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Immobilization of Anti-Galectin-3 onto Polysiloxane–Polyvinyl Alcohol Disks for Tumor Prostatic Diseases Diagnosis

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Abstract This work aimed to immobilize the antibody anti-galectin-3 onto polysiloxane–polyvinyl alcohol (POS-PVA) support, to evaluate its capacity to capture the serum antigen galectin-3 and to quantify by ELISA the antigen levels in sera from patients with prostatic adenocarcinoma (PA) and benign prostatic hyperplasia (BPH) and healthy individuals. Also, for comparative effect, the galectin-3 expression in the prostate tissue through immunohistochemistry was evaluated. The optical density (galectin-3 level) values established for the sera from PA and BPH patients were lower compared with those found for the healthy individuals. Galectin-3 immunohistochemically showed a significant increase and reduction of the cytoplasmatic protein expression in BPH and PA, respectively, compared with the normal prostate. These results showed that POS-PVA disks could be used as solid phase to immobilize serum galectins and in immunoassays procedures for the correspondent IgG anti-galectins detection in human sera.

Keywords Polysiloxane–polyvinyl alcohol · Antibody immobilization · Galectin-3 · Prostatic tumor · ELISA

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Introduction

Prostate cancer is the sixth most common cancer in the world and the third leading cause of cancer in men. In developed countries, prostate cancer develops in one in every nine men older than 65 years. Incidence of prostate neoplasm has increased 1–7% per year for the past 15 years [1]. Eventually, the emergence of new molecular markers might increase the consistency of prostate cancer diagnosis and provide more reliable guidelines to define prognosis and therapeutic modalities [2].

Galectins are a family of evolutionarily conserved human proteins, widely distributed from different tissues [3]. In the last few years, significant experimental data have been accumulated concerning their participation in different biological processes requiring carbohydrate recognition such as cell adhesion, cell growth regulation, inflammation, immunomodulation, apoptosis, and cancer [4–6].

The serum and tissue galectins expression, mainly galectins-1 and 3, recently emerged as potential diagnostic and/or prognostic markers of some cancers [7, 8], mainly prostatic cancer [9–11]. Galectin-3 is also expressed in a variety of tumors, and the intensity of the expression depends on tumor progression, invasiveness and metastatic potential [12, 13].

Alterations in the profile of cell surface epitopes are thus likely to reflect shifts in cellular parameters such as differentiation and malignancy [8]. Monoclonal anti-galectins antibodies are instrumental to detect and localize deviations from the glycosylation patterns of normal cells [14, 15].

Antibodies (and antigens) have been used for many years for the specific detection of their complementary partners. Today, immunoassay is the predominant analytical technique for quantitative measurements [16]. Immunoassays based on monoclonal antibodies are still the most important diagnostic methods widely used in clinical and research areas [17].

In the past few years, there has been a significant increase in the number of reports describing the immobilization of biological components into inorganic silicate matrices formed by the sol–gel processing methods [18]. Solid-phase immunoassays are widely used both for diagnostic purposes and basic research. The most common immunoassay is the enzyme-linked immunosorbent assays (ELISA).

The immobilization of antibodies on the solid surface is an essential step in fabricating serologic analysis tools. The immobilized antibodies on the solid supports retain their capabilities of recognizing and capturing their antigens as well as antigen-associated proteins [19].

In our laboratories, disks from a semi-interpenetrated polymeric network of polysiloxane–polyvinyl alcohol (POS-PVA) were synthesized by sol–gel process and were used as solid phase in ELISA for experimental studies in different disease models [20–23].

Here, for the first time, anti-galectin-3 antibody was covalently immobilized onto POS-PVA disks and applied in ELISA method to evaluate the serum concentrations of galectin-3 from patients with prostatic adenocarcinoma (PA) and benign prostatic hyperplasia (BPH). Also, these values were compared to the galectin-3 expression in the prostatic tissue through immunohistochemistry.

Material and Methods

Tissue and Sera Selection

Serum samples were obtained from patients with prostatic adenocarcinoma ($n=18$), benign prostatic hyperplasia (BPH, $n=15$) and healthy individuals ($n=10$) at a private laboratory

(Recife, Brazil) and private hospital (Caruaru, Brazil). Serum samples (1.0 ml) were snap-frozen and stored at −80 °C until further use. Sera from cancer patients were collected prior to treatments. The patients with prostate cancer clinically staged as T1 and T2N0M0 [24] were submitted to radical prostatectomy. The left and right lobes were separated, 3-mm transversal serial sections were obtained from each lobe and the whole tissue was submitted to histological examination.

Prostatic tissue biopsies (PA=22; BPH, n=20 and Normal=20) were obtained from the Pathology Sector archives of the Laboratório de Imunopatologia Keizo Asami (LIKA) of the Universidade Federal de Pernambuco, Brazil. 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 [25], and histological grade according to Gleason score [26]. Patients' ages varied from 45 to 80 years old and agreed to participate in this study and signed a consent declaration.

Solid Support Synthesis

POS/PVA beads were synthesized as follows: 6 ml of 2% *w*/*v* polyvinyl alcohol (MW 72,000; Reagen, Brazil), 5 ml of ethanol (Merck, USA) and 5 ml of tetraethylorthosilicate (Merck, USA) were mixed in a beaker. After raising the temperature to 60 °C, under stirring, 100 µl of 1 M HCl were added and incubation lasted for 1 h. Then, the solution was distributed into Terazaki-style microplates (10 µl/well) and allowed to solidify for about 24 h at 25 °C. Approximately 500 disks were synthesized under these conditions.

Anti-Galectin-3 Antibody Immobilization onto POS-PVA Disks

The disks (seven disks, 10 mg) were incubated with 2.5% glutaraldehyde (100 µl) and 0.1 M H_2SO_4 (900 µl) for 2 h under stirring at 25 °C. Activated disks were incubated with 1 ml of anti-galectin-3 non-marked antibody (SIGMA, St. Louis, MO, USA) for 18 h under stirring at 4 °C.

ELISA

The anti-galectin-3 antibody immobilized onto the POS-PVA disks were then distributed in microplates of 96 wells and serum (1 ml) from patients and healthy individuals, in different dilutions (1:3,200, 1:1,600, 1:800, 1:400, 1:200, and 1:100), were added and incubated for 1 h at 25 °C. Afterwards, the disks containing the immobilized antibody protein complex were incubated with 1 ml of the anti-galectin-3 antibody conjugated to peroxidase for 1 h to 4 °C [27].

Disks were washed five times with 0.1 M phosphate buffered saline (PBS) between each step. Finally, peroxidase substrates (0.325% *w*/*v* ortophenylenediamine dihydrochloride and 0.085% *v*/*v* H_2O_2, prepared in 0.3 M Tris-citrate buffer, pH 6.0, were added and the reaction was stopped after 15 min with 0.1 M H_2SO_4 (25 µl) The disks were removed and the optical density of the supernatants were determined at 450 nm in ELISA reader (BIO-RAD, USA).

Immunohistochemistry

Tissues were fixed in 10% formalin and embedded in paraffin and slices of 4 µm were obtained. Deparaffinized sections were treated with 0.3% methanol containing 0.5 mL

H_2O_2 for 30 min. Following extensive rinsing in 0.1 M phosphate buffer, pH 6.0, containing NaCl 1 M (PBS), normal swine serum (diluted 1:20) was added to the section for 30 min. Anti-galectin-3 antibody (1:100, Novocastra, Norwell MA, USA) was used according to Jakubiak-Wielganowicz et al. [28]. Streptavidine–biotin–peroxidase kit (DAKO, USA) was applied for the antigen detection and diaminobenzidine solution was used for visualization. In control sections, PBS replaced antibody solution.

Computer Image Analysis

Slices images (magnification ×100) 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 galectin-3 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 [29]. Analyses of the number of cells per area (12,234 μm^2) were developed in three random areas of stained tumor tissues. Mann–Whitney U test and Student t test ($p<0.05$) were used to statistical analysis using PRISM 3.0 software.

Results and Discussion

Galectins were detected in numerous cell and tissue types, and various functions were ascribed to them. Multiplicity and diversity of their functions make galectins intriguing enough to become emerging research attractors, not only in glycobiology, but also in Medicine and Pharmacy [3, 30].

Galectin-3, among all subtypes, has been the most studied in cancer cells. They can be considered as functional tumor markers by the emergence of insights into their role in growth control, resistance to or induction of apoptosis and invasive behavior [7, 8].

Galectin-3, one of the most studied galectins, is a pleiotropic homodimeric galectin whose monomer is a 26-kDa protein almost entirely composed of a single carbohydrate-binding site [5]. It has been implicated in several biological processes that are important during tumor progression, such as modulation of cancer cell adhesion, including extracellular matrix components [8, 9].

The galectins were found in the cytoplasm and the nucleus, on the cell surface, and in the extracellular matrix [7]. The presence of galectins outside of the cell is a consequence of secretion via non-classical pathway, since they lack a signal sequence for insertion into the endoplasmatic reticulum [30].

According to Cooper and Barondes [31], the galectins are found both in the cytoplasm and extracellular sites; none has a secretion signal peptide. Instead, several galectins have been shown to be secreted by an unorthodox mechanism. There is also evidence that galectins can simultaneously have distinct intracellular and extracellular functions. Today, the expression of galectins has been shown to be altered during neoplastic transformation of certain cell types, for instance, the galectin-3-stained benign lesions with higher frequency were not specified to be malignant.

In this study, the immunohistochemical detection of galectin-3 showed a significant increase and reduction of the cytoplasmatic protein expression in the benign prostate hyperplasia and the

prostate adenocarcinoma, respectively, compared with the normal prostate (Fig. 1 and Table 1). Ellerhorst [32] reported a significant decrease galectin-3 expression in primary carcinoma and metastatic disease compared with normal and pre-malignant tissue.

To check the usefulness of anti-galectin-3 immunohistochemistry, Jakubiak-Wielganowicz [28] studied its expression in 42 follicular adenomas and 42 follicular carcinomas. The carcinoma cases had only small foci positive with galectin-3 antibody, thirteen cases presented positivity mostly restricted to peripheral part of the tumor and 21 cases possessed diffuse and strong reaction. Also, based on the galectin-3 immunohistochemistry in the differential diagnosis of solitary thyroid nodules, Beesley and McLaren [33] concluded that galectin-3 might serve as a marker for the recognition of follicular carcinoma, particularly the minimally invasive form.

Previous studies have demonstrated expression of galectins in thyroid and endometrial carcinoma [6, 33]. Similarly, the increase of the galectin-3 expression has been correlated with the metastatic potential of several tumorigenic cells, possibly by affecting cell motility and invasion of extracellular matrices [7].

In prostate neoplasm, early experiments demonstrated that the galectin-3 could be participating in cell-extracellular matrix interactions, particularly with laminin and fibronectin [32–34]. On the other hand, they suggest that galectin-3 acts as a bridge, linking cells to the extracellular matrix

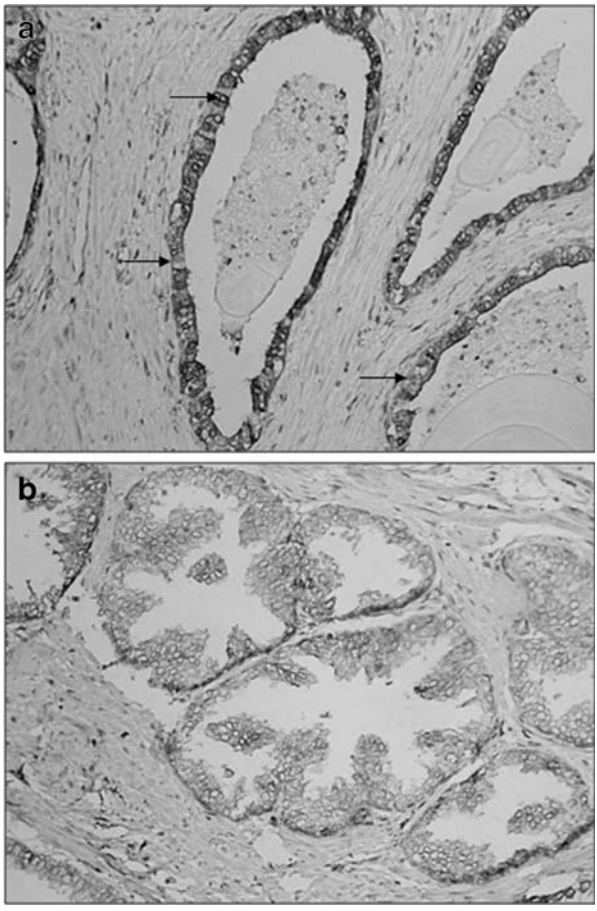


Fig. 1 Imunohistochemical expression of the galectin-3 in prostate tissues. **a** Prostate benign hyperplasia. The gland cells showing intense stain (*arrows*). **b** The glands of the prostate adenocarcinoma were not expressed the galectin-3 protein (magnification ×200)

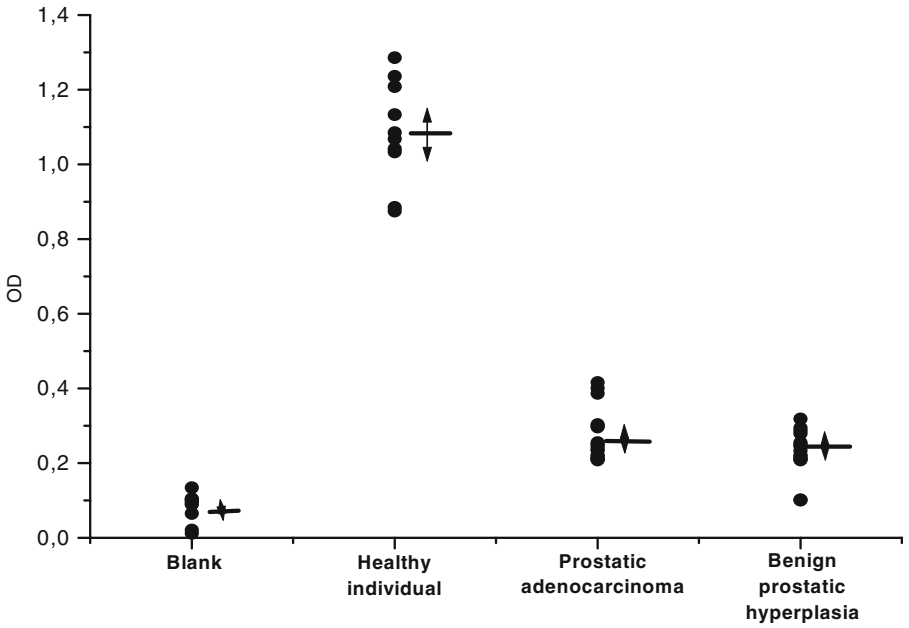
Table 1 Result of immunohistochemistry from number of positive galectin-3 cells in prostatic tumoral tissue evaluated through image analysis (total area per field=12,234 μm^2).

Tissue sample	Cells/per area (mean±SD)	*n*
Healthy individuals	238.4±27.6	16
Prostate adenocarcinoma	120.2±23.3	25
Benign prostate hyperplasia	875.9±52.1	20

$p<0.001$

or to other cells and lends credence to the theory that this protein is involved in the formation of tumor emboli and dissemination of tumor cells in the circulation [8].

Here, the anti-galectin-3 antibody was covalently linked onto POS-PVA composite via glutaraldehyde and ELISA were established for the human serum galectin-3 detection. Previous experiments established the optimal conditions for this ELISA and they were: immobilized antibody, serum and anti-human IgG conjugated to horseradish peroxidase at



Diagnosis	Mean ± SD	n
Healthy individuals	1.085 ± 0.137	10
Prostate adenocarcinoma	0.261 ± 0.078*	18
Benign prostate hyperplasia	0.228 ± 0.061**	15
Blank (PBS)	0.081 ± 0.038	10

The t-Tests presented *($t = -19.28$ and $p = 6.2 \times 10^{-17}$) and **($t = -18.43$ and $p = 2.8 \times 10^{-15}$)

Fig. 2 Optical densities of ELISA by using anti-galectin-3 antibody immobilized onto POS-PVA disks to serum titration 1:800. The means and standard deviations are displayed by *bars* and *arrows*, respectively

2.44 ng/disk; 1:800 and 1,000 to 32,000 times dilutions, respectively. Figure 2 depicts a typical galectin-3 titration under these conditions for the PA, BHP, and normal sera.

The galectin-3 levels were then estimated in all sera and the results are displayed in Fig. 3. Our results showed that the galectin-3 levels are lower in the sera from PA and BPH patients compared with those found for the healthy individuals.

Galectin-3 is found at elevated levels in a variety of neoplastic cells, and several experimental observations suggest that it is involved in tumor progression. Alternatively, galectin-3 high serum levels may reflect an immune reaction to the tumor load from inflammatory cells that are known to express that galectin [28]. However, other studies, including ours, demonstrated an opposite picture, with serum-decreased galectin-3 expression in breast [35, 36], ovary [13], and prostate tumors [10, 37 and this work].

The source of decreased serum galectin-3 in cancer patients remains unclear. According to recent studies, the removal of the tumor decreased serum galectin-3 concentrations, tumor tissues are likely to produce and secrete galectin-3 in sera [7, 11]. However, our results showed that galectins were expressed not only on malignant cells but also in stromal cells in the normal prostate tissue suggesting that circulating galectin-3 is reduced mainly by cancer cells but also from non-neoplasic tumor-like BPH.

Similar to the present study, Iurisci et al. [7] developed an immunoligand assay for the determination of soluble galectin-3 in the circulation from control and cancer patients. The assay used the galectin-3 protein immobilized onto plastics, followed by incubation with rat anti-galectin-3 antibody and peroxidase-labeled goat anti-rat IgG as detecting antibody.

Numerous questions call for elucidation about human galectins. Detailed investigations of various cell populations, tissues, and organ systems of galectin-3 are needed to tie up our knowledge of the physiological role of galectin-3. In order to clarify the precise mechanism of galectin-3 actions, it is of utmost importance that physiologically functional ligands are identified, like auto-antibodies or tumoral antigens [30, 37–39].

A particular significant step in assay design is to capture the protein antigen using a solid-phase-bound antibody and then quantified the amount of captured antigen using a second labeled antibody. This two-site or sandwich immunoassay, which relied on two

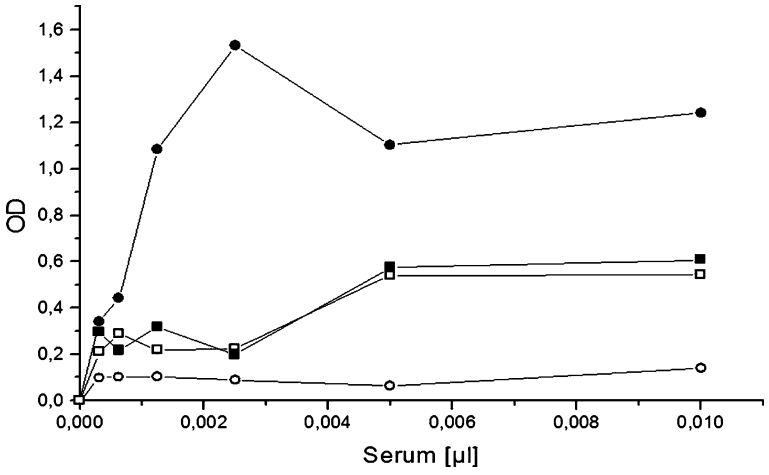


Fig. 3 Comparative serum galectin-3 titration by using anti-galectin-3 antibody immobilized onto POS-PVA disks. Healthy individuals (*filled circle*); adenocarcinoma prostate (*filled square*); benign prostate hyperplasia (*open circle*); PBS as blank (*open circle*)

antibodies recognizing different antigenic epitopes on the analyzed molecule, increased the specificity compared with the previous simple single-site assays [40].

The advantages of sol–gel technique are the homogeneity, purity of the gels, and a relatively low sintering temperature. The sol–gel technique is also an excellent method to prepare hybrid materials. The low temperature synthesis enables organic or inorganic species to be incorporated into rigid silicon oxide matrices without degradation. The resulting composite combines the chemical and the physical properties of the guest material with excellent optical, thermal, and chemical stability of the host silicon oxide matrices [41].

The alkoxide sol–gel process is an efficient method to prepare silica glass by the hydrolysis of alkoxysilanes precursors and by subsequent condensation of the remaining silanols, followed by aging and drying at room temperature. Thus, our group has been developed a support (disks) of hybrid inorganic–organic composite based on polysiloxane and PVA network using the sol–gel technique [20].

In our laboratories, several supports have been proposed for biomolecules immobilization: Dacron [42, 43]; ferromagnetic Dacron [44, 45]; polyvinyl alcohol–glutaraldehyde network [46–48]; polyaniline–Dacron composite [49]; plasticized filter paper with polyvinyl alcohol–glutaraldehyde [50].

Recently, we immobilized the antibody anti-protein S100 in the same net of semi-interpenetrated POS-PVA and concluded that there was a clear difference between tissue and plasmatic expression of S100 protein related to neoplasic prostate tissue [23]. The values for the sera of patients with prostatic adenocarcinoma were significantly lower compared to those established for the healthy individuals. In the immunohistochemistry study, no significant variations were observed in the number of positive S100 cells between prostatic adenocarcinoma and normal prostate. Here, galectin-3 did not show significant differences between tissue and serum expressions in the same tumoral diseases. These results indicate that a down regulation of those proteins is implicated in different mechanisms of biological behavior in the prostate tumors.

Various traditional studies defended a predominant idea to the classic definition of galectins as intracellular, non-immunoglobulin, non-enzymatic carbohydrate-binding proteins. Perhaps it is appropriate to shed this paradigm and search for new ones that focus on the dominant extracellular form of this family of molecules [39, 51]. Thus, further studies are required to determine biological functions and molecular mechanisms underlying the increased or reduced expression of galectin-3 protein in prostate lesions.

Conclusion

The covalent immobilization of IgG anti-galectin-3 onto disks of polysiloxane and polyvinyl alcohol composite yielded a preparation capable to capture galectin-3 from the human serum. This immobilized antibody derivative presents additional advantages of easy synthesis and low-cost reagents. ELISA based on this system allowed to quantify galectin-3 levels. Moreover, this procedure demonstrated that galectin-3 levels decrease in prostatic adenocarcinoma and benign prostatic hyperplasia patients compared with those found in healthy individuals. This contribution showed significant alterations between tumoral diseases and prostate normal in tissue and serum expression of galectin-3. Also, similar plasmatic behavior in relation to galectin-3 between BPH and adenocarcinoma was demonstrated. On the other hand, there was an evident difference in the profile expression to galectin-3 into prostate tissues.

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