Original Article
Evaluation of glycophenotype in prostatic neoplasm by chemiluminescent assay

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Abstract: This work aimed to evaluate the glycophenotype in normal prostate, bening prostatic hyperplasia (BPH) and prostatic adenocarcinoma (PCa) tissues by a chemiluminescent method. Concanavalin A (Con A), Ulex europaeus agglutinin (UEA-I) and Peanut agglutinin (PNA) lectins were conjugated to acridinium ester (lectins-AE). These conjugates remained capable to recognize their specific carbohydrates. Tissue samples were incubated with lectins-AE. The chemiluminescence of the tissue-lectin-AE complex was expressed in relative light units (RLU). Transformed tissues (0.25 cm² by 8 µm of thickness) showed statistical significant lower α-D-glucose/mannose (BPH: 226,931 ± 17,436; PCa: 239,520 ± 12,398) and Gal-β(1-3)-GalNAc (BPH: 28,754 ± 2,157; PCa: 16,728 ± 1,204) expression than normal tissues (367,566 ± 48,550 and 409,289 ± 22,336, respectively). However, higher α-L-fucose expression was observed in PCa (251,118 ± 14,193) in relation to normal (200,979 ± 21,318) and BPH (169,758 ± 10,264) tissues. It was observed an expressive decreasing of the values of RLU by inhibition of the interaction between tissues and lectins-AE using their specific carbohydrates. The relationship between RLU and tissue area showed a linear correlation for all lectin-AE in both transformed tissues. These results indicated that the used method is an efficient tool for specific, sensitive and quantitative analyses of prostatic glycophenotype.

Keywords: Chemiluminescence, lectins, carbohydrates, prostatic cancer, benign prostatic hyperplasia

Introduction

Histochemistry has been a powerful tool in cancer diagnosis and many techniques have been reported in order to identify their pathological molecular alterations. Among cell differentiation/dedifferentiation the oligosaccharide composition and distribution in the cell surface glycoconjugates is under intensive analysis [1]. Lectins have been used to characterize the cancer cell glycophenotype. The most employed visualization of the binding between lectin-carbohydrate in histochemistry is based on enzyme, particularly, peroxidase. The conventional methods and parameters adopted by the analysts in the biopsy analysis are usually based on subjective procedures [2]. It should be advisable to introduce specific and quantitative procedures to assist the morphological analysis.

In our lab, histochemistry chemiluminescence has been proposed replacing enzyme as label by luminescent compound [3-6]. Chemiluminescence has been known to be a powerful analytical technique that exhibits high sensitivity with ultra-sensitive detection limits (atto-mole-zeptomole), selectivity, development of rapid assays and a broad range of analytical applications [7].

Here, the same approach is proposed to evaluate the glycophenotype in prostatic neoplasm. Prostate fragments diagnosed as benign prostatic hyperplasia (BPH), prostatic adenocarcinoma (PCA) and normal human prostate were investigated.
Prostate cancer is the second most frequently diagnosed cancer and the sixth leading cause of cancer death in males [8]. To date, the diagnosis of PCa is based on serum prostate-specific antigen (PSA) testing, digital rectal examination (DRE) and defined by histological examination of prostate core needle biopsies defining the degree of tumor malignancy by the Gleason score [9]. However, this value depends on the analyst. For this reason some methodologies have been tested for helping this widely used technique [10].

In this work the lectins Concanavalin A (ConA), peanut agglutinin (PNA) and Ulex europaeus agglutinin (UEA-I), respectively, specific to α-D-glucose/mannose, Gal-β(1-3)-GalNac and α-L-fucose, were conjugated to acridinium ester (AE). The derivatives (lectins-AE) were used to evaluate the glycophenotype of prostate tissues (PCa, HBP and normal). Previous reports have used these lectins to evaluate the glyco-code of prostate tissues but labeling them with peroxidase [11-13]. Thus a comparison could be established between the present procedure and those contributions. Besides these are biological relevant carbohydrate residues among others [1].

Material and methods

Reagents

N-hydroxysuccinimide-activated dimethyl acridinium ester (DMAE-NHS)/1966-1-53-2/Organic Lab was kindly supplied by Dr. H. H. Weetall. Con A, PNA, UEA-I, N,N-dimethylformamide, trypsin from porcine pancreas, methyl-α-D-mannoside, D-galactose, α-L-fucose and Sephadex G-25 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Chemiluminescent detection was performed using Siemens Reagent TSH 500T (Siemens Medical Solutions Diagnostics; Malvern; PA; USA) composed of 0.5% H₂O₂ in 0.1 N HNO₃, 0.25 M NaOH. Xylene and ethanol were obtained from Merck (Darmstadt, Germany). All other chemical reagents used were of analytical grade.

Samples

Prostate fragments diagnosed as BPH (n=49), PCa (n=50) and normal human prostate (n=5) were obtained through paraffin-embedded biopsies from Tissue Bank of the Clinic Hospital at the Federal University of Pernambuco, Northeastern Brazil. The service pathologists under Dr. Mariana Montenegro de Melo Lira leadership performed the diagnosis of prostatic neoplasms. Patient’s ages for BPH and PCa varied between 47 and 89 years (mean 64) and 58 and 84 years (mean 67), respectively. The Health Science Centre Bioethical Board from Federal University of Pernambuco, Brazil CEP/CCS/UFPE No 195/09, approved this study.

Con A, PNA and UEA-I conjugations with acridinium ester (AE)

AE was conjugated to lectins according to [14]. Briefly, lectins (1 mL containing 2 mg of protein) were incubated with 10 µL of acridinium ester solution (0.2 mg diluted in 400 µL of N,N-dimethylformamide) for 1 h at 25°C under rotary stirring. The conjugate (lectin-AE) was applied to a column of Sephadex G-25 (10 × 1 cm), previously equilibrated with 10 mM phosphate buffer, containing 0.15 M NaCl (PBS) pH 7.2, and eluted with this buffer. Aliquots (1 mL) were collected and their protein content was spectrophotometrically determined at 280 nm. Chemiluminescence of the same aliquots was also assayed with solutions of 0.5% H₂O₂ in 0.1 N HNO₃ (50 µL) and 0.25 M NaOH (50 µL) 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. After conjugation, Con A-AE and PNA-AE were evaluated regarding the maintenance of its carbohydrate recognition property (hemagglutinating activity) using glutaraldehyde treated rabbit erythrocytes and human erythrocytes for UEA-I-AE (Beltrão et al., 1998). Fractions corresponding to protein and chemiluminescence peaks were pooled and protein concentration was measured [14].

Lectin histochemistry

Paraffin section (8 µm) of samples were cut, transferred to glass slides, deparaffinized in xylene (once 5 min, and three-fold 1 min) and rehydrated in graded alcohols (three-fold 100% and once 70% - 1 min each). Slides were treated with 0.1% (w/v) trypsin solution at 37°C for two minutes. Slides were washed (twice, 5 min each time) with PBS. Afterwards tissue slices
Figure 1. ConA-AE (A), PNA-AE (B), UEA-AE (C) purification profile from a Sephadex G-25 column (10 × 1 cm). Elution was carried out with 10 mM phosphate buffer, pH 7.2. Fractions (aliquots of 1 mL) were collected for protein 280 nm (○), chemiluminescence (●) and hemagglutinating activity (◆) assays.
Figure 2. Scheme of the chemiluminescent lectin histochemistry. Paraffin section (8 µm) of biopsies being cut, transferred to glass slide, deparaffinized, rehydrated, treated with trypsin, washed with PBS, incubated with lectins-AE and again washed with PBS. Afterwards the squared-shaped area of tissue section being cut and transferred to a polypropylene test tub and, finally, the chemiluminescence measured and expressed as relative light unit (RLU). A duplicate of the deparaffinized and rehydrated tissue sample was hematoxilin eosin stained for transformed tissue identification.
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were incubated with lectins-AE (100 µL containing 10 µg of protein) for 2 h at 4°C, followed by washing (three-fold 5 min) with 15 mL of PBS. The area corresponding to tissue section (square-shaped) was defined as 0.25 cm² using a homemade mold. Previous to chemiluminescent assay, slices from the selected paraffin-embedded biopsies were stained with hematoxylin eosin and examined by optic microscopy for localizing the tumor area. Then the tissues were cut with a bistoury, removed from glass slides and transferred to a polypropylene test tub containing 50 µL of PBS. Finally, RLU from tissue-squared samples were assayed as described above. Triplicate measurements were carried out throughout this study. Lectin binding inhibition assays were accomplished by incubating each lectin solution with 300 mM methyl-α-D-mannoside (Con A), D-galactose (PNA) and α-L-fucose (UEA-I) for 45 min at 25°C prior to their incubation with tissues. The following steps were those described for the binding protocol (after Lectin-AE incubation).

Area versus carbohydrate expression correlation

The profile of carbohydrate expression, revealed by chemiluminescent emission due to specific lectin binding, was also evaluated as a function of tissue area (0.125 to 1.0 cm² using appropriate homemade molds). These different tissue size samples of BPH and PCa were processed similarly as above described (lectin histochemistry) using Con A-AE, PNA-AE and UEA I-AE conjugates.

Statistical analysis

The software Origin Pro 8 (Origin Lab Corporation, One Roundhouse Plaza, Northampton, MA 01060 USA) was used for the statistical analysis and data were expressed as mean ± standard deviation (SD). Obtained data were compared using ANOVA test followed by post-hoc Tukey test for Con A-AE and UEA I-AE results and Kruskal-Wallis followed by post-hoc Conover test for the PNA-AE results (P < 0.05).

Results

A typical purification profile of Con A, PNA and UEA-I lectins conjugated to acridinium ester is demonstrated in Figure 1 (A, B and C, respectively). It can be observed that all elution profiles showed absorbance 280 nm (protein) and chemiluminescence peaks around the 10th fraction, indicating that the lectins obtained from these aliquots were conjugated to acridinium ester. The other chemiluminescence peaks without concomitant protein presence corresponded to the free ester. Furthermore, the figure demonstrates that after the process of conjugation and elution all lectin conjugates were still capable to recognize their specific carbohydrates by hemagglutinating activity assay. These results showed that the conjugation did
not cause lectin structure alterations to interfere their carbohydrate recognition sites.

Figure 2 schematically illustrates this chemiluminescent lectin histochemistry procedure. Con A-AE, PNA-AE and UEA I-AE conjugates were used to investigate the glycophenotype in prostatic normal and neoplastic tissues. The finding results are displayed in Figure 3. It was observed a lower expression of α-D-glucose/mannose residues, recognized by Con A-AE, between the BHP (226,931 ± 17,436 RLU) and PCa (239,520 ± 12,398 RLU) tissues and the normal (367,566 ± 48,550 RLU). The transformed prostatic tissues presented an expression of α-D-glucose/mannose significantly lower (p < 0.05) when compared to normal prostatic tissues. However the difference between malignant and benign tissues was not statistically significant.

The expression pattern of β-Gal(1-3)-GalNac also decreased in BPH (28,754 ± 2,157 RLU) and PCa (16,728 ± 1,204 RLU) compared to normal tissues (409,289 ± 22,336 RLU). Nevertheless, statistic significant differences were observed among the tissues (BHP and PCa) and between these two conditions and normal tissue.

In relation to α-L-fucose it was observed an increase of the expression in PCa (251,118 ± 14,193 RLU) compared to normal (200,979 ± 21,318 RLU) and BHP (169,758 ± 10,264 RLU) tissues. These differences were statistically significant.

Inhibition assays (Figure 4) was performed using methyl-α-D-mannopyranoside, D-galactose and D-fucose sugars for Con A-EA, PNA-EA and UEA-I-EA, respectively. It was observed an expressive decreasing of the RLU values for all inhibited lectins-AE. However, the inhibition of the PNA-AE was relatively lower (from 27,987 ± 1,649 to 15,242 ± 2,739) in HPB and PCa tissues (from 22,630 ± 1,119 to 14,363 ± 1,891). These decreases in RLU values suggest that non-specific binding between the lectins-EA and cell surface glycoconjugates played not relevant role.

The relationship between RLU and tissue area showed a linear correlation for all lectins-AE and transformed tissues (Figure 5). These results reveal that the intensity of emitted light is directly proportional to available tissue area as well as to the content of exposed carbohydrate residues.

**Discussion**

Glycans are involved in several physiological and pathological conditions such as host-pathogen interactions, inflammation, development and malignancy [15]. The cancer is associated with glycosylation alterations in glycoproteins and glycolipids [16]. These changes can affect interactions between tumor cell-surface glycans and endogenous lectins, which may determine the metastatic potential of the tumor cell [17]. Several common structural changes occur in tumor glycans, including increases in the level of truncation and branching of structures as well as an increased expression of unusual terminal sequences [18].

Studies presented evidences suggesting that the glycosylation of proteins was modified in both BPH and prostatic carcinoma [11-13]. However, these reports employed different approaches from this work. Lectins were
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labeled with peroxidase and the stained tissues were arbitrarily quantified by image analysis.

The present study demonstrated that the transformed prostatic tissues presented an expression of α-D-glucose/mannose significantly lower compared to normal prostatic tissues. However the difference between malignant and benign conditions was not statistically significant. These results disagree with those reported by [13], where they noted an increase of α-D-glucose/mannose expression in hyperplastic tissues compared to normal and prostatic adenocarcinoma tissues. Other study, also observed a high expression of glucose/mannose residues in prostatic tissue; however, it was not observed significant differences among the normal, benign prostatic hyperplasia and prostatic adenocarcinoma tissues.

The recognition of the pattern of Gal-β(1-3)-GalNac expression obtained by PNA-AE according to this work showed a decreasing in these glycans with the tissue transformation process. The RLU values (Gal-β(1-3)-GalNac expression) presented the following order: normal tissue > BHP > PCa. These results are consistent with those demonstrated by [13], despite the difference of methodologies. However, [11] reported different behavior. They noted that PNA presented a lower staining in normal prostate and benign prostatic hyperplasia, and a stronger staining in prostatic adenocarcinoma. It is worthwhile to call attention to the fact that RLU values for PNA-AE regarding the transformed tissues (BHP and PCa) are the lowest suggesting that Gal-β(1-3)-GalNac expression is dramatically reduced under these conditions. The tissue-lectin-AE complex inhibition by galactose was also not so intense like for the other lectins-AE inhibition studies. The analysis of the transformed prostatic tissues with PNA-AE analysis deserves further studies.

With respect the expression of L-fucose residues identified by UEA-I-AE this work demonstrated the increasing expression in PCa compared to normal tissue. However, this expression decreased in BHP that presented a lower RLU values than PCa and normal tissue. These results corroborate those reported by [11, 12]. The increased expression of L-fucose residues in the malignant condition has also been observed in the carbohydrates fraction of serum PSA from individuals with prostatic adenocarcinoma [19].

Lectins conjugated to a chromogenic label have been widely adopted as histochemical probes for investigation of glycopHENotype from various human normal and malignant tissues [20-24]. The analyses of these stained preparations were either performed through light microscopy by an observer or quantified by several morphometric proposals. Here, chemiluminescent probes (lectins labeled with acridinium ester) provided a simple, specific and efficient tool in the quantitative determination of the tissue glycode as previously reported by [3, 4] for human breast tissue. This procedure is the first report for prostatic transformed tissues.

Here, as reported in these previous works from our lab, it was observed relationship between the light emitted by the chemiluminescent reaction (RLU) and the tissue area. The nature (linear, hyperbolic and other parameters) of this relationship difference allows to study the kinetics between lectins and tissues. Therefore, the quantitative advantage of the use of chemiluminescence to establish the glycopHENotype of tissue may add other data to the analysis such as maximum carbohydrate per tissue area and association/dissociation constants.

In conclusion, normal, benign prostatic hyperplasia and prostatic adenocarcinoma tissue samples were incubated with Concanavalin A (Con A), Peanut agglutinin (PNA) and Ulex europaeus agglutinin (UEA-I) lectins conjugated to acridinium ester (Lectin-AE). The chemiluminescence of the tissue-lectin-AE complex expressed in relative light units (RLU) showed statistical signifi cant differences depending on the lectin and the tissue. The light emitted was diminished by inhibiting the interaction between tissues and lectins with their specific carbohydrates. The relationship between RLU and tissue area showed a linear correlation for all lectin-AE and prostatic tissues. These results indicate that the used method is a promising tool for specific, sensitive and quantitative analyses of prostatic glycopHENotype.

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Disclosure of conflict of interest

None.

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