Inhibition of uPAR and uPA Reduces Invasion in Papillary Thyroid Carcinoma Cells

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Objectives/Hypothesis: We analyzed the expression of urokinase plasminogen activator (uPA) and its receptor (uPAR) in papillary thyroid carcinoma (PTC) and normal thyroid tissue and examined in vitro how uPA and uPAR contribute to an invasive/metastatic phenotype, and the functional consequences of inhibiting this system.

Study Design: Retrospective chart review of PTC patients, followed by prospective study using previously obtained patient tissue and PTC cellular models.

Methods: uPA and uPAR RNA and protein levels were analyzed in PTC patient tissue samples, PTC and normal thyroid tissue culture cells, and conditioned media (CM) using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and/or Western blotting. The plasminogen-activating ability of CM was examined using dark-quenched casein fluorimetry and casein-plasminogen gel zymography. The invasive potentials of the PTC and normal thyroid epithelial cell lines were assessed using an in vitro cellular invasion/migration system.

Results: uPA and uPAR RNA and protein levels were increased in PTC patient samples and PTC cells relative to controls. uPA and uPAR RNA were also significantly higher in patients with metastatic disease. Casein-plasminogen zymography and Western blotting demonstrated increased active uPA secreted by PTC cells compared with normal thyroid cells. Fluorimetric assays revealed that the PTC cells’ CM was able to activate plasminogen, resulting in measurable casein hydrolysis. This casein hydrolysis was prevented by the addition of several specific uPA inhibitors. Finally, the in vitro invasion phenotypes of PTC cells were augmented by the addition of plasminogen, and this augmentation was reversed by inhibitory anti-uPA and anti-uPAR antibodies.

Conclusions: These data provide new functional evidence of the uPA/uPAR system’s role in PTC invasion/metastasis and demonstrate the attractiveness of uPA and uPAR as molecular biomarkers and therapeutic targets.

Key Words: Papillary thyroid carcinoma, urokinase plasminogen activator, urokinase plasminogen activator receptor, invasion, metastasis.

Level of Evidence: 5

INTRODUCTION

Papillary thyroid carcinoma (PTC) is the most prevalent endocrine and thyroid malignancy, and its incidence has more than doubled in many countries during the past 30 years.¹,² PTC has a great propensity for early invasion and lymph node metastasis.³,⁴ Furthermore, distant metastases occur in 10% to 15% of PTC cases (usually to the bone or lung), and represent the most frequent cause of thyroid cancer–related death.⁵,⁶ Currently, the only treatment for inoperable metastatic PTC is radioiodine, iodine 131 (¹³¹I); as such, it is repeatedly administered to PTC patients, even when it is not clearly effective.⁷ High cumulative activity of ¹³¹I is also associated with a number of unfavorable side effects, including pulmonary fibrosis, xerostomia, and lacrimal complications,⁸,⁹ as well as an increased risk of cancer and leukemia.¹⁰ The invasiveness of PTC and the shortcomings of treatment for metastatic disease have led to the investigation of molecular targets that directly
promote invasion and metastasis in hopes of developing safer, more specific inhibitors of metastatic PTC.

The urokinase plasminogen activator (uPA) and the urokinase plasminogen activator receptor (uPAR) are induced in a wide variety of cancers, leading to enhanced invasive and metastatic potential.\textsuperscript{11,12} Upon binding to uPAR, pro-uPA is converted to its active form, which is then capable of cleaving plasminogen to plasmin. Plasmin can then degrade components of the basement membrane and extracellular matrix components, a prerequisite for tumor cell invasion and metastasis.\textsuperscript{12} Although there are reports of uPA and uPAR overexpression in various thyroid malignancies,\textsuperscript{13–19} there has been no work dealing with this system's function or its inhibition in PTC. As the uPA/uPAR system does not appear to be essential for survival under physiologic conditions\textsuperscript{20} and its enzymatic activity is controlled by a number of endogenous inhibitors, inhibition of this system may represent an innovative and potent strategy against invasion and metastasis.\textsuperscript{12} We hypothesized that the inhibition of uPA and uPAR could reduce the invasive potential of PTC. We thus examined the expression patterns of uPA and uPAR both in clinical samples of PTC and cellular models of PTC, characterized the role of the uPA/uPAR system in degradative/invasive phenotypes of cellular models of PTC, and demonstrated the functional, anti-invasive consequences of directly inhibiting this system.

**MATERIALS AND METHODS**

**Patient Tissue Samples**

Twenty-one PTC samples were collected from patients who underwent thyroidectomy at New York Eye and Ear Infirmary (New York, NY) or Westchester Medical Center (Valhalla, NY). All samples were collected in research-study protocol approved by the institutional review boards of the respective institutions. After surgical excision, the tumor samples were frozen and stored at \(-80^\circ \text{C}\) until use. During sample collection, care was taken to ensure that the matched samples were obtained from areas of the thyroid that contained either at least 90% malignant or 90% normal tissue. To use each sample for RNA studies, each sample was divided into the required number of portions (approximately 100 mg–250 mg of tissue each) prior to processing.

**Cell Lines and Culture Conditions**

The human thyroid epithelial cell line NTHY-Or1-3-1 was generously donated by Dr. Norman Eberhardt (Mayo Clinic, Rochester, MN), and the invasive/metastatic PTC cell lines BCPAP, 8505C, and NTHY-Or1-3-1 cell cultures were harvested and washed with PBS. The cells were lysed (1 \(\times\) 10\(^6\) cells/100 mL of lysis buffer) using RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2% sodium deoxycholate, 0.1% SDS, 0.5% NP-40, and 1 \(\mu\)M Pefabloc), by incubating on ice for 60 minutes and vortexing periodically. The lysate was centrifuged at 20,000 g for 30 minutes and the supernatant was collected. Cell lysates were subjected to 12% SDS-PAGE under reducing conditions, while SFCM samples were subjected to 12% SDS-PAGE under nonreducing conditions (with and without \(\beta\)-mercaptoethanol, respectively). The proteins were transferred to Immobilon-P membranes (Millipore, Billerica, MA) at 200 mA for 1 hour and membranes were blocked with 3% dried milk in TBST [200 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween 20 added fresh/liter of 1X TBS (TBS-T)] for at least 2 hours on a shaker. Subsequently, the membrane was incubated overnight at 4\(^\circ\)C with mouse anti-human uPAR, uPA (R&D Systems), or \(\beta\)-actin antibodies (Cell Signaling Technology, Beverly, MA) in 3% milk-TBS-T on a shaker. Membranes were washed three times with 3% milk-TBS-T and incubated with a goat antimouse IgG secondary antibody (Thermo Scientific, Rockford, IL) in 3% milk-TBS-T under similar conditions. After three washes with TBS-T, membranes were developed with an electrochemical lumination system (Thermo Scientific) and detected on x-ray film.

**Analysis of uPA Activity by Casein-Plasminogen Zymography**

uPA activities of the SFCM were measured by casein-plasminogen zymography. The SFCM samples (10 \(\mu\)g of protein) concentrations were determined by spectrophotometric quantification using Bradford reagent (Bio-Rad Laboratories, Inc., Hercules, CA).

**RNA Extraction/Analysis and qRT-PCR**

Total RNA was isolated from patient samples and cell cultures using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the instructions of the supplier. RNA purity/concentration and integrity were evaluated by measuring sample absorbance at 260 nm and 280 nm, and by Tris-acetate-EDTA gel electrophoresis, respectively. High-quality RNA samples with A\(260\)/A\(280\) ratio > 1.8 and intact, degraded ribosomal 28S and 18S RNA bands were utilized for quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

The Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) and SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen) were employed for qRT-PCR. Primer sets were purchased from Integrated DNA Technologies and designed using their PrimerQuest software (IDT, Coralville, IA). The reactions were performed using the following thermocycling conditions: 50\(^\circ\)C for 5 minutes, 95\(^\circ\)C for 15 minutes, and 40 cycles of 95\(^\circ\)C for 15 seconds, 60\(^\circ\)C for 30 seconds, and 72\(^\circ\)C for 30 seconds. Samples were run in triplicate with test primer sets for uPAR (P1: 5′-AAG ATC ACC AGC CTT ACC GAG GTT-3′, P2: 5′-ATT CGA GGT ACC GCC TTC GGG AAT-3′), uPA (P1: 5′-CGA CAT TGC CTT GGT GAA-3′, P2: 5′-ATC GTT ATA CAT CGA GGG CAG GCA-3′), or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (P1: 5′-ACC ACA GTC CAT GCC ATC AC-3′, P2: 5′-TCC ACC ACC CTG TTG CTG TA-3′), which served as the endogenous control. Data were analyzed according to the comparative \(C_p\) method.
Dark-Quenched FTC-Casein Degradation Fluorimetric Assays

The ability of NTHY-Ori-3-1, BCPAP, and 8505C SFCM to activate plasminogen to functional plasmin via uPA was determined by degradation of fluorescent thiochelomoyl-labeled (FTC-labeled) casein (Thermo Scientific). Reactions were carried out in 200 mL, in triplicate, in white “half-volume” 96-well polystyrene plates at 37°C and 5% CO2. Each reaction contained 50 mM Tris-HCl pH 7.6, 150 mM NaCl, 5 mM CaCl2, 0.02% NaN3, and 8 ng/mL of FTC-casein. Fifty mL (10 ng) of SFCM from either cell line was added to each reaction, along with PBS or recombinant human plasminogen (10 ng/mL Sigma, St. Louis, MO) plus one of the following: 1) recombinant urokinase plasminogen activator inhibitor (10 ng/mL; CalBiochem, San Diego, CA), 2) mouse antihuman uPA mAb (20 ng/mL; American Diagnostica, Stamford, CT), or 3) soybean trypsin inhibitor (Roche Applied Science, Indianapolis, IN). SFCM that had been generated in the presence of an anti-uPAR mAb (R&D Systems) was also employed, as were nonconditioned media (blank) samples with recombinant plasminogen (Sigma, St. Louis, MO) plus one of the following: 1) recombinant urokinase plasminogen activator inhibitor (10 ng/mL; CalBiochem, San Diego, CA), 2) mouse antihuman uPA mAb (20 ng/mL; American Diagnostica, Stamford, CT), or 3) soybean trypsin inhibitor (Roche Applied Science, Indianapolis, IN). The fluorescent molecules were excited at 495/520 nm (5/10 nm slit width). Fluorescence was measured as arbitrary units (a.u.) in each well at 0 and 145-hour time points.

Cellular Migration and Invasion Assays

To assess cell migration in vitro, NTHY-Ori-3-1, BCPAP, or 8505C cells (25,000 cells in 500 mL RPMI 1640 without FBS) were placed in the top chamber of transwell migration chambers (8 µm BioCoat Control Inserts; Becton Dickinson (BD) Biosciences–Discovery Labware, Bedford, MA) with PBS or recombinant human plasminogen (10 ng/mL Sigma) plus one of the following: 1) mouse antihuman uPA mAb (10 ng/mL; R&D Systems), 2) mouse antihuman uPA mAb (20 ng/mL; American Diagnostica), or 3) soybean trypsin inhibitor (Roche Applied Science). The lower chamber was filled with 750 mL RPMI 1640 supplemented with 5% FBS. After 24 hours, nonmigratory cells were removed from the upper surface of the transwell membrane with a cotton swab, and migratory cells on the lower membrane surface were fixed with methanol, stained with 1% toluidine blue (Acros), photographed, and counted under ×50 magnification.

To assess invasion, in vitro invasion assays were done under the same conditions as the transwell migration assays, but in Matrigel-coated transwells (BioCoat Matrigel Invasion Chamber; BD Biosciences Labware). Invasion/migration indices were calculated as the average number of invading cells per field divided by the average number of migrating cells per field.

Statistical Analysis

Data were analyzed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA) according to the Student t test unless otherwise indicated.

RESULTS

uPAR and uPA Expression Levels Are Increased in PTC Patient Samples and Tumor Cells

We first examined the relative uPAR and uPA expression levels in clinical PTC samples and matched, normal thyroid tissue from 21 patients. The clinicopathologic characteristics of the patients, as well as the uPAR and uPA fold increases in their respective PTC tissue samples, are summarized in Table I. As shown in Figure 1A, uPAR and uPA were, on average, significantly increased in PTC tissue samples relative to their matched, normal thyroid tissue counterparts by an average of 5.5-fold (95% confidence interval [CI] = 3.0–8.1) and 2.4-fold (95% CI = 1.6–3.2), respectively. Additionally, Figure 1B shows the differences in average fold increases in uPAR and uPA RNA levels in patients with confirmed lymphatic metastases (n = 6) compared with patients with no metastatic disease (n = 15). uPAR and uPA fold increases were significantly higher (P < .05, Wilcoxon signed rank test) in patients with lymphatic metastases (11.7 and 4.3, respectively) than in patients without metastases (3.2 and 1.7, respectively). Of note is the fact that six male patients in our study displayed significantly higher-fold increases of uPAR RNA (but not uPA RNA) than the 15 female patients (11.1 and 3.4, respectively; P < .05, Wilcoxon signed rank test).

Furthermore, uPA and uPAR overexpression patterns were also seen in the PTC cell lines BCPAP and 8505C relative to the normal thyroid epithelial line NTHY-Ori-3-1. uPAR and uPA RNA levels in BCPAP cells were an average of 9.0-fold and 2.7-fold higher than those in NTHY-Ori-3-1, and uPAR and uPA RNA levels in 8505C cells were 14.0-fold and 2.3-fold higher, respectively (Fig. 1C). uPAR and uPA protein levels were increased in both PTC cell lines relative to NTHY-Ori-3-1 as determined by Western blot (Fig. 1D). uPAR and uPA protein levels in BCPAP cells were approximately 3.2-fold and 2.3-fold higher than those in NTHY-Ori-3-1, and 8505C uPAR and uPA protein levels were approximately 3.5-fold and 2.4-fold higher than NTHY-Ori-3-1.

PTC Cells Secrete uPA, Which is Responsible for Plasminogen Activation and Subsequent Degradative Potential

To assess the functionality and degradative potential of increased uPAR and uPA in the BCPAP and 8505C cell lines, we utilized a fluorometric assay that measures the relative abilities of factors present in a given cell’s SFCM to activate plasminogen to plasmin, which is then able to degrade FTC-casein. This substrate is so heavily fluorescently tagged that it is self-quenching, and fluorescence can only be detected if the substrate has been sufficiently degraded as to liberate the fluorescent molecules.

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Although NTHY-Ori-3-1 SFCM possessed negligible ability to degrade casein, regardless of the presence or absence of plasminogen, BCPAP SFCM and 8505C SFCM were able to degrade casein upon addition of plasminogen (10 ng/L), which implies an increased presence of a plasminogen activator in the BCPAP and 8505C SFCM. This plasminogen-activating ability was detectable in media dilutions containing as little as 100 ng of protein (data not shown). This degradative ability was eliminated by the addition of several specific uPA/uPAR inhibitors (inhibitory anti-uPA or anti-uPAR antibodies, and recombinant uPAI, an endogenous inhibitor of uPA), as well as by the addition of the nonspecific serine protease inhibitor soybean trypsin inhibitor (STBI), which served as an additional negative control (Fig. 2A). These data imply that the PTC cells are capable of generating functional plasmin from plasminogen via uPA, without which the SFCM would lack significant ability to degrade casein (Fig. 2A, BCPAP and 8505C controls).

Additionally, casein-substrate gels copolymerized with or without plasminogen were used to further characterize uPA activity in NTHY-Ori-3-1, BCPAP, and 8505C SFCM. As shown in Figure 2B, a lysis band appeared in the BCPAP and 8505C lanes when the gels were cast with both casein and plasminogen, but not with casein alone. These lysis bands corresponded to the migratory pattern of uPA on a Western blot that was run in parallel to the zymograms using the same SFCM from the cellular samples. Neither a significant lysis band nor a protein band was located in the NTHY-Ori-3-1 lanes of the zymograms or Western blots, respectively.

**uPA/uPAR Inhibition Reduces PTC Cell Invasion In Vitro**

To evaluate the contribution of the uPA/uPAR system on PTC cell and normal thyrocyte invasion, as well as the consequences of inhibiting this system, transwell migration and invasion assays were performed. Whereas the NTHY-Ori-3-1 cells displayed negligible invasive potential under any conditions, BCPAP and 8505C cells showed a ~2-fold increase from their baseline level of invasiveness upon supplementation with plasminogen (Fig. 3A and Fig. 3B). This increase in invasive potential was reversed with supplementation of an anti-uPAR mAb that blocked uPAR’s interaction with uPA, as well as with an inhibitory anti-uPA mAb. This reversal was also seen with the addition of the nonspecific serine protease inhibitor SBTI. Migration rates for both cell types were unaffected by any of the different conditions (data not shown). This indicates that the inhibition of the uPA/uPAR system reduces the ability of PTC cells to degrade and invade the extracellular matrix, and does not simply decrease the cells' migratory potential.

As the uPA/uPAR system has also been shown to play a role in cell signaling leading to aberrant growth and proliferation, we also sought to determine what, if any, effects the anti-uPAR mAb had on cellular health. We found that the colony-formation capacity, efficiency of plating, and cell proliferation rates for all cell lines

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**uPAR** = urokinase plasminogen activator receptor; **uPA** = urokinase plasminogen activator; **M** = male; **F** = female.
used were unaffected by the presence or absence of the anti-uPAR mAb (data not shown).

DISCUSSION

Although the majority of patients with PTC have an excellent prognosis following total or subtotal thyroidectomy, cases with extensive local invasion and metastasis frequently have worsened prognosis.\(^5,6\) Distant metastases in PTC, though less common, have a tremendous impact on the prognosis, with 5-year survival rates of \(~50\%\).\(^6,21\) The proteolytic degradation of the basement membrane and extracellular matrix is a crucial step required for cancer invasion and metastasis. Other systems, such as various matrix metalloproteinases, have been widely studied as mediators of invasion and metastasis in thyroid malignancies, as well as potential diagnostic/prognostic biomarkers.\(^22,23\) The uPA/uPAR system represents another important contributor to an invasive/metastatic tumor phenotype. Activation of pro-uPA by uPAR allows subsequent cleavage of plasminogen to its active form, plasmin, which is a serine protease.\(^12\) Several components of the extracellular matrix are substrates for plasmin, which is also involved in the fibrinolytic cascade.\(^24\) Increased uPA/uPAR activity has been shown to facilitate remodeling of the surrounding tissue in the context of tumor invasion and metastasis formation in a wide variety of cancers.\(^11,12\)

In the present study, we show that uPA/uPAR are consistently overexpressed in a group of clinical PTC samples, and that this overexpression is significantly more pronounced in patients with lymph node metastases. These results are in keeping with the various studies showing this system to be induced in numerous cancers, including various thyroid malignancies, especially highly invasive/metastatic disease.\(^11–19\) The significantly higher-fold increases of uPAR in the male PTC patients corroborate established reports of more aggressive disease course in males.\(^25,26\) The observed relationship between gender and lymph node metastasis, as well as uPA/uPAR expression patterns, is currently being further analyzed in an expanded patient population. Furthermore, we have shown that increased expression of uPAR and uPA in PTC cells was associated with increased degradative and invasive ability upon...
plasminogen supplementation, and that specific inhibition of this system was capable of abolishing these increases. Previous work has demonstrated that the uPA/uPAR system is able to drive invasion in a cellular models of follicular and/or anaplastic thyroid carcinomas, and that direct or indirect inhibition of this system reduces this invasiveness.\textsuperscript{27–30} As our results show that this is also true of PTC, it may be that uPA/uPAR overexpression and its association with invasive/metastatic behavior may be a general feature of differentiated thyroid carcinomas. To our knowledge, this is the first study that demonstrates the degree to which the urokinase system contributes to PTC invasion, as well as the functional consequences of inhibiting this system.

Of note is the fact that the concentration of plasminogen used in our fluorimetry and invasion/migration assays (10 ng/\(\mu\)L) is well below the reference range of 60 to 250 ng/\(\mu\)L for plasminogen in human serum.\textsuperscript{31} The thyroid is one of the most highly vascularized organs in the body, with blood perfusion rates of 4 to 6
As such, any thyroid malignancy over-expressing uPA/uPAR will have no shortage of plasminogen to activate, thus leading to a highly favorable environment for tumor invasion and metastasis. We postulate that, given the significance of its contribution to an invasive tumor phenotype (as well as the significance of its inhibition on invasion), inhibition of the uPA/uPAR system might represent a novel avenue for controlling and targeting invasive/metastatic PTC. Indeed, experimental synthetic inhibitors of this system devised by Wilex have shown great promise in preventing invasion and metastasis in both cellular and animal models of various cancers,\(^\text{11,33}\) and these compounds are currently being studied in clinical trials.

Fig. 3. (A) Representative micrographs (×50 magnification) of BCPAP and 8505C cells from the invasion chambers. (B) Summary of invasion/migration results. The plasminogen-supplemented BCPAP and 8505C cells’ increased invasion/migration index was statistically significant (*) when compared with the control NTHY-Ori-3-1 and BCBAP/8505C cells and all three inhibitory conditions \(P < .05\). The invasion/migration indices of the BCPAP and 8505C cells in control and inhibitory conditions were not significantly different from each other. Figures are representative of two separate experiments with similar results. Error bars represent standard deviation (SD). P = plasminogen.
CONCLUSION

Our data have shown that uPA and uPAR are increased in PTC patient samples relative to matched normal tissue, and that this increase is significantly higher in patients with lymphatic metastases. uPA and uPAR levels are also increased in our cellular models of PTC, and this induction is directly responsible for increased functional plasmin generation. Furthermore, we were able to link this increased plasmin generation with increased degradative ability and improved invasive potential of PTC cells, which we were able to completely reverse with specific inhibitors of uPAR or uPA. Taken together, these data represent new functional evidence of the uPA/uPAR system's role in PTC invasion and metastasis, as well as the attractiveness and vulnerability of this system as a molecular biomarker and therapeutic target.

Acknowledgments

We wish to thank Dr. Radha Iyer for technical assistance with the qRT-PCR. We also wish to thank Dr. David Frick and Craig Belon for the use of the Varian Cary Eclipse fluorescence spectrophotometer.

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