Separation and Characterization of Free-PSA and Its Complexes in Benign and Malignant Uterus Tumors

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Abstract

Gel filtration chromatography was used to separate free $^{125}$I-anti total PSA antibody from the complexes of $^{125}$I -anti total PSA antibody with the PSA immunoreactive isofurms [Free-PSA (F-PSA) and the complex of PSA with α1-antichymotrypsin (PSA-ACIT)] in benign and malignant uterus tumors tissues in order to estimate the ratio of (PSA: t-PSA) or F-PSA%. The data obtained revealed that these ratios are different in benign patients from those of malignant patients.

Spectrophotometric studies using UV range (200-350 nm) was performed on PSA, anti total PSA antibody, and their complexes in both cases. The results revealed the presence of more than one type of complexes from the same tissue homogenate in both cases.

Polyacrylamid electrophoresis was also performed to characterize the differences and the similarity between the PSA complexes in tissue homogenates of benign and malignant uterus tumors.

Introduction

Prostate specific antigen (PSA) is a 33-34 KD single chain glycoprotein, 3.1S sedimentation coefficient and isoelectric point at pH 6.8-7.5. At least five PSA isoforms, based on differences in isoelectric
point have been described\(^3\), two biologically active forms are differing in the degree of glycosylation ranging from non-glycosylated to fully glycosylated\(^4\), and three biologically inactive or "nicked" forms\(^5\).

PSA exists in serum in three main forms - a protein bound, complexed to α2-macroglobulin and α1-antichymotrypsin (PSA-ACT) and free unbound form (FP-PSA). The two later forms are immunologically detectable\(^6\).

Among the chromatographic techniques employed for protein purification, gel filtration is unique in that fractionation is based on the relative size of protein molecules\(^7\). Also electrophoresis of protein in polyacrylamide gel has provided to be one of the most useful analytical and preparative techniques. Hence proteins are separated on the basis of both size and charge. They have the advantages of high resolution and sensitivity\(^8\). There were many studies using polyacrylamide gel electrophoresis (PAGE) on PSA\(^9\) and (PSA-ACT) complex in serum\(^10\), prostatic fluid\(^11\), seminal fluid\(^12\), of prostatic diseases\(^13\), and in breast diseases\(^14\).

Generally, several methods have been developed to determine the structures of protein molecules in solution, such as: hydrodynamic, thermodynamic, optical and electrochemical methods, optical rotation, infrared absorption spectra and ultra violet spectra\(^15\). UV. Spectral method remain one of the most important spectral methods applied in immunology because it provide a sensitive, quantitative methodology for the study of antibody structure and specific ligand binding\(^16\).

The first part of this study was directed toward separation of unbound PSA from its complexes form in both benign and malignant uterus tumors using gel filtration technique. Electrophoresis technique were applied in order to detect any changes in the bands of PSA after the binding with its antibody in benign and malignant uterus tumors in the comparable with crude tissues homogenates. While the second part of this study deals with the structural studies of PSA, \(^17\)-antitotal PSA antibody, and their complexes using UV spectroscopic technique.

Experimental

Chemicals

All laboratory chemicals and reagents were of analar grade: Tris (hydroxy methyl) amino methane, Na-K-tartarate, EDTA, MgCl\(_2\), acrylamide, and N,N-methylene bis acrylamide were obtained from Fluka-Switzerland company, Dithiothreitol, polyethylene glycol, NaCl, ammonium persulfate, and N,N,N-tetramethylethylenediamine from BDH-UK company, bovine serum albumin (BSA) from Sigma-USA company, Urea from May & Baker company, Bromophenol blue from Hopkins & William company, Coomassie Brilliant blue from Merck company, and total PSA kit purchased from ImmunoTech-Beckman Coulter Company-Czech Republic.

Apparatus

The apparatus used during this study were: Analytic balance, LKB gamma counter type 1270-rack gamma II, Pye unican pH meter, Cooling centrifuge type Hettich, memrrent incubator, memrrent water bath, orbital shaker, and spectrophotometer ultra.

Patients

Two groups of patients were included in this study. The first group involved 6 postmenopausal patients suffering from benign uterus tumors (age 55.7 years ± 1.7) while the second group included 8 postmenopausal patients suffering from malignant uterus tumors (age 56.4 years ± 2.3). The patients were clinically diagnosed by physicians and histologically proven by laboratory reports and were not taken any type of therapy.

Collection of Specimens and Preparation of Tissue Homogenate

The tumor tissues were surgically removed from uterus tumor patients by hysterectomy. The specimens were cut off and stored immediately at -20°C. When needed, the frozen tissue was pulverized on ice bath then homogenized at 4°C in TED buffer with a ratio of 1:3 (w:v) using a manual homogenizer. (TED buffer contains 0.01M tris(hydroxy methyl) amino methane, 0.15mM EDTA, and 1.2mM dithiothreitol). The homogenate was filtered through a nylon mesh sieves in order to eliminate filters of connective tissues then centrifuged at 1600 xg for 20 min at 4°C. The supernatant was used as a source of PSA in this study.
Methods

A: Separation of PSA Immunoreactive Isomers (F-PSA) and (PSA-ACT) Complexes of Benign and Malignant Uterus Tumors Homogenates Using Gel Filtration Chromatography:

1- A volume of 100μl (250μg protein) of 125I-anti total PSA antibody was incubated with 200μl (300μg protein) of benign uterus tissue homogenate for 1.5 hr at 15°C in a final volume of 1000μl with TED buffer pH 7.6. Two additional tubes, containing 100μl labeled antibody only for total activity computation were set aside until counting.

2- At the end of the incubation, the mixture was applied to the surface of a sephadex G-200 column (1x27cm) equilibrated with TED buffer pH 7.6. Elution was carried out to separate F-PSA and PSA-ACT bound to 125I-anti total PSA antibody using the same above buffer with a flow rate of 4ml/hr and fraction volume of 1ml.

3- The Absorbance at 280nm was measured and the radioactivity of each fraction was counted in a gamma counter for 1min.

4- The experiment was repeated using 200μl (500μg protein) of 125I-anti total PSA antibody incubated with 100μl (400μg protein) of malignant uterus tissue homogenate for 2.5 hrs at 15°C using TED buffer pH 7.2.

- Void Volume Determination

The elution volume of Blue Dextran 2000 is equal to the column void volume (V_v), and was determined as follows:

A fresh solution of Blue Dextran (2mg/ml) was prepared in the eluent buffer in a sample volume of 1-2% of the total bed volume, then applied to the column with a flow rate of 4 ml/hr, fractions of 1 ml were collected and their absorbance were measured at λ=600 nm.

Calculation

1- The radioactivity (cpm) of each eluted fraction was plotted against fraction number.

2- All experiments gave three peaks profile. The first and second peaks represent the 125I-Ab bound to (PSA-ACT) and 125I-Ab bound to F-PSA, respectively while the third peak represents the unbound 125I-Ab.

3- The percent radioactivity of each peak was calculated by dividing the sum of the radioactivity of the fractions under each peak by the sum of radioactivity of all three peaks appeared in the profile:

\[
\text{Radioactivity per peak (cpm)} = \frac{\text{Sum of radioactivity under all peaks (cpm)}}{\text{Sum of radioactivity under each peak (cpm)}}
\]

4- The percentage of free-PSA (f-PSA%) in each sample was determined as follows.

\[
f-\text{PSA\%} = \frac{\text{The radioactivity under (peak2)}}{\text{The sum of radioactivity under (peak1+peak2+peak3)}} \times 100
\]

5- The percentage of bound PSA (bound PSA%) and (PSA-ACT) in each sample was determined as follows.

\[
\text{Bound - PSA\%} = \frac{\text{The radioactivity under (peak1+peak2)}}{\text{The sum of radioactivity under (peak1+peak2+peak3)}} \times 100
\]

\[
\text{PSA - ACT\%} = \frac{\text{The radioactivity under (peak3)}}{\text{The sum of radioactivity under (peak1+peak2+peak3)}} \times 100
\]

6- The percentage unbound PSA (unbound PSA%) in each sample was determined as follow.

\[
\text{Un bound- PSA =} \frac{\text{The radioactivity under (peak1)}}{\text{The sum of radioactivity under (peak1+peak2+peak3)}} \times 100
\]

B: Spectral Studies of PSA, (125I-Anti Total PSA Antibody), and Their Complexes

B-1: The UV Spectra of PSA and Its Antibody:

1- Twenty μl of PSA (100 ng/ml) were diluted to 500μl with TED buffer pH 7.4.

2- The solution was placed in a 0.5 cuvette in sample beam and the absorption spectrum was immediately measured against the same buffer in reference beam in the range (200-350 nm).

3- The experiment was repeated by using 20 μl (100 μg) of 125I-anti total PSA antibody.

B-2: The UV Spectra of the Complex of 125I-Anti Total PSA Antibody Bound to (PSA-ACT) and (F-PSA)

The gel filtration experiment for benign and malignant uterus tumors gave three peaks profile. The fractions under each peak were pooled and the absorption spectrum was measured in the area (200-350 nm) using a 0.5 cm cuvette against TED buffer pH 7.4 in the reference beam.
Results & Discussions

Figure (1): The elution profile of the $^{125}$I-anti total PSA antibody binding with PSA immunoreactive isoforms in:

A: Benign uterine tissue homogenate
B: Malignant uterine tissue homogenate from sephadex G-200 Column (1×27 cm). The void volume= 7 ml and 1 ml fraction were collected.

The trials of this experiments revealed three peaks profile. The first peak represented the antibody to (PSA-ACT) complex, the second peak represents the antibody bound to F-PSA while the third peak represents the labeled Antibody.

The results show that the PSA complexed to ACT is the predominate fraction of PSA in both benign and malignant uterine tumors homogenates while the minor fraction is PSA not associated with ACT (F-PSA). Moreover, the high molecular weight of the first peak which was expected from its low retention volume may attributed to the PSA- $^{125}$I-anti total PSA antibody polymerization and aggregation reaction which may indicate the bivalency or multivalency of PSA.$^{15,6}$

Table (1) represents the ratio of (bound PSA/total PSA) or (bound PSA%), (PSA-ACT%), (F-PSA%) and (unbound PSA%) in benign and malignant uterine tumors homogenates.

<table>
<thead>
<tr>
<th>Case</th>
<th>Bound %</th>
<th>PSA-ACT%</th>
<th>F-PSA%</th>
<th>Unbound %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign</td>
<td>77.5</td>
<td>54.79</td>
<td>22.72</td>
<td>22.49</td>
</tr>
<tr>
<td>Uterus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malignant</td>
<td>86.49</td>
<td>74.79</td>
<td>11.7</td>
<td>13.51</td>
</tr>
<tr>
<td>Uterus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The data in this table show that: the bound%, PSA-ACT%, and F-PSA% in benign groups are less than those in malignant groups, and the F-PSA% are less than the PSA-ACT% in both benign and malignant uterine tumors.
homogenates (i.e. in both cases the PSA showed high complexation to ACT).

This results are in agreement with another study on prostatic tissues homogenate, which indicated that the F-PSA% was 26.73% for benign prostate tumor patients and 10.95% for prostate cancer patients\[^{20}\] while the results are not consistent with other study which indicated that the only immunologically active type of PSA in cancerous prostatic tissue is free PSA\[^{21}\].

Many previous studies on prostate tumors emphasized the clinical significance of PSA immureactive isoforms (F-PSA) and (PSA-ACT) in detection of prostatic cancer \[^{22}\]. It have been suggested that F-PSA% allows a better discrimination between benign and malignant tumors compared with t-PSA\[^{23,24}\]. The results of this experiment indicated that the ratio of free to total PSA (and also the ratio of F-PSA to the PSA-ACT complex) may be used for differentiation between benign and malignant uterus tumors homogenates as well as the prostate tumors.

B: Spectral Studies of PSA, \(^{125}\)I- anti total PSA antibody, and Their Complexes

B-1: The U. V Spectra of PSA and Its Antibody:

The U. V spectra of PSA and its antibody were measured to determine their maximum wavelengths, and the alteration in their U. V spectra as a result of the interaction with each other.

Figure (2 A&B) illustrates the U. V spectra of PSA and its antibody \(^{125}\)I-anti total PSA antibody.

![Image](A) A: the U. V spectra of:

1. PSA
2. \(^{125}\)I- anti total PSA antibody

![Image](B) B: The U. V spectrum of the Complex of \(^{125}\)I- anti total PSA antibody Bound to (PSA-ACT) and (F-PSA):

As a result, PSA and its antibody has a characteristic spectrum and can be identified by their peaks.

Table (2) shows the \(\lambda_{max}\) values of \(^{125}\)I- anti total PSA antibody bound to (PSA-ACT) and F-PSA complexes in benign and malignant uterus tumors homogenates which were purified and obtained by gel filtration chromatography as described previously.
Table (2): The $\lambda_{\text{max}}$ values of the UV spectra of the (AbAg) complexes.

<table>
<thead>
<tr>
<th>Case</th>
<th>Anti total PSA antibody bound to (PSA-ACT) $(\lambda_{\text{max}} \text{ nm})$</th>
<th>Anti total PSA antibody bound to (F-PSA) $(\lambda_{\text{max}} \text{ nm})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign Uterus Tumors</td>
<td>257.0, 213.5</td>
<td>261.2, 218.5</td>
</tr>
<tr>
<td>Malignant Uterus Tumors</td>
<td>258.0, 212.5</td>
<td>262.5, 216.0</td>
</tr>
</tbody>
</table>

From this table we can conclude that:
- Each of PSA isoforms has characteristic spectrum that can be identified by the specific absorption coefficient (a) of its $\lambda_{\text{max}}$.
- The $\lambda_{\text{max}}$ values of the spectrum of all complexes in both benign and malignant uterus tumors homogenates are different from those of the PSA and its antibody alone.
- The $\lambda_{\text{max}}$ values of both benign and malignant uterus tumors homogenates are approximately the same. That may be due to the structure similarity between the complexes.

C: Conventional Polyacrylamide Gel Electrophoresis:

Electrophoresis on polyacrylamide gel was carried out on benign and malignant uterus tumors homogenates, the complexes of $^{125}$I - anti total PSA antibody with (PSA-ACT) which resulted from the gel filtration experiment of benign and malignant uterus tumors, and standard PSA, as described previously and shown in figure (3).

It is obvious from this figure that:
- The standard PSA have five bands, that because the PSA is not a simple molecule, existing in the serum in five isoforms (27).
- There are several protein bands in crude homogenates in comparable with that of the standard PSA, that because the tissue homogenates contained many proteins in addition of PSA and there are bands in the region of the PSA of both benign and malignant uterus tumors.
- The malignant homogenates contain proteins more than that of benign homogenates.
- The complexes migrate faster than that of the standard PSA in spite of that the complexes have larger molecular weight, that may be due to the charge of the complexes at the used pH.
- There are a similarity in the bands of complexes of both benign and malignant tumors in spite of that the concentration of proteins in benign tumors are less than that of malignant tumors, that can be dealt with that the molecular weight of the antigen in both cases are the same.

Conclusions
- PSA appears to be present in two immunoreactive isoforms (F-PSA and PSA-ACT) in both benign and malignant uterus tumors homogenates.
- The ratios of PSA isoforms in benign patients differ from those of malignant patients, this indicates that these ratios in benign and malignant uterus tumors tissues may be used as a biochemical markers for uterus tumors prognosis.
- The spectroscopic study revealed that each of PSA, anti-PSA antibody and their complex has a characteristic spectrum.
Electrophoresis on polyacrylamide gel show that there are bands in the region of the PSA of both benign and malignant uterus tumors.

Acknowledgment

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References


