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Immunological comparison between prostate-specific antigen and γ-seminoprotein

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Summary. Prostate-specific antigen (PA) and γ-seminoprotein (γ-Sm) were compared by immunocytochemical, immunodiffusion and immunoblotting methods using rabbit anti-PA antibody and rabbit anti-γ-Sm antibody. Enzyme immunoassys (EIAs) were developed for measurements of PA and γ-Sm to determine a correlation between serum PA and γ-Sm levels in patients with prostate cancer. The patterns of localization and distribution of PA and γ-Sm were identical in prostate tissue sections, including benign and cancerous human prostates. The immunodiffusion study showed that the antigens with which anti-PA antibody and anti-γ-Sm antibody reacted in seminal plasma and prostate tissue homogenates were identical to each other. In the immunoblotting study, anti-PA antibody and anti-γ-Sm antibody recognized a single antigen corresponding to a molecular weight of approximately 33,000 both in seminal plasma and prostate tissue homogenates. The EIAs developed in this study were sensitive, specific, and reproducible, and the correlation between serum PA and γ-Sm values determined by these EIAs was highly significant ($r = 0.99$, $P < 0.001$). These results indicated that PA and γ-Sm were immunologically identical and that serum PA and γ-Sm determined by immunoassays using anti-PA antibody and anti-γ-Sm antibody should be evaluated as identical tumor markers for serodiagnosis of prostate cancer.

Key words: Prostate cancer – Tumor marker – Prostate-specific antigen – γ-Seminoprotein

Prostate-specific antigen (PA) was first isolated from prostate tissues and seminal plasma as prostate tissue-associated antigen [25] and its measurement in sera from patients with prostate cancer has been reported to be useful in detecting and monitoring the disease [8, 10]. An antigen specific for human seminal plasma, designated γ-seminoprotein (γ-Sm), was isolated in immunological studies of human seminal plasma [4].

Immunocytochemically γ-Sm has been shown to originate in the epithelia of prostate gland [22] and has been also clinically evaluated as a tumor marker for prostate cancer [11, 16, 17]. PA and γ-Sm are biochemically and immunologically distinct from prostatic acid phosphatase [23, 26]. Althoug PA and γ-Sm are biochemically similar to each other in some respects [1, 3, 9, 13, 18, 24], a significant correlation between serum PA and γ-Sm in patients with prostate cancer has not been established [2, 27].

In this study, we compared PA and γ-Sm by immunocytochemical, immunodiffusion, and immunoblotting techniques and developed enzyme immunoassays (EIA) to measure PA and γ-Sm in order to determine whether there is a correlation between serum PA and γ-Sm levels in patients with prostate cancer. We report that PA and γ-Sm are immunologically identical and that there is a significant correlation between serum PA and γ-Sm levels in patients with prostate cancer.

Materials and methods

Seminal plasma and prostate tissues

Seminal fluid was collected from men who consulted Gifu University Hospital, complaining of sterility. The mixture of seminal plasma was dialyzed against distilled water and centrifuged at 1,500 rpm for 10 min. The supernatant was collected and used in this study. Human prostate tissues were obtained promptly after surgical removal and transperineal needle biopsy. The specimens from benign prostatic hypertrophy (BPH) and well-differentiated adenocarcinoma of the prostate were obtained by subcapsular prostatectomy and radical prostatectomy. Hand-cut small cubes of BPH and well-differentiated adenocarcinoma were fixed in formalin for immunocytochemical examination. The remaining tissues (BPH and adenocarcinoma) were blended for 30-s. The homogenates were centrifuged at 3.500 rpm for 15 min and the resulting supernatants were used for immunodiffusion and immunoblotting analysis. Prostate tissues of moderately and poorly differentiated adenocarcinoma were obtained by transperineal needle biopsy and fixed in formalin for immunocytochemical study.

Antibodies

Rabbit anti-γ-Sm antiserum [6] and rabbit anti-PA antiserum [25] were supplied by Chugai Pharmaceutical Co., Tokyo, Japan and by the Department of Diagnostic Immunology Research and Biochemistry, Roswell Park Memorial Institute, Buffalo, U.S.A., respectively. Horseradish peroxidase-labeled Fab' fragment of goat IgG specific for rabbit IgG was purchsaed form MBL Co., Tokyo, Japan.

Direct immunoperoxidase staining

Rabbit IgG antibodies specific for γ-Sm and PA were isolated from the antisera and cloven in Fab' and Fc' fragments. Fab' fragments of anti-γ-Sm antibody and anti-PA antibody were conjugated to horseradish peroxidase [15]. Peroxidase-labeled FAb' fragment of nonspecific rabbit IgG was prepared in an identical manner. Formalin-fixed, paraffin-embedded prostate tissue sections, including histologically diagnosed BPH, well, moderately, and poorly differentiated adenocarcinoma of the prostate, were rehydrated and treated with 5 m*M* periodic acid solution to quench any tissue endogenous peroxidase activity. The slides were washed in phosphate-buffered saline (PBS) and incubated with 10% normal rabbit serum for 30 min to reduce nonspecific protein binding. After removal of excessive rabbit serum, the slides were incubated with peroxidase-labeled Fab' fragment of anti-γ-Sm antibody, anti-PA antibody, and nonspecific rabbit IgG, respectively, for 60 min at room temperature. The slides were washed in PBS again and then soaked in freshly prepared diaminobenzidine solution containing 0.03% H_2O_2 for the color reaction. The slides were washed, counterstained with methyl green, dehydrated, and mounted for microscopic examination.

Double immunodiffusion study

Wells with a diameter of 4 mm were punched out of a 1% agarose gel plate. To each well, 10 µl of anti-PA antiserum, anti-γ-Sm antiserum, seminal plasma, or prostate tissue homogenates was added. After incubation for 18 h the agarose plate was washed in PBS and stained for protein in Coomasie blue solution.

Immunoblotting study

Seminal plasma, prostate tissue homogenates, and molecular markers which were reduced with mercaptoethanol were first subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [12] and then transferred to a nitrocellulose sheet [20]. The lane of the nitrocellulose sheet containing molecular markers was cut away and stained for protein in Coomasie blue solution. The electrophoretic blots in the remaining sheet were soaked in bovine serum albumin to saturate additional protein binding sites and then the sheet was divided into two pieces. One of them was incubated with anit-PA antibody and the other with anti-γ-Sm antibody for 30 min at room temperature. The sheet was rinsed in PBS and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody for 30 min at room temperature. After being washed in PBS, the blots were soaked in diaminobenzidine solution contianing H_2O_2 for color reaction.

Correlation between serum PA and γ-Sm in patients with prostate cancer

EIAs for measurements of PA and γ-Sm were developed using 96-well microplates (Dynatec, Denkendorf, West Germany). Wells in microplates were incubated with poly-*L*-lysine succinate solution for 1 h at room temperature [7]. After the wells were washed with distilled water, 100 µl of anti-γ-Sm IgG or anti-PA IgG solution with an IgG concentration of 10 µg/ml was added to the wells and incubated for 18 h at 4°C. After being washed with PBS, 200 µl of egg albumin in PBS was added to the wells to saturate nonspecific protein binding sites. Seminal plasma standard solution (100 µl) diluted with PBS (range 2–4,000 ng/ml in protein concentration) or sample sera diluted with the same buffer was added to the wells coated with anti-PA IgG or anti-γ-Sm IgG and incubated for 1 h at 37°C. After being washed with PBS containing 0.05% tween 20 (T-PBS), 100 µl of horseradish peroxidase-labeled Fab' fragment of anti-PA IgG or anti-γ-Sm IgG prepared as described above was added to the wells and reincubated for another hour at 37°C. After washing with T-PBS, 100 µl of the substrate solution (40 mg of *o*-phenylenediamin and 10 µl of 31% H_2O_2 in 100 ml of citrate buffer, pH 4.8) was added to the wells and incubated for color reaction for 15 min. After the enzymatic reaction was stopped by adding 50 µ of 6 *N* H_2SO_4, the absorbance of the reaction product was read at 492 nm in a spectrophotometer. All assays were performed twice. The absorbances of standard solutions of seminal plasma were plotted versus protein concentrations of seminal plasma on semi-log scale paper to construct standard curves in both EIAs for measurements of PA and γ-Sm. The quantity of PA and γ-Sm in the sample was determined from the standard curves and expressed as protein concentration of seminal plasma. The specificities of the EIAs were checked by diluting a sample serum with a female serum containing no detectable PA or γ-Sm. The accuracy of the EIAs was verified by addition of known amounts of seminal plasma in three diluted sera and calculation of the percent recoveries of PA and γ-Sm. The within-assay precision was estimated by running nine replicates of two different sera. Serum PA and γ-Sm were measured by these EIAs and expressed as protein concentration of seminal plasma in twenty sera obtained from patients with prostate cancer. A correlation between serum PA and γ-Sm values was statistically determined by means of the linear regression method.

Results

Immunocytochemical study

The localization and distribution of PA and γ-Sm was examined by a direct immunoperoxidase staining technique on formalin-fixed, paraffin-embedded prostate tissue sections, including BPH and well, moderately, and poorly differentiated adenocarcinoma of the prostate. PA and γ-Sm were localized diffusely in the cytoplasm of epithelial cells in all prostate tissue sections examined in this study. The staining patterns of PA and γ-Sm varied from prostate tissue to prostate tissue, but were identical to each other within an individual prostate tissue section (Figs. 1, 2).

Immunodiffusion study

Immunoreactivities of the antibodies specific for PA and γ-Sm with seminal plasma or prostate tissue homogenates were analyzed by double immunodiffusion tests. As revealed in Fig. 3, anti-PA antibody and anti-γ-Sm antibody reacted with the supernatant of seminal plasma, resulting in a single immunoprecipitation line in the gel plate, respectively. The lines formed between anti-PA antibody and seminal plasma and between anti-γ-Sm antibody and seminal plasma were fused to a single line.

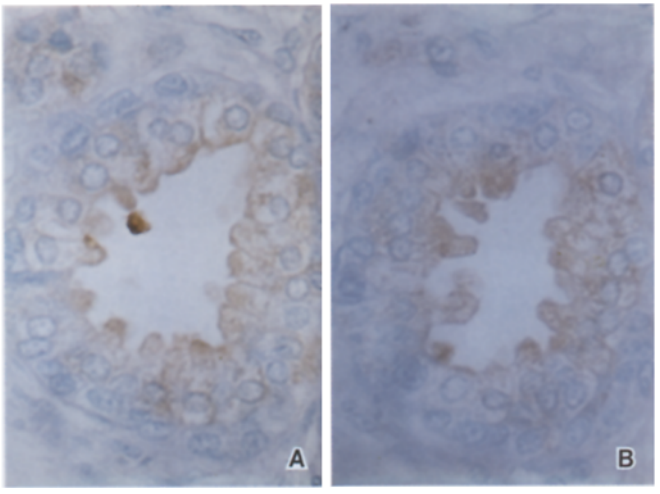

Fig. 1A, B. Immunoperoxidase staining of PA (**A**) and γ-Sm (**B**) in BPH

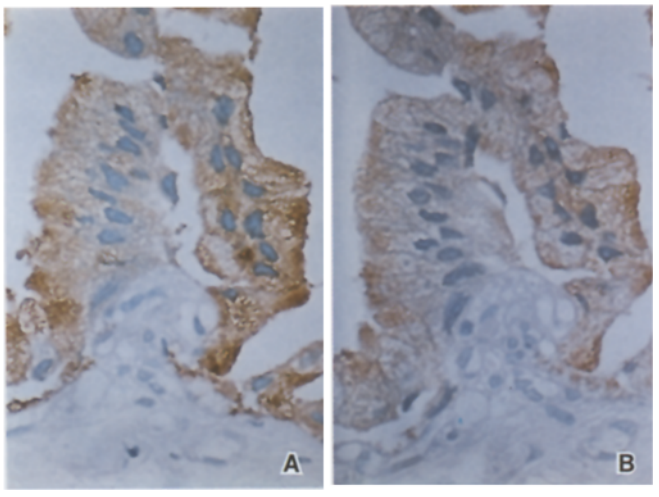

Fig. 2A, B. Immunoperoxidase staining of PA (**A**) and γ-Sm (**B**) in well-differentiated adenocarcinoma of the prostate

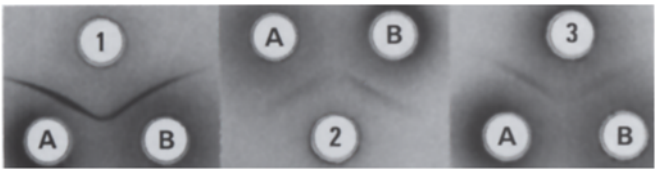

Fig. 3. Double immunodiffision. *1*, seminal plasma; *2*, homogenate of BPH; *3*, homogenate of well-differentiated prostate adenocarcinoma; *A*, anti-γ-Sm antibody; *B*, anti-PA antibody

There were identical observations in immunodiffusion tests employing the supernatants of tissue homogenates of BPH and well-differentiated adenocarcinoma of the prostate (Fig. 3).

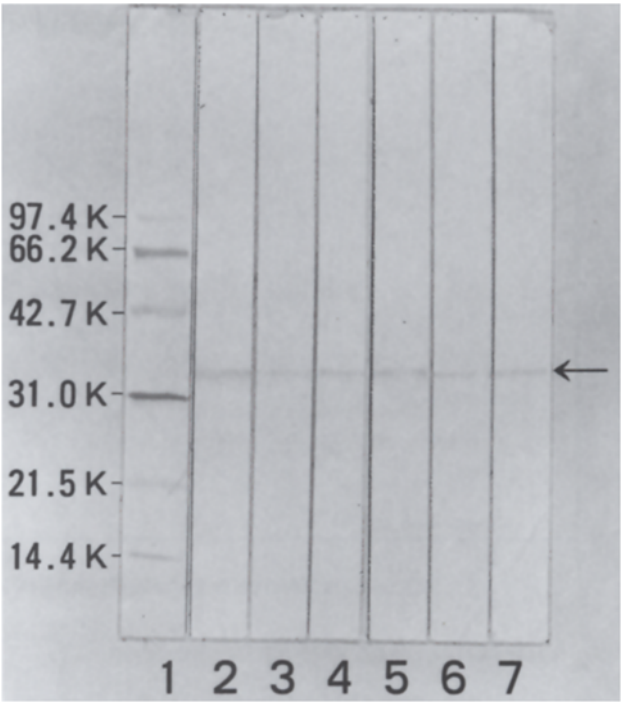

Fig. 4. Immunoblotting of PA (*lanes 2, 3,* and *4*) and γ-Sm (*lanes 5, 6,* and *7*) in seminal plasma (*lanes 2* and *5*), homogenate of BPH (*lanes 3* and *6*) and homogenate of well-differentiated prostate adenocarcinoma (*lanes 4* and *7*). *Lane 1,* molecular markers: phosphorylase b (97,400), bovine serum albumin (66,200), ovalbumin (42,700), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), lysozyme (14,400)

Immunoblotting study

Proteins in seminal plasma and prostate tissue homogenates with which anti-PA antibody and anti-γ-Sm antibody reacted were analyzed by an immunoblotting technique. As revealed in Fig. 4, the antigen recognized by anti-PA antibody or anti-γ-Sm antibody was blotted as a single band in each lane of nitrocellulose sheet to which seminal plasma. BPH, or well-differentiated prostate adenocarcinoma was subjected. The single band in each lane corresponded to a molecular weight of approximately 33,000.

Correlation between serum PA and γ-Sm determined by EIAs

EIAs for measurements of serum PA and γ-Sm were developed. Typical dose-response curves of the EIAs for PA and γ-Sm expressed as protein concentration of seminal plasma are shown in Fig. 5 with calibration curves developing in the range from 60 to 1,000 ng/ml. The curves obtained with a PA- and γ-Sm-positive serum diluted with a various amount of a female serum were parallel to standards curves of PA and γ-Sm. The averages of recoveries of PA and γ-Sm in seminal plasma added to

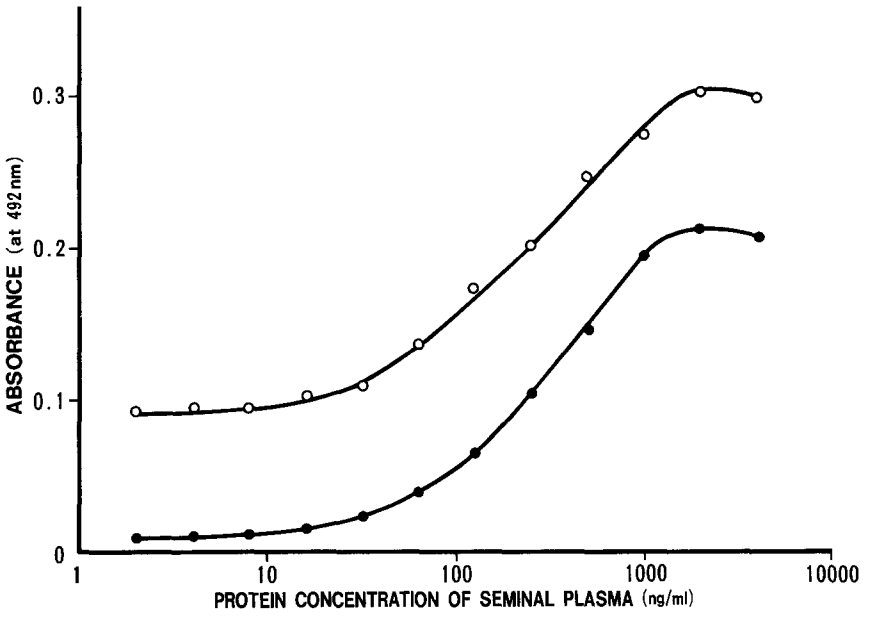

Fig. 5.Standard curves of EIAs for PA (O–O) and γ-Sm (●–●) expressed as protein concentration of seminal plasma

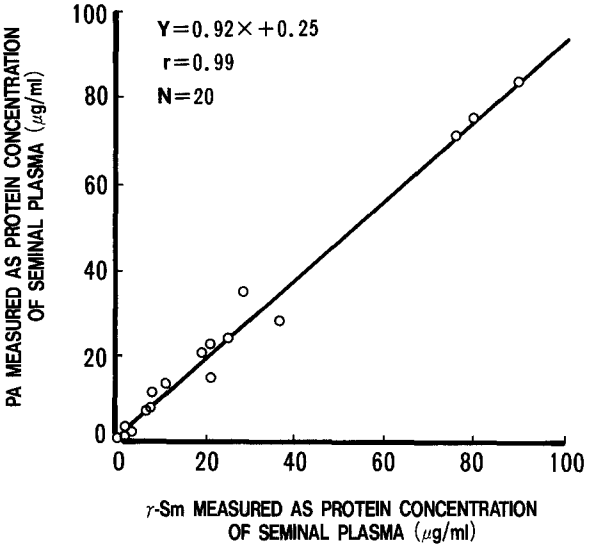

Fig. 6.Correlation between serum PA and γ-Sm values determined by EIAs and expressed as protein concentration of seminal plasma

diluted PA- and γ-Sm-positive sera were 97% and 91%, respectively. The coefficients of variation within assays ($n=9$) for PA and γ-Sm were 5.9%–13.2% and 8.9%–12.0%. Serum PA and γ-Sm values in 20 sera from patients with prostate cancer determined by these EIAs and expressed as protein concentration of seminal plasma ranged from 0.3 μg/ml to 90.8 μg/ml. The correlation coefficient between serum PA and γ-Sm values was 0.99 and there was a highly significant correlation between them ($P<0.001$). The regression equation was Y(PA) = 0.92X(γ-Sm) + 0.25 (Fig. 6).

Discussion

A human PA wa isolated from BPH prostate tissues and seminal plasma [25]. Biochemically PA is a glycoprotein with a molecular weight of 32,000–33,000 determined by SDS-PAGE and isomeric forms of PI 6.2 to 7.2 [26] and exhibits a proteolytic activity [1]. PA has been shown to localize in the epithelia of normal, benign, and cancerous human prostate [14]. Clinically PA has been commonly used as a marker for serodiagnosis of prostate cancer, and a correlation between serum PA levels and disease stage and activity has been established [8, 10]. The complete sequence of PA of 237 amino acid residues was determined and showed extensive homology with protease of the kallikrein family [13].

On the other hand, γ-Sm was first isolated from seminal plasma as an antigen specific for seminal plasma [4]. Biochemically γ-Sm is a glycoprotein with a molecular weight of 26,000 determined by the semidimentation equilibrium method [12] or with a molecular weight of 33,000 determined by SDS-PAGE [5] and with isomeric forms of PI 5.8 to 7.1 [13] and which possesses proteolytic activity [18]. The heterogeneity of purified γ-Sm has been demonstrated [3]. PAGE of γ-Sm produced five or six bands, and differences among amino acid compositions of the bands were suggested [21]. However, all the bands shared a common antigenicity [17] and SDS-PAGE produced a single band corresponding to a molecular weight of 33,000 [3, 5]. Immunocytochemically γ-Sm has been shown to originate in the prostate glands and to be distributed in the epithelia of both cancerous human prostate and normal and benign prostate [22]. As a potential tumor marker of prostate cancer, γ-Sm has been also evaluated for detecting and monitoring the disease [11, 16, 17, 23]. Schaller et al. isolated γ-Sm by ion-

exchange chromatography and determined the amino acid sequence of its main form, which consisted of 237 amino acid residues [18]. This sequence is in complete agreement with that of PA [18]. It coulcd be, therefore, considered that γ-Sm is a complex of five or six forms which have different amino acid compositions but share an identical molecular weight determined by SDS-PAGE and a common antigenicity, and that PA corresponds to a main form of γ-Sm.

In this study, we compared PA and γ-Sm immunologically. Data obtained from immunocytochemcial staining showed that the patterns in localization and distribution of PA and γ-Sm in prostate tissues were identical. The immunodiffusion and immunoblotting studies showed that the antigens recognized by anti-PA antibody and anti-γ-Sm antibody in seminal plasma and prostate tissue homogenates were identical and that their molecular weight was approximately 33,000. These results indicate that PA and γ-Sm are immunologically identical proteins with a molecular weight of approximately 33,000.

Clinically, a significant correlation between serum PA and γ-Sm levels in patients with prostate cancer has not been established, so that PA and γ-Sm have been evaluated as different tumor markers for serodiagnosis of prostate cancer [2, 19, 27]. Serum PA and γ-Sm have been measured by various commercially available immunological assays, which have not been developed in an identical manner. In this study, EIAs for PA and γ-Sm were developed to determine a correlation between serum PA and γ-Sm levels in patients with prostate cancer. The EIAs for PA and γ-Sm were prepared identically, using anti-PA antibody and anti-γ-Sm antibody, respectively, and were conducted under identical conditions. The EIAs were shown to be sensitive, specific, and reproducible by fundamental analyses of the quality of the EIAs. Serum PA and γ-Sm were simultaneously determined by these EIAs and expressed as protein concentration of seminal plasma. The correlation coefficient between serum PA and γ-Sm values was 0.99, and a highly significant correlation was demonstrated ($P < 0.001$). This would be the consequent result, since, as demonstrated in this study, PA and γ-Sm are immunologically identical. Serum PA and γ-Sm, therefore, measured by immunological assays using anti-PA antibody and anti-γ-Sm antibody should be evaluated as identicla tumor markers for serodiagnosis of prostate cancer.

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