Original Article

Immunochemiluminescent detection of galectin-3 in tumoral tissue from prostate

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Received June 24, 2013; Accepted July 23, 2013; Epub August 15, 2013; Published September 1, 2013

Abstract: This work proposes a chemiluminescent quantitative method for galectin-3 (Gal3) detection in prostate tissues. Monoclonal antibody anti-Gal3 was conjugated to acridinium ester (AE) and the complex formed with Gal3 in the prostate tissue was chemiluminescently detected. The light emission (expressed in Relative Light Unit-RLU) showed mean values higher for benign prostatic hyperplasia than normal tissues and adenocarcinoma. These differences showed to be statistically significant (p < 0.001). There was a linear relationship between RLU and tissue area. Furthermore, these values were dramatically reduced when the tissue samples were previously incubated with non labeled anti-Gal3. Finally, the anti-Gal3-AE solution in buffer stored at 4°C and the treated samples showed to be stable during a year and at least 72 h, respectively. Gal3 content in prostate tissue was higher in benign prostatic hyperplasia than normal tissues and much lower in adenocarcinoma. This quantitative, specific and sensitive method based on labeling antibody to acridinium ester can be applied to detect antigen in tissue.

Keywords: Chemiluminescence, galectin-3, prostate tissue, immunohistochemistry

Introduction

Galectins, a family of β-galactoside binding lectins, are multifunctional proteins involved in a variety of biological processes such as growth development, immune functions, apoptosis, and cancer metastasis [1]. The galectins have been widely used as tools to describe immunohistochemical changes in the tumor cell surface where these changes are associated with tumor cell growth by inducing apoptosis or metastasis [2]. Galectin-3 (Gal3) is one of the best studied galectins with many proposed functions in the immune system and cancer [3]. It is a small molecular weight (about 30 kDa) protein that is expressed in many types of human cells, in particular epithelial and immune cells [4]. Saraswati et alii have reported that Prostate-specific antigen (PSA) regulates Gal3 in human semen and may regulate Gal3 function during prostate cancer progression [5]. They presented evidences that PSA is a chymotrypsin-like serine protease secreted by the prostatic epithelium and normally functions in liquefaction of semen following ejaculation. Furthermore, PSA is implicated in the promotion of localized prostate tumors and bone metastases by its roles in immunomodulation, invasion, and apoptosis. The Gal3 is highly expressed in normal but not in malignant cells [6].

In prostate cancer, histopathological analysis has a great clinical relevance. However, immunohistochemical methods qualitatively analyzed have often shown great disparity and variability of results among different observers. Thus, in order to provide a numerical scale and reproducible patterns of marking tissue, increasing sensitivity and quality control analysis, it has been increasingly technological refinements using automated morphometric methods [7]. Another approach could be the use of chemiluminescent procedure. The chemiluminescence, a process in which excited atoms or molecules through chemical reactions release their excess energy as light has been
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The focus of research to broaden its application in various areas including in immunoassays [8].

The aim of this study was to identify the Gal3 in normal, benign and malignant prostate tissues by using a specific antibody labeled to acridinium ester (AE). Afterwards the antigen-antibody complex was revealed by chemiluminescence that is capable to detect in the range of femtomoles or atomoles (10^{-15} to 10^{-18} mol) [9]. Additionally, Gal3 was evaluated in these prostate tissues by immunohistological staining using antibody conjugated to peroxidase and digital image analysis as comparison.

Materials and methods

Human prostate specimens

Formalin-fixed and paraffin-embedded benign hyperplasia (15); adenocarcinoma (15) and normal (15) prostate tissues were obtained from the archives of the Clinical Hospital of the Federal University of Pernambuco, Brazil. These diagnostics were established by one of the authors (CABL). The Gleason histological grade ranged from 5 to 9, with the mean of 6.7 and median of 6. Clinical staging was defined according to the American Joint Committee on Cancer classification [10] and histological grade according to Gleason score [11]. Patients’ ages varied from 45 to 80 years old.

Anti-gal3 antibody conjugation with acridinium ester

Acridinium ester was conjugated to anti-gal3 antibody (diagnostic biosystems: clone 9C4) according to Weeks et al. [12]. Briefly, anti-Gal3 (500 µl containing 2 mg of protein) was incubated with 15 µl of acridinium ester solution (0.2 mg diluted in 400µl of N,N-dimethylformamide) for 1 h at 25°C. The conjugate (anti-Gal3-acridinium ester) was applied to a column of Sephadex G-25 (10 x 1 cm), previously equilibrated with 10 mM phosphate buffer, pH 7.2 (100 ml) and eluted with this buffer. Aliquots (1 ml) were collected and their protein content was spectrophotometrically determined at 280 nm and chemiluminescence assayed. The aliquots containing protein and chemiluminescence were pooled, dialyzed overnight against 10 mM phosphate buffer, pH 7.2, and kept at -10°C until use.

Chemiluminescent immunoassay

Paraffin sections (8 µm) of samples were cut, transferred to glass slides, deparaffinized in xylene (1st for 5 min and 10 dips in 4 successive containers with xylene) and hydrated in graded alcohols (3 x 100% and 1 x 70% - 10 dips each). Afterwards tissue slices were incubated with anti-gal3-AE (100 mL - 100 mg mL^{-1}) for 2 h at 4°C, followed by washings (2 x 5 min) with 5 mL of 10 mM phosphate buffer, containing 0.15 M NaCl (PBS), pH 7.2. Then the tissues were transferred to polypropylene test tubes

Figure 1. The light emission (RLU) from normal, benign prostatic hyperplasia (HPB) and adenocarcinoma (AP) tissues (n = 15 and triplicates). The inhibited columns were obtained by incubating the tissues with non-labeled antibody previous to anti-Gal3-AE incubation. The statistical analyses showed significance between the mean values (p < 0.001).
with a volume of 50 mL of PBS. Finally, solutions of 0.5% H\textsubscript{2}O\textsubscript{2} in 0.1 N HNO\textsubscript{3} (50 µl) and 0.25 M NaOH (50 µl) were added for chemiluminescent measurement using a luminometer Modulus Single Tube 9200-001 (Turner BioSystems, USA). The emission intensity was determined as relative light units (RLU) with a counting time of 5 seconds per sample. Triplicate measurements were carried out throughout in this study. Anti-Gal3-AE binding inhibition assays were accomplished by incubating the tissues with non labeled Anti-Gal3 (100 mL - 100 mg mL\textsuperscript{-1}) for 2 h at 4°C prior to its incubation with anti-Gal3-AE. Following steps were as described previously for the binding protocol.

**Immunohistochemical staining**

The immunoreactivity of anti-Gal3 conjugated to peroxidase (Sigma, USA) was established according to the protocol proposed by Hsu et al. [13]. Briefly, the tissues were cut (4 mm), transferred to glass slides and deparaffinized as described above. The sections were incubated with citrate buffer at 100°C for 30 min under water vapor, washed (5 min) with PBS and incubated with lyophilized bovine serum (to avoid cross-reactions), then incubated with H\textsubscript{2}O\textsubscript{2} for 5 min and washed with PBS, incubated with a solution containing monoclonal antibody anti-Gal3 (at 1:50 dilution) for 1 h at 25°C. The prostate tissues were incubated with biotinylated secondary antibody (DAKO LSAB Kit, USA). The revelation of the antibody was obtained by reaction of peroxidase visualized by incubating the tissues in a solution containing the substrate (diaminobenzidine-DAB plus H\textsubscript{2}O\textsubscript{2}). The sections were counter stained with hematoxylin and examined under a light microscope (Olympus BH-2, Japan).

**Digital image analysis**

Slices images (magnification 100x) were captured using a digital video-camera (Sony, Japan) connected to a microscope and processed using OPTIMAS™ software version 6.1 (Optimas Corporation, USA). Antibody staining patterns revealed by DAB-peroxidase reaction were obtained in gray value. Image analysis of Gal3 positive cells was carried out adjusting the equipment to consider positive viable cells only those presenting nucleus and visible staining. A correction factor (CF) was used to minimize distortions in values due to the presence of non-stained cells, according to the equation CF = s/S where s means relative surface area and S the total area measured [14]. Analyses of the number of cells per area (12,234 µm\textsuperscript{2}) were developed in three random areas of stained tumor tissues.

**Statistical analysis**

The software OriginPro 8 (OriginLab Corporation, One Roundhouse Plaza, Northampton, MA 01060 USA) was used for the chemiluminescence data processing (expressed as mean ± standard deviation) and statistical analysis (Mann-Whitney U-test and Student t-test; p < 0.05).
Results

Here, AE was conjugated to the anti-Gal3 antibody and used to identify the Gal3 in prostate tissues. Figure 1 shows the RLU mean values obtained for normal (1.083 x 10^6 ± 81044 RLU), benign prostatic hyperplasia (1.38 x 10^6 ± 67799 RLU) and adenocarcinoma (214,234 ± 22,172 RLU). These mean values showed to be statistically significant (p < 0.001). These values were dramatically reduced when the tissue samples were previously incubated with non labeled anti-Gal3 antibody yielding values of 10,617 ± 4,496 RLU; 8,837 ± 969 RLU and 5,687 ± 1,011 RLU, respectively. It is important to call attention to the small standard deviations of these mean values although samples were collected from different individuals. Furthermore, Figure 2 shows that RLU is directly proportional to the tissue areas for all those investigated, namely, normal, benign prostatic hyperplasia and adenocarcinoma.

The anti-Gal3-AE solution in buffer stored at -10°C showed to be stable during a year presenting (50 µl) chemiluminescence of 16,804,400 ± 431,263 (2.6%). Furthermore, the tissue samples treated with the anti-Gal3-AE were also stable for 72 h at least yielding RLU values of 1,087,250 ± 39,728 (6%); 1,311,200 ± 40,035 (3%) and 211,059 ± 12,977 (6%) for the normal, hyperplasia benign prostatic and prostatic adenocarcinoma samples, respectively.

The immunohistochemical staining studies demonstrated significant differences in the patterns of Gal3 expression in the tissues (Table 1). Benign prostatic hyperplasia presented higher average stained area than the prostatic adenocarcinoma cells and normal prostate. These results corroborate the chemiluminescence data.

Discussion

In our lab, the use of chemiluminescence has been used to reveal the glycocode of normal, fibroadenoma and invasive duct carcinoma tissues using lectins (Concanavalin A and Peanut agglutinin) labeled with AE [15, 16]. Photon emission observed during the breakage of the chemical bond between lectin and AE was quantified, expressed in RLU and correlated to the labeling of the normal and transformed tissues. The lectin chemiluminescence was higher in fibroadenoma and IDC than in normal tissue for both lectins tested. The relationship RLU emission versus tissue area described a linear and hyperbolic curve for IDC and fibroadenoma, respectively, using Con A whereas hyperbolic curves for both transformed tissues using PNA. Recently, immunochemiluminescence detection method for the human epidermal growth factor receptor 2 (HER2) in breast tumors was proposed [17].

The antibody anti-galectin-3 was also immobilized onto polysiloxane–polyvinyl alcohol semi-interpenetrated network and the antibody-matrix was capable to capture the serum antigen galectin-3 [18]. ELISA procedure was set up to quantify the Gal3 levels in sera from patients with prostatic adenocarcinoma and benign prostatic hyperplasia and healthy individuals. The optical density (Gal3 level) values established for the sera from patients were lower compared with those found for the healthy individuals. Also, for comparative effect, the Gal3 expression in the prostate tissue through immunohistochemistry was evaluated. Gal3 showed a significant increase and reduction of the cytoplasmatic protein expression in benign prostatic hyperplasia and prostatic adenocarcinoma, respectively, compared with the normal prostate.

Qualitative and quantitative changes in the glycoprotein components of cell membranes are highly significant in the development and progression of many neoplastic processes [19]. In cancer cells the increased expression of surface carbohydrates has been widely documented by histochemistry and immunohistochemis-

Table 1. Result of the immunohistochemical staining from number of positive Gal3 cells in prostatic tissues evaluated by image analysis (total area per field = 12,234 µm²)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Average area (µm²)</th>
</tr>
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<tbody>
<tr>
<td>Benign prostatic hyperplasia</td>
<td>854.6 ± 49.7</td>
</tr>
<tr>
<td>Prostatic adenocarcinoma</td>
<td>118.5 ± 21.8*</td>
</tr>
<tr>
<td>Normal prostate</td>
<td>242.9 ± 24.6</td>
</tr>
</tbody>
</table>

*p < 0.001.

Figure 3 presents typical microscopic view of histological specimens of prostate tissues stained with anti-Gal3-peroxidase/DAB and counter stained with hematoxylin.
try [20]. The galectins, in particular, are being tested as sensitive tools, stable and easy to use to distinguish transformed cells and non-transformed [21-24].

Galectins are found in many cell types and tissues, and various functions are described to them. The galectins have received increasing scientific attention due to its various functions, not only in biochemistry, but also in medicine with possible pharmacological activity. Among the most extensively studied galectins are Gal3, a protein with diverse biological roles [25].

About functional properties of galectin-3, described so far, it is clear that this one has several roles in the pathogenesis of cancer, proliferation and dissemination of metastases. In addition, changes were found not only in the expression of galectin-3 but also in their intracellular distribution in certain types of cancers [26, 27].

Our results are consistent with those described in the literature on prostate cancer, showing that a reduced expression of galectin-3 (Figure 3C) occurs as the disease progresses [25, 28]. And conversely, a higher expression of this protein in tissue fragments of normal when compared to the malignant counterpart [6].

Several evidences have demonstrated the great importance of interactions between cancer cells and residual carbohydrates during cancer progression [21], suggesting the existence of a large number of molecules involved in this biological event.

In the present study analysis based on immunohistochemical staining identified a cytoplasmic expression of Gal3 more intense in cases of benign prostatic hyperplasia (Figure 3A) and normal tissue (Figure 3B). This finding corroborates previous reports [6, 21].

From the results above one can conclude that there was a statistically significant reduction in tissue expression of Gal3 in prostatic adenocarcinoma cells compared to normal tissue. Benign prostatic hyperplasia showed a significant increase in cellular expression of Gal3 when compared with the counterpart malignant and normal tissue. Furthermore, there was a linear relationship between the Gal3 chemiluminescent detection and tissue area in all examined prostate samples. The anti-Gal3-AE conjugate and the treated samples showed to be stables for one year and 72 h, respectively. Thus the antibodies conjugated to acridinium ester can be used as tool to quantify the changes, minimizing the subjectivity of the analysis. This method demonstrated that immunochemiluminescence can be value in tissue analysis, showing high sensitivity and perspective of application in early diagnosis due to its detection at the molecular level.

Acknowledgements

The authors would like to thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo a Ciência de Pernambuco (FACEPE) for financial support.

Disclosure of conflict of interest

None.

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