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Full Length Research Paper

Erythrina velutina and *Bryothamnion seaforthii* lectins binding to proteins of primary central nervous system tumors

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This research was done to examine the interaction of lectins with glycoconjugates expressed on Primary CNS tumors using lectins from marine algal *Bryothamnion seaforthii* (BSL) and seeds of *Erythrina velutina* (EVL) Fluorescein isothiocyanate (FITC)-labeled. Samples of different tumor types obtained from the Neurology Service of Sobral-Brazil were processed for histopathologic analysis. Samples were marked with complex lectin-FITC as follows: without any marking (G-I); or incubated with BSA-FITC (G-II), EVL-FITC (G-III), BSL-FITC (G-IV), EVL-FITC + 0.1 M galactose (G-V) or BSL-FITC denatured by heat (G-VI). The quantification of tumor marking was performed by fluorescence intensity. EVL-FITC became the images, more fluorescent than the negative control. There was difference ($p < 0.01$) in fluorescence intensity between G-II/G-IV and G-III/G-V. Comparing G-III/G-IV, higher fluorescence was observed in G-IV ($p < 0.01$). There was no difference ($p > 0.05$) when comparing G-II/G-V or G-IV/G-VI. These lectins have a potential as prognostic marker in primary central nervous system (CNS) tumor.

Key words: Marine algal lectins, central nervous system (CNS) tumor, protein-carbohydrate interactions.

INTRODUCTION

Changes in glycosylation of proteins are associated with many diseases and may be useful as markers of disease states. Both structure and the concentration of carbohydrate in a protein may present themselves changed during the course of a disease, which allows its use as diagnostic information (Chrostek et al., 2011).

The expression of an oligosaccharide can for example, contribute to the metastatic behavior of a tumor cell, as observed when increased expression of GalNAc in hamster kidney cells (BHK) was induced by polyoma virus The result of this change translates into large amount of tetra-antenary oligosaccharides, modifying considerably, the cell surface glycosylation (Yamashita et al., 1985).

Lectins have become well established tools for understanding various aspects of cancer and metastasis, since several studies have demonstrated the modification of cell surface carbohydrates (Pinto et al., 2009; Blonski et al., 2007; Moisa et al., 2007). In oncology, the proposed application to lectins includes its use as diagnostic probes, biological response modifiers and cytotoxic drug carriers for target-directed (Frakking et al., 2006). In general, some factors contribute to the development of detection techniques using lectins, as they seem to have better results when compared to conventional immunological tests using antibodies as a tool (Lopes et al., 2005).

The use of lectins in the therapy of many diseases has

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been discussed elsewhere (Mody et al., 1995; Ofek et al., 2003) but this potential is still far from being fully exploited. On the other hand, these proteins have become well established means for understanding various aspects of cancer and metastasis, given that several studies have shown the modification of surface carbohydrates upon malignant transformation, tumor cell differentiation, and metastasis (Lu and Chaney, 1993; Lu et al., 1994), and lectins are, in several cases, good candidates for detecting such changes.

In the present study, we use fluorescence microscopy to examine the interaction of lectins with glycoconjugates expressed on Primary central nervous system tumors, using lectins from marine algal *B. seaforthii* (BSL) and seeds of *E. velutina* (EVL) FITC-labeled, respectively. Bovine serum albumin (BSA) FITC-labeled was used as negative control in the assays.

MATERIALS AND METHODS

Lectins

Purified BSL was obtained after ion exchange chromatography on a Diethylaminoethyl (DEAE)-cellulose column (Ainouz et al., 1995). EVL was obtained after affinity chromatography on a guar gun column (Moraes et al., 1996). The agglutinating fractions of each lectin were pooled, dialyzed and lyophilized. The purity was confirmed by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). FITC-labeling (Molecular Probes, Inc) of the lectins was performed in 2.0 ml of 0.1 M carbonate/ bicarbonate buffer, pH 9.3, and ethylene glycol (3:1 v/v), using a lectin/FITC ratio of 1:100. The mixture was submitted to constant mixing for 5 h, at 4°C, in the dark. After incubation, the lectin-FITC complex was separated from non complexed FITC by molecular exclusion chromatography using a PD 10 column (Amersham Bioscience) equilibrated with water containing 5% N-butanol. The fractions containing the labeled-lectins were recovered, dialyzed and lyophilized.

Histological processing of the tumors

We studied 60 samples of five different tumor types: astrocytoma, oligodendroglioma, ependymoma, meningioma and medulloblastoma obtained from patients treated at the Neurology Service of Santa Casa de Misericordia de Sobral-Brazil. Samples were taken during surgery and fixed using formaldehyde. The tumor fragments were dehydrated with ethyl alcohol at 70, 80, 90 and 100% (10:1/v:w). The clarification was made with xylene (3 times). The impregnation occurred with paraffin at 56°C. The tumor fragments contained in the paraffin blocks were placed in histological cassettes and finally processed in microtome.

Marking of tumors with complex lectin-FITC

After removal of paraffin, the slides containing samples of each tumor were incubated in phosphate buffer (PBS) 0.1 M, pH 7.4 containing 0.1% BSA. After 10 min, the excess of PBS was removed on each slide. Sequentially, it was added over the entire length of each tumor fragment to a solution containing 10 g/ml lectin-FITC, which remained under constant stirring in shaker for 45 min at 100 cycles/minute. After shaking, the slides were washed 3 times (5 min shaking each) in glass cell with 0.1 M PBS pH 7.4. After washing, the slides were covered with coverslip for further evaluation using a fluorescence microscope Zeiss Axioskop 40, coupled to the source MBQ52 with magnification of 40 and 100 times. For negative control were obtained images of tumor fragments without marking (*in natura*); incubated only with BSA-FITC, incubated with EVL-FITC diluted in a solution containing 0.1 M galactose and BSL-FITC heated in a water bath (100°C for 3 min).

Quantification of tumor marking (fluorescence intensity)

Images of tumor fragments *in natura*, incubated with BSA-FITC, EVL-FITC, EVL-FITC in 0.1 M galactose solution, BSL-FITC and BSL-FITC-denatured by heating, were used to determine the mean fluorescence intensity. First, the images were converted to grayscale range (8 bits) using the relationship described by Gonzalez and Wintz (1987):

$$Gray(x, y) = 0.299 \times Red(x, y) + 0.587 \times Green(x, y) + 0.114 \times blue(x, y)$$

where, Gray (x, y) is the value of gray intensity (digital number from 0 to 255) from the pixel coordinate xy image; Red (x, y) is the value of the intensity of red component (digital number from 0 to 255) from the pixel coordinate xy image; Green (x, y) is the intensity value of green component (digital number from 0 to 255) from the pixel coordinate xy image; Blue (x, y) is the value of intensity of blue component (digital number 0 to 255) from the pixel of the xy coordinate of the image. By means of filters and mathematical operations of mean subtraction on the 8-bit, images were subtracted from the background effects. The same parameters in the processing of images were kept to avoid artifacts in the fluorescence.

The fluorescence of each 8-bit image was quantified by determining the arithmetic mean of the distribution of gray values within the fluorescent region, according to the equation: Fluorescence intensity = $[\sum_y \sum_x \text{Gray}(y,x)]\, n^{-1}$, where n is the total number of pixels of the fluorescent region. The program Image J 1.44o (Wayne Rasband, National Institute Health, USA) was used for the processing and calculation of fluorescence.

Statistical analysis of fluorescence intensity

For statistical analysis of fluorescence intensity measurements, the 60 samples from 5 tumors were divided into six groups: Group I, two samples of each tumor fragments without any marking; Group II, two samples of each tumor fragments incubated with BSA-FITC; Group III, two samples of each tumor fragments incubated with EVL-FITC; Group IV, two samples of each tumor incubated with BSL- FITC; Group V, two samples of each tumor fragments incubated with EVL- FITC with 0.1 M galactose and; Group VI, two samples of each tumor fragments incubated with BSL-FITC denatured by heat (Δ).

Kolmogorov-Smirnov and Shapiro-Wilks tests were used to determine the distribution pattern of the samples (normal or not). We used the nonparametric test Kruskal-Wallis test for determining the existence of significant differences between the means of the following sets: set 1 (Groups I, III and V) and set 2 (Groups II, IV and VI).

The nonparametric Mann-Whitney test was applied for determining the existence of significant differences between the means of the following groups: Group I (*in natura*) and Group II (BSA-FITC); Group II (BSA-FITC) and Group III (EVL-FITC); Group II (BSA-FITC) and Group IV (BSL-FITC); Group II (BSA-FITC) and Group V (EVL + FITC-Gal 0.1 M); Group II (BSA-FITC) and Group

VI (BSL-FITC + Δ); Group III (EVL-FITC) and Group V (EVL + FITC-Gal); Group IV (BSL-FITC) and Group VI (BSL-FITC + Δ). All tests were performed with a significance level equal to 0.01 (α = 0.01).

RESULTS

The interaction between the lectin-FITC complex and the different tumor types evaluated in this study was described in Table 1. It showed the values of fluorescence expressed in arbitrary units, obtained from fragments of Primary Tumors of Central Nervous System. The fluorescence intensity of the 60 tumor samples was assessed using the Kolmogorov-Smirnov and Shapiro-Wilks tests, which revealed that the values of fluorescence did not follow a normal distribution ($p < 0.01$), suggesting the use of nonparametric tests. Thus, the Mann-Whitney and Krukal-Wallis tests were used to evaluate the fluorescence intensity of the samples. There was no statistically significant difference between the negative controls: *in natura* and BSA-FITC ($p > 0.05$, with $z = 0.454$ and confidence interval between 2.576 and -2.576, Mann-Whitney). This finding revealed that there was therefore the difference between the fluorescence images of tumors with no tumor marker and exposure to BSA-FITC, which ensured the use of BSA as a standard negative control.

Comparison (Mann-Whitney´s test) between the images of Groups II and III showed a statistically significant difference ($p < 0.01$). EVL-FITC became the images of the tumor samples, which was more fluorescent than the negative control (BSA-FITC). Similarly, there was a statistical difference ($p < 0.01$, with $z = -3.780$ and confidence interval of 99% between 2.576 and -2.576) between the groups II and IV (BSL-FITC). When comparing groups III and IV, higher fluorescence was observed in the latter group (BSL-FITC), with difference statistically significant ($p < 0.01$).

In assessing the Group III and V (EVL-FITC solution with galactose), there was a statistically significant difference ($p < 0.01$) in fluorescence intensity. When comparing the negative control; Group II and V, nonparametric Mann-Whitney did not show statistical difference ($p > 0.05$, with $z = -0.529$ and confidence interval between 1.960 and -1.960). Thus, the action of galactose in the samples of Group V made this group similar to the negative control (Group II), indicating that this sugar was able to block the binding of the lectin to the structures of tumor cells, strongly suggesting that the binding tumor-lectin was a biological activity.

There was also a significant difference ($p < 0.01$) between the images of Group IV and Group VI (BSL-FITC solution submitted to heat). However, there was no similarity between the negative control (Group II) and Group VI ($p < 0.01$), which could be explained by partial thermal inactivation.

The Kruskal-Wallis' test (KW) was significantly different ($p < 0.01$) between the mean fluorescence intensity of the components of the following sets: Set 1 (Groups II, III and V) and set 2 (Groups II, IV and VI).

DISCUSSION

To the best of our knowledge, this report is the first full investtigation demonstrating composition differences in Primary Central Nervous System Tumor membranes detected by agglutinating proteins from marine algae. In addition, according to our results, *B. seaforthii* (BSL), as well as *E. velutina* (EVL) exhibit distinct profiles for the tumor assayed. These differences could be due to the presence of distinct glyco-receptors, different levels of their expression on the cell surface, or even by discrepancy in the affinity profile of these lectins for these receptors.

Lectins are glycoproteins that can bind reversibly and specifically to carbohydrate structures present in the cell membrane. This property is essential for the identification and differentiation of a neoplastic cell, since the change of surface is directly proportional to genetic changes. Therefore, a tool capable of detecting one malignant cell with high sensitivity and specificity is presented as ideal test for the screening of neoplastic changes.

Several methods of early detection of tumors are under development. There are currently, for clinical use, methods based on biochemistry, immunohistochemistry, MRI and more recently, nanotechnology. The experimental model using immunohistochemical plant lectins for the identification of glycoproteins and neoplastic change related to metastatic spread is under development in our laboratory (Pinto et al., 2009). Combined with this, the biodiversity of the flora is presented as a source of new lectins. This set of regional factors favored the development of a particular model of low cost and is highly promising.

Immunohistochemistry allows accurate characterization and diagnosis of solid tumors, hematological malignancies and infections (Coleman and Tsongalis, 1997). A growing number of antibodies directed against normal and abnormal cellular proteins, as well as infectious agents, are available to diagnose different diseases. With this method, the products of genes are analyzed in tissue sections, allowing the characterization of tumor cell populations as benign or malignant, the determination of cell line and to determine the nature of the molecular genetic changes, leading to a specific process. Despite the rapid development of molecular genetic techniques, immunohistochemistry remains the most important tool in the diagnosis of tumors, adjacent to the recognition of morphological features and clinical correlation (Heim-hall and Yohe, 2008).

The immunohistochemical method may also be useful in the treatment of tumors. It was observed in a study using wheat germ lectin (WGA) and bean (PHA-L) coupled to agarose beads, that the tumorigenic cells were immobilized (Heinrich et al., 2005). There are descriptions

Table 1. Fluorescence values (expressed in arbitrary units) obtained from fragments of primary central nervous system tumors labeled with lectin-FITC.

Tumor	Group I	Group II	Group III	Group IV	Group V	Group VI
	in natura	BSA-FITC	EVL-FITC	BSL-FITC	EVL-FITC + Gal	BSL-FITC + Δ
Astrocytoma	5.965	5.853	12.403*	19.621**	3.401***	8.094#
	3.947	3.527	12.853*	22.466**	3.612***	5.352#
Oligodendroglioma	3.016	3.543	19.120*	31.305**	3.005***	7.677#
	2.795	3.145	16.206*	26.364**	3.842***	6.783#
Ependymoma	3.190	3.199	16.425*	33.114**	4.144***	7.218#
	4.098	4.057	13.509*	19.773**	3.744***	14.208#
Medulloblastoma	2.116	2.776	19.903*	28.912**	3.486***	9.485#
	2.102	2.893	11.981*	34.724**	3.245***	12.122#
Meningioma	4.286	5.608	19.129*	31.853**	6.307***	15.005#
	6.608	6.947	19.679*	37.825**	6.748***	9.903#

*p < 0.01 comparing Groups II and III; **p < 0.01 comparing Groups II and IV; ***p < 0.01 comparing Groups II and V; #p < 0.01 comparing Groups II and VI.

of two ways in which lectins may interact: (1) using a non-toxic lectin coupled to an anticancer drug or; (2) using an anticancer lectin (Banchonglikitkul et al., 2002). In both cases, lectin binds to carbohydrates on the cell surface as a first step. Therefore, cells devoid of carbohydrate residues to which the lectin can bind are not affected by the lectin (Goreli et al., 2001).

The discussion on the use of lectins as recognition molecules and differentiation of glycoconjugates implies the ability of the lectin to recognize and interact with molecules on the surface and within cells, and physiological fluids. This interaction has been extensively studied in different disciplines, from basic science to clinical studies. This multidisciplinary corroborates the importance of developing new methods for studying lectin-saccharide interactions with potential diagnostic applications (Reuter and Gabius, 1999).

In several studies (Fox and Flynn, 1992; Koizumi et al., 1976; Strand, 1995) there is evidence of a biologically active role for albumin, as oxidation of lipids, for example. Comparing the anti-mutagenic effect of BSA, soy protein, total serum protein, the hydrolyzed casein and lactoglobulin, it was observed that only the hydrolyzed casein and BSA were able to protect against genotoxic compounds (Bosselaers et al., 1994). On the other hand, the model used in our study, there was no significant change in the intensity of fluorescence of the samples by the action of BSA. The results showed statistical similarity in fluorescence intensity between the tumor samples, without marking (Group I) and those incubated with BSA-FITC (Group II). This finding validates the use of BSA-FITC as a negative control for this model study.

There was a significant difference ($p < 0.001$) related to fluorescence values between samples treated with BSA-FITC and those treated with lectins conjugated to FITC. Between two lectins (BSL and EVL), BSL-FITC marked more intensely studied tumor fragments ($p < 0.01$), suggesting the presence of aberrant glycoproteins in the membranes of the tumors studied. Similar results were obtained by Pinto et al. (2009) who observed that the lectin BSL coupled to FITC was able to mark cell cultures of human colon cancer, which are rich in variety of Lewis antigens and mucin type O.

Significant result was the reduction of fluorescence levels of tumor samples incubated with FITC-EVL inhibited by 0.1 M galactose. The addition of galactose, known inhibitor of EVL (Moraes et al., 1996), was able to inhibit its binding to the tumor, showing fluorescence values similar to control - BSA-FITC (Figures 1 and 2). This data suggests that more intense labeling of tumor fragments by Group III (EVL-FITC) is due to biological activity of the lectin, since the addition of its inhibitor reduced the intensity of fluorescence values similar to those observed for Group II (BSA-FITC). This outcome also suggests the expression in tumor cell surface, carbohydrate, glycoprotein which binds to EVL as galactose and N-acetylgalactosamine. The presence of glycoproteins containing these sugars was reported in breast cancer tumors (Osinaga et al., 2000).

There is no known monosaccharide able to inhibit specifically the BSL, so heat treatment was performed in BSL-FITC solution to abolish the biological activity of the lectin. However, comparison of Group VI (BSL-FITC denatured by heat) with Group II (BSA-FITC) showed

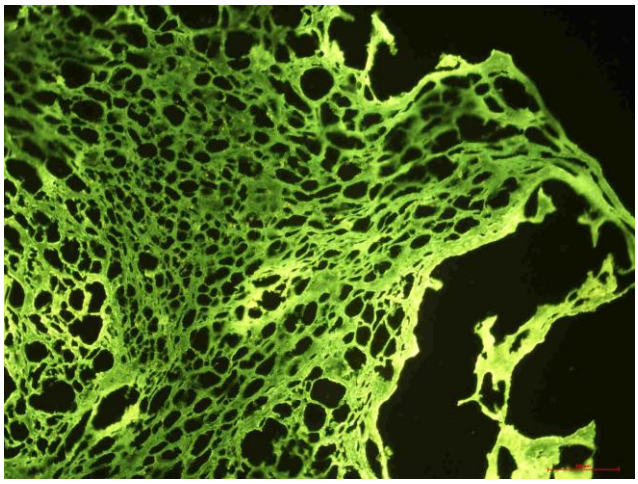

Figure 1. Photomicrograph of medulloblastoma fragment incubated with EVL-FITC (10 µg/ml for 45 min); magnified by 100×.

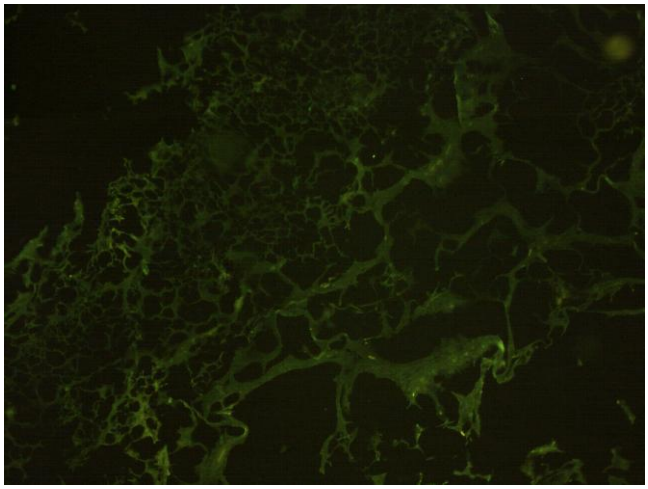

Figure 2. Photomicrograph of medulloblastoma fragment incubated with FITC-EVL + 0.1 M Gal (10 µg/ml for 45 min); magnified by 100×.

significant difference ($p < 0.01$). It is possible that the heat has not completely denatured BSL, remaining biologically active molecules, which is due to the ability of some proteins having to return to its initial structure after lowering the temperature, recovering thus its biological activity. It is known that biochemical inhibition with an antagonist, as occurred between the EVL and galactose is more specific, and results in effective inhibition by a physical agent such as increased temperature, in the case of BSL.

According to data analyzed and discussed, it was found that there was a clear difference between the groups. The lectin isolated from the marine algae *B. seaforthii* was able to interact with the tumor samples far more intensively than the lectin extracted from seeds of *E. velutina*. The comparison between the fluorescence intensity of the Group V (EVL + FITC-Gal 0.1 M) and the Group II (BSA-FITC) strongly suggests that there is an interaction between the lectin and structures present in the tumor membranes.

Outcomes also show that thermal inhibition can be used an alternative to block/reduce the biological activity of BSL, since this lectin is not inhibited by simple sugars. However, it should be emphasized that the thermal inhibition of a protein depends on multiple variables, such as heating time and the presence of disulfide bonds. Even having two disulfide bonds in its structure was more appropriate to use the heating, since the use of reducing agents resulted in high turgor of the solution.

Conclusion

Results presented support the hypothesis of interaction between the tumor surface and the lectins studied. There is no record in the medical literature about this possible interaction of these lectins and the tumor types studied. It is an important step to be able to get the exact description of the molecular nature of ligand structure on the surface of tumor cells evaluated. This finding could lead to the development of new tools for the diagnosis of specific markers of primary tumors of the central nervous system.

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