Isolation & Partial Purification of Testosterone Receptors in Benign & Malignant Prostatic Tumors

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Abstract:

Background: A gel filtration technique has been used for the isolation and purification of soluble testosterone receptors from benign and malignant prostatic tumors**.** Two types of testosterone receptors from benign and malignant prostatic tumors were eluted from the sephadex G-200 column. This work was carried out to characterize and quantify human nuclear androgen receptors from benign and malignant prostatic tumors.

Methods: The study involved twenty five patients with benign prostatic hyperplasia (BPH) and thirteen patients with prostatic adenocarcinoma (PCA) attending Al-Kadhimiya teaching hospital from the period of November 2005 till july 2006.

Results: The purification folds of two benign separated receptors (BI & BII) were 11.588 and 19.582 fold respectively whereas for the two malignant separated receptors (MI & MII) were 24.280 and 29.111 fold respectively.The choice of most appropriate conditions of the binding of ^{125}I-testosterone with its receptors were also carried out.The concentrations of binding sites and the equilibrium dissociation constants for the binding between ^{125}I-testosterone and its purified receptors have been determined using Scatchrad analysis and the specificity of the binding has been examined. The concentrations of two benign separated receptors (BI & BII) were 0.931 and 1.140 pmole/mg protein respectively whereas separated malignant receptors (MI & MII) have 1.056 and 2.163 pmole/mg protein respectively at 37ºC.

Conclusions: Gel filtration technique and Scatchard analysis confirmed the presence of two types of testosterone receptors in each tumor type. The first eluted receptor (I) has a relatively higher molecular weight with a lower affinity constant for testosterone binding than the other(II).

Key Words: Benign prostatic hyperplasia, Prostatic adenocarcinoma, Testosterone receptor, Scatchard ananlysis.

Introduction:

Androgens play a crucial role in several stages of male development and act on their target cells via an interaction with the androgen receptor resulting in direct regulation of gene expression [1,2]. One of the target organs of androgen is the prostate. Development and maintenance of differentiated function of the normal prostate gland require androgen[3,4].

Androgen has also been implicated in the abnormal growth, since neither cancer nor hyperplasia develops in castrates. The understanding of the hormonal regulation of normal and diseased human prostates is incomplete [5].

The androgen receptor belongs to a large family of DNA-binding zinc-cluster proteins, which also includes other steroid hormone receptors, thyroid hormone receptors and retinoid receptors [6]. Preliminary reports of androgen receptor purification scheme have been published [7], that

involves the following sequence of procedures: DNA-cellulose chromatography, isoelectric focusing and polyacrylamide gel electrophoresis [8,9].

A new androgen receptor purification method using gel exclusion chromatography with sephadex G200 was presented in this paper. A method of radioreceptor assay for purified nuclear testosterone receptors was also developed using ^{125}I-testosterone and found to be suitable for assessment of these receptors in human prostatic tumors. Furthermore, the maximum number of binding sites (B_{max}) and the apparent association constant (K_a) for purified testosterone receptors were measured in benign prostatic hyperplasia (BPH) and prostatic adenocarcinoma (PCA) specimens, these measurement were carried out using Scatchard analyses [10] of binding data obtained with a dextran-coated charcoal (DCC) technique [9].

Patients and Methods:

- **Patients**

The benign prostatic hyperplasia (BPH) patients group comprised twenty five men, aged 64-68 years [mean age 65.96±1.15 (SD) years], while prostatic adenocarcinoma (PCA) patients consisted of thirteen men, aged 72-75 years [mean age 73.66±0.62 (SD) years]. All tumors are without any type of prostatitis. All patients underwent transurethral resection prostatectomy (TURP). Non of the patients had a history of chronic illnesses like diabetes mellitus, hypertension and cardiovascular diseases.

- **The preparation of nuclear salt extracts**

The human prostatic tumor tissues were weighed, pulverized finely with a scalpel in Petri dish standing on ice bath, and then homogenized at 4°C in TEMG (b) buffer solution with a ratio of 1:5 (weight : volume) using a manual homogenizer. The homogenate was filtered through four layers of nylon gauze to remove tissue clumps and fibers of connective tissues. The filtrate fluid was transferred by a Pasteur pipette to low-speed centrifuge tubes and prepare a crude nuclear pellet by centrifugation at 2000 xg for 15 min. The supernatant was decanted, and the pellet was resuspended in 10 volumes of TEMG (b)-NaCl buffer pH 7.8 for 15 min. Nuclei were allowed to swell at 4°C for 30 min in the same buffer. The nuclei were then ruptured by exposing them to sonic waves for forty 30 seconds intervals. The tubes were kept immersed in ice during the entire procedure. Sonically ruptured nuclei solution was then sedimented in a refrigerated centrifuge at 2000 X g for 60 min. The supernatant was then used as a source of nuclear testosterone receptors [9,11,12].

- **Buffers and reagents**

All buffer solutions were prepared [13] by dissolving the appropriate amount of salt in distilled water and the required pH was adjusted.

1. Tris/HCl buffer at different pH values was prepared as follows:
   Solution A: 0.2 M Tris (2.4228 g tris [hydroxymethyl] aminomethane) in 100 ml of distilled water.
   Solution B: 0.1 N HCl
   Working buffers pH (7.2-9) were prepared by mixing 25 ml of solution A with an appropriate amount of solution B to adjust the required pH, then the volume was made up to 100 ml with distilled water.
2. TEMG buffer (pH 7.4): 0.01 M Tris buffer containing 1.5 mM Na_2-EDTA, 2 mM β-mercaptoethanol and 10% glycerol. The buffer was prepared by an appropriate dilution of the stock solution to 250 ml.
3. Citric acid/phosphate buffer was prepared as follows:
   Solution A: 0.1 M Citric acid (21.01 g $C_6H_8O_7.1H_2O$) in 100 ml distilled water.

Solution B: 0.2 M Disodium phosphate (3.560 g $Na_2HPO_4.2H_2O$) in 100 ml distilled water.
Working buffers pH (2.2–7.8) were prepared by mixing appropriate volumes of solution A and B to reach the required pH in a final volume of 100 ml. The buffer was also contained 1.5 mM Na_2-EDTA, 2mM □-mercaptoethanol and 10% glycerol.

4. Dextran–coated charcoal (DCC) suspension:
This suspension was prepared by dissolving the following compounds: 1.25 g charcoal, 0.625 g dextran T-70 and 0.2 g gelatin in 100 ml of TEMG buffer pH 7.4.

- **Gel preparation and column packing [14,15]**

The gel was allowed to swell in excess of buffer (A) pH 7.8 (50 ml buffer/g of gel) and left to stand for three days (72 hrs) at room temperature without stirring, the gel slurry were degassed by suction for 1 hr, then the swollen gel was poured carefully into a vertical glass-column down the wall using a glass-rod. After the gel has settled the column was equilibrated with buffer (A) pH 7.8 for 24 hrs with the dimension of (0.7 × 28 cm).

- **Purification procedure**

Half milliliter of the nuclear salt extract (3.5 mg protein) was applied to the surface of sephadex G-200 column (0.7 × 28 cm) equilibrated with buffer (A). The sample was eluted using the same buffer, fractions of 1 ml were collected at a flow rate of 5 ml/hr. The absorbances of the fractions collected were measured at 280 nm and the protein contents were determined by the method of Lowry et al [16].

The preliminary test of the binding of ^{125}I-testosterone to the purified fractions separated by gel filtration

Fifty micro liters of purified fractions were added to 100 □l (17.42 PM) of ^{125}I-testosterone with and without the addition of 250 fold excess of unlabeled testosterone in a final volume of 1 ml completed with TEMG (b) buffer. The tubes were incubated for 16 hrs at 37°C, the bound testosterone was estimated by adding 200 □L of DCC, then the tubes were shaken for 10 min and centrifuged at 2000xg for 10 min. at 4°C. 600 □L was taken from each supernatant and counted by □-counter. It represents the bound testosterone.

Solutions

Buffer (A): TEMG (b) buffer pH 7.8 containing 0.02 % sodium azide.

TEMG (b)-NaCl buffer: TEMG (b) buffer pH 7.8 containing 1M-NaCl.

TEMG (b) buffer pH 7.8: 0.01 M tris contain 1.5 mM Na_2-EDTA, 2.5 mM □-mercaptoethanol, 10% glycerol, 0.1 M NaCl and 25mM $CaCl_2$.

The choice of most appropriate conditions of ^{125}I-testosterone binding to its purified nuclear receptors

- **The effect of different purified testosterone receptor concentration**

One hundred micro liters (17.42 PM) of ^{125}I-testosterone were added to 50 □l of increasing amounts (50, 100, 150, 200, 250 □g) of purified nuclear testosterone receptors (BI and BII from benign tumors, MI and MII from malignant tumors) in a final volume of 0.6 ml completed with TEMG buffer pH 7.8 with and without the addition of 250 fold excess of unlabeled testosterone. At the end of incubation (16 hrs) at 37°C, the bound testosterone was estimated by adding 200 □l of DCC, then the tubes were shaken for 10 min and centrifuged at 2000 X g for 10 min at 4°C. Six hundred micro liters was taken from each supernatant and counted by □-counter. It represents the bound testosterone.

- **The choice of most appropriate ^{125}I-testosterone concentration for the binding with its purified nuclear receptors**

Increasing concentrations (28.998-87 PM) of ^{125}I-testosterone was each added to 50 □l (250 □g BI and MI-protein, 200 □g BII-protein, 150 □g MII-protein) in the first set of tubes with a final volume of 0.6 ml completed with TEMG buffer pH 7.8. The second set of tubes consists of the same

reactants plus 250 fold excess of unlabeled testosterone. After incubation for 16 hrs at 37°, the bound testosterone then was estimated.

- **The effect of pH on the binding of ^{125}I-testosterone to its purified nuclear receptors**

Fifty micro liters of purified nuclear fractions (250 µg BI and MI-protein, 200 µg BII-protein, 150 µg MII-protein) were added to 150 µl (43.5 PM) for MII purified fraction and to 200 µl (57.997 PM) for BI, BII and MI purified fractions of ^{125}I-testosterone with and without the addition of 250 fold excess of unlabeled testosterone. The volumes of the mixtures were made up to 0.6 ml with TEMG buffer of different pHs ranging from 7.8 to 9.5 and with citric acid/phosphate buffer of pHs ranging from 6.4 to 7.8. The tubes were incubated at 37°C for 16 hrs. After the incubation, the bound testosterone was estimated as mentioned previously.

- **The effect of incubation time on the binding of ^{125}I-testosterone to its purified nuclear receptors**

Fifty micro liters of purified nuclear fraction (250 µg BI and MI-protein, 200 µg BII-protein, 150 µg MII-protein) were added to 43.5 PM of ^{125}I-testosterone for MII purified fraction and to 57.997 PM for BI, BII and MI purified fractions with and without the addition of 250 fold excess of unlabeled testosterone. The volumes of the mixtures were completed to 0.6 ml with TEMG buffer (pH 7.8 for BI and MII fractions, pH 8.6 for BII fraction) and with citric acid–phosphate buffer (pH 7 for MI fraction). The tubes were incubated at 37°C for different time intervals (1,2,4,6,10 and 14 hrs). At the end of incubation, the bound testosterone was estimated as described previously.

- **Temperature dependency of testosterone binding to its purified nuclear receptors**

The experiment was carried out at the optimum conditions of each purified receptor of pH, ^{125}I-testosterone concentration and protein concentration. The tubes were incubated for (2hrs for BI fraction, 6hrs for BII and MI fractions and 14 hr for MII fraction). The experiment was performed at different temperatures (4,10,25,37 and 45°C), the bound testosterone was then estimated.

Determination of the concentration of purified nuclear testosterone receptors and the affinity constant of testosterone association with its purified nuclear receptors

Purified nuclear receptors were measured by using of increasing concentrations (8.71-34.84 PM) of ^{125}I-testosterone. The experiment was carried out at the optimum conditions of protein concentration, incubation time and pH for each purified fraction. It was performed at different temperatures (4,10,25,37 and 45°C). The bound testosterone was then estimated.

Results:

Purification and isolation of nuclear testosterone receptors were performed by gel exclusion chromatography technique. Benign and malignant homogenates were applied to sephadex G-200 (0.7 × 28 cm) column. The void volume of this column was 6 ml as predicted from the elution profile of the blue dextran as shown in Figure (1A). The resultant fractions of each homogenate type were collected, detected for the binding with ^{125}I-testosterone pooled, concentrated and then subjected to protein determination. This experiment revealed as shown in Figure (1B&C) the presence of two different eluted components (I & II), these two components eluted with different elution volume corresponding to their different molecular weights. From benign tumors homogenate, the first one (BI) eluted with the void volume (V_o) while the second one (BII) eluted with about 2.5 V_o. From malignant tumors homogenate, (MI) eluted with one fraction after the void volume (V_o) while the second one (MII) eluted with 2.5 V_o.

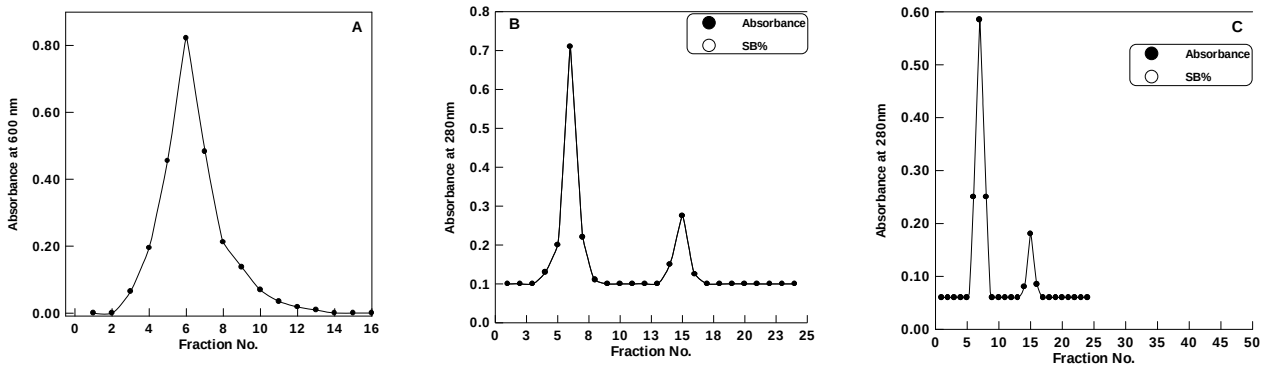

Figure (1): Elution profiles of: A) Blue dextran 2000, B) testosterone receptors from BPH homogenate, C) testosterone receptors from PCA homogenate.

Table (1) illustrates the purification parameters for the different purified receptor forms isolated by gel exclusion chromatography technique. The elution profiles result from our experiment as shown in Figure (1B&C) were nearly similar to that obtained previously by many investigators worked mainly on rat prostate[24-37].

Table (1): Purification data of testosterone receptors isolated by gel filtration technique.

Receptor type	*Total proteins (□gm)*	*Specifically bound ^{125}I-testosterone (fM)*	*Specific binding Fmole ^{125}I-testosterone /mg protein*	*Purification factor (fold)*
Crude benign prostatic tumors homogenate	250	3.480	13.920	1.000
BI purified fraction	200	32.261	161.308	11.588
BII purified fraction	100	27.258	272.586	19.582
Crude malignant prostatic tumors homogenate	100	1.110	11.100	1.000
MI purified fraction	85	22.908	269.515	24.280
MII purified fraction	70	22.620	323.143	29.111

Figure (2) shows the effect of increasing amounts of purified receptors on the binding with ^{125}I-testosterone. The results revealed that 250□gm protein was the most appropriate concentration of the binding of BI and MI purified fractions while 200 □gm of protein for BII purified fraction and 150 □gm protein was the most appropriate concentration of the binding of MII purified fraction.

Figure (3) shows that purified nuclear receptors were saturated with testosterone concentrations equal to 72.498 PM for BI purified fraction, 57.997 PM for BII, MI purified fractions and 43.5 PM for MII purified fraction.

Figure (4) shows the effect of increasing pH on the binding of ^{125}I-testosterone to its purified receptors. These results revealed that the optimum pH for BI and MII purified fractions for the binding with testosterone was 7.8 while 7 was the optimum pH for the binding of testosterone with MI purified receptor and 8.6 for BII purified receptor binding with testosterone.

Figure (5) shows that at 37°C the apparent equilibria of the ^{125}I-testosterone binding were reached in 2 hrs for BI purified receptor, 10 hrs for MI purified receptor, 6 hrs for BII and MII purified receptors.

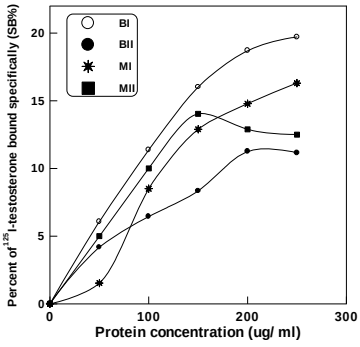

Figure (2): The effect of protein concentration on ^{125}I-testosterone binding to its purified nuclear receptors.

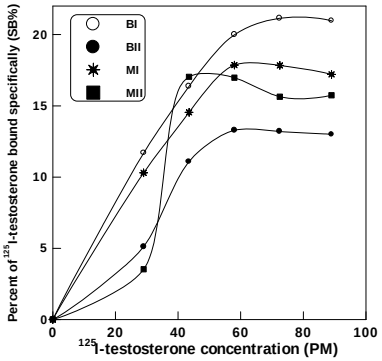

Figure (3): The effect of different ^{125}I-testosterone concentrations on the binding to its purified nuclear receptors.

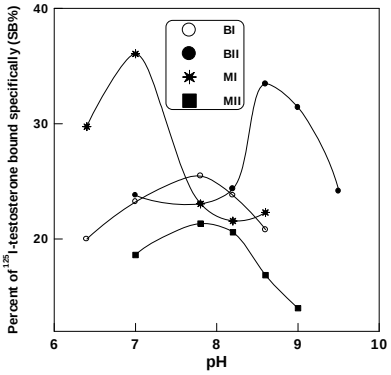

Figure (4): pH dependency of ^{125}I-testosterone binding with its purified nuclear receptors.

The temperature dependency of the testosterone binding to its purified nuclear receptors was investigated. Figure (6) shows that the optimum temperature of the binding of ^{125}I-testosterone was 10°C with BI purified receptor, 25°C with BII purified receptor, 4°C for MI purified receptor and 45°C with MII purified receptor.

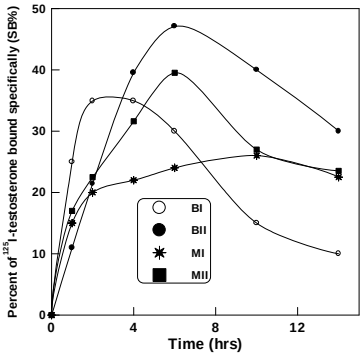

Figure (5): The effect of incubation time on the binding of ^{125}I-testosterone with its purified nuclear receptors.

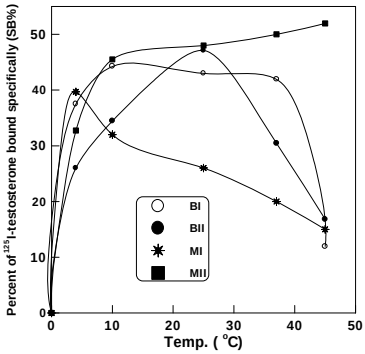

Figure (6): Effect of temperature on the binding of ^{125}I-testosterone with its purified nuclear receptors.

Scatchard analysis (figure (7)) gave a straight line for each purified receptor at 4, 10, 25, 37 and 45 ºC indicating the presence of only a single class of receptor. Table (2) lists maximum number of binding sites ($B_{max.}$) and affinity constants(Ka) for each type of purified testosterone receptors in benign and malignant prostatic tumor tissues.

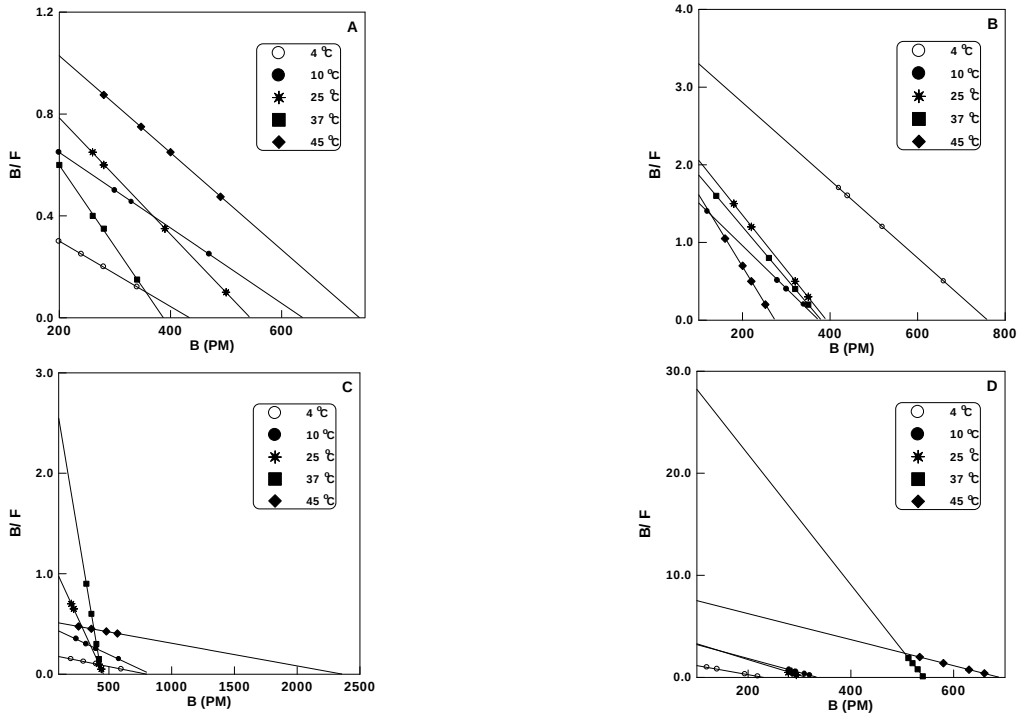

Figure (7): Scatchard plot of ^{125}I-testosterone binding with its purified nuclear, A) BI-receptor, B) BII-receptor, C) MI-receptor, D) MII-receptor at different temperatures.

Table (2): Concentrations and affinity constants of purified nuclear testosterone receptors in benign and malignant prostatic tumors.

Temp(℃)	*BI-purified receptor*			*BII-purified receptor*			*MI-purified receptor*			*MII-purified receptor*		
	B_{max}	k_a	k_d	B_{max}	k_a	k_d	B_{max}	k_a	k_d	B_{max}	k_a	k_d
4	1.036	1.318	0.758	2.280	4.96	0.201	1.870	0.294	3.401	0.920	8.81	0.113
10	1.920	1.571	0.636	1.122	5.48	0.182	2.008	0.592	1.689	1.344	13.80	0.072
25	1.305	2.313	0.432	1.173	7.04	0.142	1.099	2.792	0.358	1.183	34.00	0.029
37	0.931	3.211	0.311	1.140	6.69	0.149	1.056	7.590	0.131	2.163	65.10	0.015
45	1.757	1.922	0.520	0.822	9.38	0.106	1.466	0.229	4.366	2.768	12.50	0.080

B_{max}: in (Pmole/mg protein, k_a : x 10^9 (M^{-1}), k_d : x 10^{-9} (M)

Discussion:

From the results listed in this study, it was concluded that these components are capable of binding to the testosterone with different affinities and in general receptors type II have higher affinities for the binding than those of receptors type (I). The first eluted component (I) with a higher molecular weight is an aggregated complex of testosterone receptor and nuclear matrix. The nuclear matrix is a chromatin–depleted and salt–washable, proteinaceous (non–histonic), intra–nuclear structure or may be defined as nuclear scaffolding proteins which provide functional organization for DNA. Many biological functions reported to be associated with the nuclear matrix include steroid hormone binding, DNA–replication sites, RNA synthesis and processing.In the last two decades, great interest has developed in the molecular characterization of the matrix bound

androgen receptors in the normal and diseased prostate glands1 of different species and different ligand used[9,12,17,18].

An indication of such complex formation is available from previous studies by Mainwaring, Irving and others [9,19-21].

The second component (II) represents the purified testosterone receptors with a lower molecular weight than the first one (about 110 kDa) [22,23]

Figure (2) shows that the binding of ^{125}I-testosterone with its purified receptors BII and MII needed lower amounts of these receptors to get the equilibrium compared with the amounts required for BI and MI purified receptors. This may be due to cell hyperproliferation or increase in these receptors affinities for testosterone.

As shown in figure (3), BII and MII purified fractions were saturated with smaller concentrations of ^{125}I-testosterone than these required for BI and MI. Thus it was concluded that BII and MII purified receptors have higher affinities (but not concentrations) toward testosterone than BI and MI purified fractions.

The differences in the optimum pHs of different purified receptors may suggest the differences in the binding sites of these purified receptors. Also it was found that BII purified receptor binding site contains basic amino acid residues more than that of MII purified receptor.

Figure (6) illustrates the effect of temperature on the binding of testosterone to its different purified receptors. In general the loss of specific binding activity above the optimum temperature of BI, BII and MI purified receptors may be due to degradation of these receptor molecules or to the irreversible dissociation of the testosterone receptor complexes.

Scatchard plot analysis gave a straight line as shown in Figure (7A, B, C&D) for each purified receptor at each temperature (4,10,25,37 and 45°C) indicating the presence of only a single class of receptor site, or more but with the same affinity and number of binding sites, these results were summarized in Table (2). Concentrations and affinity constants of purified nuclear testosterone receptors in benign and malignant prostatic tumors were listed in table(2). Many reports indicate the possibility of using the nuclear testosterone receptor content as a possible marker of responsiveness to hormonal therapy in prostatic carcinoma [38,39]. Many investigators worked on rat prostate tumors reported that the concentration of matrix bound nuclear androgen receptors may represent the functional intranuclear androgen receptor in prostate cancer and characterization of these sites may also provide an understanding of the etiology of benign prostatic hyperplasia and cancer of the prostate. Possibly, the combined quantitation of testosterone receptor (II) content and matrix–bound nuclear testosterone receptors (I) is necessary for accurate prognosis and prediction of androgen–dependence of prostatic cancer specimens [9,12,17,18,38,40,41]. Gonor et al (1984), underlined that nuclear matrix bound androgen receptor could accurately identify those patients who should receive chemotherapy early in the progression of aggressive androgen–independent disease when the tumor burden is less, in the hope that this would increase both patient tolerance and tumor response to this treatment [18].

The results in Table (2) show that k_a value at 37°C for BII–purified receptor is about two times that of k_a value for BI–purified receptor while the k_a value at the same temperature for MII–purified receptor is about 8.5 times that of MI–purified receptor and about 20.2 times that of BI–purified receptor. In general, it was found that testosterone receptors interact with testosterone with higher affinity than the interaction of testosterone with nuclear matrix.

Conclusions

This study provides a useful information about the concentrations and affinity constants of testosterone receptors in benign and malignant prostatic tumors. It supports previous studies concerned with rats and mice models about the increased androgen receptor content in malignant tumors compared with benign tumors. Also affinity constant of testosterone binding was increased significantly in malignant tumors compared with benign tumors.

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