Production of Milligram Concentrations of Free Prostate Specific Antigen (fPSA) From LNCaP Cell Culture: Difference Between fPSA From LNCaP Cell and Seminal Plasma

James T. Wu,* Bradley W. Lyons, Grace H. Liu, and Lily L. Wu

*Department of Pathology and Associated Regional University Pathologists (ARUP),
University of Utah Health Science Complex, Salt Lake City, Utah*

We have established a procedure for the production of milligrams of free PSA (fPSA) from LNCaP cells derived from a human carcinoma of the prostate. By growing LNCaP cells in a serum-free medium in the presence of a synthetic androgen (R1881) and taking advantage of the special design of the Micro-mouse Hollow Fiber Bioreactor, relatively pure fPSA could be obtained. We found that columns containing either Sephacryl S-100 or S-200 could be used to remove the small amount of bovine serum albumin (BSA) and PSA- α 1-antichymotrypsin complex (PSA-ACT) from the preparation. More than 90% of the PSA from LNCaP cell cultures are fPSA. Like fPSA from seminal plasma, two fractions of fPSA differing in protease activity can be separated by DEAE-Sephacryl

chromatography. Based on the band pattern exhibited on the Western blot following sodium dodecyl sulfate-polyacrylamide electrophoresis separation, fPSA from LNCaP contains more inactive PSA isoforms. This was confirmed by chromatofocusing: the isoelectric point (pI) of the major PSA isoforms from the LNCaP cell culture were higher (6.8 and 6.6) than that (6.4 and 6.1) of fPSA from seminal fluid. We conclude that the LNCaP cell culture is a reliable source for obtaining large quantities of pure fPSA both for the preparation of assay calibrators and controls and for studying the difference in fPSA between benign prostate disease and prostate cancer. *J. Clin. Lab. Anal.* 12:6–13, 1998.
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INTRODUCTION: PRODUCING RELATIVELY PURE fPSA

Prostate specific antigen (PSA) is a protease produced by epithelial cells and secreted into the prostatic ductal system (such as the seminal fluid). Only a very small fraction of the PSA enters the general circulation (1,2). It was found only recently that the majority of PSA detected in the blood circulation exists in the form of a complex, such as that formed from the combination of PSA with protease inhibitor α 1-antichymotrypsin (ACT) forming the PSA-ACT complex. A small portion of the serum total PSA remains as free molecule (fPSA) (3,4). Assay for free PSA and expressed fPSA as percent fPSA has been found to be valuable for the differentiation of BPH from prostate cancer during prostate cancer screening (5,6).

Human seminal fluid is the most common source for obtaining fPSA for the preparation of anti-fPSA antibody, calibrator, and control for the fPSA assay. Concentration of fPSA in the seminal fluid averages ~ 0.5 mg/mL. Because of the presence of other proteins in the seminal plasma, extensive

purification is required in order to obtain fPSA of reasonable purity. The yield is not always satisfactory and the method recommended for fPSA purification is still being debated. We found that fPSA also can be produced by LNCaP cell cultures. Since LNCaP was derived from human metastatic adenocarcinoma of the prostate, the fPSA produced could be more similar in properties to that of fPSA found in the serum of patients with prostate cancer (9–11).

Under normal growth conditions, only very low concentrations of PSA can be detected in the cell medium. However, increased secretion of PSA can be achieved from the cells by introducing androgen or synthetic androgen R1881 (methyltrienolone, 17 β -Hydroxy-17 α -methyl-estra-4,9,11-trien-3-one) into the growth medium (12–13). In the present study we made an effort to optimize the growth conditions in order to obtain large quantities of fPSA from the LNCaP

*Correspondence to: James T. Wu, Dept. of Pathology, University of Utah Health Science Complex, Salt Lake City, Utah

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medium. By growing LNCaP cells in the micro mouse hollow fiber bioreactor in the presence of R 1881, we found that we could produce milligrams of relatively pure fPSA.

MATERIALS AND METHODS

LNCaP cells clone FGR (cat. # CCL-1740) at passage 14 were purchased from American Type Culture Collection (ATCC, Rockville, MD). Anti-ACT polyclonal antibodies and horseradish peroxidase (HPR)-conjugated antirabbit antibody were from Dako (Carpinteria, CA). Hybritech Tandem E kits were purchased from Hybritech (San Diego, CA) and RPMI-1640 medium from Gibco (Grand Island, NY). Bovine serum albumin (BSA) was from Sigma (St. Louis, MO). Synthetic androgen R1881 (methyltrienolone, 17 β -Hydroxy-17 α -methyl-estra-4,9,11-trien-3-one) was obtained from New England Nuclear (Boston, MA). Sephacryl S-100, S-200 gels, DEAE Sepharose FF, PBE 94 gel, polybuffer 74, Mono P HR 5/5 prepac column were all obtained from Pharmacia (Alameda, CA). BCA protein assay reagent (bicinchoninic acid) was purchased from Pierce (Rockford, IL). AuroProbe BLplus GAR (goat antirabbit IgG) and IntenSE BL silver enhancement kit were obtained from Amersham (Arlington Heights, IL).

Cell Culture in 24-well Microplate

Our earlier studies of the effect of various compositions of growth medium on PSA production by LNCaP cells were performed in 24 multiwell dishes at a cell density of 2.5×10^4 cells/well in RPMI-1640 medium with 5% fetal calf serum (FCS) medium. After the cells were plated out in the multiwell microplate, the cells were allowed to attach for 2–3 days, followed by the addition of synthetic androgen R 1881. After 3 days incubation, the medium was refreshed and after an additional 3 days, the cells were harvested. The conditioned media of the last 3 days of culture were collected, centrifuged, and subjected to PSA determination.

Normally, LNCaP cells were maintained in RPMI 1640 supplemented with 1 μ M ferrous sulfate, 1 mM sodium pyruvate, and 0.1% bovine serum albumin (BSA). The cell lines were maintained in a monolayer culture in minimal essential medium supplemented with glutamine, 10% fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 μ g/mL). All cell cultures were incubated at 37°C in 5% CO₂ in air, and the medium was replaced every second day.

Micro Mouse Hollow Fiber Bioreactor

The apparatus was used to grow LNCaP cells for the production of large quantities of PSA under our optimized growth conditions. The apparatus was purchased from Unisyn Technologies (Hopkinton, MA). It has two liters intracapillary space and 12 mL extracapillary space. The molecular weight cutoff of the hollow fiber was 10,000 kDa. The intracapillary space was filled with a medium that consisted of RPMI-1640,

4.5 g/L glucose, 5% FetalClone III (a synthetic serum replacement for fetal bovine serum from HyClone Laboratories, Logan, UT), penicillin (5×10^6 units/L), and streptomycin (5 g/L). The extracapillary space was also filled with RPMI-1640 containing 4.5 g/L glucose, 0.5% BSA, 5×10^6 units/L penicillin and 5 g/L streptomycin.

Essentially, 1×10^8 LNCaP cells was seeded in the extracapillary space in a total of 12 mL medium. The entire bioreactor was placed inside an incubator at 5% CO₂ in air at 37°C. The bioreactor was attached to a peristaltic pump and the flow rate was set at 150 mL/min. The medium in the bioreactor was replaced with fresh medium every 7 days. After 4 weeks of culture, medium containing 2.5 nM of R1881 was used to stimulate PSA production. Then, ~ 10 mL liquid in the extracapillary space, containing secreted PSA, was withdrawn and was withdrawn again at every 7th day culture. The media withdrawn were used for PSA isolation. All new fresh media used for replacement contained 2.5 nM R1881.

The detail procedure of all chromatographies, including Sephacryl S-200 and S-100 gel filtration chromatography, DEAE column chromatography, chromatofocusing, polyacrylamide gel electrophoresis (PAGE), and Western blot performed in this study, were described previously (14). Specific conditions for these chromatographies are also described in the legends of individual figures.

Total PSA, PSA-ACT Specific, and fPSA Assays

Assays and assay characteristics for the total serum PSA, fPSA, and the assay specific for the PSA-ACT complex were also described in our earlier publications (15,16). All are microplate assays following the sandwich format and were established in house. Both assays used an anti-PSA coated microplate to trap the analytes. In the PSA assay, polyclonal anti-ACT antibodies were used to react with the captured PSA-ACT complex, whereas a different monoclonal anti-PSA antibody was used to react with the captured PSAs (free and complexed PSA) in the total PSA assay.

RESULTS

Effect of Composition of Growth Media

The LNCaP cells, growing normally in the presence of 10% calf serum, produced ~ 100–200 ng/mL PSA based on the Hybritech Tandem E assay. Since the yield was too low to meet our needs and extensive purification was required to obtain reasonable pure fPSA, we decided to study various growth conditions, not only in an attempt to improve the overall yield of PSA, but also to avoid any need for extensive purification.

First, we replaced the bovine fetal serum with a less expensive synthetic product of FetalClone III. We also tested whether the use of charcoal stripped fetal bovine serum (FBS) would increase the yield of PSA production. Then, we tried

the serum-free condition and the use of the human cord serum (see the results in Fig. 1). It is apparent that the use of synthetic androgen equivalent (R 1881) invariably increased the amount of PSA secreted by the LNCaP cells. The low yield found in the presence of human cord serum might be due to the presence of many inhibitors in the serum, since it was only a semipurified preparation. The use of charcoal stripped FBS did not result in any advantages compared to the use of FetalClone III. It was encouraging to find that even while growing LNCaP cells under the serum-free conditions, the amount of PSA secreted was not much less than that found in the presence of 10% FetalClone III. The only protein contained in the serum-free medium was bovine serum albumin (BSA). It should be noted that since the production of PSA for these experiments was monitored by a total PSA kit from Hybritech, both fPSA and PSA-ACT were detectable.

In Figure 2 we extended the incubation time from 10 to 20 days. It appears that there was no advantage of growing LNCaP cells longer than 15 or 20 days. We also found that the amount of FetalClone III had an effect on the PSA production; 5% FetalClone III was sufficient to maintain maximum production of PSA. Therefore, some gains were achieved by not using the 10% FetalClone III. Apparently the growth of LNCaP cells and the secretion of PSA by the cells were not easy to control precisely, at least in our attempts. We found that less PSA was produced the second time in the serum-free conditions than that shown in Figure 1 and also that less PSA

Fig. 1. Enhanced production of PSA by LNCaP cells by the addition of synthetic androgen, R1881. These studies were carried out in a 24-multiwell microplate at a cell density of 2.5×10^4 cells/cm² in the absence or presence of 2.5 nM R 1881. Invariably the addition of R 1881 in the growth medium resulted in increase of PSA production.

Fig. 2. Effect of the concentration of FetalClone III on the production of PSA by the LNCaP cells. The growth condition was identical to that described in Figure 1. LNCaP cells growing in the presence of 5% FetalClone III gave the highest yield of PSA.

was produced by cells grown in the presence of 10% FetalClone III.

It was found during our early investigations that the PSA-ACT complex was also detectable in the cell medium. However, we had no idea whether the PSA-ACT found in the media were entirely formed outside the cell after fPSA was secreted from the LNCaP cells or preformed inside the cells before secretion. In our earlier report (17), we proved that fPSA secreted from the cells formed PSA-ACT complexes with ACT contained in bovine calf serum. The complex was not detectable by the PSA-ACT assay for human equivalents but could be measured by the total PSA assay. However, the position of the PSA-active peak appeared in the elution profile of the gel filtration chromatography indicated that it corresponded to PSA-ACT. In the present study, however, there was a small amount of PSA-ACT complex measurable in the medium even under serum-free conditions and with no ACT present outside the cells. The concentration of PSA-ACT increased with the time of incubation (Fig. 3). Conceivably, a small amount of PSA-ACT can be formed inside the cells before secretion. Evidence of the presence of PSA-ACT complex in the medium also can be found in the PAGE-Western blot pattern: a band corresponding to the PSA-ACT complex can be discerned among the fPSA bands. The PSA-ACT complex is known to have a higher electrophoretic mobility than most fPSA isoforms.

The fact that it is possible for fPSA secreted into the medium to form the PSA-ACT complex with ACT in the me-

Fig. 3. Detection of low concentrations of PSA-ACT complex in the medium under serum-free condition. No ACT was present in the medium dur-

ing cell growth. Apparently PSA-ACT was synthesized inside the LNCaP cells and was then secreted into the medium.

dium provides us an opportunity to obtain both fPSA and PSA-ACT isoforms from the LNCaP cell culture. Both free and complexed PSA are in great demand for use in antibody preparation and as controls and calibrators. To find out whether we could make PSA-ACT in the medium while growing LNCaP cells, we added semipurified human ACT to the various media (see results in Fig. 4). As indicated from the PSA-ACT measurements, significant amounts of PSA complex were formed during the early periods of incubation. However, for reasons not yet clear, the growth of LNCaP cells and the production of PSA at the same time were suppressed. In the presence of added ACT, the production of PSA leveled off early at 4–5 days of incubation.

Production of PSA From LNCaP Cells Growing in the Bioreactor

For large-scale production of fPSA, we used the micromouse hollow fiber bioreactor to grow the LNCaP cells. The unique design of the hollow fiber allowed us to deposit FetalClone III through the holes of the hollow fiber into the intraspacer, which provided all the nutrients and growth factors required for cell growth without contaminating the PSA preparation. The extraspacer outside the hollow fiber where the LNCaP cells were seeded was maintained in a serum-free environment. The only foreign protein presented around the cells was the 0.5% BSA. As shown in Figure 5, microgram per milliliters of fPSA can be obtained from growing LNCaP cells in this appa-

Fig. 4. Formation of PSA-ACT outside the cell by the addition of ACT to the growth media; 1.4 mg/mL of semipurified ACT was included in the growth media in the presence of various concentrations of FetalClone III and in the presence of 2.5 nM of R 1881. ACT added outside the cells apparently formed PSA-ACT with the fPSA secreted by the LNCaP cells. As shown in Figure 3, only few ng/mL of ACT was detected in the medium. However, close to 100 ng/mL of PSA-ACT in the medium was detectable when ACT was added in the medium. However, the overall yield of PSA was suppressed in the presence of added ACT.

Fig. 5. Production of fPSA by the LNCaP cells growing in the micromouse hollow fiber bioreactor in the presence or absence of R 1881; 2.5 nM R 1881 was routinely added to the growth medium to enhance the production of fPSA. The harvest could be continued even after 28 days of culture. Each harvest involved withdrawing 10 mL of medium from the extraspaces of the bioreactor.

rat. At the end of 30 days of incubation, in the presence of R 1881, close to 80 $\mu\text{g/mL}$ of fPSA was obtained. Since 10 mL medium was withdrawn during every refreshment of the growth medium, > 1 mg of PSA can be accumulated in 30 days. We have not had a chance to test how much longer we could continue to produce PSA in this manner. Conceivably, it should be possible to obtain several milligrams of free PSA in one continuous growth of LNCaP cells in this apparatus. In fact, it is also possible to scale up the PSA production by using an apparatus with a 270 mL volume in the extraspaces. Therefore, when there is a greater demand for fPSA, we should have few problems in increasing the production to several hundred milligrams per month.

Purification

Since BSA is the only other protein besides PSA to be found in the extraspaces of the bioreactor, practically no purification is needed if the fPSA produced will be used only as calibrator or control for the fPSA assay. In other words, the whole medium containing the fPSA can simply be concentrated, dialyzed to remove all small molecules, and at the same time be

equilibrated to the buffer desired and used directly as calibrator or control. The concentrated fPSA also can be used to form the PSA-ACT complex by incubating it with ACT in vitro for use in the PSA-ACT assay.

If pure fPSA is desired, a simple gel filtration chromatography on either Sephacryl S-100 or S-200 column can be performed to remove the small amount of PSA-ACT complex and BSA (Fig. 6).

Characterization

In general, the fPSA obtained from the LNCaP cell culture does not appear to be different from the fPSA isolated from seminal plasma. The size of the fPSA from both sources was found to be the same; both are capable of binding to ACT to form the PSA-ACT complex, and both appear to exhibit a similar PAGE band pattern (14). However, when closely examined, minor differences can be detected. We have characterized the fPSA from LNCaP cells by the following techniques:

Fig. 6. Gel filtration chromatography of the cell medium withdrawn from the extraspaces of the bioreactor on columns containing either Sephacryl S-200 or S-100. 10 mL medium withdrawn from the extraspaces of the bioreactor, after it was concentrated by lyophilization, passed through either S-100 or S-200 column (2.5 $\times$ 55 cm) to remove BSA and a small amount of PSA-ACT complex. The buffer, 0.01 M phosphate containing 0.5 M NaCl, pH 7.2, was used for dialysis, equilibration, and elution.

DEAE sepharose chromatography

As with fPSA isolated from seminal plasma, both fractions of active and relatively inactive fPSA could be found in the fPSA produced by the LNCaP cells. Even though they are not completely separated by the DEAE anion exchange column (Fig. 7), they can be identified in the DEAE elution profile. More than a fourfold difference in enzyme activity can be measured between these two fractions. The fPSA from seminal plasma apparently contains more active fPSA (14). The majority of the fPSA eluted at high salt concentration corresponded to higher protease activity; a larger fPSA peak eluted at lower salt concentrations was found in the elution profile for the LNCaP medium corresponding to fPSA of lower enzymatic activity.

Western blot

Multiple bands, similar to that of seminal plasma, can be observed in the PAGE-Western blot experiment (Fig. 8). Upon closer examination, the band intensity of the first two fPSA bands of slower electrophoretic mobility is much heavier than the intensity revealed by the fPSA from seminal plasma. According to our earlier investigation (14), these bands correspond to inactive fPSA. Therefore, the result of the PAGE-Western blot agrees with the result by the DEAE sepharose chromatography, which shows that there is more inactive fPSA in the fPSA preparation secreted from LNCaP cells.

Chromatofocusing

To confirm our observations from the above two techniques, we also performed chromatofocusing for the LNCaP cell

Fig. 7. DEAE sepharose column chromatography. Cell medium (10 mL) withdrawn from the extraspaces of the bioreactor containing 0.5 mg/mL of BSA and ~ 3.5 mg of fPSA was dialyzed against 0.01 M phosphate buffer, pH 7.0 before applying to a column (2.6 × 65 cm) containing DEAE Sepharose FF. A linear gradient of 0–0.2 M NaCl in the equilibration buffer was used for elution at 4°C. Protease activity of the eluates were determined as described earlier (14).

Fig. 8. Pattern of fPSA from LNCaP cells revealed by PAGE-Western blot. Purified fPSA isolated from DEAE-sepharose chromatography (see Fig. 7) containing 0.8 mg fPSA/mL and 5 µL of seminal plasma containing 1 µg fPSA were subjected to electrophoresis by SDS-PAGE (8% gel) at 100 volts for ~ 90 min. Hoeffer transphor electrophoresis unit was used to transfer proteins from PAGE gel to the nitrocellulose sheet. The transfer buffer was 0.02 M Tris-buffer containing 0.0198 M glycine, 20% methanol, and 0.01% SDS. Transfer was performed at 80 volts for 30 min. After the transfer, the nitrocellulose sheet was blocked with 2% skim milk in PBS for 30 min at room temperature. The fPSA bands were visualized by polyclonal rabbit antihuman PSA antibody and Auroprobe GAR with silver enhancement. L refers to fPSA from LNCaP cell medium and SP to fPSA from seminal plasma.

medium and seminal plasma. After chromatofocusing the eluates collected from two separated runs, one for cell medium and the other for seminal plasma, were measured for their fPSA content. The pH measured at the peaks of the fPSA profile provided us the approximate pI for these fPSA molecules derived from two different sources. As shown in Figure 9, the pI of the major two fPSA peaks from LNCaP cell medium were found to be 6.8 and 6.6, which are higher than the pI determined for the fPSA peaks derived from the seminal plasma (pH 6.4 and 6.1). These results are in good agreement with the DEAE elution profiles and the PAGE-Western patterns. It is well known that molecules with high pI values carry less negative charges and thus are eluted at lower salt concentrations by anion exchange chromatography and are associated with slower electrophoretic mobility.

DISCUSSION

The cell culture condition that we finally adopted for growing LNCaP cells and PSA production was derived from our initial studies using the 24-well microplate. We learned to use the synthetic androgen, R 1881, for enhancing the PSA production. We also learned not to add ACT in the medium and to avoid the use of charcoal stripped FBS. As far as PSA production is concerned, the use of charcoal stripped FetalClone did not show any increase in PSA production. The most important decision we made was to take advantage of the micromouse hollow fiber bioreactor to grow LNCaP cells. The design of the bioreactor allows the LNCaP cells to be

Fig. 9. Combined chromatofocusing elution profiles of free PSA from LNCaP cells and from seminal plasma. Free PSA from LNCaP cells and human seminal plasma were subjected to chromatofocusing in two separate runs under identical conditions. Mono P HR 5/5 prepacked column from Pharmacia was used. The starting buffer was 0.025 M imidazole-HCL, pH 4. Pharmacia Polybuffer 74 was used as elution buffer after it was diluted with water and adjusted to pH 4 at room temperature. The run was performed on FPLC also at room temperature. Both seminal plasma and LNCaP cell medium were diluted with starting buffer to ~1–2 μ g fPSA per mL and were injected directly into the column (200 μ L) without purification. The flow rate was maintained at 0.25 mL/min. Aliquot of 10 μ L eluate was taken for fPSA determination.

maintained in a serum-free environment, but without the shortage of supply of all necessary growth factors and nutrients from FetalClone III. The presence of 0.5% BSA in the extraspase not only avoids the severe problems of protein contamination, but also stabilizes the fPSA secreted from the LNCaP cells. It is known that fPSA is not stable in a protein-free matrix (18).

The fact that we can obtain relatively pure fPSA from the LNCaP cell medium without going through extensive purification is a major advantage. We know that seminal plasma contains high concentrations of fPSA; however, multiple chromatographic steps are required in order to obtain reasonable pure fPSA from that source. The instability of fPSA at diluted concentrations, along with the problem of recovery associated with every purification step, makes it difficult to obtain pure fPSA from seminal plasma. Because of the large difference in molecular size between fPSA and BSA, it is relatively easy to remove both BSA and the small amount of PSA-ACT from fPSA by gel filtration chromatography with good recovery.

The assay for fPSA is now considered valuable for the differentiation of BPH from prostate cancer. During screening of prostate cancer, many unnecessary biopsies performed on patients with BPH could be avoided if we determined the % fPSA in addition to the total PSA test. Our recent study also

suggested that %fPSA may help detect occult prostate tumor for prostate cancer patients with normal serum total PSA. It also appears from our study that % fPSA determination seems useful for predicting prognosis. As pointed out by Catalona et al. (5), patients with low % fPSA were usually associated with prostate cancer, whereas higher % fPSA indicated good prognosis and was frequently free of cancer. Therefore, there is no doubt that measuring fPSA is probably not only equally important, but more important clinically than the measurement of total PSA. However, there is one problem: how to differentiate the fPSA of prostate tumors from that of benign prostate diseases. Naturally, it is the fPSA from benign diseases that is associated with a good prognosis, not the fPSA from prostate cancer. If we can measure specifically the fPSA derived from benign prostate diseases, a more accurate prediction should be possible.

It is very likely that fPSA isolated from seminal plasma is related to the fPSA appearing in the serum of benign prostate diseases, whereas the fPSA obtained from the LNCaP cell medium is similar to fPSA found in the serum of cancer patients. It is not clear to us why fPSA isolated from two distinct sources, so different in protease activity, both appeared to bind to ACT to the same extent (14). The knowledge of associations between %fPSA and prostate cancer and between %fPSA and benign prostate diseases suggests that less binding should occur between fPSA from seminal plasma and ACT. Less binding to ACT is also expected between relatively inactive fPSA and ACT. Our failure to find a difference between fPSA from the two different sources and between enzymatically more active and less active fPSA may be due to the qualitative methods we employed (Western blot) (14).

The present study did reveal for certain that the fPSA from seminal plasma is different from the fPSA from LNCaP cells. The higher content of relatively inactive fPSA and the association of fPSA from the cancer cell line with overall less protease activity was expected for fPSA from prostate tumor. It is also expected for ACT, a protease inhibitor, to bind to inactive fPSA poorly. But it is unclear why less fPSA is found in sera from cancer patients and much higher % fPSA are associated with sera from benign prostate diseases. Apparently, much more work will be needed in the future before we can develop an ideal assay for fPSA. We hope that the availability of large quantities of fPSA from prostate cancer cell lines and the fPSA from seminal plasma will allow us to carry out further studies, which will allow us eventually to answer fully the questions that for now we can only thoughtfully pose.

REFERENCES

1. Oesterling JE: Prostate-specific antigen. Improving its ability to diagnose early prostate cancer. *JAMA* (Editorial) 267:2236–2238, 1992.
2. Wang MC, Papsidero LD, Kuriyama M, et al.: Prostate antigen: A new potential marker for prostatic cancer. *Prostate* 2:89–96, 1981.
3. Wu JT: Assay for prostate specific antigen (PSA): Current problems and possible solutions. *J Clin Lab Anal* 8:51–62, 1994.

4. Wu JT, Zhang P, Wang T, et al.: Evaluation of free PSA isoforms, PSA complex formation and specificity of anti-PSA antibodies by HPLC and PAGE-immunoblotting techniques. *J Clin Lab Anal* 9:1–14, 1995.
5. Catalona W, Smith D, Ratliff T, et al.: Measurement of prostate-specific antigen in serum as a screening test for prostate cancer. *N Engl J Med* 324:1156–1161, 1991.
6. Catalona WJ, Smith DS, Wolfert RL, et al.: Evaluation of percentage of free serum prostate-specific antigen to improve specificity of prostate cancer screening. *JAMA* 274:1214–1220, 1995.
7. Wu JT, Zhang P, Bandhauer ME, et al.: Purification of PSA-ACT complex: characterization of PSA-ACT complex by various chromatographic procedures. *J Clin Lab Anal* 9:25–31, 1995.
8. Wu JT, Wilson L, Zhang P, et al.: Correlation of serum concentrations of PSA-ACT complex with total PSA in random and serial specimens from patients with BPH and prostate cancer. 9:15–24, 1995.
9. Gleave ME, Hsieh J-T, Wu H-C, et al.: Serum prostate specific antigen levels in mice bearing human prostate LNCaP tumors are determined by tumor volume and endocrine and growth factors. *Cancer Res* 52:1598–1605, 1992.
10. Gleave ME, Hsieh J-T, von Eschenbach AC, Chung LWK: Prostate and bone fibroblasts induce human prostate cancer growth in vivo: Implications for bi-directional tumor-stromal cell interaction in prostate carcinoma growth and metastasis. *J Urol* 147:1151–1159, 1992.
11. Langelier EG, van Uffelen CJC, Blankenstein MA, et al.: Effect of culture conditions on androgen sensitivity of the human prostatic cancer cell line LNCaP. *Prostate* 23:213–223, 1993.
12. Hsieh J-T, Wu H-C, Gleave ME, et al.: Autocrine retulation of prostate-specific antigen gene expression in a human prostatic cancer (LNCaP) subline. *Cancer Res* 53:2852–2857, 1993.
13. Esquenet M, Swinnen JV, Heyns, Verhoeven G: Control of LNCaP proliferation and differentiation: Actions and interactions of androgens, $1\alpha,25$ -dihydroxycholecalciferol, all-trans retinoic acid, 9-cis retinoic acid, and phenylacetate. *Prostate* 28:182–194, 1996.
14. Wu JT, Zhang P, Wang T, et al.: Evaluation of free PSA isoforms, PSA complex formation and specificity of anti-PSA antibodies by HPLC and PAGE-immunoblotting techniques. *J Clin Lab Anal* 9:1–14, 1995.
15. Wu JT, Wilson LW: Development of a microplate ELISA for free PSA and PSA-ACT complex in serum. *J Clin Lab Anal* 9:252–260, 1995.
16. Wu JT, Stephenson RA: Microplate assay for free PSA, PSA-ACT and total PSA: Assay characteristics, selection of calibrators and in vitro stability studies. Proceeding for the 1st International Consultation on Prostate Cancer, 1997, p 159–168.
17. Wu JT, Zhang P, Astill ME, et al.: PSA immunoreactivity detected in LNCaP cell medium, breast tumor cytosol and female serum. *J Clin Lab Anal* 9:243–251, 1995.
18. Wu JT, Liu GH, Zhang P: Effect of denaturation and curve fitting on the accuracy of microplate fPSA assay (submitted).
19. Wu JT, Liu GH, Zhang P, Stephenson RA: Determination of percent free prostate-specific antigen may identify occult prostate tumor and provide early detection. (submitted).