Systematic Characterization of Human Prostatic Fluid Proteins with Two-Dimensional Electrophoresis

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We present a systematic analysis of human prostatic fluid with two-dimensional gel electrophoresis (the iso-DALT system) and a characterization of normal and disease-related protein patterns. A reference map for prostatic fluid proteins was established by analysis of pooled prostatic fluids from 80 men (age = 50 years) without prostatic lesions. Proteins in prostatic fluid that shared immunogenicity with serum proteins were identified by use of antibody to whole human-serum protein in an affinity-column fractionation of a reference pool and differential analysis of the absorbed (serum components) and unabsorbed (non-serum components) fractions. Individual prostatic fluids from 30 patients (eight with prostatic cancer, 10 with prostatitis and benign prostatic hyperplasia, six with benign prostatic hyperplasia alone, and six with asymptomatic chronic prostatitis) were scored qualitatively with respect to the presence or absence of 57 major prostatic fluid proteins. Statistically significant, disease-correlated alterations were observed for at least eight of the proteins so scored.

Additional Keyphrases: "map" of proteins for normal individuals · prostatic cancer · prostatitis · benign prostatic hyperplasia · screening

Prostatic cancer has the second highest incidence of all cancer diseases and ranks third as a cause of death from malignancy in males in the United States (1, 2). Yet an early and reliable method for detection of prostatic cancer in asymptomatic men is still lacking after many years of intensive research.

Prostatic fluid is secreted by prostatic epithelial cells; therefore, the composition of the fluid should reflect metabolic activities of the secreting cells. Thus, one might expect that a disease-related change in the function of prostatic epithelial cells might be observed when the biochemistry of prostatic fluid is studied. Biochemical analyses of prostatic fluid reported previously suggest that the determination of the LDH-5/LDH-1 ratio (isoenzyme ratio of lactate dehydrogenase, EC 1.1.1.27), complement components C3 and C4, transferrin, and prostatic acid phosphatase (EC 3.1.3.2) may assist in identifying individuals with increased risk of carcinoma of the prostate (3, 4). Although these assays are indeed reasonably sensitive, they are still less than optimally specific in distinguishing various prostatic diseases.

Two-dimensional gel electrophoresis, with its well-documented resolving capabilities, provides a more comprehensive screening method. Two-dimensional mapping of seminal plasma has been reported previously by Edwards et al. (5). We have continued the study of the secretions of male accessory sex organs by describing our initial mapping of the proteins of human prostatic fluid, both serum derived and non-serum derived, with the iso-DALT system (6). In addition, we present a preliminary analysis of the prostatic fluid protein patterns of 30 patients: eight with prostatic cancer, six with benign prostatic hyperplasia (BPH), 10 with prostatitis and BPH, and six with asymptomatic chronic prostatitis.

Materials and Methods

Samples

Pooled “normal” prostatic fluid. This was a pooled specimen of prostatic fluid from about 80 men, 50 years old or younger, with no recognized prostatic lesions, who were seen in the Urology Clinic of Northwestern University Medical School for other disorders of the urogenital tract.

Patient groups. The diagnoses of carcinoma and BPH were confirmed by histological study of tissue removed at prostatectomy or at needle biopsy. The prostate was not histologically examined in patients with both prostatitis and BPH or with asymptomatic chronic prostatitis. Criteria for symptomatic prostatitis were results of culture of urine or prostatic fluid and the presence of 10 or more leukocytes per high-power microscopic field.

Methods

Prostatic fluid collection. Prostatic fluid, obtained by rectal massage 10–30 min after voiding, was collected on a glass slide. After transfer to a plastic vial, the fluid was frozen immediately in either liquid nitrogen or in a freezer at –20 °C.

Sample preparation. After the sample of prostatic fluid was thawed and centrifuged (6500 × g, 30 min, 4 °C) to remove particulates, 4 µL was solubilized with two parts of sodium dodecyl sulfate (SDS) mix (7), then heated in a water bath at 95 °C for 5 min before loading on the iso gel. In addition, aliquots of the prostatic fluid were used to determine the LDH-5/LDH-1 ratio, and the amounts of complement C3 and transferrin (4).

Anti-serum protein affinity-column fractionation. We conjugated 15 mg of rabbit antibodies (IgG fraction, anti-human serum protein; DAKO Immunoglobulins Ltd., c/o Accurate Chemical & Scientific Corp., Hicksville, NY 11801) to 1 g of CNBr-activated Sepharose 4B beads (Pharmacia Fine Chemicals, Piscataway, NJ 08854) according to the manufacturer’s recommended procedure. One milliliter of pooled prostatic fluid, diluted with 20 mL of a pH 8.3 solution containing 0.1 mol of NaHCO3 and 0.5 mol of NaCl per liter, was batch absorbed onto the antibody-conjugated matrix overnight at 4 °C on an end-over-end rotator. After the unabsorbed fraction was eluted, the column was washed with 150 mL of a pH 8.3 solution containing 10 mmol of NaHCO3, 0.15 mol of NaCl, and 2 g of Triton X-100 detergent per liter. The absorbed fraction was then eluted

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with 30 mL of 0.2 mol/L glycine HCl, pH 2.5. Both fractions were dialyzed extensively against de-ionized, distilled water at 4 °C, then lyophilized and stored at -20 °C.

Two-dimensional electrophoresis. Two-dimensional gel electrophoresis was performed according to the ISO-DALT manual (7).

Focusing in the first dimension ISO gel was done for 14 000 V · h in 30 g/L polyacrylamide containing, per liter, 1.8 g of N,N-methylenebisacrylamide as cross-linker, 8 mol of urea, 20 g of total ampholytes (8 g of Servalyt P 3–10, 8 g of Pharmalyte pH 3–10, and 4 g of LKB Ampholine pH 5–7 per liter), and 17 g of Nonidet P-40 detergent. Separation of proteins in the first dimension with the BASO system was performed according to Willard et al. (8), with 20 g of Servalyt pH 2–11 per liter and the ampholyte.

For the second-dimension separation, based on molecular mass, we used slab gels consisting of a linear polyacrylamide gradient (90–180 g/L, containing 8 g of crosslinker per liter) in a mixture of 10 g of SDS and 0.37 mol of Tris HCl per liter, pH 8.5. The pH 8.6 electrophoretic buffer contained 24 mmol of Tris, 0.2 mol of glycine, and 1 g of SDS per liter. Proteins in gels were detected by the silver stain method of Guevara et al. (9).

Data analysis and photography. We made permanent black-and-white transparencies and color slides for silverstained gels, using X-ray duplicating (XRD) image technique (10) and Ektachrome 64 professional film, respectively. Scoring of protein occurrence was done directly on the gels, manually, on a fluorescent illumination light box. Qualitative measures were the presence or absence of protein or alterations in charge-train microheterogeneity with respect to the map for the pooled "normal" prostatic fluid. Noticeable changes in quantity were scored semiquantitatively.

Results

Analytical variables such as ampholyte composition, duration of electrofocusing, volume of sample loaded, choice of denaturant, and intrinsic protease digestion were evaluated systematically during a series of preliminary optimization experiments (data not shown). We compared the two-dimensional patterns of pooled prostatic fluid as analyzed by both the ISO-DALT and the BASO-DALT systems. No additional proteins were present in the pattern of the BASO-DALT gel (pH 9–11); therefore we used the ISO-DALT system with wide-range ampholytes for all further prostatic fluid analyses. We also compared the urea mix and the SDS mix (7) as sample denaturants. Six to seven families of proteins were either drastically or moderately decreased when prostatic fluid was solubilized in the urea mix. Therefore, we routinely used the SDS mix with the prostatic fluid analyses. To determine whether proteolysis was an important concern during sample preparation, two prostatic fluid samples were stored in liquid nitrogen immediately after collection, then thawed and incubated at room temperature. We saw no differences in pattern for samples incubated 0, 1, 3, 5, 10, 30 or 60 min, 24 h, or 48 h.

We included serum reference preparation and pooled prostatic fluid in each of the ISO-DALT runs, as controls. Within-run and between-run reproducibility of the pattern was excellent, primarily because of our close attention to quality-control procedures.

Figure 1 shows the pattern for the pooled prostatic fluid. To differentiate the serum and non-serum components of the prostatic fluid proteins, we used an anti-human-serum protein affinity column as described above. Figures 2 and 3 show the patterns of the serum and the non-serum components of prostatic fluid, respectively. A schematic diagram of

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**Fig. 1.** Two-dimensional electrophoretic pattern of pooled "normal" prostatic fluid

**Fig. 2.** Two-dimensional electrophoretic pattern of the serum-derived components of pooled prostatic fluid

Thirty micrograms of the serum fraction solubilized in SDS mix was applied on the iso gel
cautiously when considering diagnosis-related alterations. Figures 5 and 6 show diagnosis-related alterations in sections of prostatic fluid patterns of three prostatic cancer patients and three asymptomatic chronic prostatitis patients. The proteins in these patterns are highlighted in boxes and numbered according to the reference map of Figure 4.

**Discussion**

Our investigation of intrinsic protease activity during sample preparation ensured that the handling procedures we routinely used were not responsible for the protease-related differences we observed. The stability of the samples contrasts with a prior study of seminal plasma (5) in which substantial proteolytic activity in ejaculatory fluid was observed under similar conditions.

Our pattern for prostatic fluid is similar to that of Carter and Remick (15), who used a single-concentration (140 g/L) polyacrylamide gel for the second dimension and detected proteins with Coomassie Blue stain. Our patterns revealed many more proteins, owing to the greater sensitivity of the silver stain method.
Protein no. 23, a prominent family of spots that stain heavily, can be absorbed on an anti-serum affinity column—but the corresponding spots are not visible on the two-dimensional pattern for serum. This family of proteins has a pI, distribution of charge trains, and color on staining similar to that of α1-antitrypsin. Thus we speculate that it may be a cleavage product of α1-antitrypsin, approximately 5000 Da different in mass. This hypothesis is supported by the fact that electrophoretically-recognizable α1-antitrypsin is absent from the 2D pattern of normal pooled prostatic fluid and the fraction thereof that is serum derived.

Table 1 shows that six proteins (nos. 1, 4, 24, 37, 38, 54) were significantly absent in the prostatic fluid from cancer patients. Of these six proteins, five were not of serum origin, and only no. 24 (prostatic acid phosphatase) is identified (5). The finding that prostatic acid phosphatase is significantly absent in fluid from prostatic cancer patients agrees with the report of Grayhack and Wendel (16), who showed by both histochemical and quantitative studies that the concentration of prostatic acid phosphatase is decreased in prostatic carcinoma cells. Because of the small sample size, we did not subdivide the prostatic cancer patients by clinical stage or histological grade.

For the groups of proteins evaluated here, no statistically significant alterations were evident in the samples from
patients with BPH, but protein no. 49 is absent from fluid of patients with both prostatitis and BPH. Protein no. 56, very possibly a granulocyte-specific "calcium-regulated protein" (Calgon:1) [12], is absent from patterns for patients with asymptomatic chronic prostatitis (Figure 6).

Many proteins unique to the prostatic fluids of individual patients yet not apparent in the pooled "normal" prostatic fluid were observed in our study. Examples may be seen in Figure 5d (to the right of no. 31 as a series of long oval-shaped spots) and in Figure 6d (series of "cairn-like" spots above no. 54). Because of the limited number of patients samples studied thus far, it is premature to discuss these individual differences.

Prostatic cancer antigen-1 (PCA-1) was found by Edwards et al. [17] in the urine of prostate cancer patients. This antigen had electrophoretic properties very similar to those of an antigen in the ISO-DALT system. We have tentatively identified protein no. 29 as antigen on the basis of its location on the map as compared with that of the serum standard. We have observed protein no. 29 in the prostatic fluid patterns of all 30 patients (Table 1). Protein no. 29 is also observed as a series of heavily stained spots in the serum-derived fraction of prostatic fluid (Figure 2) and rather weakly stained spots in the non-serum fraction (Figure 3). However, we did notice an increased intensity of the spot for protein no. 29 in the pattern for prostatic fluid of four of eight prostatic-cancer patients (for example, see Figure 5). Whether protein no. 29 is indeed antigen or the putative PCA-1, or both, remains to be answered by biochemical studies.

Table 1 should be considered preliminary, owing to the small sample size. We intend to enlarge our diagnosis-related data base, to follow up the present study. Nevertheless, this preliminary study has illustrated that two-dimensional electrophoresis can be useful to detect potential markers of disease.

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