Glycotyping of prostate specific antigen

Sadhana Prakash and Phillips W. Robbins
Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

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Measurement of serum levels of the prostate specific antigen (PSA) is now widely used for the diagnosis of prostate cancer and benign prostate hyperplasia. This serum marker is of value since it is derived only from the tissue of interest, but increased levels of PSA in serum do not allow a completely clear cut diagnosis of benign versus malignant changes. Since PSA is a glycoprotein with one asparagine linked oligosaccharide, and since malignant transformation often leads to an increased branching of such oligosaccharides, we initially studied the asparagine linked structures on PSA made by a cell line derived from malignant metastatic prostate tissue. We observed that unlike normal PSA, which bears only biantennary oligosaccharides, PSA from the metastatic cell line has a mixture of biantennary and triantennary oligosaccharides. Further experiments will reveal carbohydrate differences derived from the PSA from sera or, prostate tissue of normal versus prostate cancer patients, and of the utility of such carbohydrate differences as a possible diagnostic marker for prostate cancer.

Key words: carbohydrate/metastasis/oligosaccharides/prostate specific antigen/tumor marker

Introduction

Prostate cancer is the second leading cause of male cancer death. Clinical cancer of the prostate can only be cured if localized, and then it is usually asymptomatic. One of the biggest problems in this field is to distinguish normal prostate and benign prostatic hyperplasia from cancer. Thus efforts are being made to improve the efficiency of diagnosis, to better distinguish BPH from prostate cancer and to identify indolent prostate cancer.

Prostate specific antigen (PSA), a glycoprotein secreted by the prostatic epithelium has been demonstrated to be clinically important for the detection and monitoring of prostate cancer (Hudson et al., 1989). It is believed that PSA has the highest validity of any circulating cancer screening marker discovered thus far. Elevated serum concentration has become a common tool for detecting early stage prostate cancer and monitoring therapy of this disease (Staney et al., 1987; Catalona et al., 1991). However, the use of this screening method remains controversial.

Since oncogenesis is often correlated with a change in carbohydrate structure (Fukuda, 1985; Dennis and Lafarté, 1989), a number of carbohydrate markers have been used for clinical characterisation of human carcinomas (Hakomori, 1991a,b). In the current study, we sought to determine if the carbohydrate moiety on normal PSA is distinguishable from the carbohydrate moiety of PSA derived from cancerous prostatic epithelium. As an initial effort we investigated the PSA derived from a cell line (LnCaP) which was established from the lymph node of a patient with metastatic prostate carcinoma (Horoszewicz et al., 1980). We report here that the carbohydrate derived from the metastatic cell line (LnCaP) is different from that derived from normal PSA. Further investigations will reveal whether these differences are useful for clinical diagnosis.

Results

Purification of PSA derived from the LnCaP cell line

PSA from the cell line was purified by immunoprecipitation. Normally the cell derived supernatants, upon stimulation with DHT (dihydrotestosterone) contained PSA in the range of 0.5 to 2.0 µg/ml. Initial calibration of the gel showed that 20 µl of the gel could bind at least 1–2 µg of PSA from conditioned medium. Approximately 25–30% of the bound PSA was recovered using the procedure outlined. Figure 1 shows SDS-PAGE of PSA purified by immunoprecipitation. Normal PSA migrates as a 30 kDa protein (data not shown). Samples purified from the cell line resolved as protein bands with a mobility of approximately 55 kDa, 30 kDa, and 22–25 kDa (lanes b and c). The band at approximately 22–25 kDa may be a degradation product of PSA. When analyzed by Western blotting, the lower bands (30 kDa and 22–25 kDa) react with the polyclonal anti-PSA antibody (data not shown). We suspect that the band at approximately 55 kDa corresponds to the heavy chain of the immunoglobulin or possibly other proteins that may copurify.

The PSA band with the mobility of ∼30 kDa was analyzed for carbohydrate composition.

Fluorophore assisted carbohydrate electrophoresis of PSA

PSA (normal) and that derived from the cell line was labeled with ANTS and separated by PAGE. Oligosaccharides released from as little as 2 µg of the purified protein can be detected by this method. Our initial experiments showed that the oligosaccharides released from transformed PSA migrated with a different mobility than those from normal PSA (Figure...
The difference in intensity of the glycan band derived from native PSA (lanes 3 and 4) and the transformed cell line (lanes 5 and 6) is due to a difference in the amount of material loaded on the gel. Since the electrophoretic mobility of the oligosaccharide bands is different when charged groups are present, both normal PSA and PSA from the transformed cell line were treated with neuraminidase (III) (Figure 3). After removing the sialic acid residues, most of the oligosaccharide derived from normal PSA (lane 5) had the same electrophoretic mobility as the asialobiantennary standard (lane 3). The majority of the oligosaccharide from the transformed PSA is biantennary, but some had the mobility of triantennary and also tetraantennary oligosaccharide (lane 6).

Discussion

Prostate specific antigen (PSA) is a widely accepted tumor marker for prostate cancer (Guinan et al., 1987; Lange et al., 1989). It contains a single polypeptide chain 237 amino acids long with a molecular mass of 26,079 Da. By ion spray mass spectrometry (ISMS) and analysis of its carbohydrate moiety by NMR spectroscopy (Lange et al., 1989), it has been shown that the predominant PSA species has a molecular mass of (M_r) of 28,430, suggesting that PSA contains a carbohydrate residue of M_r 2351. Apparently the protein contains only one N-linked chain at asparagine-45, and it has been proposed that the carbohydrate structure is a biantennary N-linked oligosaccharide of the N-acetyl lactosamine type with a sialic acid group at the end of the two branches (Bélanger et al., 1995). Approximately 70% of the PSA molecules contain a fucose group.

Our data using the ANTS labeled (GLYKO FACE) analysis of the carbohydrate moiety of the normal PSA (Figures 2, 3) is in agreement with the previously determined structure of the carbohydrate moiety of PSA. Upon treatment with neuraminidase, a majority of the carbohydrate appears to be fucosylated biantennary oligosaccharide. In comparison, carbohydrates derived from the PSA of a metastatic prostate carcinoma cell line is a mixture of biantennary, triantennary, and possibly tetraantennary oligosaccharides. It is possible that oncogenic transformation of the prostate epithelium may differentially affect the N-linked glycan processing of the prostate specific antigen.

Other studies have shown that oncogenic transformation of cells can profoundly affect the processing of some glycosylation sites—yielding higher levels of tri- and tetraantennary oligosaccharides (Feizi, 1985; Fernàndez et al., 1991; Matsumoto et al., 1992). However, the regulation of this
Materials and methods

Cell culture of LnCaP cells

The LnCaP cell line (Horoszewicz et al., 1980) was obtained from ATCC (Rockville, MD). It was propagated in RPMI 1640 containing 10% heat inactivated FBS (GIBCO, Grand Island, NY) supplemented with penicillin and streptomycin (50 U/ml) and glutamine (50 µg/ml). Data provided by the manufacturer for the single lot of serum used in our experiments indicated that concentrations of testosterone, 17 β-estradiol, cortisol were sufficiently low so as not to influence the culture conditions. DHT (Dihydrotestosterone) was obtained from Sigma Chemical Co. (St. Louis, MO). Semiclonal monolayers of LnCaP cells were cultivated in 75 cm² plastic flasks (Falcon Plastics, Oxnard, CA). In some instances, the cells were grown in polystyrene-coated flasks or 6-well culture dishes (Collaborative Research, Becton Dickinson, Franklin Lakes, NJ). For propagation, cells were detached by treatment with trypsin (0.02%): EDTA (0.02%) solution (GIBCO) and counted. Cells were inoculated into new flasks at a density of 10⁵ cells/ml; the cells were grown undisturbed for at least 48 h and then were stimulated with DHT at a concentration of 10–50 nm. Supernatants were collected from semiconfluent cultures approximately 7–10 days later. In some cases, the LnCap cells were grown in medium containing bovine serum albumin (BSA, 0.3 mg/ml; Sigma) The amount of PSA in the cell culture supernatant was assessed using the Hybritech Kit (Hybritech Inc., San Diego, CA). This kit uses a standard Immunoenzymetric method for detecting PSA in clinical samples. Using these culture conditions, the cells produced 1–2 µg/ml of PSA on average. Normal PSA used in the experimental procedures was prepared from seminal plasma and was a gift from Dr. G.P. Murphy (Murphy et al., 1995).

Purification of PSA by immunoprecipitation

PSA was purified by use of anti-PSA antibody linked gel. A polyclonal rabbit anti human PSA antibody (AXL 685, Accurate Chemical & Scientific Corp.) was cross linked to Protein G Sepharose using an Immunopure crosslinking kit (Pierce, Rockford, IL). Before crosslinking, protein G Sepharose was equilibrated with Immunopure binding buffer and then mixed with anti PSA IgG at a concentration of 3–4 mg IgG/ml of gel. The solution was mixed by gentle inversion at room temperature. After 30–60 min, gel was washed with wash buffer and the antibody was bound using a solution of DMP (dimethylpimelimidate·2HCl) for 1–2 h at room temperature; the remaining active sites were blocked using Immunopure blocking buffer. Unbound IgG was eluted with glycine-HCl (pH 2.5), gel was washed and then stored in PBS containing 0.02% NaN3. For immunoprecipitation, approximately 15–20 ml of medium containing PSA derived from hormone stimulated LnCaP cells was incubated with 0.3–0.4 ml of washed anti-PSA bound gel. After incubation at room temperature for 30–60 min, unbound fraction was withdrawn and the gel was washed 3–4x with PBS. Bound PSA was eluted in a batchwise procedure using an equal volume of 100 mM acetic acid. Resulting fractions (3 or 4) were collected and concentrated using a Speed Vac. Concentrated fractions were then resolved by SDS-PAGE for carbohydrate analysis. In some cases, the fractions eluted were placed in tubes containing 50 µl of...
Tris-HCl (pH 8.5). These fractions were used for estimating concentrations of recovered PSA.

**SDS polyacrylamide gel electrophoresis**

Samples were subjected to electrophoresis through 10% separating, 5% stacking precast gels (Bio-Rad, Richmond, CA) under reducing conditions according to the method of Laemmli (Laemmli, 1970). Gels were stained for protein by a silver stain using a Bio-Rad kit. Transfer to PVDF (Immobilon-P) was as described previously (Towbin et al., 1979). Transfers were done for 1.5–2 h at a constant voltage of 85–90 V at 4°C. The PVDF membranes were stained with Ponceau S (Bio-Rad; 0.1% v/v in 1% acetic acid) destained with 1% acetic acid and then washed with water. The appropriate bands were excised and either stored at −70°C or used immediately for releasing oligosaccharides.

**Preparation and analysis of oligosaccharides from PSA**

PSA bands were excised from the stained membranes and placed in tubes containing 35 µl of sodium phosphate buffer (50 mM, pH 7.7) and 0.5 µl of SDS (5%). Samples were denatured for 5 min at 100°C and cooled on ice; 2.7 µl of 7.5% NP-40 was added to each tube, followed by 4 µl of PNGase F (New England Biolabs, Beverly, MA). Samples were incubated at 37°C for 2 h; control samples were treated as above without the addition of PNGase F. For treatment with NaN3 (Glyko), samples were acidified by addition of 1–2 µl of acetic acid; ~2 µl of the enzyme (Glyko) was added subsequently. The samples were incubated for a duration of 2 h at 37°C, and the digests were dried in a Speed-Vac. The released oligosaccharides were labeled for 18 h at 37°C using reagents from Glyko with fluorophore, 8 aminonapthalene-1,3,6 trisulfonate (ANTS), by reductive deamination (Friedman and Higgins, 1995) and sequencing of carbohydrates.

**References**


