Substrate gelatin zymography analysis of matrix metalloproteinase-2 and -9 (gelatinase A and B) in sera from patients with benign and malignant prostate disease

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Abstract

Background: it is widely recognized that the measurement of serum prostate-specific antigen (PSA) levels as a biomarker of prostate cancer is imperfect, in that it can have many false positive elevations attributable to benign hyperplasia and subclinical prostatic inflammation. There is increasing data that support a positive correlation between gelatinases (MMP-2 and MMP-9) activity and tumor cell invasion and tumor aggressiveness.

Objectives: we evaluated gelatinolytic activities in the sera of patients with prostate carcinoma (PCa) and benign prostate hyperplasia (BPH) in order to verify whether MMP-2 and MMP-9 might have a potential as non-invasive biomarkers.

Methods: by gelatin zymography, we verified MMP activity in a total of 42 patients. Of these, 8 had benign prostate hyperplasia and 34 had carcinoma. A total of 16 normal healthy volunteers with no concomitant illnesses were used as controls.

Results: four dominant proteinases were detected migrating at ~ 240, 130, 92 and 72 kDa. The most abundant lytic activity is at 92 kDa (MMP-9); whereas MMP-2 is present in smaller quantities. MMP-9 activity is significantly enhanced in the sera from patients with cancer compared with control individuals (p=0.003). Moreover, MMP-9/MMP-2 ratio was able to discriminate cancer patients from healthy subjects as well as from BPH, and the ROC curve showed that the ratio was a significant predictor for prostate cancer with sensitivity of 80% and specificity of 87%.

Discussion: these results suggest that the inexpensive measurement of MMPs in serum may serve as a suitable supplementary tool to distinguish patients with prostate cancer from patients with BPH, and the addition of these enzymes to currently available PSA and/or f-PSA/t-PSA ratio might provide clinicians additional objective information on prostate carcinomas.

KEY WORDS: matrix metalloproteinase 2, matrix metalloproteinase 9, prostatic hyperplasia, prostate cancer, serum.

Introduction

The gelatinase or collagenase type IV MMP-2 (gelatinase A, 72 kDa) and MMP-9 (gelatinase B, 92 kDa) are two members of the large group zinc-containing endopeptidases (MMPs family) with a central role in the degradation of all types of extracellular matrix (ECM) components. It is becoming increasingly recognized that MMPs are a multifunctional group of biologically important molecules with diverse roles in normal biological and pathological processes such as arthritis, cardiovascular diseases, nephritis, neurological diseases, breakdown of blood-brain barrier, skin ulceration, etc. (1-3).

The gelatinases are the most complex family members in terms of domain structure and regulation of their activities, being that they are also endowed with functions other than cleaving the collagen type IV. They are also important in creating an environment that supports the initiation and maintenance of growth of primary and metastatic tumors (3, 4). It has been reported that MMP-2 and MMP-9 are associated to the aggressive biological nature and accordingly, with the ungovernable clinical course in several types of human neoplasias (3-6). Therefore, they are used as markers of the malignant phenotype. However, it is not known which of the two gelatinases has a more determining role in the progression of cancer, since their role often varies in defining the prognosis in different types of cancer (7). Moreover, the expression of MMP-2 and MMP-9 is not necessarily co-ordinated in all neoplasias, and it is also not clear what the most reasonable method to assay the gelatinases in the biological fluids would be (e.g. immunological assay or gelatin-zymography). As it concerns prostate neoplasias, preclinical studies have demonstrated that: i) MMP-9 is linked to the ability of cultured prostate carcinoma cells to produce lung metastasis in animal models (8); ii) MMP-2 and MMP-9 regulate the invasiveness of human metastatic prostate cancer cells (9) and clinical studies showed that over-production of
these two enzymes is associated with the progression of prostatic neoplasia and with more aggressive phenotypes (10, 11). Furthermore, we have previously showed that, in the urine of prostate cancer patients, MMP-9 is present in higher quantities, whereas MMP-2 is present in smaller quantities, and that MMP-9 activity is enhanced in the urine from patients with benign prostate hyperplasia compared with cancer patients (12).

In this preliminary study, we determined MMP-2 and MMP-9 activity levels in the sera from patients with prostate carcinoma, benign prostatic hyperplasia and normal healthy individuals (HC) using gelatin zymography in order to analyze the serum gelatinolytic activities and to verify whether serum content of MMPs might have a potential as non-invasive biomarkers in providing additional useful clinical information in prostate cancer.

Materials and methods

Patients

Patients chosen for the study underwent biopsy or radical prostatectomy at our Institution. Inclusion criteria were: no evidence of active infection or inflammatory disease, no neo-adjuvant androgen therapy, no 5α-reductase inhibitory treatment. Pathological diagnosis was performed by usual clinical laboratory criteria and confirmed postoperatively by a histopathological findings. The tumors were classified for Gleason score and pathological UICC 2002 pTNM stage by genitourinary pathologist (13, 14). Approval for the study was provided by institutional ethics committee. The study was performed in accordance with the ethical standards laid down in 1964 Declaration of Helsinki and its later amendments.

During a 1-year period, a total of 42 patients with prostate disease were evaluated. Of these, 8 had benign prostate hyperplasia (BPH) and 34 had carcinoma (PCa). The tumor samples were: 3 cases with Gleason 5, 13 patients with Gleason 6, 14 patients with Gleason 7, 1 patient with Gleason 8, and 3 patients with Gleason 9. All patients were untreated before surgery. A total of 16 normal healthy volunteers with no concomitant illnesses were untreated before surgery. A total of 16 normal healthy individuals (HC) using gelatin zymography in order to analyze the serum gelatinolytic activities and to verify whether serum content of MMPs might have a potential as non-invasive biomarkers in providing additional useful clinical information in prostate cancer.

Serum samples

Informed consent for venous blood sampling was obtained from all individuals. Native serum was prepared by using plastic tubes without coagulation accelerators, to prevent the release of gelatinase during platelet activation. Tubes were centrifuged at 1600 g for 10 min, 30 min after blood collection. For each sample, determination of protein concentration was performed by using the Bradford method (15). Sera were aliquoted and stored at -20°C until use. Each aliquot was used only once in order to prevent enzyme activation due to freeze-thawing processes.

Materials

Gelatinase A and gelatinase B were purchased from Hoffmann-La Roche Ltd (Basel, Switzerland). Triton X-100, Calcium chloride (CaCl₂) glycercyl, gelatin, ethylenediaminetetraacetic (EDTA), phenylmethylsulphonyl fluoride (PMSF) were from Sigma Chemical Co. (St. Louis, MO, USA). Total prostate specific antigen (t-PSA) and free prostate specific antigen (f-PSA) were determined using the Immulite analyzer with a commercial kit (Diagnostic Products Co., Los Angeles, CA, USA). All other reagents were available from commercial sources.

Gelatin zymography

Zymography was performed using 7.5% (w/v) polyacrylamide gels containing 0.1% (w/v) of gelatine as previously described (16). Briefly, total protein (25μg) was mixed with sample buffer (10 mM Tris-HCl pH 6.8, 12.5% SDS, 5% sucrose, 0.1% bromophenol blue) and applied directly without prior heating or reduction to the gel. After removal of SDS from the gel by incubation in 2.5% (v/v) Triton X-100 for 1 hour, the gels were incubated at 37°C for 18 hours in 50 mM Tris-HCl pH 7.6 containing 0.2 M NaCl, 5 mM CaCl₂, and 0.02 % (w/v) Brij 35. Gels were stained for 1 hour in 30% methanol, 10% glacial acetic acid containing 0.5% (w/v) Coomassie Brilliant Blue G 250 and destined in the same solution without dye. The gelatinolytic activity of each gelatinase was evident as a clear band against the blue background of stained gelatin. The molecular size of bands displaying enzymatic activity were identified by comparison with pre-stained standard protein, as well as with purified gelatinase A or gelatinase B. To normalize the possible difference between zymograms, an internal blood sample (pool serum) was incorporated in every gel (Fig.1, lane 2).

Figure 1 - Gelatin zymography of serum specimens from patients with PCa. Molecular weights standards are shown on the left. In all samples, 25 μg of protein was loaded onto the gel. Lane 1, internal control (pool serum) in presence of 20 mM EDTA; lane 2, internal control (pool serum); lane 3, pT2cN0M0 6 (3+3); lane 4, pT2aN0M0 6 (3+3); lane 5, pT2cN0M0 9 (5+4); lane 6, pT2cN0M0 7 (3+4); lane 7, pT3aN0M0 6 (3+3); lane 8, pT2aN0M0 6 (3+3); lane 9, pT2aN0M0 6 (3+3) in presence of 0.1 mg/ml of PMSF; lane 10, pT2cN0M0 7 (3+4).
Control gels for MMPs

Control gels contained either of the MMP selective inhibitors, 20 mM EDTA or 10 mM 1,10 phenanthroline, in the MMP incubation buffer to confirm that lysis band were the results of MMPs. Furthermore, the character of proteolytic bands was analyzed by incubating the identical zymograms in 0.1 mg/ml of PMSF, a serine protease inhibitor; or 2 mM Pefabloc, an irreversible serine protease inhibitor.

Analysis of the gels

Following zymography, the degree of gelatin digestion was quantified as previously described (12). Briefly, we used an image analysis software (ImageQuant TL, Amersham Bioscience, Chicago, IL, USA) according to the manufacturer’s specifications. The image of the gel was inverted to reveal dark bands on a white background. The molecular weight, volume and background of each band were determined. The relative amounts of the different forms of the sera gelatinases were expressed as the integrated density x 10^{-3} (volume) of all the pixels above the background of each band.

Statistical analysis

Quantitative variables were characterized by using mean ± S.D. or median (25th – 75th percentile). Summaries for qualitative variables were expressed as frequencies and percentages. Comparisons between groups (HC, BPH and PCa) were based on ANOVA followed by Bonferroni adjusted Student T test for unpaired samples in case of normally distributed variables; otherwise, difference in locations were evaluated by means of the Kruskall-Wallis test followed by Bonferroni adjusted Mann Whitney U test. Normality assumption was tested using Shapiro Wilk Test, whereas Levine Statistics was adopted to test for the homoscedasticity assumption in the Student T test. The discrimination ability of biomarkers was further assessed by calculating the areas under the receiver operating characteristic curves (ROC’s AUC) and the corresponding confidence intervals. Sensitivity and specificity values were thus computed for the optimal cut points, i.e. those closer to the upper left vertex of the ROC quadrant. Correlations were based on the non-parametric Spearman rho. All analyses were two-sided, and significance was set at a P value of 0.05. Statistical analyses were performed with the R environment (version 3.01; R Foundation for Statistical Computing, Vienna, Austria).

Results

Clinical characteristics of the study population are listed in Table 1. Thirty-four subjects had carcinoma, of these 53% had Gleason score >6; 50% had T2 tumor stage, and 26.5% had T3 tumor stage. Mean age of the overall population was 62.6 ± 7.9 (range 34-74), with healthy controls significantly younger than PCa patients. Percentage values of f-PSA/t-PSA were significantly lower in PCa patients as compared to BPH subjects, whereas t-PSA and f-PSA values were not different between the two groups (Tab. 1).

Table 1 - Clinical characteristics of study population.

<table>
<thead>
<tr>
<th></th>
<th>HC (n=16)</th>
<th>BPH (n=8)</th>
<th>PCa (n=34)</th>
<th>Omnibus p-value</th>
<th>HC vs.BPH</th>
<th>HC vs.PCa</th>
<th>BPH vs.PCa</th>
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<tr>
<td>Age, years°</td>
<td>56.88 ± 11.35</td>
<td>64.38 ± 2.72</td>
<td>64.85 ± 5.04</td>
<td>0.002</td>
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<td>f-PSA, ng/ml *</td>
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<td>1 [0.25 - 1]</td>
<td>0 [0 - 1]</td>
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<td>f-/PSA/t-PSA, % *</td>
<td>/ 20.5 [10.75 -34.5]</td>
<td>11 [7.75 - 17]</td>
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<td>0.038</td>
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<td>Pathological Stage</td>
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<td>T1</td>
<td>8 (23.5)</td>
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<td>T2</td>
<td>17 (50)</td>
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<td>T3</td>
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<td>Gleason Sum</td>
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<td>≤ 6</td>
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<td>7</td>
<td>14 (41.2)</td>
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<td>&gt; 7</td>
<td>4 (11.8)</td>
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HC= Healthy Control; BPH=Benign Prostate Hyperplasia; PCa=Prostate Carcinoma; n.s.= non significant

°Data are expressed as mean ± standard deviation. Group comparison was based on ANOVA test followed by Bonferroni adjusted Student t test for unpaired samples. *Data are expressed as median [25th-75th percentile]. Group comparison was based on Mann Whitney U test.
To investigate gelatinolytic activity present in sera substrate gel zymography was performed. This method allows the detection of the metalloproteinases that exhibit significant gelatinolytic activity (gelatinase A and B). Representative zymography results are shown in figures 1-3. Polyacrylamide gels were evaluated for the presence of clear zone representing degradation of gelatine by proteolysis. The nature of lytic bands was confirmed by inhibition assay with selective inhibitor of MMPs (Fig. 1, lane 1) and with selective inhibitor of serine proteases (Fig. 1, lane 9).

Four dominant proteinases were reproducibly detected migrating at ~ 240, 130 and 92 kDa (MMP-9, gelatinase B), and 72 kDa (MMP-2, gelatinase A). Moreover, few samples (two BPH subjects, and three cancer patients) showed a lytic band at about 220 kDa. Comparison of these gelatinolytic bands with pre-stained standard protein and purified gelatinase A and gelatinase B clearly identified the MMP-constituting bands as gelatinase A (72 kDa) and gelatinase B (92 kDa) (data not shown). The clear zones with molecular weight >92 kDa may represent complexes of MMPs that are not dissociated in zymography. In fact, MMP-9 can be associated with a 25 kDa protein (lipocalin 2) giving a band at ~125 kDa (17, 18) and can form a complex with its endogenous inhibitor TIMP-1 giving a band at ~140 kDa (19). Furthermore, MMP-9 can form dimer or multidimer giving lytic bands at approximately 220 and 240 kDa (20). Also, several MMPs together can form complexes of high molecular weight gelatinase species that can only be identified with specific antibodies in Western blot analysis. However, because zymography is much more sensitive than Western blot analysis, it has been difficult to find antibodies that were sensitive enough to detect small amounts of MMPs. Following gelatine zymography, the proteolytic bands were subjected to densitometric analysis and the data, normalized to an internal serum standard, were expressed as the integrated density of all the pixels of each band (volume x 10^-3).

Considering the volume average of each individual band, we observed that MMP-9 is the most abundant of all the bands, whereas MMP-2 is present in smaller quantities. The second point is that the 92 kDa band is higher in the sera from cancer patients compared with those of BPH patients as well as with HC (p=0.003). On the contrary the 72 kDa band is similar in the three groups (Tab. 2). MMP-9/MMP-2 ratio was able to discriminate PCa patients from both healthy controls (p=0.045) and from BPH subjects (p=0.003) (Fig. 5).

The discriminatory ability (PCa vs BPH) of the MMP-9/MMP-2 ratio was further assessed by ROC curve (Fig. 6).

The results showed that the ratio was a significant predictor of PCa with an AUC=0.86; 95% CI, 0.73-0.99; p= 0.02. The optimal cut-off point for the ratio was 2.15, providing a sensitivity of ~80% and a specificity of ~87%.
Finally, a negative correlation between the MMP-9/MMP-2 ratio and the % f-PSA was found with a Spearman rho of -0.335 (p=0.03) (Fig. 7).

Discussion
Carcinoma of the prostate is the commonest solid-organ malignancy diagnosed in men (21). Current screening relies on a digital rectal examination associated with a serum prostate-specific antigen test. PSA is a sensitive marker for prostate cancer but it produces a false-positive result in many patients because of BPH and subclinical prostatic inflammation. Numerous reports have showed that free-to-total PSA ratio is relatively lower in men with prostate cancer compared to men with benign disease (22, 23). However, discrepancies have been reported for the f-PSA/t-PSA cut-off ratio (24). Thus, more accurate tests are needed to discriminate between BPH and prostate cancer. One of the strategies to improve this situation is the identification of biomarkers in serum or urine whose levels are sensitive to detect tumor and to monitor for disease progression. Among protein markers the MMP-2 and MMP-9 have gained wide acceptance. These two enzymes have been investigated, using diverse techniques, in tissue and body fluids at different molecular levels (e.g. the RNA and protein levels) with variable results. In prostates removed by radical prostatectomy, Lichtinghagen et al. found that on the mRNA level, MMP-2 expression was decreased and MMP-9 was unchanged in cancerous tissue compared to the normal counterparts, whereas on the protein levels, MMP-2 expression was unchanged and MMP-9 was significantly higher in tumor tissue (25). Others
vestigators found that MMP-9 expression was higher in cancer tissues than in BPH tissues (25-27). By immunohistochemistry, several studies showed that MMP-9 was cytoplasmatic (28, 29), and expressed at different levels in cancer, stromal, and benign epithelial cells (11). High levels of MMP-9 expression by prostate cancer cells was strongly associated with high Gleason score; and MMP-9 expression levels by any cell type was not associated with cancer disease-free survival (11). But surprisingly other researchers did not find any case with immunohistochemical MMP-9 expression (30). Probably, these discrepancies are due to some limitations of the technique. In fact, immunohistochemistry is semi-quantitative and highly dependent on a range of variables such as choice of antibody, antibody concentration, fixation techniques, variability in the interpretation and stratification criteria, and inconsistency in specimens handling and technical procedure.

As it concerns body fluids, the gelatinases have been investigated both in urine and sera. In particular, Roy et al. reported that MMP-9 was detected with higher frequencies in the urine of patients with prostate cancer compared with those of bladder cancer (19) and we have previously shown that urinary MMP-9 values were higher in BPH than in PCa patients (12). In peripheral blood, Castellano et al. measured MMP-2 and -9 levels with immunoassay (ELISA) and zymography and found that MMP-9 levels were significantly higher in patients with PCa compared with those from BPH and HC (31).

In this study, we measured gelatinolytic serum levels of MMP-2 and -9 by zymography. These zymographic tests have some advantages over immunological assays such as lower cost, a more rapid time of execution and the possibility of simultaneously detecting multiple forms of the same enzyme. The results shown here indicate that MMP-9 is the most abundant form of MMPs and its content is statistically significant enhanced in patients with cancer compared with the healthy specimens. In addition, the MMP-9/MMP-2 ratio is able to distinguish cancer patients from both BPH and HC patients. Therefore, according to Castellano et al. the results of the current study support the hypothesis for the significance of MMP-9 in prostate cancer, whereas MMP-2 does not appear to be important.

Taking these observations together, we suggest that the inexpensive measurement of sera MMP-9/MMP-2 ratio may serve as a suitable supplementary tool to distinguish between patients with prostate cancer and patients with BPH, and the addition of MMP-9 to currently available PSA and/or f-PSA/t-PSA ratio might provide clinicians additional objective information on prostate carcinomas. Furthermore, these observations suggest that MMP-9 should be considered as a drug development target for the treatment of prostate cancer. Nevertheless, our study has some limitations: i) the small number of patients included in this observational study, ii) we performed our experiments on sera and we did not confirm the presence of MMPs in tumor tissue. Therefore, in our future research, the next step will be the analysis of tissue content of MMP-2 and -9 by in situ zymography. In conclusion, we showed that MMP lytic activities are easily detected in sera specimens by zymographic technique. We believe that the dosage of gelatinase A and B in the sera of prostate disease patients could be a useful non-invasive tool for oncologists in the management of these patients.

References


