Tumor-associated Antigens in the Urine of Patients with Bladder Cancer

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INTRODUCTION

Human bladder cancer cells may express one or more TAAs. Evidence for this expression appears to be provided by a number of studies of patient responses to these putative antigens. For example, positive reactions have been noted when lymphocytes from bladder cancer patients have been reacted with transitional cell tumors or tumor extract in assays measuring cell-mediated cytotoxicity (3, 19, 20) and migration inhibition factor (14). Cells from control individuals were generally unreactive. In addition, sera from some bladder cancer patients can induce antibody-dependent cell-mediated cytotoxicity of tumor cells in vitro (10). Troye et al. (24) found that IgG fractions conferring transitional cell-specific cytotoxicity could be prepared from the sera of patients with bladder cancer but not from the sera of healthy donors or patients with prostatic carcinoma. While these studies do suggest the existence of TAA in bladder cancer, these types of assays are difficult to interpret, particularly in light of the recent understanding of the action of natural killer cells in vitro systems.

ABSTRACT

Previous work in our laboratory has demonstrated that antisera to proteins found in the urine of bladder cancer patients can distinguish between urine samples from these patients and those from normal individuals when tested in a complement-fixation assay. Antisera which were reactive with individual tumor-associated antigens were used to detect the antigens in the urine of bladder cancer patients and control individuals. One antigen was found in 66% of the bladder cancer patients (n = 38) and 25% of control individuals (n = 20). This antigen is a glycoprotein with a molecular weight of about 200,000 and β-electrophoretic mobility and is distinct from carcinoembryonic antigen. A second molecule detected by an antisera to urine fractions proved to be completely identical to serum C-reactive protein. C-Reactive protein was found in the urine of 72% of the bladder cancer patients (n = 39) and 32% of the control population (n = 32). Although a third protein detected with these antisera proved to be a normal urinary component, two of these tumor-related proteins appear to have potential as diagnostic markers for bladder cancer.

MATERIALS AND METHODS

Collection and Processing of Urine Specimens. Twelve-hr, overnight urine samples were collected from patients with proven cases of bladder cancer (papilloma and various grades of carcinoma), patients with proven urinary tract infections, and various hospitalized patients, including those with a variety of disorders which might predispose them to proteinuria. In addition, urine samples were collected from proven normal kidney donors and volunteers. The samples were collected in sterile containers and stored at 4° during collection, discarding any with gross hematuria. After collection, the samples were centrifuged to remove cells and debris and either frozen at −20° or processed immediately by ultrafiltration, saving an aliquot at −80° for future analysis.

Using a thin-channel ultrafiltration unit (Model DC 2; Amicon Corp., Lexington, Mass.) equipped with a HIX50 cartridge, an initial concentration of the urine samples was made, retaining solutes with molecular weights greater than approximately 50,000. Further concentration of urinary protein as required for chromatography of other procedures was accomplished with B15 Minicon concentrator cells (Amicon Corp.) or Immersible-CX ultrafiltration unit separators (Millipore Corp., Bedford, Mass.). The protein concentration of urine specimens and the ultrafiltration fractions was determined by the dye-binding assay of Bradford (2).

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1 The abbreviations used are: TAA, tumor-associated antigen; CU, concentrated urine from bladder cancer patients; HAP, hydroxypatite; CRP, C-reactive protein; CEA, carcinoembryonic antigen; anti-CU, antiserum to a gel filtration fraction of bladder cancer urine; anti-HAP6, antiserum to a hydroxypatite adsorption chromatography fraction of bladder cancer urine; anti-HAP3C, antiserum made using bladder cancer urine protein adsorbed to hydroxypatite crystals.
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Our laboratory has taken an alternative approach, investigating the possibility that TAAs are released into the urine of bladder cancer patients. Initially, we observed that rabbit antiserum prepared against bladder tumor tissue and then absorbed with normal tissue reacted in a gel diffusion assay not only with tumor extract but also with CU. Thus, it appeared that some tumor-derived substance could be detected in urine by immunological techniques. Subsequently, antiserum prepared using gel filtration fractions of urine was used in complement fixation and gel diffusion assays of CU and urine from control patients (9). CU samples, even those from patients with low-grade neoplasms, showed high reactivity with this antiserum. Most samples from normal individuals did not react, but a moderate degree of reactivity was noted in the population of control patients with urinary tract infection.

Because it is possible that multiple components were being detected in these assays, we have more recently been concerned with further fractionation and identification of urinary TAAs. In the present work, antiserum to individually characterized TAAs were tested against CU and urine from control populations.
HAP Adsorption Chromatography. HAP (Bio-Gel HT; Bio-Rad Laboratories, Richmond, Calif.) was equilibrated with 0.1 M sodium phosphate, pH 6.75 (starting buffer), and the slurry was degassed at 85°C for 15 min. The crystals were packed into a 2.5 x 85-cm column, and a sample of approximately 45 mg of CU protein, equilibrated with the starting buffer, was applied at room temperature. After unbound material was washed through the column with starting buffer, elution was begun with a linear phosphate gradient (0.1 M sodium phosphate to 0.3 M potassium phosphate). Eluted fractions were concentrated in the B15 units and tested by immunoelectrophoresis.

Affinity Chromatography on Immobilized Concanavalin A. A 30-ml slurry was packed with 12-ml concanavalin A-Sepharose (Pharmacia Fine Chemicals) equilibrated with 0.1 M phosphate buffer containing 0.5 M NaCl (pH 7.2). After applying 14 mg of CU protein, unbound material was eluted and concentrated for analysis by immunoelectrophoresis. Concanavalin A-bound material was eluted by flushing the column with 0.5 M (1-methyl-β-D-mannoside) in the phosphate buffer.

Antiserum. Antiserum to CRP was purchased from Miles Laboratories, Inc., Elkhart, Ind. Antiserum to CEA was the generous gift of Dr. H. S. Hansen, Hoffman-LaRoche Inc., Nutley, N. J. This antiserum was also reactive with normal cross-reacting antigen.

One antiserum to TAAs, designated anti-CU6, was produced by injecting a San Juan strain rabbit with fractions from the second peak area of G-200 gel filtration of CU (Chart 5). The priming dose of approximately 4 mg of protein was emulsified with Freund’s complete adjuvant and injected s.c. into multiple sites. After 2 weeks, the animal was boosted, administering one half of the dose i.v. and the other half s.c., and then bled the following week.

Anti-HAP 6 was prepared using material recovered from a batch modification of the HAP chromatography method referred to above. Approximately 25 mg of CU protein were added to 25 ml of HAP in 0.1 M sodium phosphate (pH 6.75). Unadsorbed protein was washed away by centrifugation, and the molarity of the buffer was increased in these steps: 0.2 and 0.275 M sodium phosphate; and 0.6 M potassium phosphate. Material eluting in the 0.6 M wash was concentrated and used for immunization, following the immunization schedule described.

HAP crystals in the above fractionation, which had been washed in 0.275 M phosphate but not yet eluted with the 0.6 M buffer, were used directly for immunization. A slurry made by adding 0.25 ml HAP crystals to 0.25 ml 0.275 M sodium phosphate and 0.6 M potassium phosphate. Material eluting in the 0.6 M wash was concentrated and used for immunization.

Antiserum was adsorbed using insoluble immunoabsorbents prepared by coupling proteins to cyanogen bromide-activated Sepharose (Pharmacia Fine Chemicals) and stabilizing this coupling with glutaraldehyde by the method of Kowal and Parsons (12). Separate absorbents were made with normal plasma and with concentrated proteins from normal urine and urine from patients with urinary tract infections, adding at least 10 mg protein per g dry activated Sepharose. For adsorption, antiserum was added to an equal volume of drained gel and incubated with gentle rocking at 37°C for 30 min. The antiserum was drained from the gel by centrifugation through a porous support.

Assay Methods. Immunoelectrophoresis performed as described previously (6) on antigen-containing samples (5 µl) concentrated to >10 mg/ml protein in the Minicon units.

Gel slabs prepared for immunoelectrophoresis were also used for analysis by gel diffusion. Antigen and antibody wells 4 mm in diameter were filled with 25 µl of reactants. After 24 hr, the gels were washed and stained as described.

The microcomplement fixation assay of Levine (15) was used to measure levels of TAAs in urine samples. All tests were incubated overnight at 4°C before adding indicator sheep RBC. Following incubation at 37°C, the degree of complement fixation was determined.

RESULTS

Detection of Bladder Tumor-related Antigens by Immunoelectrophoresis. When concentrated CU was studied by immunoelectrophoresis using absorbed antisera to CU fractions, 3 apparently tumor-related precipitin bands were detected. Two precipitin bands with β-electrophoretic mobility were detected using adsorbed anti-CU6. One, designated ‘β’ was detectable in several CU samples. The second was readily detectable in both CU specimens and samples from patients with urinary tract infections, and is referred to as ‘βu’. Anti-HAP 3C was specific for βu after treatment of the antiserum with the normal plasma immunoabsorbent.

Anti-HAP 6, adsorbed with plasma and normal urine, precipitated a band with γ-electrophoretic mobility from several CU samples and also reacted with the βu-antigen.

Occurrence of TAAs in CU and Urine of Control Patients. A more sensitive assay for the occurrence of these antigens, complement fixation, was used to determine the frequency with which these TAAs could be detected in CU and control samples.

Anti-CU6, adsorbed extensively with normal plasma, normal urine, and urine from patients with urinary tract infection, was used to detect the β-antigen in an assay in which 50% complement inhibition was taken as the end point (Chart 1). Urine samples which produced complement-fixation at relatively low total protein concentrations were considered to be highly active, while samples which did not give at least 50% fixation at concentrations greater than 250 µg/ml were considered negative. When CU was studied in this way, 66% of the samples tested as positive, including those from patients with papilloma or carcinoma in situ. Of the 20 control samples (including normal individuals, patients with other cancers, nonmalignant urogenital disease, and various conditions with associated proteinuria), only 25% were positive. By Yates x2 analysis, this assay is able to distinguish between the bladder cancer and control population at better than a 99% confidence level.

Similar results were obtained when adsorbed anti-HAP 6 was used to detect the γ-antigen in the complement fixation assay (Chart 2). Of 39 CU samples, 72% were positive, while only 32% of the control samples (n = 22) were positive. The differences in reactivity between the bladder cancer and control populations was again statistically significant, in this case, at better than a 95% level.

A total of 43 samples was tested with both antisera, and many of the CU samples were positive for both the β- and the γ-antigen (Chart 3). However, if reactivity with either of the 2 antisera is considered, the bladder cancer detection rate is increased to 82% (n = 28), while the false-positive rate (20%, n = 15) is not increased above that for either antiserum used alone.

The distribution of the βu-antigen was studied by complement fixation with anti-HAP 3C absorbed so as to be unreactive with normal plasma. Although it was difficult to remove reactivity to βu from antiserum by absorption with normal urine or urine from patients with urinary tract infection, this assay revealed βu to be normal urinary component, since all 45 urine speci-
Urinary Tumor-associated Antigens in Bladder Cancer

Chart 1. Complement-fixation assay for the β-antigen of urine samples from patients with various grades of bladder cancer (cancer in situ (IN SITU), papilloma (PAP), Grades I to III (I, II, III)) and from control individuals. The control population included normal individuals (NOR), kidney transplant donors (TRANS DONOR), and patients with urinary tract infection (UTI), adenocarcinoma of the bladder (ADENOCA BLADDER), undiagnosed infertility (INFERT) diabetes mellitus, prostatic carcinoma (PROSTATE CA), gastric by-pass surgery, urinary tract infection and prostatic hypertrophy (UTI + PROST HYPER), breast cancer (BREAST CA), and obstructive jaundice (OBSTR JAUNDICE). Urine samples giving end point fixation of 50% (50% CF) at low concentrations of total protein were considered highly reactive. Samples not giving 50% fixation at protein concentrations greater than 250 ng/ml were considered negative.

Chart 2. Complement-fixation assay for the γ-antigen, which proved to be CRP, of samples from CU and urine of control patients as in Chart 1. Additional categories of control patients included are obesity (OBESE), endometriosis with bladder involvement (ENDOMET BLADDER), and inguinal hernia.

Chart 3. Reactivity in complement-fixation (CF) for the β-antigen vs. reactivity for CRP. Note that most CU samples tested contain either the β-antigen or CRP.

Partial Characterization of the TAAs. CU was subjected to gel filtration, and the eluted fractions were concentrated and analyzed by immunoelectrophoresis. The TAAs eluted as shown in Chart 4. By comparison with molecular weight standards, the molecular weight of the β-antigen was estimated at 200,000 and that of the γ-antigen was estimated at 110,000.

HAP adsorption chromatography had been used as a step in the preparation of CU samples for antibody production. Chromatography of CU samples at pH 6.75, followed by analysis with immunoelectrophoresis, revealed that the β component has little affinity for this material, eluting at very low phosphate concentrations (<0.01 M; Chart 5). However, the γ- and βγ-antigens bound tightly to HAP, being eluted only at very high phosphate concentrations (>0.2 M). These antigens could be separated quite efficiently from the bulk of normal proteins in CU by this method.

The electrophoretic mobility and molecular weight of the γ-antigen were reminiscent of the properties of the acute-phase reactant protein, CRP. During immunoelectrophoresis, commercial antiserum to CRP precipitated from CU a molecule identical to that of the γ-antigen, while anti-HAP 6, adsorbed to be unreactive with normal plasma, precipitated a band with γ-mobility from serum samples from patients with elevated CRP. The serum and urinary molecules showed complete immunological identity on gel diffusion with either antiserum. Finally, when immunoelectrophoresis was performed in the absence of Ca²⁺ by including EDTA (0.01 mM) in the gel buffer, the mobility of the γ-band precipitated by anti-HAP 6 shifted to a β position, as has been reported for serum CRP (8). Thus, one of the TAAs we have detected in CU is CRP, and this urinary molecule is similar to serum CRP in molecular weight, antigenicity, and ability to bind calcium.

When CU was passed through a column packed with concanavalin A bound to Sepharose, the βγ-antigen and CRP did not bind. The β-antigen did bind and could be subsequently...
samples from a number of patients with papilloma and carcinoma in situ were positive in these assays is of particular interest in light of difficulties involved in diagnosing these by urinary cytology and cystoscopy. Therefore, it does not appear in normal plasma or in measurable quantities in the urine of many of the control patients tested. Its binding to concanavalin A suggests that it is a glycoprotein. Gel diffusion studies and immunoelectrophoresis following perchloric acid treatment indicate that it is not CEA. Additionally, the molecular weight of the $\beta$-antigen (approximately 200,000) distinguishes it from the very large tumor-associated molecule (>1,000,000) (22) and the smaller molecule (21,000) (23) found in the urine of patients with a variety of other types of tumors.

A second urinary protein detected in CU samples, $\beta_{\text{HbU}}$, which initially appeared to be tumor related in that antibody to it was difficult to absorb out with normal antigens, proved to be a normal urinary nonplasma component.

A third substance was detected in CU which was neither a normal plasma nor urinary protein and was identified as the acute-phase reactant protein, CRP. The molecule found in CU appears to be identical with native serum CRP; no antigenic dissimilarities were noted, and the molecular weight and $\text{Ca}^{2+}$-binding properties of urinary and serum CRP are similar (8).

CRP is normally present in serum in very low concentrations, but greatly elevated levels are associated with a number of disease states involving inflammation and tissue damage, including some types of cancer (5). Elevated serum CRP in 13 of 17 patients with bladder cancer was noted by Rodriguez Torres et al. (21) using a semiquantitative capillary precipitation method. Postoperatively, there was some decline in CRP levels in many of these patients. Bastable et al. (1), while studying serum acute-phase reactant proteins in bladder cancer patients, noted that the frequency of elevated CRP levels was strongly correlated with stage; only about 13% of patients with T1 or T2 tumors exhibited elevated CRP levels, while frequency of this abnormality rose to 45% in T3 and 69% in T4 bladder cancers. O’Quigley et al. (18) have extended these findings by showing that pretreatment levels of acute-phase proteins, especially CRP, have considerable prognostic significance for T3 and T4 bladder cancer patients.

Relatively little is known of the occurrence of CRP in urine. Chun et al. (4) have reported that CRP can be found in the urine of patients undergoing rejection of kidney grafts. In a study of patients with elevated serum CRP, Mukin et al. (17) noted that these individuals did not excrete CRP, even if their serum levels were very high, unless they were also somewhat proteinuric. These authors noted that a threshold concentration of urinary protein of 6.6 mg/100 ml was necessary for the CRP to spill into the urine. The protein concentrations of most of our urine samples, both CU and urine from control patients, was well above this threshold (65% of bladder cancer, 72% of control cases, data not presented).

However, many of the patients in our study whose urine was CRP positive had relatively low-grade cancers (papilloma, carcinoma in situ, and Grade I). As shown by Bastable et al. (1), patients with low-stage tumors tend to have normal serum CRP levels. Because there is a general correlation between stage and grade of the disease (16), by inference it might be expected that many of these patients with urinary CRP actually did not have elevated serum CRP. CRP is known to localize in damaged tissue (7, 13), apparently because of its affinity for phospholipid groups exposed in the membranes of damaged cells (25). It is possible that CRP might selectively concentrate in bladder tumor tissue and then be shed into urine. Further studies to elucidate this point are in progress.
In conclusion, we have demonstrated that the occurrence in the urine of CRP and the \( \beta \)-antigen is associated with bladder cancer. Like CRP, the \( \beta \)-antigen may have a nonspecific origin, being, for example, produced or shed in response to tissue injury. Nevertheless, these substances appear to have potential as diagnostic markers, particularly if assays for both markers are utilized.

REFERENCES

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