Expression of the Plasminogen Activation System in Kidney Cancer Correlates with Its Aggressive Phenotype

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ABSTRACT

Malignant tumors contrast with benign ones in their ability to invade adjacent tissue and to metastasize. The urokinase plasminogen activator is a proteolytic enzyme that can facilitate these processes. In many carcinomas, the concentration of the urokinase plasminogen activator system is high. The high expression of these enzymes is related to tumor grade. In this study, we have investigated whether secretion of the urokinase plasminogen activator, urokinase plasminogen activator receptor, and plasminogen activator inhibitor 1 in normal kidney tissue and kidney cancer tissue follows this pattern. We have found that urokinase plasminogen activator, urokinase plasminogen activator receptor, and plasminogen activator inhibitor 1 were expressed in higher levels in kidney cancers (squamous cell carcinoma and renal cell carcinoma) than in normal kidney tissue and that these differences were statistically significant (P ≤ 0.05). In renal cell carcinomas, we have observed differences between normal kidney tissue and renal cell carcinomas in males and Caucasians but not in females and African Americans (P ≤ 0.05). Expression of the urokinase plasminogen activator system was also higher in grade III tumors when compared with lower-grade tumors or normal tissue.

INTRODUCTION

The processes of tumor cell invasion and metastasis require proteolytic enzymes to promote the hydrolysis of the surrounding extracellular matrix and basement membrane. Frequently, cancer cells express elements of the u-PA system, but other proteases such as collagenase,stromelysin, and gelatinase are also overexpressed by different cancers (1–8). The u-PA and t-PA convert latent plasminogen into active plasmin, which hydrolyzes many different proteins and could also activate other latent proteolytic enzymes. The t-PA mainly mediates intravascular thrombolysis, whereas u-PA is involved in pericellular proteolysis during cell migration, wound healing, and tissue remodeling under physiological and pathological conditions (9). The t-PA is not relevant to carcinogenesis (10). PAI-1 controls the activity of u-PA and t-PA. PAI-1 belongs to the serpin superfamily of proteins and rapidly interacts with u-PA and t-PA and thus can modulate their activity (11).

The u-PAR is a glycosyl phosphatidyl inositol-linked single chain glycoprotein that is expressed on the surface of the tumor (up to 1 million per malignant tumor cell) and serves as a binding site for u-PA. Plasminogen is also able to bind to tumor cells through its receptor and could be activated when bound (12–14). Such configuration creates areas of high proteolytic activity in the vicinity of cancer cells, protecting these cells by polarizing this activity out from the surface of the cancer cells (15).

In the present study, we have investigated the expression of u-PA, u-PAR, and PAI-1 by ELISA in seven types of kidney cancer tissue and compared this with normal kidney tissue. We have found that u-PA, u-PAR, and PAI-1 were expressed in higher levels in kidney cancers (squamous cell carcinoma and RCC) than in normal kidney tissue, and that these differences were statistically significant (P ≤ 0.05). In RCCs, we have observed differences between normal kidney tissue and RCC in males and Caucasians but not in females and African Americans (P ≤ 0.05). Expression of the u-PA system was also higher in grade III tumors when compared with lower-grade tumors or normal tissue.

MATERIALS AND METHODS

Tissue Samples. All tissue samples were purchased from National Cancer Institute/Cooperative Human Tissue Network (Midwestern OSU Comprehensive Cancer Center, Western Division; Eastern Division) and The University of Alabama at Birmingham/Cooperative Human Tissue Network Southern Division-Comprehensive Cancer Center. Tissue specimens were obtained from radical nephrectomy of patients of both genders with an age range for females of 3–80 years and males 3–79 years, including Caucasians and African-Americans. The kidney tissues used in this study were defined as normal kidney tissue (n = 28), kidney cancer tissue with the border of normal tissue (n = 4), RCC (n = 52), squamous cell carcinoma (n = 4), renal cell carcinoma; AA-RCC, African-American RCC group; W-RCC, Caucasian RCC group; M-RCC, male RCC group; F-RCC, female RCC group.
transitional cell carcinoma (*n* = 5), von Hippel-Lindau tissue (*n* = 2), sarcomatoid cell carcinoma (*n* = 3), and Wilms’ tumor tissue (*n* = 10). Two surgical pathologists independently graded all samples. All samples were stored at −80°C. Three samples of kidney tissue were removed from the study due to incomplete or nontraceable records.

**Reagents and Chemicals.** ELISA kits (Imubind u-PA kit, u-PAR kit, and PAI-1 kit) were from American Diagnostica, Inc. (Greenwich, CT). Micro Protein determination kits, BSA, and sodium chloride were purchased from Sigma Chemical Co. (St. Louis, MO); Triton X-100 was purchased from Aldrich (Milwaukee, WI). The ImmunoPure Metal Enhanced DAB substrate kit was purchased from Pierce (Rockford, IL).

**Tissue Extraction.** Tissue samples weighing 100–300 mg were suspended in 1.8 ml of TBS buffer (120 mM NaCl, 2.7 mM KCl, and 10 mM Tris-base, pH 8.5) and homogenized for 30 s using a Tissue Tearor (model 985-370; Biospec Products, Inc.). For detergent extraction, 0.2 ml of 10% Triton X-100 in TBS (pH 8.5) was added to the homogenate. The mixture was gently shaken for 12 h at 4°C in the dark. Cell debris was separated by centrifugation at 10,000 × *g* for 20 min at 4°C, and tissue extract was removed and stored in liquid nitrogen prior to assay.

**ELISA.** The u-PA, u-PAR, and PAI-1 antigens were determined by ELISA. Microplate wells were coated with murine monoclonal antibody against human u-PA, u-PAR, and PAI-1 as the capture antibody. In all three cases, 100 μl of the antibody was diluted 1:20 in sample buffer (1% solution of the BSA in PBS) and were incubated in Micro-Test wells overnight at 4°C in a cold room. u-PA, u-PAR, and PAI-1 were detected by the addition of 100 μl of the second detection antibody, biotinylated anti-human u-PA, or u-PAR, or PAI-1, followed by 100 μl of solution of the streptavidin conjugated with horseradish peroxidase and 100 μl of perborate/3,3′,5,5′-tetramethylbenzidine substrate to generate the blue color of the solution. The sensitivity of the reaction was increased by the addition of 50 μl of 0.5 M sulfuric acid. The absorbance was measured within 30 min after the addition of sulfuric acid at *λ* = 450 nm using a Thermo-Max micro-plate reader (Molecular Devices, Inc.). Antigen levels were quantified by measuring the absorbance of the solutions and comparing the values with a standard curve. All measurements were performed in duplicate.

**Determination of Protein Content in Tissue Extracts.** Protein content in tissue extracts was determined by the Ohnishi and Barr (16) modification of the Micro Lowry method. Briefly, this method is based on the reaction of diluted Biuret reagent with peptide bonds of the protein to yield a purple-blue complex. The addition of Folin and Ciocalteu’s phenol reagent intensifies this method is the reaction of diluted Biuret reagent with peptide bonds of the protein to yield a purple-blue complex. The addition of Folin and Ciocalteu’s phenol reagent intensifies the reaction with diluted Biuret reagent intensifies the reaction with biuret, and 100 μl of Biuret reagent at room temperature for 10 mm. After incubation, 0.1 ml of Folin and Ciocalteu’s phenol reagent were added, and the solutions were incubated at room temperature for 30 min. Absorbance was measured using a Response spectrophotometer (Gilford, Inc.) at *λ* = 725 nm within 30 min, and protein concentrations were determined from the calibration curve.

**Immunohistology.** Adjacent serial sections of frozen tissue were mounted on poly-L-lysine-coated glass slides and used for immunohistology, using the peroxidase/diaminobenzidine procedure as described by Wordinger et al. (17). Briefly, the tumor sections were blocked for endogenous peroxidase activity in 0.3% *H*2O2 methanol for 5 min. Next, sections were rehydrated in PBS (pH 7.4) and blocked in blocking solution for 5 min (blocking solution contains carrier solution: 96 ml of PBS, 1 g of crystalline bovine serum albumin, and 3 ml of 10% Tween). Then, sections were incubated with primary antibodies in the carrier solution for 20 min at 37°C. Affinity-purified murine monoclonal antibody against u-PA, class IgGI (10 μg/ml), was used. Antibody 3689 was directed against an epitope within the β-chain of u-PA and recognizes pro-u-PA and high molecular weight u-PA. This antibody recognizes free and complexed u-PA and does not cross-react with tPA. Murine antibody 3936, class IgG2a (10 μg/ml), was also used to detect u-PA. The antibody recognizes noncomplexed and complexed u-PA; however, saturation of the receptor with high molecular weight u-PA and pro-u-PA might result in a reduction (~50%) of binding monoclonal antibody 3936 to the receptor (18). Another murine monoclonal antibody, IgGI 3937, was used to detect u-PAR. This antibody does not prevent binding of u-PA to u-PAR, and presumably, the presence or absence of the ligand on the receptor should not affect its binding to u-PAR. Murine antibody 3785, class IgG2a (10 μg/ml), was used to detect free and complexed PAI-1. All primary antibodies were supplied by American Diagnostica, Inc. Subsequently, the sections were incubated with goat anti-mouse antibody (Pierce; 10 μg/ml) for 20 min at 37°C. The brown reaction products of peroxidase identified antigen staining with diaminobenzidine. Negative controls consisted of incubation with the secondary antibody only, with the remaining steps unchanged.

**Image Analysis.** Color pictures of immunostained histological samples were scanned and saved on a computer as *.tif* files. Color images were converted into black/white, and contrast was enhanced. In this format, the highest staining is represented by the number 255 (white), whereas the lowest is 0 (black). Because the human eye distinguishes rather poorly between different grades of gray, all images are shown in false colors (rainbow striped) using the Transform2 program from Fortner.

**Statistical Analysis and Boxplot Graph.** Statistical analysis was performed by the one-way ANOVA test and the modified LSD (Bonferroni) test (19). All statistical calculations were performed using of the SPSS computer program, and significance was established at the level of *P < 0.05.*

A boxplot graph consists of a box, whiskers, and outliers. The line drawn across the box is the median. The bottom of the box is at the first quartile (*Q*1), and the top is at the third quartile (*Q*3). The whiskers are the lines that extend from the top and bottom of the box. The top whisker extends from the box to the highest observed value that is less than *Q*1 + 1.5 × IQR (where IQR is the interquartile range, *Q*3−*Q*1, or the height of the box). The bottom whisker extends from the box to the lowest observed value that is greater than *Q*1 − 1.5 × IQR. Outliers are points outside the lower and upper limits of the whiskers and are plotted with circles or asterisks.

**RESULTS**

**Expression of u-PA, u-PAR, and PAI-1 by Tissue Extracts.** In the first statistical analysis, we analyzed three groups of normal kidney tissue: tissue from cancer-free kidney...
Fig. 1 Boxplot representation of the expression of the u-PA system in normal and cancerous kidney tissue. C, control group; TWBN, kidney tumor tissue with a border of normal tissue; WT, Wilms' tumor; TCC, transitional cell carcinoma; SCC, squamous cell carcinoma; RCC, renal cell carcinoma; VHL, von Hippel-Lindau tissue; SRCC, sarcomatoid cell carcinoma.

Fig. 1a shows that the highest expression of u-PA was noted in squamous cell carcinoma, cancer tissue with a border of normal tissue, and RCC. However, only the difference between the control group and RCC ($P = 0.01$) and squamous cell ($P = 0.0001$) groups were statistically significant. Wilms' tumor, von Hippel-Lindau tissue, and sarcomatoid

$(n = 3)$; normal tissue from kidney with cancer $(n = 18)$; and normal kidney tissue adjacent to cancer $(n = 7)$. Statistical analysis revealed that there were no differences in the expression of u-PA, u-PAR, and PAI-1 among these three types; therefore, we decided to join them together into one group under the name control group.
cell carcinoma expressed less u-PA than normal kidney tissue.

Fig. 1b shows that differences in the expression of u-PAR between the control group and cancer groups were statistically significant for squamous cell carcinoma ($P = 0.0001$) and RCC ($P = 0.007$). The remaining kidney cancer tissues showed higher expressions of u-PAR than normal kidney tissue, but those were not statistically significant.

The differences in the expression of PAI-1 between the control group and the cancer groups, shown in Fig. 1c, were statistically significant for squamous cell carcinoma ($P = 0.0001$) and RCC ($P = 0.0001$). Other kidney cancer tissues expressed more PAI-1 than the control group, whereas von Hippel-Lindau tissue showed a similar level of expression as normal tissues, but these differences were not statistically significant.

Expression of u-PA, u-PAR, and PAI-1 in RCC Depends on Gender, Race, and Tumor Grade. Because of the large number of samples of RCC (n = 52), we were able to test the possibility that the expression of the u-PA system could depend on race, gender, and/or tumor grade. Indeed, statistical analysis results revealed that expression of these antigens were different in males and females, African Americans and Caucasians, and also in different grades of tumors. Fig. 2 shows the differences between the male control group and the RCC group in the expression of u-PA ($P = 0.022$), u-PAR ($P = 0.001$), and PAI-1 ($P = 0.005$), which were statistically significant. The female control group expressed more u-PA than the F-RCC group. Expression of u-PA and PAI-1 was higher than in the control group, but the differences were not statistically significant.

Overall, as shown in Fig. 3, RCC samples from Caucasians expressed more u-PA, u-PAR, and PAI-1 than samples from African Americans but statistical analysis showed that only differences for u-PAR and PAI-1 ($P = 0.0036$ and $P = 0.0035$, respectively) were statistically significant. Additionally, the W-RCC group expressed more u-PA, u-PAR, and PAI-1 ($P = 0.004$, $P = 0.0001$, and $P = 0.0001$, respectively) than the control group. These differences were statistically significant. The AA-RCC group expressed less u-PA than the AAC group, but the expression of the u-PA and PAI-1 by the AA-RCC group was higher than the AAC group. There were no statistically significant differences in this group.

Analysis of the expression of u-PA, u-PAR, and PAI-1 (Fig. 4) in different tumor grades showed that RCC samples classified in stage III expressed more of these enzymes than any other stage ($P = 0.004$, $P = 0.0001$, and $P = 0.0001$, respectively), and differences were statistically significant if compared to normal kidney tissue. For stage I, only PAI-1 ($P = 0.0042$), and in stage IV, u-PAR ($P = 0.003$) and PAI-1 ($P = 0.013$), expressed statistically significant differences in comparison with the control group.

Immunohistological Staining of u-PA, u-PAR, and PAI-1. We have immunostained 16 randomly chosen tissues of RCC and all squamous cell carcinoma. The malignant kidney tumors showed a wide spectrum of staining intensities. In general, we found diffuse cytoplasmic staining, and in some cases, strong cell membrane staining for u-PA (Fig. 5, a, b, i, and m).

Malignant kidney tumors showed a variety of u-PAR intensities, mostly distinct or strong intensity of staining within the tumors. Distinct staining was observed on the cell membrane and occasionally in the nucleus of cancer cells (Fig. 5, b, c, f, g, j, k, n, and o). Virtually no positivity was detected in extracellular spaces. In general, the antibody 3937 showed stronger intensity of staining (Fig. 5b) than antibody 3936 (Fig. 5c), which is typical for the u-PA receptor occupied by its ligand. However, little staining was observed for u-PA by antibody 3689 (Fig. 5i), no significant difference in staining was observed between antibody 3936 and antibody 3937 (Fig. 5, j and k).

Kidney tumor tissues stained with different intensities, but the normal noncancerous and distant tissues frequently stained strongly for PAI-1 as well (Fig. 5, d, h, l, and p).

Some distinct staining for u-PA, u-PAR, and PAI-1 of scattered macrophages was also detected occasionally.

DISCUSSION

Over the past few years, the expression of u-PA, u-PAR, and PAI-1 has been investigated extensively in many types of malignant tumors. Numerous studies showed the relationship between the level of expression of u-PA, u-PAR, PAI-1, and their aggressive phenotypes of the cancer (1, 10, 11, 20, 21). In general, malignant tumors originating from the brain, colon, stomach, uterus, ovary, breast, and prostate express higher amounts or activity of these enzymes than their normal or benign counterparts (5, 20, 22-27). Also, more aggressive tumors showed higher amounts of u-PA system elements than less aggressive malignant tumors. Consequently, it was established that in extensively investigated tumors such as breast cancers, the expression of u-PA and PAI-1 could be used as prognostic marker predicting the outcome of the disease (11, 25-27). Our studies show a similar pattern, e.g., more aggressive type of cancer (squamous cell carcinoma or RCC grade III and some grade IV) expresses more u-PA, u-PAR, and PAI-1 than the control group or different, less aggressive kidney tumors. Literature on expression of the u-PA system in kidney cancers is rather small, but we have found a good agreement between our findings and work reported previously (8, 20, 28). For example, in several studies by Hofmann et al. (8, 28), who used the same ELISA method, we found very good agreement in range and mean of detected antigens.

We investigated differences in u-PA, u-PAR, and PAI-1 expression by race, gender, and tumor grade by RCC only, because it has been the only group with a sufficient numbers of samples to conduct meaningful statistical analysis. Our results showed that W-RCC samples had a higher expression of either u-PA, u-PAR, or PAI-1 than AA-RCC samples. There have been reports about differences in expression of the u-PA system in cancers depending on race and gender. However, the study of Frist et al. (29) revealed that cultured human umbilical cord endothelial cells from African Americans expressed more t-PA but less PAI-1 than from Caucasians. Also, higher fibrinolytic activity was shown in African Americans than in Caucasians. In another study, Iso et al. (30) revealed that concentrations of t-PA and PAI-1 in the blood plasma of Caucasian males were higher than in males of Asian origin; however, no differences were observed between females of both races.

Our gender analysis showed that the M-RCC group had a
higher expression of u-PA, u-PAR, and PAI-1 than the F-RCC group. Control groups showed that males had a higher expression of u-PAR and PAI-1, but not u-PA, than females. RCC usually affects those over 40 years of age, and the incidence of RCC increases between 40 and 50 years of age, regardless of sex, but occurs more frequently among men (as much as twice) than among women (31, 32). Additionally, the incidence of RCC is higher in Caucasians than in African Americans (33). It is interesting that a similar pattern was observed in the expression of the plasminogen activation system. It is, therefore,
possible that this system is responsible for aggressive properties of the renal cancers.

Curiously, PAI-1 is overexpressed in kidney cancers, in some cases in 10–20 times higher molar ratio than u-PA, as detected by the ELISA method. That contradicts our hypothesis and could indicate that u-PA will be completely inhibited and as such could not function in metastasis and invasion. Immunohistochemical data reveal that u-PA is contained on cancer cells with...
Fig. 4 Boxplot representation of the expression of the u-PA system in RCC that depends on tumor grade. C, control group; S-I, stage one; S-II, stage two; S-III, stage three; S-IV, stage four.

an intensity frequently much higher than the diffused staining of PAI-1 that is spread through large areas of tissue. Therefore, local concentration of u-PA on individual cancer cells could be higher than the concentration of PAI-1, allowing cancer cells to invade and to metastasize, as we hypothesize. Similar instances were reported for other types of cancer (1, 4, 5, 34).

Additionally, immunohistological staining shows that all elements of the urokinase system are located in areas where...
Fig. 5 Immunostaining of RCC (a–h) and squamous cell carcinoma (i–p) with antibody against: u-PA (a, e, i, and m); u-PAR free and bound with u-PA (b, f, j, and n); u-PAR free (c, g, k, and o); and PAI-1 (d, h, l, and p). False color images (e–h and m–p) were produced by enlargement of the images above (approximate location is shown by a red rectangle), followed by a conversion to false color where red represents the highest intensity and blue the lowest intensity of immunostaining.

would expect them. In particular, u-PA was present on cancer cell membranes and in the cytosol where it is synthesized, recycled, and degraded after the u-PA/u-PAR complex binds to PAI-1. The receptor was detected predominantly on the cancer cell membrane. Similarly, PAI-1 was detected in the same cell fragments, because it is synthesized, recycled, and degraded in a similar way as u-PA is. Receptors are rapidly recycled to the cell membrane, and little u-PAR could be found in the internal part of cells, as detected by antibodies. All of these facts very strongly support the authenticity of immunostaining for all detected elements of the urokinase plasminogen activation system (2, 9, 11, 12).

Therefore, we conclude that the u-PA system is overexpressed in kidney malignant tumors, and the amount of these proteins in more aggressive cancers is higher than in less aggressive cancers.

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