoptosis was promoted, as determined by the increase in cytoplasmic nucleosomes and caspase-3 activity. Treating the cells simultaneously with PD098059 and uPA- or uPAR-specific antibody did not further promote apoptosis, compared with either reagent added separately, supporting the hypothesis that uPAR and ERK are components of the same cell growth/survival-regulatory pathway. The ability of uPA to signal through uPAR, maintain an elevated basal level of activated ERK and inhibit apoptosis represents a novel mechanism whereby the uPA-uPAR system may affect breast cancer progression in vivo.

Key words: Urokinase-type plasminogen activator, uPAR, MAP kinase/extracellular signal-regulated kinase, Apoptosis, Breast cancer

INTRODUCTION

In breast cancer, the mitogen-activated protein kinases, extracellular signal-regulated kinase (ERK)-1 and ERK-2, are frequently hyperexpressed and exhibit increased activity (Sivaraman et al., 1997). This is important because activated ERK controls many processes that are central to cancer progression, including cell growth, apoptosis and cell migration (Cho and Klemke, 2000). Activated ERK may also promote cancer cell invasion by upregulating expression of proteinases and associated receptors that are involved in this process, including urokinase-type plasminogen activator (uPA), its cell-surface receptor uPAR (Seddinghzadeh et al., 1999; Simon et al., 1996; Lengyel et al., 1997) and matrix metalloproteinases (Reddy et al., 1999).

In many types of cancer, ERK activation reflects the activity of mutated forms of Ras; however, in breast cancer, activating *Ras* mutations are rare (Rochlitz et al., 1989; Miyakis et al., 1998). Instead, any one of the three major *Ras* genes (*H-Ras*, *K-Ras*, *N-Ras*) may be overexpressed and this process correlates with cancer progression (Miyakis et al., 1998; Shackney et al., 1998; Smith et al., 2000). Because wild-type Ras is subject to regulation by GTPase activating proteins and guanine nucleotide exchange factors, irrespective of expression level (Rebollo and Martinez, 1999), it is crucial to understand the upstream factors that control Ras and ERK activity in these cells.

We and others have shown that binding of uPA to uPAR activates ERK (Kanse et al., 1997; Konakova et al., 1998; Nguyen et al., 1998; Tang et al., 1998; Nguyen et al., 1999; Aguirre-Ghiso et al., 1999). Furthermore, in MCF-7 cells, uPA-promoted ERK activation is entirely dependent on Ras (Nguyen et al., 1999). We hypothesized that in breast cancer, uPA produced by neoplastic or non-neoplastic cells may bind to malignant epithelial cell uPAR and thereby regulate the steady-state level of activated ERK in these cells. There is evidence that the equivalent autocrine/paracrine pathway may be operational in cell culture. For example, when receptors in the LDL receptor family (LRFs) are neutralized so that catabolism of uPA and uPAR is inhibited, increased binding of endogenously produced uPA to uPAR is observed and the basal level of activated ERK is increased (Webb et al., 1999; 2000). In epidermoid carcinoma cells, the basal level of activated ERK correlates with uPAR expression, probably reflecting the

activity of a signaling-receptor complex in which uPAR is associated with the integrin $\alpha_5\beta_1$ (Aguirre-Ghiso et al., 1999).

MDA-MB-231 cells provide a model of aggressive human breast cancer. These cells express high basal levels of activated ERK, which is necessary for increased cyclin D1 expression, rapid cell growth and increased uPA gene transcription (Seddinghzadeh et al., 1999). In the present study, we demonstrated that an autocrine pathway, in which endogenously produced uPA binds to uPAR, is a major determinant of the high basal level of activated ERK in MDA-MB-231 cells. The increase in activated ERK, which resulted from the endogenous uPA-uPAR system, was essential not only for promoting rapid growth, but also for suppressing apoptosis. These studies suggest a novel mechanism whereby the uPA-uPAR system may promote breast cancer progression in vivo.

MATERIALS AND METHODS

Reagents and proteins

Single-chain uPA and two-chain uPA were kindly provided by Jack Henkin and Andrew Mazar (Abbott Laboratories). Two-chain uPA was inactivated with diisopropyl fluorophosphate to form DIP-uPA, as previously described (Nguyen et al., 1998). Rabbit IgG-specific antibody-peroxidase conjugate, goat IgG-specific antibody-peroxidase conjugate, purified nonimmune rabbit IgG and mouse nonimmune IgG were from Sigma. IODO-BEADS were from Pierce. $Na^{125}I$ and [α-^{32}P]dATP were from Amersham. Both the uPA-specific monoclonal antibody that recognizes the amino-terminal receptor-binding domain of uPA (#3471) and the uPAR-specific polyclonal antibody 399R were from American Diagnostica. These antibodies block uPA-binding to uPAR (Nguyen et al., 1999). Human uPA-specific polyclonal antibody 776 was from Chemicon. The mitogen-activated protein (MAP) kinase kinase (MEK) inhibitor, PD098059, and polyclonal antibody that recognizes phosphorylated extracellular signal-regulated kinase (ERK) were from Calbiochem. Polyclonal antibody that recognizes total ERK was from Zymed. [Methyl-^{3}H]thymidine was from NEN Life Science Products. The Cell Proliferation Kit I, which detects viable cells using a substrate for succinyl dehydrogenase, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), and the Cell Death Detection ELISA^{PLUS} kit were from Roche Molecular Biochemicals. Caspase-3 fluorogenic substrate *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (Ac-DEVD-AFC) and caspase 3 inhibitor *N*-acetyl-Asp-Glu-Val-Asp-CHO aldehyde (Ac-DEVD-CHO) were from PharMingen International. Effectene^{TM} transfection reagent kit was from Qiagen.

Cell culture

MDA-MB-231 cells were purchased from the ATCC and cultured in L-15 medium (Life Technologies, Inc.) supplemented with 10% FBS, 100 units/ml penicillin (Life Technologies, Inc.) and 100 μg/ml streptomycin (Life Technologies, Inc.). Cells were passaged at subconfluence with Cell Dissociation Buffer (Enzyme Free, Hank's-based, Life Technologies, Inc.). Low-passage MCF-7 cells were cultured as previously described (Nguyen et al., 1998).

uPA-binding to MDA-MB-231 cells

Cell-surface uPAR was determined by measuring the specific binding of ^{125}I-DIP-uPA at 4ºC. DIP-uPA was radioiodinated to a specific activity of 1-2 μCi/μg using IODO-BEADS. To dissociate endogenously produced uPA from uPAR, the MDA-MB-231 cells were subjected to a mild acid wash. The acid-wash protocol involved treating confluent cultures in sequence with: Earle's balanced salt solution (EBSS), 10 mM HEPES, pH 7.4 for 2 minutes; 50 mM glycine-HCl, 100 mM NaCl, pH 3.0 for 30 minutes; and 0.5 M HEPES, 0.1 M NaCl, pH 7.5 for 2 minutes. The cells were then washed three times with EBSS, 10 mM HEPES, pH 7.4 with 10 mg/ml bovine serum albumin (EHB medium). All of the steps in the acid-wash procedure were executed at 4ºC.

uPA-binding was studied 30 minutes after completing the acid-wash protocol. Increasing concentrations of ^{125}I-DIP-uPA were incubated with the MDA-MB-231 cells in EHB for 4 hours at 4°C. A 100-fold molar excess of nonradiolabeled DIP-uPA was added to some cultures to distinguish specific and nonspecific binding. At the end of an incubation, cultures were washed three times with EBSS, 10 mM HEPES, pH 7.4. Cell associated radioactivity was then recovered in 0.1 N NaOH, 1% (w/v) SDS and measured in a γ-counter. Cellular protein was determined by bicinchoninic acid assay. K_D and B_{max} values were determined by fitting specific-binding isotherms to the equation for a rectangular hyperbola and by Scatchard transformation. To express the B_{max} in terms of uPA-binding sites/cell, the average mass of the MDA-MB-231 cell was determined. In four separate experiments, suspended MDA-MB-231 cells were counted using a hemocytometer. The protein content of each preparation was then determined. The mass was 0.51±0.08 ng/cell.

uPA accumulation in conditioned medium

Confluent cultures of MDA-MB-231 cells were washed extensively and cultured for 24 hours in serum-free L-15 medium without phenol red. In some cultures, uPA-specific antibody 3471, which blocks uPA-binding to uPAR, was included in the medium. Conditioned medium (CM) was recovered and concentrated 20-fold using Centricon concentrators with 10 kDa exclusion filters (Amicon). To determine the uPA concentration in CM, samples were subjected to SDS-PAGE and compared with known concentrations of purified scuPA. Proteins were transferred to nitrocellulose membranes and probed with goat anti-human uPA polyclonal antibody followed by anti-goat IgG-peroxidase conjugate. Secondary antibody was detected by enhanced chemiluminescence (ECL).

Analysis of ERK phosphorylation

Phosphorylated and total ERK were detected by immunoblot analysis, as previously described (Nguyen et al., 1998). MDA-MB-231 cells were grown in 6-well plates until 50% confluent and washed three times with 20 mM sodium phosphate, 150 mM NaCl, pH 7.4 (PBS). The cells were then cultured in serum-free medium, supplemented with uPA-specific antibody (25 μg/ml), nonimmune mouse IgG (25 μg/ml) or PD098059 for 24 hours. Some cultures were subjected to a mild acid wash, as described in the uPA-binding experiments, except that the incubation at pH 3.0 was conducted for 3 minutes at 22ºC. The cells were then allowed to recover for 18 hours in serum-containing medium, washed and incubated for 24 hours in serum-free medium with uPAR-specific antibody (50 μg/ml) or nonimmune rabbit IgG (50 μg/ml). At the end of each incubation, the medium was aspirated and replaced with ice-cold PBS containing 2 mg/ml sodium vanadate. The cells were then extracted with 1% Nonidet P-40, 50 mM HEPES, 100 mM NaCl, 2 mM EDTA, 1 μg/ml leupeptin, 100 KIU/ml aprotinin, 0.4 mg/ml sodium vanadate, 0.4 mg/ml sodium fluoride and 5 mg/ml dithiothreitol, pH 7.4. The extracts were subjected to SDS-PAGE on 12% slabs. Proteins were transferred to nitrocellulose membranes and probed with antibodies that detect phosphorylated and total ERK.

Cell growth assays

Viable cell number was determined by MTT assay using the Cell Proliferation Kit I. MDA-MB-231 cells were plated at the indicated densities in 96-well plates. Twelve hours later, the cells were washed with serum-free medium. Selected cultures were subjected to a mild acid wash and allowed to recover for 18 hours. Acid-washed cultures were treated with uPAR-specific antibody, rabbit nonimmune IgG, PD098059 or DMSO (the vehicle for PD098059) in serum-free medium for 24 hours. The cultures that were not acid-washed were

treated with uPA-specific antibody, mouse nonimmune IgG, PD098059 or vehicle in serum-free medium for 24 hours. MTT hydrolysis was determined as directed by manufacturer and detected on the basis of the absorbance at 570 nm.

DNA synthesis was assessed by measuring [^{3}H]thymidine incorporation. 4×10^4 cells were seeded into 48-well plates and cultured overnight. Some cultures were subjected to a mild acid wash and allowed to recover for 18 hours. The cells were then treated with uPA-specific antibody, uPAR-specific antibody, nonimmune IgG, PD098059 or DMSO in serum-free medium for 30 hours. Each culture was pulse-exposed to [^{3}H]thymidine (1 μCi/ml) for 1 hour at 37°C and washed twice with EBSS, 25 mM HEPES, pH 7.4. The cultures were then treated with 10% trichloroacetic acid (TCA) for 10 minutes at 4°C, washed and treated again with TCA at 22°C. Radioactivity that remained cell-associated was recovered in 1.0 N NaOH. After neutralizing the pH, samples were combined with Ready-safe scintillation fluid. Radioactivity recovery was determined using a Beckman LS6500 scintillation counter.

Chromosome fragmentation analysis

In apoptosis, activated endonucleases cleave chromosomal DNA at internucleosomal linker regions, leading to the accumulation of intracytoplasmic DNA- and histone-containing mono- and oligonucleosomes (Hockenberry, 1995; Duke and Cohen, 1986). Intracytoplasmic nucleosomes were quantitated, in MDA-MB-231 cells, using the Cell Death Detection ELISA^{PLUS} Kit (Roche Molecular Biochemicals). This kit provides separate antibodies directed against DNA and histones so that quantitative sandwich-immunoassays may be performed. MDA-MB-231 cells were incubated with uPA-specific antibody or PD098059 for 24 hours at 37°C in 96-well plates. Some cultures were acid-washed, allowed to recover for 18 hours and then treated with uPAR-specific antibody or PD098059 for 24 hours. Cytoplasmic fractions were prepared, as directed by the manufacturer. ELISA assays were executed in quadruplicate.

Caspase-3 activity assays

MDA-MB-231 cells (5×10^5) were cultured in 6-well plates and treated with uPA-specific antibody or with PD098059 in serum-free medium for 24 hours. Some cultures were subjected to mild acid wash, allowed to recover and treated with uPAR-specific antibody or PD098059. The medium was then aspirated. Dead cells or cell fragments in the medium were recovered by centrifugation at 11,000 ***g*** with extracts of intact cells from the monolayer, which were obtained by scraping in 20 mM HEPES, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol and 0.1% CHAPS, pH 7.2 at 4ºC. Equal amounts of cell protein (50 μg) were incubated with the caspase-3 fluorogenic substrate Ac-DEVD-AFC (20 μM) in 20 mM HEPES, 100 mM NaCl, 1 mM EDTA, 10 mM dithiothreitol, 0.1% CHAPS, 10% (w/v) sucrose, pH 7.2. Release of 7-amino-4-trifluoromethylcoumarin was monitored continuously for 3 hours using a Cytofluor 2350 spectrofluorometer. The excitation wavelength was 390 nm and the emission wavelength was 530 nm. To confirm that substrate hydrolysis was due to caspase-3, some cell extracts were incubated with the caspase-3 inhibitor, Ac-DEVD-CHO (0.1 μM) for 30 minutes at 37°C before adding Ac-DEVD-AFC. Ac-DEVD-CHO inhibited substrate hydrolysis by >95% in all of our experiments. Velocities of Ac-DEVD-AFC hydrolysis were always linear in the 3 hour monitoring period.

uPA antisense oligonucleotide treatments

MDA-MB-231 cells were treated with antisense oligodeoxynucleotides, which were designed to hybridize with the initiation codon of the uPA mRNA (5′ CAG CAG GGC TCT CAT GGT 3′). A sense uPA oligodeoxynucleotide (5′ ACC ATG AGA GCC CTG CTG 3′) was used as one control. A GenBank search confirmed that the oligodeoxynucleotide sequences are homologous only with the uPA mRNA. A nonsense oligodeoxynucleotide with the same G+C content (5′ GAT CGG ATC GGC GAT CAG 3′) was prepared as a second control. The oligodeoxynucleotides were modified with phosphorothioate linkages, rendering them nuclease resistant and delivered to MDA-MB-231 cells, in 6-well plates, using Effectene transfection reagent (8 μl of Effectene per 1 μg of oligodeoxynucleotide). The cells were incubated with the DNA-Effectene mixture for 24 hours, washed twice and maintained in fresh serum-supplemented medium for 24 hours. An equal number of cells from each preparation was transferred into serum-free medium and incubated for another 24 hours. uPA levels in serum-free CM were determined by immunoblot analysis. The number of viable cells at the end of the 24 hour incubation period was determined by MTT assay. Chromosome fragmentation was determined by ELISA.

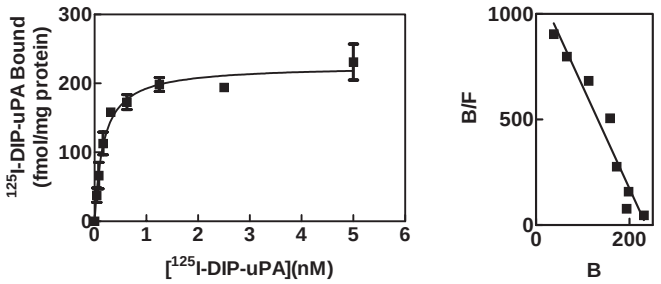

Fig. 1. Equilibrium binding of ^{125}I-DIP-uPA to MDA-MB-231 cells. MDA-MB-231 cells were acid-washed and then incubated with increasing concentrations of ^{125}I-DIP-uPA for 4 hours at 4°C. The specific binding isotherm is shown in panel A. Each point represents the mean ± s.e.m. of results from two separate experiments, each with triplicate determinations. The Scatchard transformation of the same data is shown in panel B. *B/F*, bound/free.

Northern blot analysis of uPA and uPAR mRNA expression

MDA-MB-231 cells were cultured until confluent in T-75 flasks. The cells were then washed three times with PBS and cultured in serum-free medium supplemented with PD098059 (10 or 50 μM) or vehicle (0.1% DMSO, v/v) for an additional 24 hours. Total cellular RNA was purified using the RNeasy Mini Kit (Qiagen). Equal amounts of RNA (20 μg) were subjected to electrophoresis in 1.0% (w/v) agarose-formamide gels and transferred to nitrocellulose membranes (Biorad). The membranes were sequentially probed to detect mRNA for uPA, uPAR and phosphoglyceraldehyde dehydrogenase (PGAD) using DNA probes that were labeled with [α-^{32}P]dATP using the Rediprime II random prime labeling system.

RESULTS

uPAR expression by MDA-MB-231 cells

Holst-Hansen et al. (1996) reported that MDA-MB-231 breast cancer cells express high levels of uPA and uPAR, which are critical for cell invasion through Matrigel. By contrast, MCF-7 breast cancer cells are poorly invasive. We previously examined the binding of radioiodinated DIP-uPA to MCF-7 cells and reported that these cells express approximately 3400 copies of cell-surface uPAR (Nguyen et al., 1998; Webb et al., 1999). In the present study, we examined the binding of ^{125}I-DIP-uPA to acid-washed MDA-MB-231 cells (Fig. 1). ^{125}I-DIP-uPA-binding was specific and saturable. The K_D was 0.17±0.02 nM, in good agreement with K_D values determined for uPA-binding to other cell types (Mignatti and Rifkin, 2000). The B_{max} was 225±7 fmol/mg of cell protein, which

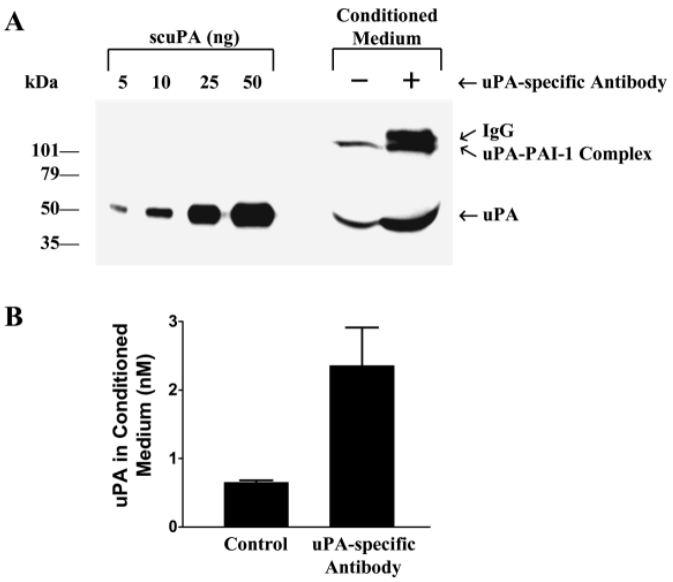

Fig. 2. Accumulation of uPA in MDA-MB-231 cell-conditioned medium. (A) Immunoblot analysis was used to detect uPA in serum-free medium conditioned by MDA-MB-231 cells in the presence or absence of uPA-specific antibody for 24 hours. A standard curve was generated using known amounts of purified uPA (5-50 ng). The low mobility band is assumed to represent uPA-PAI-1 complex, due to the apparent molecular mass. (B) Levels of UPA in CM were quantitated by densitometry, in comparison with the standard curve (*n*=3).

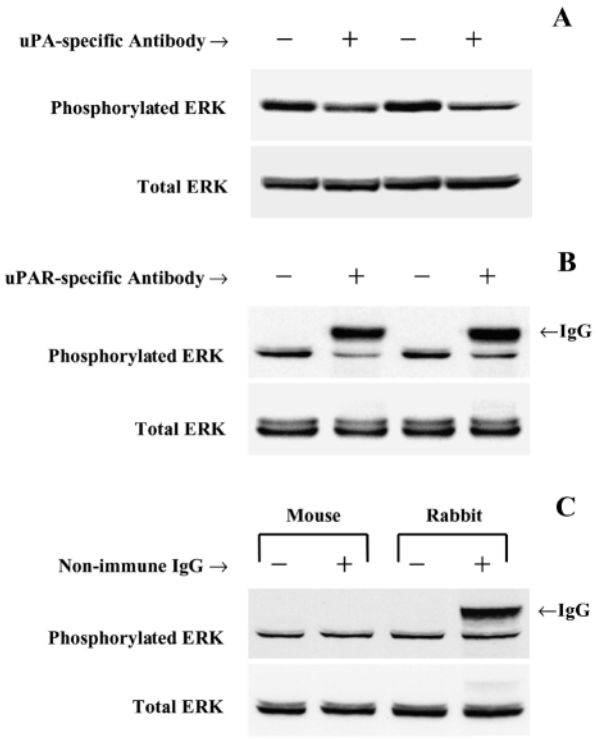

Fig. 3. ERK phosphorylation in MDA-MB-231 cells treated with uPA- or uPAR-specific antibody. Cells were treated for 24 hours with uPA-specific antibody (panel A), uPAR-specific antibody (panel B) or nonimmune IgG from mouse or rabbit (panel C). Lanes labeled '–' were treated with vehicle. Cell extracts were subjected to SDS-PAGE and electrotransfered to nitrocellulose membranes, which were probed to detect phosphorylated ERK, stripped and then re-probed to detect total ERK. Duplicate determinations from separate cultures are shown in panels A and B.

corresponds to approximately 70,000 receptors/cell. The level of uPAR detected in MDA-MB-231 cells by ^{125}I-DIP-uPA binding was approximately one half of that determined by ELISA (Holst-Hansen et al., 1996); however, the ELISA detects cell-surface and intracytoplasmic pools of uPAR.

^{125}I-DIP-uPA-binding experiments were also performed using MDA-MB-231 cells that were not acid-washed. Under these conditions, little or no specific binding was detected. We interpret these results to indicate that MDA-MB-231 cell-surface uPAR is largely saturated by endogenously produced uPA under our standard culturing conditions.

uPA accumulation in MDA-MB-231 cell conditioned medium

MDA-MB-231 cells were allowed to condition serum-free medium for 24 hours. uPA in the CM was detected by immunoblot analysis and quantitated by comparison to a standard curve, generated using known quantities of purified uPA. As shown in Fig. 2, MDA-MB-231 cell CM contained uPA and a lower-mobility species with an apparent molecular mass consistent with the predicted mass for uPA-plasminogen activator inhibitor-1 (PAI-1) complex. The concentration of free uPA in the CM was 0.7±0.1 nM (*n*=3), which is considerably higher than the K_D for uPA-binding to MDA-MB-231 cell uPAR. The uPA-PAI-1 complex also may have contributed to the total available pool of ligand for uPAR as uPA-PAI-1 complex and free uPA bind to uPAR with equivalent affinity (Jensen et al., 1990).

To determine whether uPA-binding to uPAR depletes CM of uPA, we conditioned serum-free medium for 24 hours in the presence of uPA-specific antibody, which blocks uPA-binding to uPAR. The uPA concentration in the CM was increased more than threefold, suggesting that cellular uptake of uPA affects accumulation in CM. In addition to uPAR, cellular uptake of uPA may involve LRFs, which bind free uPA with low affinity and uPAR-associated uPA that has reacted with PAI-1 (Kounnas et al., 1993; Heegaard et al., 1995). It is not known whether uPA-specific antibody 3471 affects uPA-binding to LRFs.

The uPA-uPAR system controls the basal level of phosphorylated ERK in MDA-MB-231 cells

MDA-MB-231 cells were cultured for 24 hours in the presence of uPA-specific antibody (25 μg/ml) to inhibit binding of endogenously produced uPA to uPAR. We previously demonstrated that the concentration of antibody used in this study is sufficient to inhibit ^{125}I-DIP-uPA binding to uPAR by more than 95% (Nguyen et al., 1999). Fig. 3A shows that uPA-specific antibody decreased the level of phosphorylated ERK in the MDA-MB-231 cells. In four separate experiments, phosphorylated ERK was decreased by 55±4%. Nonimmune mouse IgG, at the same concentration, did not affect ERK phosphorylation (Fig. 3C). These results suggest that autocrine signaling through uPAR is a major determinant of the basal level of ERK phosphorylation, in MDA-MB-231 cells, in the absence of exogenous stimuli.

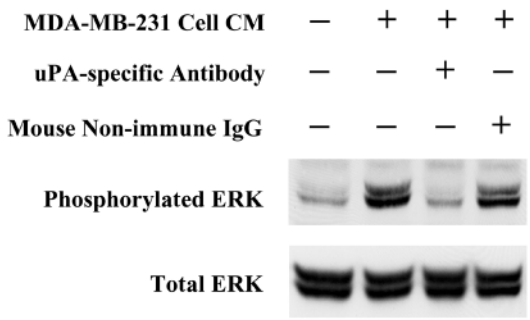

Fig. 4. Activation of MCF-7 cell ERK by MDA-MB-231 cell CM. MDA-MB-231 cells were allowed to condition serum-free medium for 24 hours. The CM was subjected to centrifugation at 15,000 ***g*** for 10 minutes to remove debris and then added to cultures of MCF-7 cells that had been serum-starved for 12 hours. Some samples of CM were pre-incubated with uPA-specific antibody (25 μg/ml) or mouse nonimmune IgG (25 μg/ml) for 20 minutes at 37°C before being added to the MCF-7 cell cultures. ERK phosphorylation was assessed 1 minute after addition of the CM.

To confirm that uPA-specific antibody decreased ERK phosphorylation by inhibiting uPA-binding to uPAR, similar experiments were performed using uPAR-specific antibody 399R. We found that this antibody was optimally effective when the MDA-MB-231 cells were subjected to a mild acid wash to dissociate endogenously bound uPA and then allowed to recover for 18 hours. As shown in Fig. 3B, basal levels of phosphorylated ERK were decreased in MDA-MB-231 cells that had been cultured in the presence of uPAR-specific antibody (50 μg/ml). In four separate experiments, the decrease in ERK phosphorylation was 68±6%. Because antibody 399R is polyclonal, control experiments were performed in which we demonstrated that nonimmune rabbit IgG (50 μg/ml) does not affect ERK phosphorylation (Fig. 3C).

As further evidence for the activity of MDA-MB-231 cell-produced uPA as a regulator of ERK phosphorylation, MDA-MB-231 cells were allowed to condition serum-free medium for 24 hours. The CM was then added to cultures of MCF-7 cells that had been serum-starved for 12 hours, as previously described (Nguyen et al., 1998). As shown in Fig. 4, ERK phosphorylation was substantially increased within 1 minute of adding the MDA-MB-231 cell CM. When the CM was pre-treated with uPA-specific antibody, ERK phosphorylation was blocked. Nonimmune IgG had no effect on the ability of the CM to stimulate ERK phosphorylation. These results suggest that uPA is the principal ERK-activating agent that accumulates in the MDA-MB-231 cell CM.

The endogenous uPA-uPAR system controls MDA-MB-231 cell growth

uPA does not promote MCF-7 cell growth (Nguyen et al., 1998; Nguyen et al., 1999). To assess the activity of the endogenous uPA-uPAR system in MDA-MB-231 cells, cultures were treated for 24 hours with uPA-specific antibody. Fig. 5 shows that the antibody inhibited MDA-MB-231 cell growth, as determined by MTT assay and [^{3}H]thymidine incorporation. PD098059 (10 μM) also decreased [^{3}H]thymidine incorporation; the magnitude of the effect was slightly greater than that observed with the highest

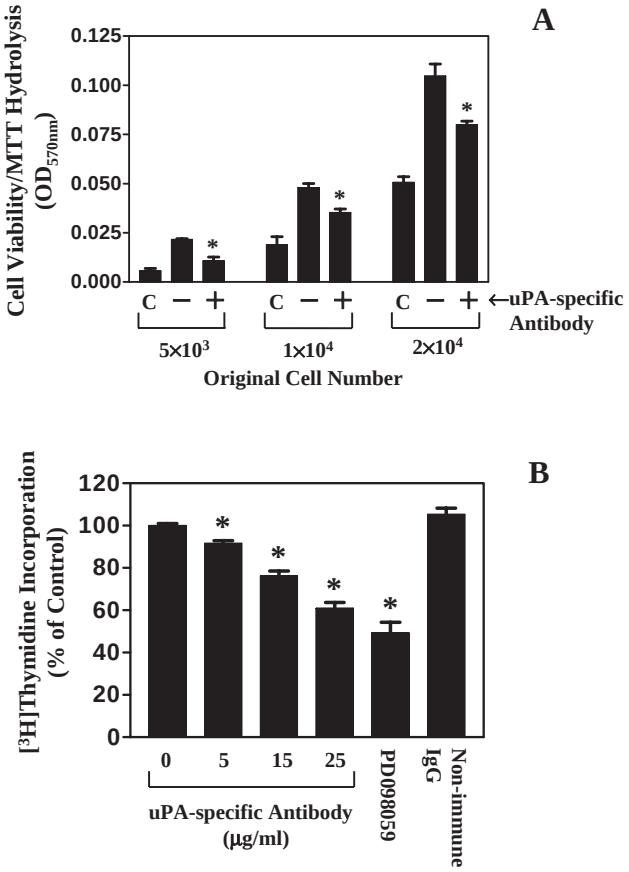

Fig. 5. uPA-specific antibody inhibits the growth of MDA-MB-231 cells. (A) The indicated number of MDA-MB-231 cells was plated in 96-well plates, in triplicate, and cultured overnight. The cultures were then transferred into serum-free medium and incubated for 24 hours with uPA-specific antibody (+) or vehicle (–). Cell growth was determined by MTT assay. The number of viable cells in each culture prior to the 24 hour culturing period was determined as a control (labeled 'C'). The asterisk indicates that antibody significantly altered cell growth compared with vehicle (unpaired *t*-test, $P<0.05$, $n=3$). (B) 4×10^4 MDA-MB-231 cells were plated in 48-well plates, in triplicate, and cultured at 37°C for 24 hours. The cells were then incubated with increasing amount of uPA-specific antibody, PD098059 (10 μM) or mouse nonimmune IgG (25 μg/ml) for 30 hours. [^{3}H]thymidine incorporation was measured. The asterisk indicates that the value is significantly different from the control (unpaired *t*-test, $P<0.01$, $n=3$).

concentration of uPA-specific antibody (25 μg/ml). In control experiments, nonimmune IgG did not affect MDA-MB-231 cell [^{3}H]thymidine incorporation. Furthermore, the effects of uPA-neutralizing antibody on MDA-MB-231 cell growth were blocked when the antibody was pre-incubated for 20 minutes with 0.25 μM DIP-uPA (results not shown).

When cell-growth experiments were performed with uPAR-specific antibody instead of uPA-specific antibody, equivalent results were obtained (Fig. 6). The highest concentration of uPAR-specific antibody (50 μg/ml) decreased [^{3}H]thymidine incorporation comparably to PD098059. Importantly, when cells were treated simultaneously with uPAR-specific antibody and PD098059, cell growth was decreased, as determined by MTT assay and [^{3}H]thymidine incorporation; however, the

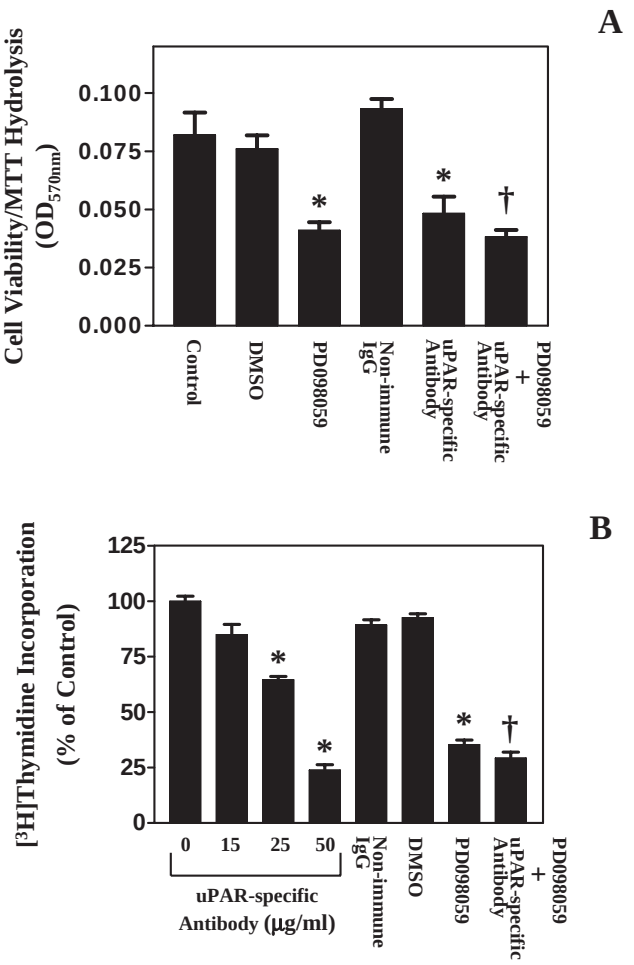

Fig. 6. uPAR-specific antibody inhibits the growth of MDA-MB-231 cells. (A) 5×10^3 cells were plated in 96-well plates, in triplicate, and incubated for 24 hours. The cells were subjected to a mild acid wash and incubated with DMSO (0.1% v/v), PD098059 (10 μM), rabbit nonimmune IgG (50 μg/ml), uPAR-specific antibody (50 μg/ml) or PD098059 (10 μM) plus uPAR-specific antibody (50 μg/ml). Cell growth was measured by MTT assay. The asterisk indicates that the treatment is significantly different from control (unpaired *t*-test, $P<0.015$, $n=3$). The dagger indicates that this result is not significantly different than the result obtained with uPAR-specific antibody or PD098059 alone. (B) An identical series of incubations was executed and cell growth was measured by the [^{3}H]thymidine incorporation method. Asterisks/daggers are as for part A.

magnitude of the effect was no greater than that observed with either reagent alone.

The endogenous uPA-uPAR system prevents apoptosis in MDA-MB-231 cells

A number of studies have demonstrated that activated ERK inhibits apoptosis (Bonni et al., 1999; Xia et al., 1995; Guillonneau et al, 1998). Thus, we hypothesized that the endogenous uPA-uPAR system may prevent MDA-MB-231 cell apoptosis. In support of this hypothesis, Kin et al. (2000) previously demonstrated an increased rate of apoptosis in glioblastoma cells that were transfected to express uPAR antisense RNA. To test the effects of the endogenous uPA-uPAR system on apoptosis, we treated MDA-MB-231 cells with antibodies that block uPA-binding to uPAR or with PD098059. Apoptosis was detected by measuring intracytoplasmic nucleosomes and caspase-3 activity.

Fig. 7A shows that uPA-specific antibody induced a concentration-dependent increase in the cytoplasmic nucleosome level. PD098059 (10 μM) also increased the level of cytoplasmic nucleosomes. Fig. 7B shows that uPA-specific antibody (25 μg/ml) and PD098059 (10 μM) significantly increased caspase-3 activity. In control studies, nonimmune IgG and the PD098059 vehicle (DMSO) had no effect on caspase-3 activity. When uPA-specific antibody and PD098059 were added in combination, the increase in caspase-3 activity was not significantly greater than that observed with either reagent added separately.

Culturing MDA-MB-231 cells with uPAR-specific antibody (50 μg/ml) or with PD098059 increased the cytoplasmic nucleosome level (Fig. 7C) and caspase-3 activity (Fig. 7D). In both assays, simultaneous addition of PD098059 and uPAR-specific antibody was no more effective than either reagent added separately. These results suggest that the autocrine uPA-uPAR system functions to inhibit MDA-MB-231 cell apoptosis and that the mechanism involves the ability of this system to regulate ERK activation.

uPA antisense oligodeoxynucleotides inhibit cell growth and induce apoptosis

uPA antisense oligodeoxynucleotides have been successfully used to reduce uPA expression by cells in culture (McGuire et al., 1993; Engelhard et al., 1996). To further test the role of the uPA-uPAR system in MDA-MB-231 cell growth and apoptosis, phosphorothioate-modified oligodeoxynucleotides corresponding to the translation start-site of the uPA mRNA were introduced into MDA-MB-231 cells. Accumulation of uPA in CM was assessed from 48-72 hours after introduction of the oligodeoxynucleotides. This time period was chosen in consideration of the reported half-life for uPA mRNA in MDA-MB-231 cells, which is 17 hours (Nanbu et al., 1997).

The antisense oligodeoxynucleotides reduced uPA accumulation in CM by 72±3% ($n=4$) (Fig. 8). The uPA level was reduced less than 20% by the sense oligodeoxynucleotides and by less than 5% by the nonsense oligodeoxynucleotides. In MTT assays, antisense oligodeoxynucleotide-treated cells showed a significant decrease in cell growth, whereas sense- and nonsense-oligodeoxynucleotide-treated cells did not. The antisense oligodeoxynucleotide-treated cells also showed a significant increase in intracytoplasmic nucleosomes, suggesting increased apoptosis. As an additional control, sense and antisense oligodeoxynucleotides were mixed at equimolar concentration, heated to 85°C and allowed to hybridize at room temperature. This preparation decreased MDA-MB-231 cell growth by less than 5%, as determined by MTT assay (data not shown). These results support our model regarding the role of the uPA-uPAR system in cell growth and survival.

ERK regulates uPA and uPAR expression in MDA-MB-231 cells

When cells are treated with uPA, ERK functions downstream of uPAR to promote cell migration (Nguyen et al., 1999); however, in many cell types, agents that regulate ERK activation also regulate expression of uPA and uPAR (Seddinghzadeh et al., 1999; Simon et al., 1996; Lengyel et al.,

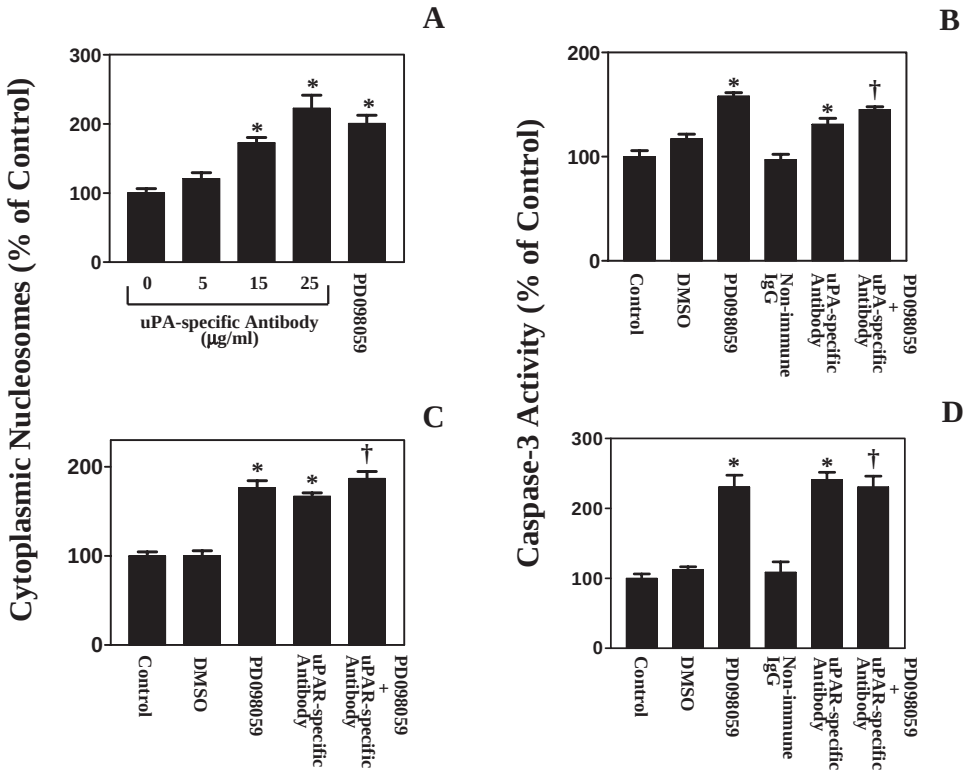

Fig. 7. uPA-and uPAR-specific antibodies induce apoptosis in MDA-MB-231 cells. (A) MDA-MB-231 cells were cultured in 96-well plates and then incubated in serum-free medium, supplemented with uPA-specific antibody or PD098059 (10 μM) for 24 hours. Cytoplasmic nucleosomes were detected by ELISA. (B) MDA-MB-231 cells were cultured in 6-well plates and treated with DMSO (0.1% v/v), PD098059 (10 μM), mouse nonimmune IgG (25 μg/ml), uPA-specific antibody (25 μg/ml) or uPA-specific antibody (25 μg/ml) plus PD098059 (10 μM) for 24 hours. Caspase-3 activity was then measured. (C) MDA-MB-231 cells were subjected to mild acid wash and then cultured in serum-free medium containing DMSO (0.1% v/v), PD098059 (10 μM), uPAR-specific antibody (50 μg/ml) or uPAR-specific antibody (50 μg/ml) plus PD098059 (10 μM) for 24 hours. Cytoplasmic nucleosomes were detected by ELISA. (D) MDA-MB-231 cells were subjected to a mild acid wash and then cultured for 24 hours in serum-free medium containing DMSO (0.1% v/v), PD098059 (10 μM), rabbit nonimmune IgG (50 μg/ml), uPAR-specific antibody (50 μg/ml) or uPAR-specific antibody (50 μg/ml) plus PD098059 (10 μM). Caspase-3 activity was determined. The asterisk indicates a significant difference compared with control (unpaired *t*-test, $P<0.05$, $n=3$). The dagger indicates that uPA- or uPAR-specific antibody in combination with PD098059 did not significantly alter apoptosis compared with either reagent alone.

1997). Thus, ERK may affect cell growth and apoptosis indirectly by its effects on expression of uPA and uPAR. To test whether ERK regulates the expression of uPA and uPAR in MDA-MB-231 cells, cultures were treated with PD098059 in serum-free medium for 24 hours. As shown in Fig. 9A, PD098059 substantially decreased uPA accumulation in CM, levels of cell-associated uPAR and levels of soluble uPAR in the CM.

To determine whether the decreased levels of uPA and uPAR reflected changes in gene transcription or mRNA stability,

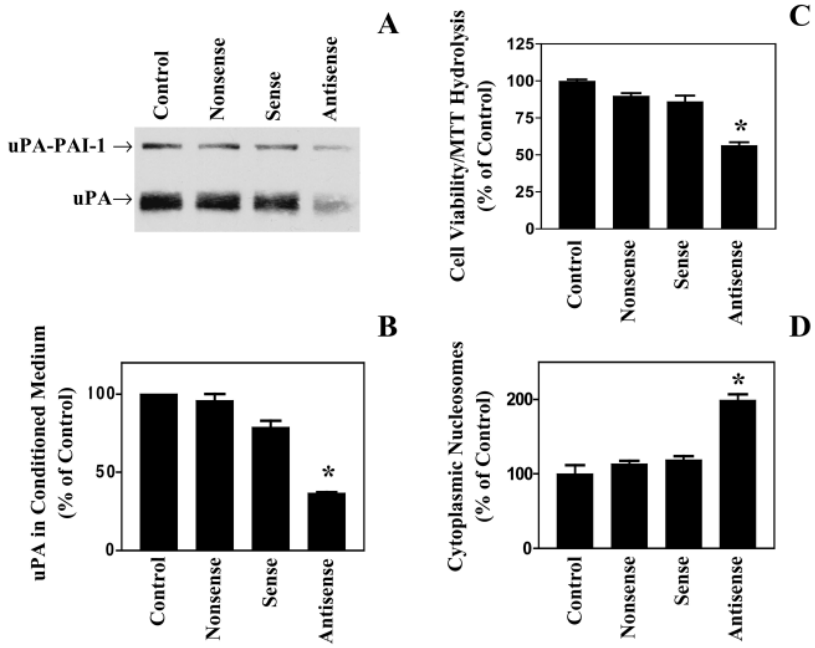

Fig. 8. uPA antisense oligodeoxynucleotides inhibit MDA-MB-231 cell growth and promote apoptosis. (A) MDA-MB-231 cells were treated with uPA antisense, sense or nonsense oligodeoxynucleotides for 24 hours, cultured in serum-containing medium for 24 hours and then in serum-free medium for 24 hours. uPA accumulation in serum-free conditioned medium was assessed by immunoblot analysis. (B) uPA levels in CM were quantitated by densitometry and standardized to the level observed in control cultures. (C) MDA-MB-231 cells were treated with oligodeoxynucleotides according to the protocol used to measure uPA levels. Cell growth was determined by MTT assay. The asterisk indicates a significant difference compared with the control (unpaired *t*-test, $P<0.05$, $n=3$). (D) MDA-MB-231 cells were treated with oligodeoxynucleotides. Cytoplasmic nucleosomes were detected by ELISA. The asterisk indicates a significant difference compared with the control (unpaired *t*-test, $P<0.05$, $n=3$). Values in B-D represent mean±s.e.m.

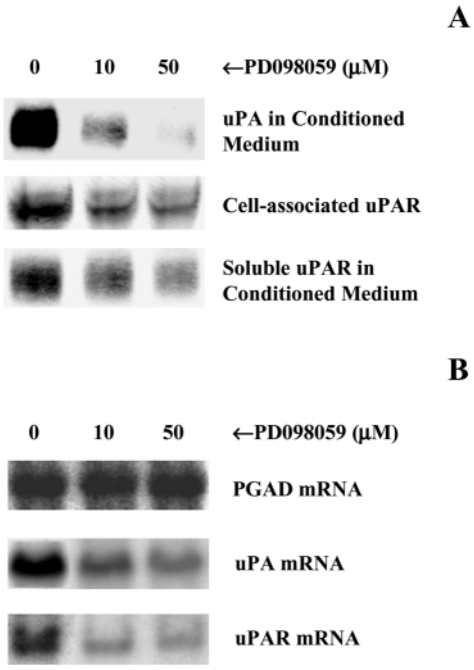

Fig. 9. Expression of uPA and uPAR is regulated by ERK in MDA-MB-231 cells. MDA-MB-231 cells were treated with PD098059 (10 μM or 50 μM) or with vehicle for 24 hours in serum-free medium. (A) Levels of uPA, cell-associated uPAR and soluble uPAR in CM were determined by immunoblot analysis (representative of three separate experiments). (B) PGAD, uPA and uPAR mRNA levels were analyzed in total RNA isolates.

northern blot analyses were performed. As shown in Fig. 9B, uPA and uPAR mRNAs were both decreased by PD098059, whereas PGAD mRNA was unchanged. From these results, a model emerges in which the endogenous uPA-uPAR system plays an essential role in determining the basal level of activated ERK, whereas ERK regulates expression of uPA and uPAR. The endogenous uPA-uPAR system regulates MDA-MB-231 cell growth and apoptosis by a mechanism that depends on activated ERK by one of two non-mutually exclusive mechanisms: ERK modification of downstream substrates involved in cell survival and/or the ability of ERK to sustain expression of uPA and uPAR.

DISCUSSION

The ability of uPA to activate the Ras-ERK signaling pathway by binding to uPAR is well documented (Kanse et al., 1997; Konakova et al., 1998; Nguyen et al., 1998; Tang et al., 1998; Nguyen et al., 1999; Aguirre-Ghiso et al., 1999). This process may depend on the integrity of a signaling receptor complex that includes uPAR and plasma membrane-adaptor proteins, such as integrins and caveolin (Blasi, 1999; Chapman et al., 1999). In Hep3 carcinoma cells, uPAR expression is correlated with the ability of the cells to grow on chorioallantoic membranes, apparently reflecting uPAR binding to $\alpha_5\beta_1$, which controls the basal level of activated ERK (Yu et al., 1997; Aguirre-Ghiso et al., 1999). An autocrine signaling pathway, in which endogenously produced uPA binds to uPAR and thereby activates ERK, has been demonstrated in HT 1080 and MCF-7 cells; however, in these cells, the pathway is observed only when LRFs are neutralized (Webb et al., 1999; Webb et al., 2000).

In this study, we examined MDA-MB-231 breast cancer cells as a model of aggressive breast carcinoma. MDA-MB-231 cells are highly invasive, express high levels of uPA and uPAR and have high basal levels of activated ERK (Seddighzadeh et al., 1999; Holst-Hansen, 1996). We demonstrated that the endogenous uPA-uPAR system plays a critical role in maintaining the high level of activated ERK in these cells. Furthermore, we showed that activated ERK is necessary to maintain uPA and uPAR expression. This positive-feedback loop may be critical in determining the aggressive nature of MDA-MB-231 cells.

An important consequence of ERK regulation by the uPA-uPAR system in MDA-MB-231 cells was regulation of cell growth and apoptosis. For the first time we have implicated uPAR-initiated cell signaling as playing an important role in suppressing apoptosis. PD098059, uPA-specific antibody, uPAR-specific antibody and uPA mRNA-specific antisense oligodeoxynucleotides all increased apoptosis in MDA-MB-231 cells. The role of ERK in preventing apoptosis has been observed previously. Activated ERK counteracts apoptosis following nerve growth factor-β withdrawal in PC-12 cells (Xia et al., 1995) and plays an important role as a downstream mediator of the anti-apoptotic activity of acidic fibroblast growth factor (Guillonneau et al., 1998). In BaF3 cells, activation of both ERK and phosphatidylinositol 3-kinase is necessary to suppress caspase-3 activation following interleukin-3 withdrawal (Terada et al., 2000). The anti-apoptotic activity of ERK may reflect its ability to activate kinases in the Rsk family, which then phosphorylate BAD and the pro-survival transcription factor CREB (Bonni et al., 1999). Both of these Rsk-catalyzed reactions oppose apoptosis.

The hypothesis that the uPA-uPAR system and ERK function in the same pathway to inhibit apoptosis is supported by our studies in which PD098059 and either uPA-specific or uPAR-specific antibody increased apoptosis to the same extent as when these reagents were added separately. In addition to activating Rsk and perhaps other substrates that promote cell survival, activated ERK increases the expression of uPA and uPAR. Because of this positive feedback loop, we cannot rule out the possibility that other activities of the uPA-uPAR system are responsible for the apoptosis observed with uPA-specific antibody or PD098059. Possible alternative mechanisms whereby uPA and uPAR may oppose apoptosis include signal transduction through pathways other than the Ras-ERK pathway, modulation of cell adhesion and regulation of cell-surface proteolysis.

To measure MDA-MB-231 cell growth, we executed MTT assays and [^{3}H]thymidine incorporation experiments. Although [^{3}H]thymidine incorporation measures DNA synthesis in S-phase and is thus an index of cell proliferation, a decrease in [^{3}H]thymidine incorporation may be caused entirely by increased apoptosis, as the number of cells available to proceed through the cell cycle is decreased. In a previous study, Dumler et al. (1999) reported that uPA-binding to uPAR activates casein kinase 2 and that a signaling pathway downstream of casein kinase 2 is necessary for uPA-promoted cell growth. Although we did not test the role of casein kinase

2 in MDA-MB-231 cell growth, it is quite possible that uPA-binding to uPAR activates complementary signaling pathways that must be simultaneously activated in order to induce mitogenesis.

In breast cancer, elevated levels of uPA and uPAR are associated with a negative prognosis (reviewed in Andreasen et al., 1997). The reason for this is not clear and some complex issues have arisen, including the fact that many malignant epithelial cells in breast cancers express low levels of uPAR. To understand the role of uPA and uPAR in cancer, it will be important to elucidate further the diverse properties of this system. The ability of uPAR and ERK to function in a positive feedback loop and suppress apoptosis represents a novel mechanism whereby the uPA-uPAR system may promote cancer progression.

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