ASSESSMENT OF INDIRECT HEMAGGLUTINATION AND ZYMOGRAPHY PROCEDURES IN EVALUATION OF GELATINASE A IN PATIENTS WITH BENIGN AND MALIGNANT PROSTATE HYPERPLASIA

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ABSTRACT

There are increasing data on novel tumor markers such as gelatinase A, which play a key role in tissue invasion and metastasis. Since prostate cancer is one of the common malignancies, we designed a simple and applicable Indirect Hemagglutination (IHA) test for determination of total gelatinase A in serum samples. In this study, we have analyzed the circulating form of gelatinase A (MMP-2) in patients suffering from either benign prostate hyperplasia (n= 54) or prostate cancer (n= 26) and normal individuals as control (n= 26). The gelatinolytic activity was determined by zymography followed by densitometric analysis. PSA was quantified by using a standard ELISA technique. Correlation of densitometric analysis of gelatinase A activity and IHA titer was significant at 0.01 level ($p< 0.01$, $r = 0.916$). Correlation of PSA and IHA titer was significant at 0.01 level ($p< 0.01$, $r = 0.746$). Border line IHA titer in patients with prostate cancer was 512 ± 1 tube titer, in benign prostate hyperplasia patients was 128 ± 1 tube titer, and the titer in normal individuals was 8 ± 1 tube titer. These results demonstrate that IHA compared to zymography may be a better and simpler procedure in monitoring and screening patients with prostate cancer.

Keywords: Gelatinase A, IHA, PSA, Zymography.

INTRODUCTION

Prostate cancer is the second common cause of cancer death in men. Whereas metastasis, once occurred, has no curative treatment, screening of these patients is important. Although prostate specific antigen (PSA) is the best tumor marker available today, but use of serum PSA as a staging and monitoring tool, the specificity of isolated PSA determination remains low.

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The matrix metalloproteinases (MMPs) are a family of $Zn^{2}+$ endopeptidases that can digest various extracellular matrix macromolecules. More than 200 members have been identified and classified into five major types of structurally and functionally related metalloproteinases.[1,2] Among members of this family of human MMPs, gelatinase A and B (MMP-2, MMP-9) degrade basement membrane collagens type IV, gelatin and other proteoglycan component of extracellular matrix. MMPs have been reported to play significant roles in either physiological (e.g. aging, wound healing, and angiogenesis) or pathological (e.g. allergic contact dermatitis, tumor invasion, and metastasis) processes.[3,4]

Recent reports have indicated that gelatinase A plays a key role in the proteolytic cascade leading to extracellular matrix (ECM) degradation during tumor invasion and metastasis.[5-10] Since molecules released as a result of tissue alterations are often found in body fluids, the determination of MMPs in blood or urine has been recommended as a diagnostic measure to characterize the processes occurring in tissue.[11] Numerous methods have been developed to assess the levels of active and latent forms of gelatinase A (MMP-2) in biological fluids. One of the commonest methods is zymography, which is an electrophoretic technique that provides useful quantitative information on the enzyme as well as an estimation of molecular weight,[12,15] but it is time consuming and laborious.

In the present study, we designed a semi-quantitative and simple indirect hemagglutination test (IHA) to assess the circulating form of gelatinase A (MMP-2) in 24 cases of prostate cancer (PC) versus 50 serum samples of benign prostate hyperplasia (BPH) as compared to 26 serum samples of control individuals.

MATERIALS AND METHODS

Patients and Sampling

Based on pathological needle biopsies confirming the involvement of either adenocarcinoma or benign prostate hyperplasia (BPH), 26 prostate cancer (PC), 54 BPH and 4 prostatitis patients were included in this study. Twenty six normal individuals were also included as controls. A written consent was taken from each patient for inclusion of his record in the study. Patient's sera, taken from a 5ml clotted blood, was aliquoted and cryopreserved for further analysis.

Materials

Fresh sheep Red Blood Cell (SRBC), Glutaraldehyde (Merck), Gelatin A (Merck), Bovin Serum Albumin (Biotest), PBS buffer (pH= 7.2 & pH= 6.4), Tannic acid (Fluca), Precast 7% polyacrylamide gels containing 0.5% gelatin A.

Methods

Zymography. Serum samples for analysis were prepared by dilution into zymogram sample buffer consisting of 0.4 M Tris, pH 6.8, 5% SDS, 20% glycerol, and 0.03% bromophenol blue. The samples were loaded into the wells (each well contained 20 µg protein) of a precast gel and the electrophoresis was carried out at 20 mA constant current for 2.5 to 3.0 h, at which time the bromphenol blue dye front had reached the bottom of the gel. The gel was removed and incubated for 1 h at room temperature in 100 ml of 2.5% Triton X-100 on a rotary shaker. The Triton X-100 solution was decanted and replaced with 100 ml of enzyme buffer (50 mM Tris, pH 7.5,200 mM NaCl, 5 mM CaCl2, and 0.02% NaN3). The gel was then incubated at 37°C overnight. Staining and destaining were carried out at room temperature on a rotary shaker. Each gel was stained with 100 ml of 0.5% coomassie blue G-250 in 30% methanol, 10% acetic acid for 1h and then destained with three changes of 30% methanol, 10% acetic acid (for 15, 30, and 60 min of destain time, respectively, for each change). After this step areas of digestion appeared as non-staining regions of the gel. The degree of digestion was quantified using UVI Pro gel documentation system (GDC- 8000 System) on the basis of grey levels. Each serum (PC, BPH, and Normal) was examined in triplicate and the average value of the integrated density for a particular band was used for further calculation.[15]

Indirect Hemagglutination (IHA). *Preparation of gelatin A coated SRBC.* SRBC were collected in Alsever's solution and were washed three times in PBS, pH 7.2, before use. Washed SRBC were treated with tannic acid (4°C, 30 min). Then they were washed twice with PBS, pH 7.2, and one more time with PBS, pH 6.4. A final 5% suspension of washed cells was prepared in PBS, pH 7.2. One volume gelatin A solution (final concentration 4 mg/mL in PBS, pH 6.4) was added to one volume of 5% SRBC suspension and incubated over night in 4°C. The coated cells were washed three times with PBS, pH 7.2, containing 1% BSA (PBS- BSA) as blocker. Gelatin coated SRBC were made into a 1% suspension in PBS containing 1% BSA.

Assay procedure. The IHA reaction was performed on plastic trays with 96 multiple "V" bottoms wells. For each serum sample, as well as control positive (free FCS supernatant of HT 1080 cell culture media), two fold serial dilutions were prepared with diluent (PBS-BSA) and 50µL aliquots transferred to a series of test wells in one row. To each well 25µl of a 1% reagent cell suspension was added. The plate was shaken for 10 minutes and the end point titers were visually read after settling over night.[13]

PSA determination. The serum level of PSA was determined by solid phase, non-competitive assay based upon the direct sandwich ELISA. 50 µl of standards, control, and patient samples were incubated for 1 hour while shaking at room temperature together with the anti PSA antibody in Streptavidin coated microtiter strips. The strips were then washed with washing solution followed by incubation with HRP (HorseRadish Peroxidase) labelled anti PSA antibody. After washing, buffered Substrate/ Chromogen reagent was added to each well and the enzyme reaction was allowed to proceed, and it was then incubated for 30 min at room temperature with constant shaking. Finally 100µL of HRP

Stop solution was added to each well and then absorbance was read at 405 nm in a microtiter plate reader.[14]

Statistics. Statistical significance was determined by the Spearman correlation coefficient (rho), and p values less than 0.01 were considered significant.

RESULTS

Comparison of densitometric analysis of gelatinase A (MMP-2) activity and IHA titer demonstrated that gelatinolytic activity of MMP-2 had correlation with quantity of enzyme in PC patient vs. BPH patient as compared with normal individuals. Spearman correlation coefficient (rho) between IHA and gelatinolytic activity of MMP-2 was 0.916, indicating that correlation was significant at the 0.01 level ($p < 0.01$) (Figure 1). Border line of IHA reciprocal titer in PC patients was 512 ± 1 tube titer, in prostatistis patients was 256 ± 1 tube titer, in BPH patients was 128 ± 1 tube titer, and in normal individuals was 8 ± 1 tube titter (Table I) (Figure 2, 3).

Furthermore, Spearman correlation cofficient (rho) between PSA (Prostate Specific Antigen) and IHA was 0.874 ($P < 0.01$) (Figure 4).

DISCUSSION

The principle objective of this study, namely the development of a sensitive and simple test for detecting total MMP-2, was achieved as shown by data in

Table I. Comparison of IHA titers in normal individuals (Normal), benign prostate hyperplasia patients (BPH), Prostatistis patients (prostatistis), and patients with prostate cancer (PC).

IHA Titer*	Serum Samples Normal	 BPH	 Prostatitis	 PC	Total
4	3				3
8	19				19
16	2				2
32	2	3			5
64		10			10
128		25			25
256		11	3	5	19
512		4	1	12	17
1024		1		9	10
total	26	54	4	26	110

*Reciprocal of serum dilution at end point.

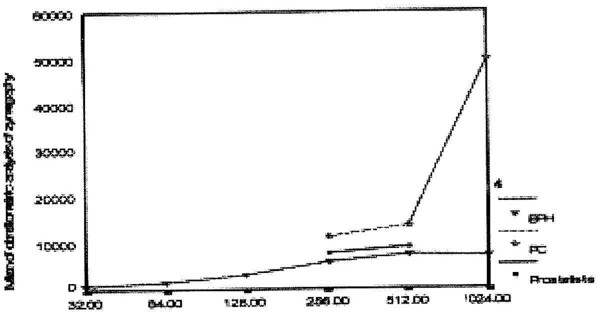

Fig. 1. Correlation of IHA titer and mean of gelatinolytic activity. 50 μL of each sample was analysed and titrated for the presence of gelatinase as indicated in materials and methods. The titers were plotted against densitometric depiction of gelatinolytic activity and the correlation was estimated.

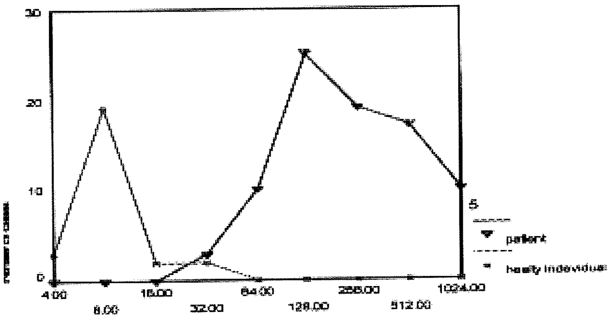

Fig. 2. Comparison of IHA titer in patients and normal groups. 50 μL of each sample was analysed and titrated for the presence of gelatinase by IHA test as indicated in materials and methods. The titers were plotted against number of cases.

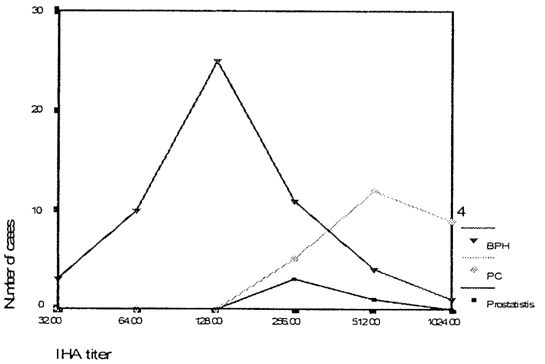

Fig. 3. Comparison of IHA titer in patients groups. The IHA titer was plotted against number of patients in Three groups: PC, BPH, and Prostatistis

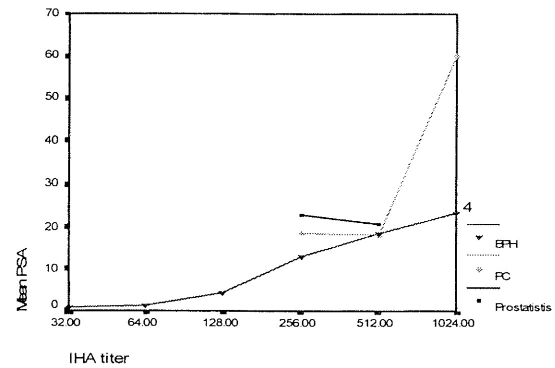

Fig. 4. Correlation of IHA titer and mean of PSA patients groups. The IHA titers were plotted against mean of total PSA.

Table I.

It may seem paradoxical that while others use sensitive tests such as ELISA and Fluorometric techniques,[16,17,18] we have chosen an indirect hemagglutination system to detect total MMP-2. With regard to sensitivity, in comparison with ELISA and Fluorometric procedures that are very expensive and unavailable, IHA is simple and available.

With respect to technique details, what is considered as a "positive pattern", here is binding of MMP-2 to gelatin- coated SRBCs. Quantitative assessment was accordingly made by two- fold serial dilutions of serum samples. Nonspecific factors might interfere with MMP-2 determination. In the present study, we evaluated the limits of specificity and sensitivity of the technique at three points: 1) Serum samples were diluted with EDTA 0.5 m, pH 8, before two fold serial dilutions, to demonstrate that what we had quantified was total MMP-2.

2) Absorption of non-specific antibodies that could intervene with the reaction, by incubation of serums with packed SRBC before doing the test. 3) Different controls were tested to eliminate other factors in serum that might interfere in examination.

The specificity of the method was demonstrated by zymography tests in serum samples of patients (BPH, PC, and Prostatistis) and control groups (control individuals).

Since the discovery of prostate- specific- antigen (PSA), detection and treatment of prostate cancer have changed dramatically. Adenocarcinoma of the prostate is now the most common noncutaneous malignancy diagnosed in the world, with a lifetime risk of nearly 1 in 6.[19] The results of large screening program have demonstrated that diagnostic evaluation of elevated serum PSA levels improves early detection and the likelihood of identifying organ- confined disease.[20, 21] Although the incorporation of PSA into practice began more than 20 years ago, optimal screening parameters are not fully established. This may due to the variable sensitivity of PSA in men of different races and ages.[21]

Global records show that prostate hyperplasia (benign and malignant) next to lung cancer is the most commonest malignancy in the world.[22, 23] The present study, in agreement with some other reports[24, 25, 26] demonstrated that evaluation of gelatinase A was a reliable alterative for prostate cancer screening. Therefore, we designed a simple, semi quantitative and specific IHA procedure to determinate total gelatinase A. Our findings indicate that IHA titer between patients and control groups and also, intra patients group (means BPH, PC, Prostatistis) are significantly different, and this test compared to zymography, could be a valuable means for screening and monitoring the patients suffering from prostate cancer.

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