

Proteins of Human Urine. II. Identification by Two-Dimensional Electrophoresis of a New Candidate Marker for Prostatic Cancer

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A protein series common to the urine and prostatic tissue of 16 of 17 patients with prostatic adenocarcinoma has been identified by high-resolution two-dimensional gel electrophoresis. These proteins, designated PCA-1, have a relative molecular mass in sodium dodecyl sulfate of about 40 000. Analyses of urines from eight age-matched controls, seven patients with other types of urogenital malignancies, two patients with benign prostatic hyperplasia, and five patients with malignancies not associated with the urogenital system failed to show PCA-1 in the patterns. These preliminary findings suggest that this protein should be systematically investigated as a candidate marker for prostatic adenocarcinoma in man.

Additional Keyphrases: *prostatic cancer antigen (PCA) · actin · electrophoresis, polyacrylamide gel · urinary proteins · ISO-DALT*

Successful treatment of cancer depends largely on early diagnosis, and in the case of prostatic adenocarcinoma, early detection has usually depended on discovery of palpable abnormalities of the gland during rectal examination. Hudson et al. (1), however, find palpation to be of limited value as a screening test. Thus, it is important to search systematically for biochemical "markers" that might be present in serum, urine, or prostatic fluid. Prostatic fluid is a complex apocrine or mesocrine secretion product of epithelial cells (2), and its composition may reflect pre-neoplastic or malignant changes.

Intensive effort has been applied to the assessment of several indicators that have a possible direct relationship to the early detection and disease management of prostatic malignancy. Increases in serum acid phosphatase (3-5), RNase (3), and the BB isoenzyme of creatine kinase (6); increased concentrations of C3, C4, and transferrin in prostatic fluid (7); and changes in the ratio of prostatic lactate dehydrogenase isoenzymes (8) have all been explored as markers of prostatic malignancy. The value of these assays for early detection or for the evaluation of therapy is limited by inconsistencies in the values obtained (9), by antigenic similarities between the protein markers and normal proteins (10), by false positives due to infection (8, 11) or other causes, and by lack of specificity for prostatic cancer (7, 12).

This paper reports our efforts to identify by electrophoretic analysis the presence of protein markers in urine that may reflect the existence of prostatic adenocarcinoma early in the disease progression. We have utilized two-dimensional electrophoresis to resolve the complex mixture of proteins in urine and prostatic tissue and to compare the patterns normally found with those of patients having adenocarcinoma of the prostate. The ISO-DALT two-dimensional electrophoresis

system (13, 14) permits multiple sample analysis by a technique first developed by Stegemann (15) and modified for higher resolution by O'Farrell (16), and it has been used to prepare two-dimensional maps of complex mixtures of proteins, including those concentrated from human urine (17). The resolution obtained by this technique in the separation of proteins far exceeds more traditional methods, and for this reason we elected to use the ISO-DALT system to search for protein indicators in the urine of prostatic cancer patients. A group of proteins, designated PCA-1 (Prostatic Cancer Antigen one), was observed in the urine of patients with prostatic cancer, but not in the urine of normal, age-matched donors or in patients with other diseases. The PCA-1 group of proteins also appears to be present in homogenates of prostatic tissue. This evidence suggests the discovery of a new protein indicator for prostatic cancer. A preliminary report of these findings has appeared.³

Materials and Methods

Preparation of urine and tissue. Fresh urine samples, collected at the M. D. Anderson Hospital and Tumor Institute, were cleared of cells and insoluble material by centrifugation at $200 \times g$ for 10 min. The urine was decanted into a graduated container and sodium azide added to a final concentration of 0.2 mg/L to inhibit bacterial growth. If the sample was not processed immediately, it was stored at -80°C .

Urinary proteins were prepared by a modification of the methods previously described (17). Up to 200 mL of urine was applied to a 6×40 cm column of Bio-Gel P-6 (Bio-Rad Laboratories, Richmond, CA 94804) that had been equilibrated with distilled water. Elution with distilled water freed the protein components of salt and other low-molecular-mass material. The portion of the eluate corresponding to the protein peak was collected, shell frozen, and lyophilized. The protein was resuspended in 5 mL of distilled water and applied to a second, smaller (2.5×25 cm) column of Bio-Gel P-6, equilibrated with water. Just before the sample, we applied a 5-mL volume of 1 mol/L NaCl to the column. The sample was allowed to pass through this zone of high salt concentration to remove adsorbed ultraviolet-absorbing materials. The column was eluted with distilled water and the portion of the eluate corresponding to the protein peak was collected, frozen, and lyophilized. The resulting powder was weighed and diluted with water to give a concentration of 50 g/L. Aliquots were frozen in Microfuge tubes, lyophilized, and stored at -80°C until analyzed. For two-dimensional electrophoresis, the dry powder was dissolved to give a final concentration of 100 g/L in a solubilizing buffer containing, per liter, 9 mol of urea, 50 mL of mercaptoethanol, and 20 mL of ampholytes in the pH range 3.5-10 (LKB, Bromma, Sweden).

Prostatic tissue was obtained by transurethral surgery at the M.D. Anderson Hospital and Tumor Institute, where the

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Fig. 1. Two-dimensional ISO-DALT electrophoretic patterns of urine from a 62-year-old normal man (A) and a 62-year-old man with stage C, grade 2 adenocarcinoma of the prostate (B)

A 750- μ g sample of urinary protein in 7.5 μ L of buffer was applied to each gel. The gel is oriented with the acidic side to the left. Abbreviations: ALB, albumin; TF, transferrin; PCA-1, Prostatic Cancer Antigen-one. Molecular-mass standards at the left of each panel are a one-dimensional separation of rat heart muscle as previously described (20) with actin (ACT) identified, for reference. Numbers are molecular mass $\times 10^{-3}$

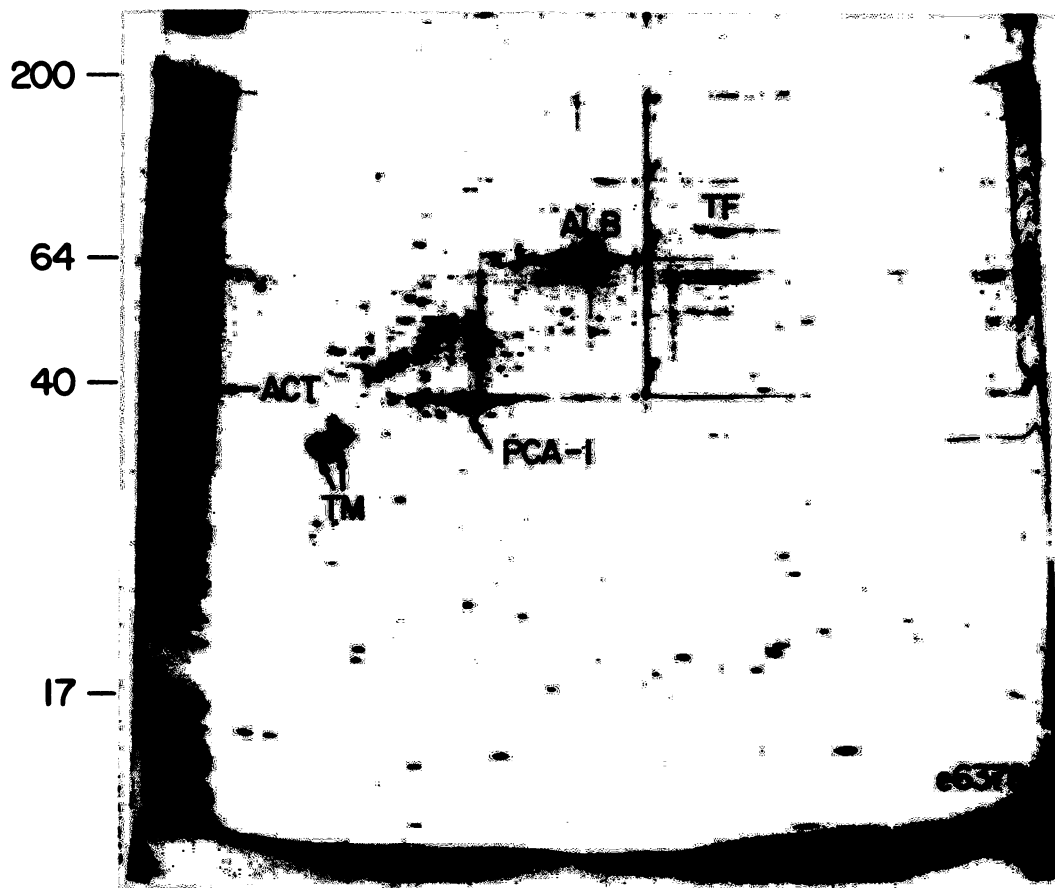


Fig. 2. Two-dimensional ISO-DALT electrophoretic pattern of proteins from prostatic tissue of a 67-year-old man with prostatic adenocarcinoma

Gel orientation and molecular-mass standards as in Figure 1. Abbreviations: ALB, albumin; TF, transferrin; TM, muscle tropomyosin; PCA-1, Prostatic Cancer Antigen-one

pathological conditions were diagnosed. The samples were then frozen in liquid nitrogen and shipped to Argonne National Laboratory for analysis. The tissues were prepared for electrophoresis by cutting 1-mm³ pieces of tissue and allowing

them to thaw in Microfuge tubes. One hundred microliters of the solubilizing buffer described above was added to the tube, and the tissue was homogenized with a small metal pestle. Insoluble material was removed by centrifuging for 3 min

(Microfuge B; Beckman Instruments, Inc., Palo Alto, CA 94304).

In some cases, fluid was expressed from the tissue immediately after surgical removal and frozen separately. The fluid was thawed and diluted with an equal volume of the solubilization buffer just before electrophoresis.

Two-dimensional electrophoresis. The procedures for two-dimensional electrophoresis were as previously described for the ISO-DALT system (13, 14).

The first-dimension isoelectric focusing was done for 10 000 V·h in 40 g/L polyacrylamide gels containing 2.4 g of *N,N'*-methylenebisacrylamide per liter as crosslinker together with, per liter, 9 mol of urea, 20 mL of ampholytes (pH range 3.5–10), and 20 mL of Nonidet P-40 (a surfactant from Particulate Data Labs., Elmhurst, IL 60126).

The separation in the second-dimension was done in slab gels consisting of a linear concentration gradient (100 to 200 g/L) of polyacrylamide made from a stock solution containing 300 g of acrylamide and 8 g of *N,N'*-methylenebisacrylamide per liter. We used a dodecyl sodium sulfate-Tris-glycine buffer system previously described (14). The gels were stained, destained, and photographed as described elsewhere (18).

Results

The two-dimensional electrophoretic pattern for urinary proteins from a normal individual is compared with that for a patient with adenocarcinoma of the prostate in Figure 1. The patterns of the samples, although complex, show a great deal of similarity. However, in Figure 1B the urine of the cancer patient contains a series of spots, labeled PCA-1, which are not seen in the pattern of normal urine (Figure 1A). With the denaturing conditions utilized for the separations, the spots in this series are slightly acidic and each protein represented by a spot has a relative molecular mass of about 40 000. These PCA-1 spots were seen in the urine maps of 16 of 17 patients between 57 and 76 years of age with adenocarcinoma of the prostate but were not present in detectable amounts in the urine maps of eight age-matched controls, two patients with testicular cancer, five patients with bladder cancer, two patients with benign prostatic hyperplasia, or in five patients with cancers not associated with the urogenital system. PCA-1 was also not seen in the urine of one prostatic-adenocarcinoma patient who had undergone prostatectomy. Analyses of seminal plasma from six normal individuals between the ages of 24 and 50 years also showed no evidence of the protein series. Although other differences were recognized in the protein patterns of urine, from prostatic-cancer patients and from normal controls, PCA-1 was the only protein that appeared to be of prostatic origin.

To determine whether the PCA-1 series of proteins was of prostatic origin, we analyzed sections from surgically obtained prostatic tissue. The protein pattern of malignant prostatic tissue (Figure 2) shows a series of spots at the same two-dimensional location in the tissue map as the PCA-1 spots in the map of urine of a prostatic-cancer patient. Although labeled as PCA-1, the location of these protein spots on the electrophoretic map is similar to that of muscle (including prostatic tissue) actin (20). Thus we wondered if these urinary proteins might also be actin. Results of preliminary analysis by double immunodiffusion of urine vs antisera to human skeletal muscle actin were inconclusive. Also, the proteins were not removed from urine when samples were passed over a DNase column according to the procedure of Lazarides and Lindberg (21). The relative molecular mass of the PCA-1 proteins in urine also appeared to be slightly less than that of muscle actin.

To determine whether the provisional marker PCA-1 proteins are characteristic only of malignant tissue, we analyzed prostatic tissue and urine from patients with benign prostatic

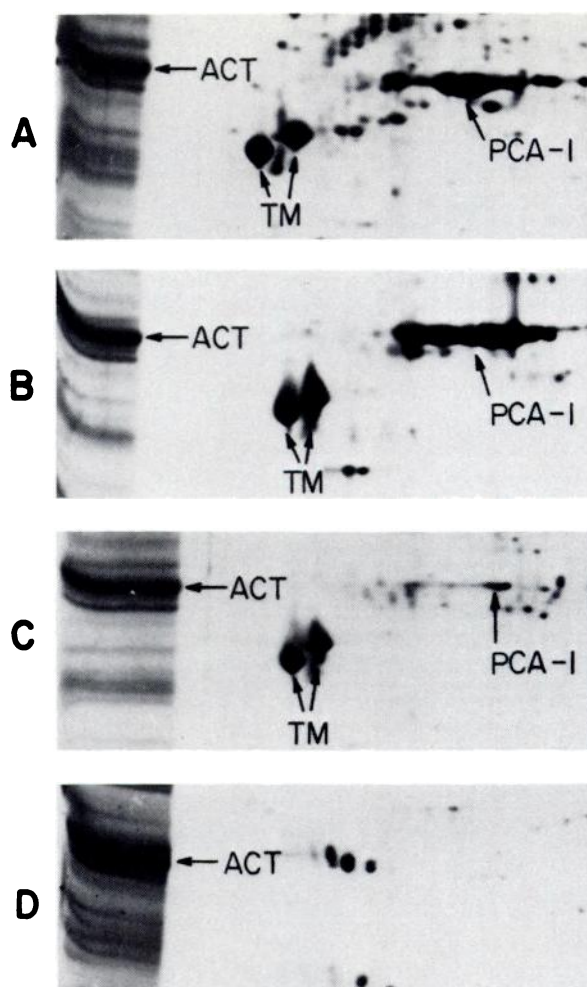


Fig. 3. Matching sections of two-dimensional ISO-DALT electrophoretic patterns

(A) Prostatic adenocarcinoma whole homogenate from a 67-year-old patient; (B) benign hyperplastic tissue whole homogenate from a 79-year-old man; (C) soluble material pressed from benign hyperplastic tissue; and (D) urine from a 73-year-old patient with benign prostatic hyperplasia. Gel orientations, abbreviations, and molecular-mass standards as in Fig. 2

hyperplasia. Figure 3, which shows parts of two-dimensional gels corresponding to the appropriate region, illustrates that the proteins in question are a component of both malignant and benign hyperplastic tissue (Figure 3A and 3B). However, they are virtually absent from a sample of soluble material expressed from the prostatic tissue with benign hyperplasia (Figure 3C) and were not detected in the urine of a patient with this disease (Figure 3D). These findings suggest that the protein may be a nonsecreted component of prostatic tissue that is released into the urine after transition to malignancy.⁴

Discussion

At present, the presence of carcinoma of the prostate cannot be detected before macroscopic growth becomes symptomatic. If malignant transformation of the prostate is reflected by changes in cellular biochemical mechanisms, then tumor markers conceivably can be identified that will make possible

⁴ *Note added in proof:* In reference to the possibility that PCA-1 may in fact be prostatic acid phosphatase, a long-recognized marker of prostatic cancer, we have located the position of prostatic acid phosphatase (EC 3.1.3.2) in the two-dimensional electrophoretic map by immunological procedures and by the migration of purified enzyme (2). The map positions of prostatic acid phosphatase (EC 3.1.3.2) and PCA-1 do not correlate, indicating that they are separate entities.

the detection of cancer before overt structural changes occur (22).

High-resolution two-dimensional electrophoresis on acrylamide gel has been applied to the analysis of urinary proteins of patients with adenocarcinoma of the prostate. An indicator of malignancy has been tentatively identified, demonstrating the usefulness of the technique as a tool for searching for cancer markers.

This paper presents evidence for the presence of a series of proteins, designated PCA-1, in the urine of patients with prostatic adenocarcinoma that is not detected in urine from controls. It is possible that the proteins designated PCA-1 are present in quantitatively lower amounts in the urine of normals or other disease states, including non-malignant diseases of the prostate, but can be observed only with more sensitive detection methods.

The present study is too small to allow definitive conclusions and more control subjects, especially those with clinically diagnosed benign prostatic hyperplasia, must be analyzed. The analysis of these samples must be interpreted cautiously, because the relationship between benign hyperplasia of the prostate and cancer of the prostate is not clearly defined. One study (23) reports a fourfold increase in cancer risk among patients with benign prostatic hyperplasia.

We plan to analyze more urine samples from both control and cancer patients, and to produce antisera against PCA-1 to determine if the urinary and tissue proteins are indeed the same. Such antisera will also allow the development of immunological tests that will enable the diagnostic specificity, sensitivity, and predictive value of this candidate marker to be assessed.

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References

1. Hudson, P. B., Finkle, A. I., Hopkins, J. H., et al., Prostatic cancer. XI. Early prostatic cancer diagnosed by arbitrary open perineal biopsy among 300 unselected patients. *Cancer* 7, 690-699 (1954).
2. Edwards, J. J., Tollaksen, S. L., and Anderson, N. G., Proteins of human semen. I. Two-dimensional mapping of human seminal plasma. *Clin. Chem.* 27, 1335-1340 (1981).
3. Chu, T. M., Wang, M. C., Kuciel, R., et al., Enzyme markers in human prostatic carcinoma. *Cancer Treat. Rep.* 61, 193-200 (1977).
4. Choe, B. K., Pontes, E. J., McDonald, I., and Rose, N. R., Immunohistochemical studies of prostatic acid phosphatase. *Cancer Treat. Rep.* 61, 201-204 (1977).
5. Choe, B. K., Pontes, E. J., Dong, M. K., and Rose, N. R., Double-antibody immunoenzyme assay for human prostatic acid phosphatase. *Clin. Chem.* 26, 1854-1859 (1980).
6. Feld, R. D., and Witte, D. L., Presence of creatine kinase BB-isoenzyme in patients with prostatic carcinoma. *Clin. Chem.* 23, 1930-1932 (1977).
7. Grayhack, J. T., Wendel, E. F., Oliver, L., and Lee, C., Analysis of specific proteins in prostatic fluid for detecting prostatic malignancy. *J. Urol.* 121, 295-299 (1979).
8. Hein, R. C., Grayhack, J. T., and Goldberg, E., Prostatic fluid lactic dehydrogenase isoenzyme patterns of prostatic cancer and hyperplasia. *J. Urol.* 113, 511-516 (1975).
9. Vihko, P., Kontturi, M., and Korhonen, L. K., Purification of human prostatic acid phosphatase by affinity chromatography and isoelectric focusing. Part 1. *Clin. Chem.* 24, 466-470 (1978).
10. Lam, W. K. W., Yam, L. T., Wilbur, H. J., et al., Comparison of acid phosphatase isoenzymes of human seminal fluid, prostate, and leukocytes. *Clin. Chem.* 25, 1285-1289 (1979).
11. Homburger, H. A., Miller, S. A., and Jacob, G. L., Radioimmunoassay of creatine kinase B-isoenzymes in serum of patients with azotemia, obstructive uropathy, or carcinoma of the prostate or bladder. *Clin. Chem.* 26, 1821-1824 (1980).
12. Chretien, P. B., Matthews, W., Jr., and Twomey, P. L., Serum ribonucleases in cancer: Relation to tumor histology. *Cancer* 31, 175-179 (1973).
13. Anderson, N. G., and Anderson, N. L., Analytical techniques for cell fractions. XXI. Two-dimensional analysis of serum and tissue proteins: Multiple isoelectric focusing. *Anal. Biochem.* 85, 331-340 (1978).
14. Anderson, N. L., and Anderson, N. G., Analytical techniques for cell fractions. XXII. Two-dimensional analysis of serum and tissue proteins: Multiple gradient-slab electrophoresis. *Anal. Biochem.* 85, 341-354 (1978).
15. Stegmann, H., Proteinfractionierungen in Polyacrylamid und die Anwendung auf die genetische Analyse bei Pflanzen. *Angew. Chem.* 82, 640 (1970).
16. O'Farrell, P. H., High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250, 4007-4021 (1975).
17. Anderson, N. G., Anderson, N. L., and Tollaksen, S. L., Proteins of human urine. I. Concentration and analysis by two-dimensional electrophoresis. *Clin. Chem.* 25, 1199-1210 (1979).
18. Edwards, J. J., Anderson, N. G., Nance, S. L., and Anderson, N. L., Red cell proteins. 1. Two-dimensional mapping of human erythrocyte lysate proteins. *Blood* 53, 1121-1132 (1979).
19. Giometti, C. S., Anderson, N. G., Tollaksen, S. L., et al., Analytical techniques for cell fractions. XXVII. Use of heart proteins as reference standards in two-dimensional electrophoresis. *Anal. Biochem.* 102, 47-58 (1980).
20. Giometti, C. S., Barany, M., Danon, M. J., and Anderson, N. G., Muscle protein analysis. II. Two-dimensional electrophoresis of normal and diseased human skeletal muscle. *Clin. Chem.* 26, 1152-1155 (1980).
21. Lazarides, E., and Lindberg, U., Actin is the naturally occurring inhibitor of deoxyribonuclease I. *Proc. Natl. Acad. Sci. USA* 71, 4742-4746 (1974).
22. von Eschenbach, A. C., Cancer of the prostate. In *Current Problems in Cancer*, 5, R. C. Hickey, Ed., Year Book Medical Publishers, Inc., Chicago, IL, 1981, pp 13-15.
23. Armenian, H. K., Lilienfeld, A. M., Diamond, E. L., and Bross, I. D. J., Relation between benign prostatic hyperplasia and cancer of the prostate. *Lancet* ii, 115-117 (1974).