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Full Length Research Paper

Lectins isolated from Brazilian beans as markers of membrane glycoconjugates of human colon cancer cells

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In this preliminary study the carbohydrate-binding activity of 10 plant lectins from the Diocleinae subtribe Canavalia ensiformis (Con A), Canavalia brasiliensis (Con Br), Canavalia bonariensis (Con Bo), Canavalia grandiflora (Con Gr), Canavalia maritima (Con M), Dioclea grandiflora (DGL), Dioclea guianensis (Dgui), Dioclea virgata (Dvir), Dioclea violacea (Dvio) and Dioclea rostrata (Dros) was studied to determine their capacity to differentiate human colon carcinoma cell variants with respect to cell membrane glyco-receptors. Flow cytometry and confocal microscopy were used to identify the interactions between PITC-labeled lectin and cells. The lectins interacted with cells assayed in a dose-dependent manner. In addition, the fluorescence spectra of Con Bo clearly differentiated the EB3 cells. Furthermore, confocal microscopy showed that Con Bo and Dvio seem to bind greatest to cell variants 8W. These preliminary data suggest a possible use of Diocleinae lectins as tools to identify quantitative and qualitative alterations in membrane glycoproteins during the oncogenic process, facilitating the diagnosis and improving the treatment. However, further experiments involving the use of appropriate controls are in progress.

Key words: Diocleinae lectins, colon carcinoma cells, protein-carbohydrate interactions.

INTRODUCTION

Histochemical studies have demonstrated that quantitative and qualitative alterations are observed in glycoproteins during the oncogenic process (Bafna et al., 2008). In colon cancer, these alterations include a decrease in the carbohydrate content of the glycocalyx which modifies the relation between carbohydrates and proteins. These changes could be observed in any part of the tridimensional structure of the polypeptide chain. Therefore, it is not surprising that altered glycosylation constitutes a universal path for malignant transformation and tumor progression (Cavada et al., 2001). Monoclonal antibody (Mab) studies have made it possible to observe that many tumor-specific Mabs are, in fact, directed toward glycoprotein epitopes, usually oncofetal antigens commonly found in embryonic tissues and tumor cells (Mangiacasale et al., 2003).

Lectins have the ability to recognize specific sugars and make them ideal candidates for detecting modifycations in cell surface carbohydrates upon malignant transformation, tumor cell differentiation and metastasis (Gemeiner et al., 2009). In fact, these proteins have been

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used to understand varied aspects of cancer and metastasis (Lu and Chaney, 1993). Within this context, lectins can be used to investigate alterations in glycosylation during normal and pathological processes and to generate new knowledge, contributing with new techniques to the detection of these alterations. In addition, there are several reasons for the development of detection techniques using lectins as tools; for instance, these proteins show better results when compared to conventional Mabs-based immunological techniques (Mody et al., 2005). In fact, in the last years, the immunomodulating effects of plant lectins have been the focus of considerable interest for application in the treatment of cancer (Andrade et al., 2004; Bains et al., 2005).

Diocleinae lectins are proteins with highly-related amino acid sequences that recognize glucose-mannose residues. Despite those similarities, it has been established that these lectins express distinct biological activities in several different biological models (Cavada et al., 2001). In this preliminary study, 10 plant lectins from the Diocleinae subtribe, obtained from seeds of Canavalia and Dioclea species, were used to investigate their capacity to differentiate variants of cancer cells with respect to cell membrane glyco-receptors.

MATERIALS AND METHODS

Purified plant lectins from seeds of Canavalia ensiformis (Con A), Canavalia brasiliensis (Con Br), Canavalia bonariensis (Con Bo), Canavalia grandiflora (Con Gr), Canavalia maritima (Con M), Dioclea grandiflora (DGL), Dioclea guianensis (Dgui), Dioclea virgata (Dvir), Dioclea violacea (Dvio) and Dioclea rostrata (Dros) were obtained by affinity chromatography on a Sephadex G-50 column following the procedure described by Cavada et al. (1996). The fractions that agglutinated the rabbit blood cells of each protein were pooled, dialyzed and lyophilized. The purity was monitored by 12.5% SDS-PAGE according to (Laemmli, 1970). Phenilisothilcyanate-labeling (PITC labeling from Molecular Probes, Inc) of the lectins were 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/PITC ratio of 1:100. The mixture was submitted to constant agitation for 5 h, at 4°C, in the dark. After incubation, the lectin-PITC complex was separated from non complexed PITC 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.

The human colon adenocarcinoma cell line LS-180 was obtained from Deutsche Krebsforschungzentrum, Heidelberg, Germany. The LS-180 cells were originated from a 58-year old Caucasian female with colon carcinoma Duke's type B. The cells produce a high level of carcinoembryonic antigen (CEA) and express on their surface the tumor-associated carbohydrate antigenic epitopes Lewis X, sialyl Lewis X and Lewis Y. The endothelial cell line HPLNEC.B3 - human microvascular endothelial cells from a peripheral lymph node of a patient with Hodgkin's lymphoma - was isolated and characterized as reported by Kieda and colleague (2002). Cell lines and the further selected variant sublines were propagated in OptiMEM medium supplemented with 3% fetal bovine serum (all reagents from Gibco, Grand Island, N.Y.). Cells were grown in 25 cm^2 tissue culture flasks at 37°C in 5% CO_2, 95% humidified air (Falcon or Costar) and passage weekly, using 0.25% trypsin/0.05% EDTA solution (Gibco, Grand Island, N.Y.).

The in vitro selection of LS180 (EB3) cells with increased affinity for human HPLNEC.B3 microvascular endothelial cells was carried out according to (Nowak et al., 2002). The EB3 variant was subjected to further selection by passing the cells in vivo, in male athymic NCr nu/nu mice, by various routes of inoculation (intravenously, intrasplenically or orthotopically), to select differentially metastasizing variants, essentially as described by Opolski et al. (1998). After repeating passages, several highly metastatic cell variants were obtained. For the lectin binding experiments, LS 180, EB3 and three variants were chosen: the first variant, LS-180EB3 5W (5 W), metastasizing preferentially to the liver, after orthotopic (into intestinal wall) transplantation; the second one, LS-180EB3 3LNLN (3LNLN), metastasizes into peripheral lymph nodes after intravenous inoculation; and the third one, LS-180EB3 (8 W) variant, mainly results in metastasis to the liver, after intrasplenic inoculation. The cells were propagated using OptiMEM medium supplemented with 5% fetal bovine serum and 2 mM glutamine (all reagents from Gibco, Life Sci.) at 37°C, in 5% CO_2, 95% humidified atmosphere. The cells were cultured in 25 cm^2 flasks (Falcon) and later passage using 0.25% trypsin/0.05% EDTA. Since Mycoplasma contamination of cell culture systems causes major problems in basic research, samples of each cell variant were screened for contamination with a Mycoplasma detection kit enzyme immunoassay (Boehringer Mannhaeim, Germany).

In choosing the concentration of lectins to be used in these assays, different doses of each lectin were initially evaluated (1, 2, 5, 10, 15, 20 and 25 µg/ml). It was observed that the lectin-cell interaction increased proportionally with lectin concentration, especially between 10 and 15 µg/ml. These assays were also carried out using glucose, to investigate the inhibition of the activity. The use of sugar that inhibits the activity of lectin is required to ensure that lectins-cells interaction involves the sugar recognition. This is important, since the lectins used in this study are glycoproteins and could be recognized by structures present in the membrane of tumor cells.

For lectin-cell-interaction analysis, cells were collected using 0.05% EDTA solution in PBS and suspended (2 x 10^5 cells) into 200 µl of 10 mM phosphate-buffered saline, pH 7.4 (PBS) containing 0.1% BSA. The cells were incubated with different concentrations of PITC-labeled lectin in 200 µl of PBS/0.1% BSA for 1 h at 4°C, then centrifuged (1000 x g for 5 min) and later washed to eliminate the non-interacting PITC-labeled lectins. The cells were recovered in 1.0 ml PBS and analyzed in a FACSCalibur flow cytometer (Becton-Dickinson). Live cells (5000 counts) were acquired for each data file. Data were processed and the mean fluorescence intensity calculated by the Cell Quest Software (Becton-Dickinson). The values of fluorescence expressed in FU, corresponding to a unit of fluorescein and were compared with the standard immunobrite (GIBCO).

Confocal microscopy analysis was performed using aliquots containing 50,000 cells in 250 µl PBS/BSA 0.1% to which were added 150 µl of PBS containing lectin (25 µg/ml for BODIPY-lectin binding and 10 µg/ml for PITC-lectin binding). The resultant material was then divided in two glass tubes and incubated, separately, at 4 and 37°C, for 45 min in the dark. After incubation, the suspension was centrifuged (1500 rpm) for 5 min at 4°C. The supernatant was eliminated and the pellet recovered in 100 µl of PBS containing 1% paraformaldehyde and incubated for 45 min at room temperature. After vortexing the material, an aliquot of 10 µl of cell suspension was placed on a glass slide and cover slipped. The interaction was analyzed approximately 4 h after the assembly, using a ZEISS

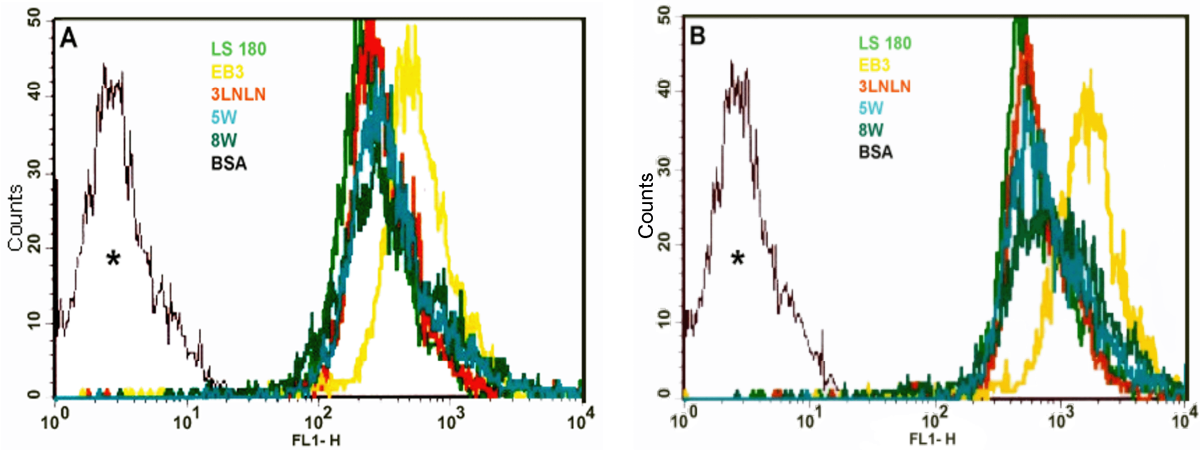

Figure 1. Analysis by flow cytometry showing: A - interaction of Con Bo/PITC-labeled at 37°C with colon carcinoma cells with sugar inhibition (glucose 0,15 M) and B - without sugar inhibitor. In this assays lectins were used at 10 µg/ml, (*) negative control BSA/PITC-labeled.

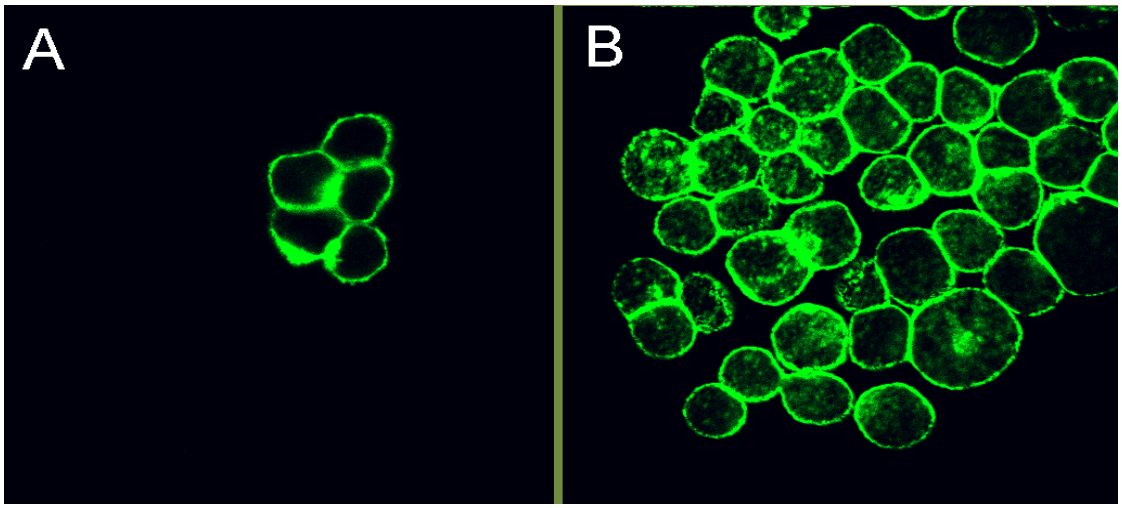

Figure 2. Confocal microscopy showing: A - interaction of 8 W colon carcinoma cells variant with Dvio/PITC-labeled at 4ºC and B at 37ºC. The interaction was analysed approximately 4 h after the assembly by confocal microscope ZEISS Axiovert S 100, containing source Laser MRC 1024, coupled to microprocessor BIO-RAD.

Axiovert S 100 microscope, with a Bio-Rad MRC 1024 confocal system equipped with an argon 488 nm laser.

RESULTS

Flow cytometry results showed that the Diocleinae lectins recognized specific carbohydrate residues present on the cell surface of the cells studied (Figures 1A and B). The interaction observed by flow cytometry was confirmed by confocal analysis at 4ºC and 37ºC. Under these conditions was observed that Dvio and Con A binding strongly to the variant 8 W (Figures 2A, 2B and 3). Among the Canavalia lectins tested, Con A showed the highest affinity for the glycans of the cells studied, especially for the 8 W variant, while the other lectins of the same genus displayed low affinity. However, Con Bo showed to be effective to differentiate the variant EB3 from the other cells, exhibiting ten times more fluorescence units (FU) (Figure 3). Among the lectins of the genus Dioclea, Dvio appeared to recognize especially the variant 8 W, while DGL showed no affinity for any of the cells studied, with FU values almost comparable to those of the negative control (BSA). The interaction of the PITC-labelled lectins with the variants of the human cancer

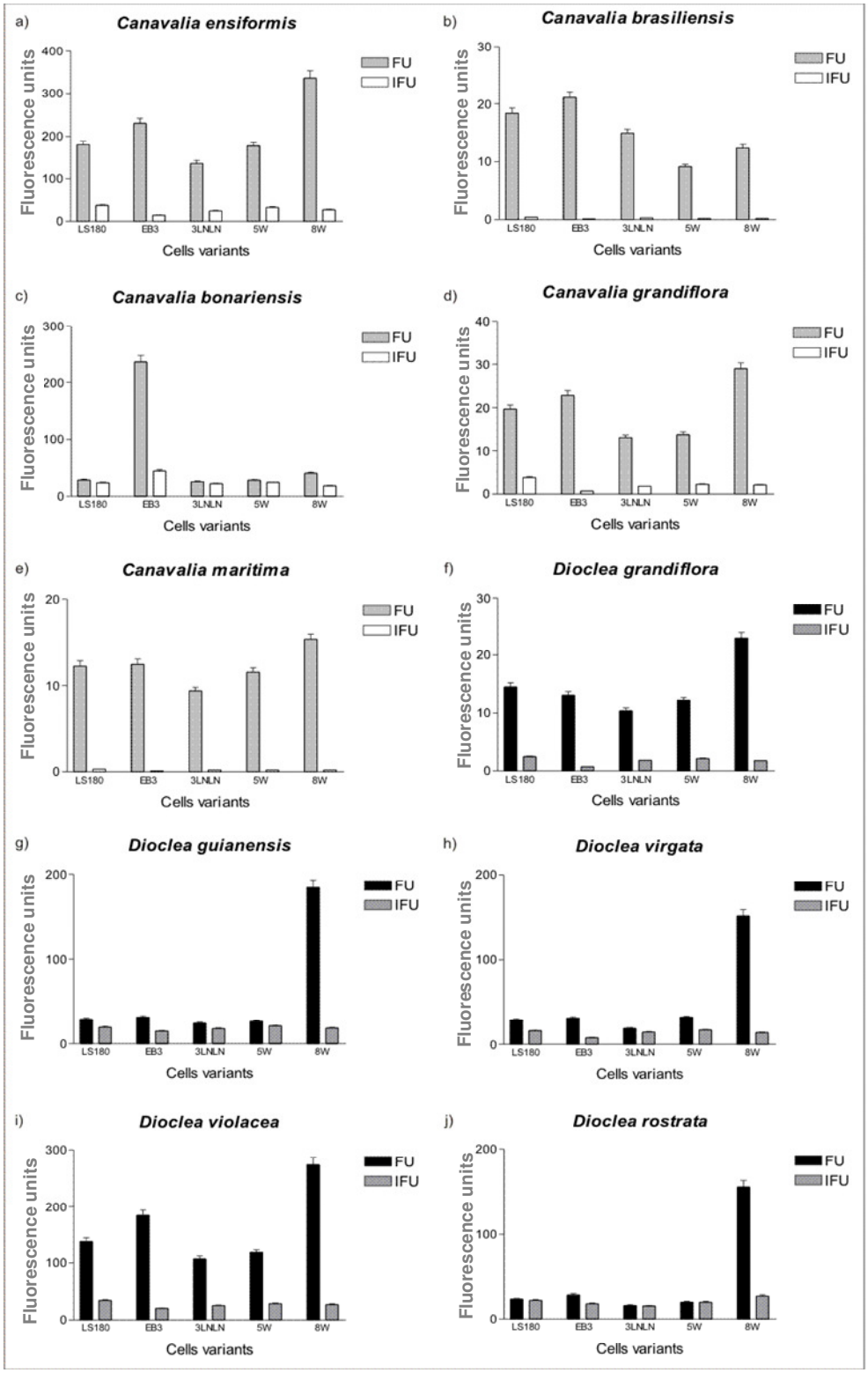

Figure 3. Interaction of the PITC-lectin complexes with variants of human colon cancer cells, carried out at 37°C, without and under presence of glucose 0.15. * FU - fluorescence units detected by FACSort with the goal of 5000 events (mean ± s.d.; $P < 0.05$), corrected using standardization with Immuno-Brite (GIBCO). ** IFU - inhibition of fluorescence units by glucose 0.15 M.

cancer colon cells was also investigated in the presence of a lectin sugar inhibitor, glucose (Figures 1A and B and Figure 3). In general, glucose was able to inhibit the interaction of the lectins with the cell variants, especially Con A and Dvio. Among the Canavalia lectins, the inhibition of glucose depends of the cell studied. For example, Con A, showed values that varied from 179.9 ± 8.9 to 36.8 ± 1.9 (FU) and 230.5 ± 11.5 to 14.1 ± 0.7 (FU) - LS 180 and EB3, respectively, while Con Br and Con M showed that cellular recognition is strongly inhibited by glucose. Under these conditions, Con Bo also differentiated the variant 8 W from the other cells. Among the Dioclea lectins, glucose was able to abolish the recognition mediated by DGL (LS 180) and Dvir (EB3).

The interaction observed by flow cytometry was confirmed by confocal analysis at 4ºC and 37ºC. Under these conditions was observed that Dvio and Con A binding strongly to the variant 8W (Figures 2A, 2B and 3).

DISCUSSION

Diocleinae lectins are characterized by a high degree of homology and they possess a quite conserved three-dimensional structure (Rouge et al., 1991). These proteins exhibit a pH dependent dimer-tetramer equilibrium. At physiologic pH, they constitute a mixture of predominantly full-length polypeptide monomer lectin (chain α), of only one chain (237 amino acid residues) and 40% formed by chain β (residues 1 to 118) and chain α (residues 119 to 237). However, only the tetrameric form is able to cross-react with glycans of the cell membrane, initiating a process of signal transduction (Calvete et al., 1999). Although, these proteins show a high structural similarity, their biological activities seem to be different when they interact with the same biological system and under the same conditions (Barral-Netto et al., 1992; Alencar et al., 1999; Cavada et al., 2001).

According to our results in this preliminary study, Diocleinae lectins exhibit distinct profiles of interactions for the cell types examined (Figures 1A and B). Some of them are able to distinguish the cells studied, which suggest that these proteins seem to recognize subtle alterations present in the membrane glycans (Cavada et al., 2001). These differences could be caused by the presence of distinct glycoreceptors, different levels of their expression on the cell surface, or still by a discrepancy in the affinity profile of these lectins in relation to these receptors. On the other hand, no significant interaction was observed among the negative control (BSA) and cells. BSA was added because it is a protein with well known physicochemical characteristics, without lectin activity and that it is able of binding with PITC. In relation to the Canavalia species, Con Br, Con B, Con Bo, Con Gr and Con M showed low similarity based on he cell variants. The high affinity expressed by Con A to the cell variants may be due to the fine specificity of this lectin. In fact, when examined in other cellular systems, the biological activities of these proteins have been shown to be quite varied (Barral-Netto et al., 1992; Cavada et al., 1996; Cavada et al., 2001; Huan-Yao Lei and Chih-Peng, 2009). In previous studies, it was observed that homologous lectins can produce different responses in a certain cellular system, by varying the potency of the action or even exerting antagonistic effects (Cavada et al., 1996; Cavada et al., 2001).

The carbohydrate-binding activity of lectins has been very useful in studying alterations in cell membrane glycosylation, although, the complexity of this problem cannot be managed by a single approach. In fact, lectin binding to carbohydrate-derived self-assembled mono-layers, showed that the same lectins may switch from one carbohydrate ligand to another as the surface density of the carbohydrate-ligands increases (Horan et al., 1999). This fact exposes the dimension and com-plexity of the problem and suggests that to study or map natural or aberrant cell surface glyco-receptors, not one, but a set of well-characterized lectins with similar ligand capacity should be used. Recently we demonstrated that the algal lectins BSL and BTL were capable of differentiating human colon carcinoma cell variants with respect to their cell membrane glyco-receptors (Pinto et al., 2009).

The finding that the binding of the lectins to the carcinoma cells and the observation of its internalization is quite appealing since they could be used as carriers in cancer-targeted therapy (Mody et al., 2005). There is evidence that drugs that bind better to cancer cells than to normal ones are internalized (Heinrich et al., 2005), but if this drug is bound to a lectin, which is internalized, not only the specificity, but the potency of the drug could be enhanced.

In summary, we demonstrated that the Diocleinae lectins were capable of differentiating human colon carcinoma cell variants with respect to their cell membrane glyco-receptors and could be exploited as a valuable tool to investigate structural modification of cell membrane glycoconjugates in cancer cell systems. In addition, we showed that the binding of these lectins to the carcinoma cells results in their internalization, which is an interesting property that could be used in future applications, such as drug delivery. Thus, we are conducting new experiments, including the use of cells obtained from healthy tissues in order to determine the ability of these lectins to differentiate membrane glycoproteins.

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