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HISTOLOGICAL AND IMMUNOHISTOCHEMICAL EVALUATION OF SARCOMA 180 IN MICE AFTER TREATMENT WITH AN α-D-GLUCAN FROM THE LICHEN *Ramalina celastri*

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ABSTRACT

The antitumoral activity of an α-D-glucan from the lichen *Ramalina celastri* was investigated using the tumor Sarcoma 180 (S-180). Mice were inoculated with the tumor and 24 h later received a single dose of α-D-glucan (200 mg/kg). Thirty-five days after inoculation the mice were sacrificed and the tumors were examined histopathologically. Morphological analyses showed that the tumor was invasive and that it produced typical and atypical mitoses, neovascularization and an infiltration of inflammatory cells. In treated mice, inflammatory cells were more frequent and the presence of nuclear fragments suggested tumor cell death by apoptosis. The tumors of control and α-D-glucan treated mice were negative for laminin but expressed fibronectin, the intensity and distribution of which varied in the connective tissue surrounding the tumor and between tumor cells. Fibronectin expression was more intense in the connective tissue surrounding the tumor mass, in treated mice than in control mice, but in tumor cells, the expression was greater in control mice. The results indicate that α-D-glucan can inhibit tumor growth and affect host defense cell responses. The differences in fibronectin distribution between the control and α-D-glucan treated mice, suggest that this protein may play an important role in limiting the invasiveness of malignant cells.

Key words: α-glucan, antitumoral activity, fibronectin, inflammatory process, Sarcoma 180

Abbreviations: FN, fibronectin; LN, laminin; TNF-α, tumor necrosis factor alpha; S-180, Sarcoma 180; EHS, Engelbreth-Holm-Swarm

INTRODUCTION

In recent years, many studies have focused on the abnormal regulation of growth in neoplasic cells. The major distinction between malignant and benign tumors is the invasiveness of the former and their ability to spread to other regions of the body in a process known as metastasis [2,8,9]. The progression of a tumor and its metastasis depend on the development of an adequate vasculature, through angiogenesis [7]. The interactions between tumor cells and the extracellular matrix are critical during the invasive process [29] and may be influenced by endogenous mediators, such as tumor necrosis factor-α, which can increase the expression of integrins to enhance cell adhesion and migration [13]. Collagen fibers, dense reticular fibers or well-developed cytoplasmic processes, such as frequently observed in myoid liposarcomas in some patients, may have a role in limiting the invasiveness of malignant cells [10]. Since fibronectin and laminin are important in tumor cell migration, adhesion and proliferation [11,23,17], the occurrence and arrangement of extracellular matrix molecules could be a useful parameter for evaluating possible alterations in tumor development.

Polysaccharides from plants and other natural sources exert antitumor, immunomodulatory,

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anticoagulant, and other biological activities. Research over the past 30 years has shown that lichen polysaccharides have similar biological activities and a low toxicity. Lichens have been used for medicinal purposes throughout the ages, and their beneficial effects have been correlated with their polysaccharide content [19]. All lichen species investigated so far contain polysaccharides which account for up to 57% of their total mass [1], and many of them exhibit antitumoral and other biological activities.

Polysaccharides from different sources have antitumoral activity *in vivo* [16,21,31]. The nature of the antitumoral action of most polysaccharides is not entirely clear, but may involve enhancement of the immune system, i.e., they behave as immunomodulators or have cytotoxic activity [3,18,19,24]. Thus, in HeLa cells, an α-D-glucan extracted from the lichen *Ramalina celastri* produced cytoplasmic vacuoles containing acid-like material and a general cellular appearance similar to that of mitotic cells. These modifications were accompanied by the formation of cytoplasmic blebs [6].

Sarcoma 180 (S-180), also known as Crocker's tumor, grows rapidly in most (≥ 90%) animals in which it is inoculated, but regresses in about 8-10% of the cases [25]. This regression rate can be increased significantly by treatment with certain chemicals or biological products [4,12]. In this study we examined the effect of a (1→3), (1→4)-linked α-D-glucan isolated from *R. celastri*, on S-180 tumors *in vivo*. Tumor growth and the expression of extracellular matrix molecules were assessed using light and electron microscopy.

MATERIAL AND METHODS

Glucan

A specimen of the lichen *R. celastri* is deposited as no. 30911 in the Herbário Municipal de Curitiba. The α-D-glucan of *R. celastri* was extracted and purified as described by Stuelp *et al.* [26]. Briefly, samples of lichen were extracted with 2% aqueous KOH at 100°C for 1 h. The resulting solution was neutralized with acetic acid, dialyzed, and subjected to Fehling precipitation. The resulting supernatant was deionised, concentrated to a small volume, and the resulting α-D-glucan was freeze-dried.

Animals

Fifty female Swiss albino mice (6-8 weeks old,18-22 g) were used. The mice were fed Purine® chow and water *ad libitum*. The experiments were done in accordance with current legislation for the use and care of animals in research.

Tumor

The ascitic form of Sarcoma 180 (S-180), obtained from the National Institute of Cancer, Rio de Janeiro (Brazil), was maintained in mice by intraperitoneal inoculation (i.p.). For the present experiments, 100 µl of ascitic fluid containing 5.0 x 10^7 cells/ml in 150 mM NaCl was inoculated into the right dorsal region of mice [14,20,32].

Therapeutic test

Twenty-four hours after the inoculation of S-180, a single dose of α-D-glucan (200 mg/kg) was administered i.p. in 25 mice. The control mice (n= 25) received 150 mM NaCl under the same conditions. Tumor growth was monitored by measuring the tumor size with a pachymeter over a five week serial, after which the mice were sacrificed.

Light microscopy

Thirty-five days after inoculation, the tumors were collected, fixed in Bouin, and embedded in paraffin, sectioned and stained in H.E. For immunohistochemistry, sections 4 µm thick were incubated with rabbit polyclonal monospecific antibodies against fibronectin (diluted 1:500) and laminin (1:500) and developed with a goat anti-rabbit secondary antibody conjugated to peroxidase (1:50). The primary antibodies were diluted in 1% BSA and 0.01% Tween 20 in PBS at pH 7.2, and the secondary antibodies were diluted in PBS pH 7.2. The primary antibodies, which were produced in rabbits and purified according to Ribeiro *et al.* [22], were a gift from Dr. Silvio Sanches Veiga (Laboratório de Matriz Extracelular, Departamento de Biologia Celular, Universidade Federal do Paraná). The secondary antibody was purchased from Jackson Immunoresearch Laboratories Inc (West Grove, PA, USA). The stained sections were observed using an Olympus BHS photomicroscope.

Electron microscopy and immunohistochemistry

Tumors not used for light microscopy were fixed for 2 h in 0.1 M cacodylate buffer, pH 7.2 containing 0.5% glutaraldehyde, 4% paraformaldehyde and 1.0 mM $CaCl_2$, then dehydrated in acetone and embedded in LRWhite. Ultrathin sections were incubated with sodium metaperiodate and then in PBS, pH 7.2, containing 1% BSA and 0.01% Tween 20 for 30 min to block non-specific sites. Antibody to fibronectin was diluted 1:500 in PBS and incubated with the sections overnight at 4°C, followed by rinsing in this buffer prior to incubation with gold-A protein. The ultrathin sections were contrasted with Reynold's solution and uranyl acetate and observed using a Jeol (JEM-200, Japan) transmission electron microscope at the Electron Microscopy Center, Federal University of Paraná.

Semi-quantitative analyses

The presence of inflammatory cells was scored semi-quantitatively as mild (+), moderate (++) and severe (+++), based on the frequency of these cells compared to control mice. Fibronectin expression was scored using the same scale based on the intensity of staining (light microscopy) or number of gold particles (electron microscopy).

RESULTS

Effect of α-D-glucan on the growth of S-180

On the 35th day after inoculation, 8% of the mice in the control group showed no tumor mass, whereas in the α-D-glucan-treated group the percentage of mice without tumors increased to 36%.

Histopathological and immunohistochemical observations

A large portion of the central area of the tumors showed necrosis after 35 days, with some foci of calcification in control and treated mice. Typical and atypical mitoses (Fig. 1) were seen in both groups. Some of the tumor cells formed binuclear or multinuclear giant cells which became swollen and had an intensely eosinophilic cytoplasm. Isolated tumor cells were seen in contact with blood vessels. In treated mice, fragmentation of the tumor cells into probable apoptotic bodies (Fig. 2) was observed and the infiltration of inflammatory cells around the tumor was more intense (+++) than in control mice (Fig. 3A and 3B). The tumors in control and treated mice were negative for laminin but fibronectin was expressed in both groups. In control mice, there was intense fibronectin expression in the extracellular matrix between tumor cells (+++) while in treated mice this expression was mild (+) (Fig. 4). However, in connective tissue delimiting the tumors fibronectin expression was greater in the treated (+++) than in the control mice (+) (Fig. 5).

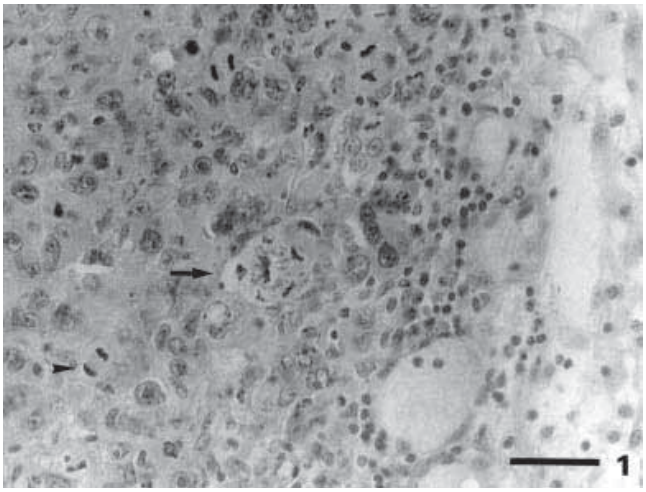

Figure 1. Sarcoma 180 showing typical (arrowhead) and atypical (arrow) mitoses. Bar: 25 µm.

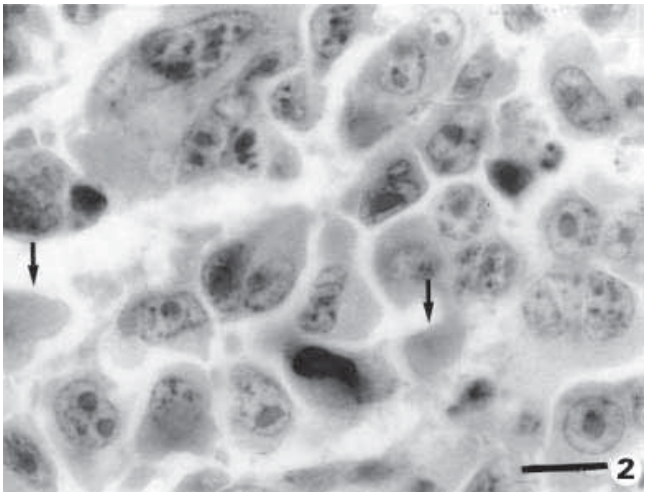

Figure 2. Sarcoma 180 showing apoptotic bodies (arrows). Bar: 10 µm.

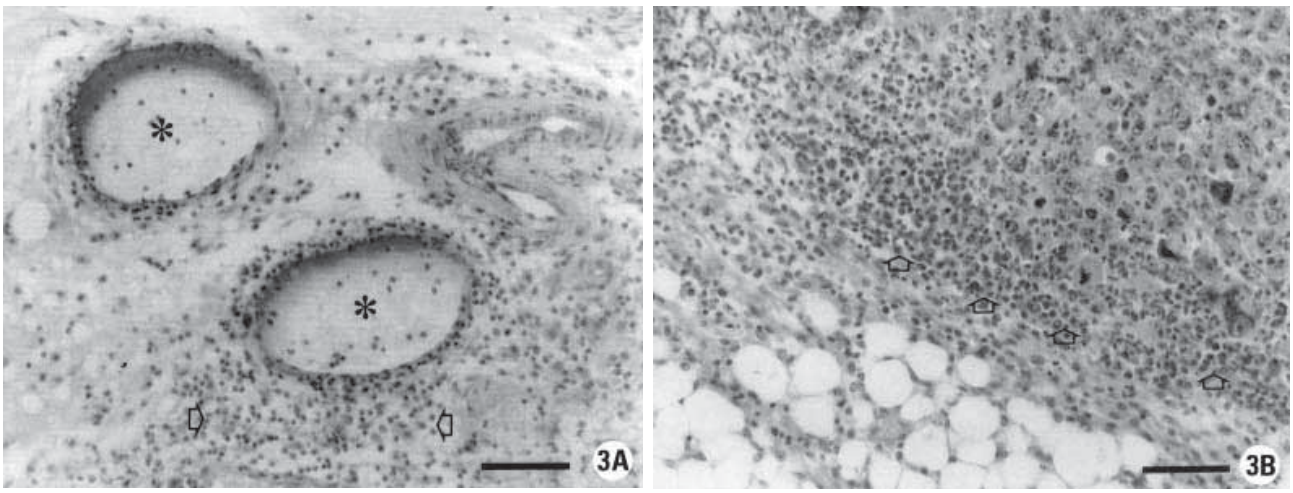

Figure 3. Infiltration of inflammatory cells in control (**A**) and S-180 treated (**B**) mice. Asterisks = blood vessels, arrows = inflammatory infiltrate. Bars: 100 µm (A), 50 µm (B).

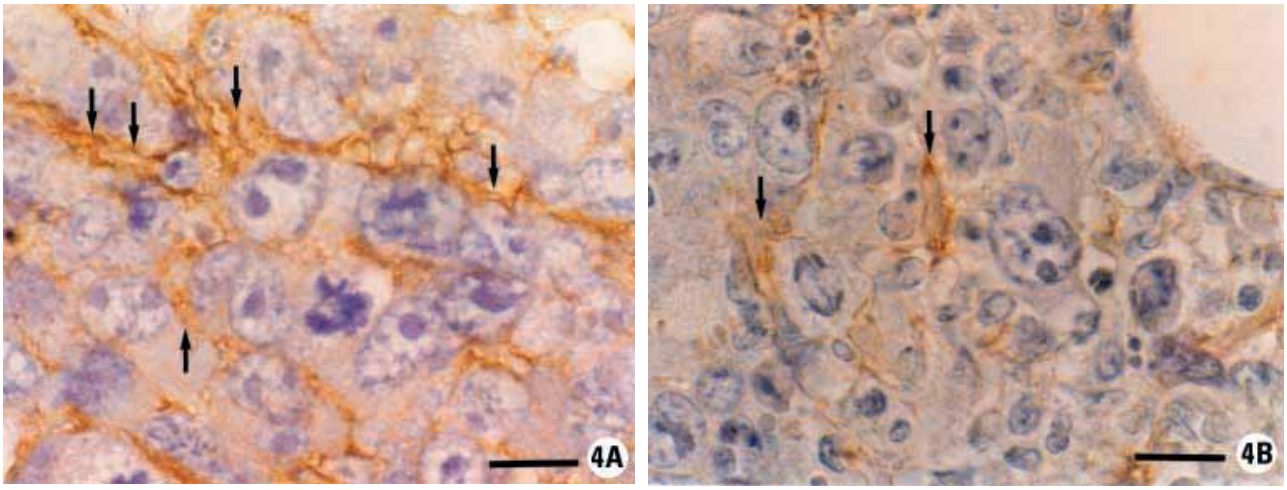

Figure 4. Immunohistochemistry of fibronectin among S-180 tumor cells. Note the intense fibronectin expression in control tissue (**A**). The reaction was mild in treated mice (**B**). Arrows: fibronectin expression. Bars: 10 μm.

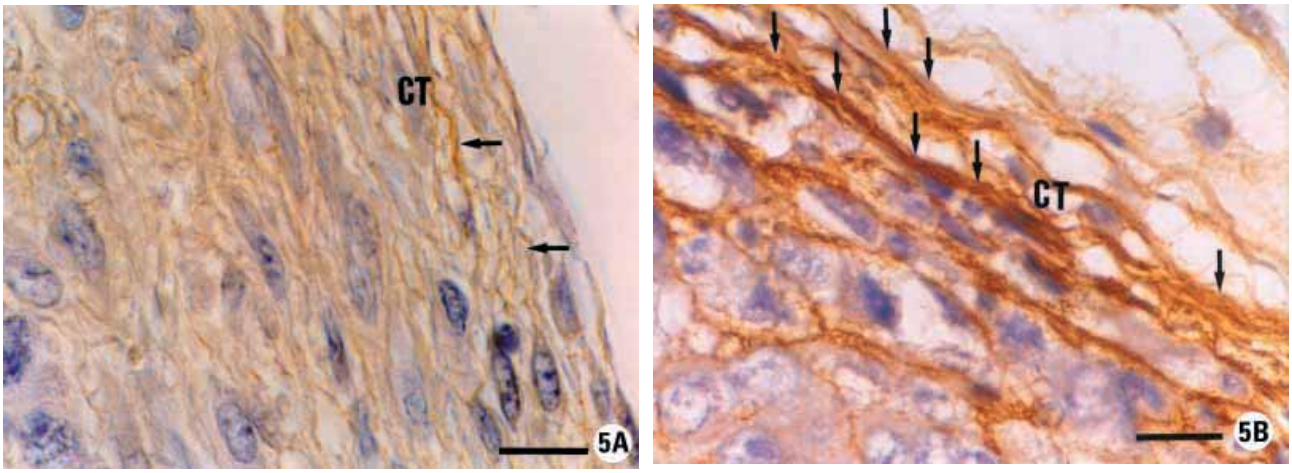

Figure 5. Immunohistochemistry of fibronectin in connective tissue surrounding S-180 tumor cells. The expression of fibronectin in mice treated for 35 days with α-D-glucan from *R. celatri* (**B**), was greater than in the control group (**A**). CT= connective tissue; arrows = fibronectin expression. Bars: 10 μm.

Electron microscopy and ultrastructural immunohistochemistry

Tumor cells were labeled with anti-fibronectin antibodies more in control than in treated mice. Colloidal gold particles were observed in cytoplasmic vesicles and at the cell surface. Fibronectin occurred in the extracellular matrix, in agreement with the results obtained in light microscopy. There were more gold particles between tumor cells in control mice (+++) than in treated animals (+) (Fig. 6). The anti-laminin antibody did not react in either group.

DISCUSSION

The α-D-glucan of *R. celastri* markedly increased the proportion of mice in which there was no development of S-180 tumors, although this result does not imply that a similar effect would occur if the polymer were administered after tumor establishment [26]. Nevertheless, the action observed was antitumoral [15,27,28]. This α-D-glucan and its sulphated derivative produced cell death by apoptosis in HeLa cells, as shown by light and electron microscopy [6].

Preliminary studies using various doses of this α-D-glucan against S-180 showed that the most significant effects occurred at 200 mg/kg, with the tumors being ~80% smaller than in non-treated animals. Light microscopy of S-180 treated with the α-D-glucan of *R. celastri* revealed apoptotic bodies in the extracellular space and an infiltration of inflammatory cells around the tumor. These findings

suggested a role for the polysaccharide in this process. These results agree with other work showing the presence of a plasma and lymphoid cell infiltration, as well as liquefactive necrosis in S-180 cells treated with this α-D-glucan [26,30]. The importance of neovascularization for tumor cell growth and metastasis has been the subject of intensive investigation. New vessels are necessary to supply nutrients needed for the development of these cells [2,9,17]. The observation of this phenomenon in both groups of mice implied that the polysaccharide did not contribute to this mechanism.

The tumors of control and treated mice were negative for laminin, perhaps because of the non-epithelial origin of S-180. Fibronectin was expressed in both groups, although its intensity and distribution varied in the connective tissue surrounding the tumor and in the tumor itself. Fibronectin expression was more intense in the connective tissue surrounding the tumor mass in treated mice than in control mice. However, among tumor cells, fibronectin expression was more severe in the control group than in the treated group. This observation suggested a role for fibronectin in the development of S-180 and that α-D-glucan affected the distribution of this molecule. These findings also indicated that fibronectin may be involved in inflammatory cell adhesion and migration and may limit the invasiveness of malignant cells, as proposed by others [10,29]. Although the mechanisms by which polysaccharides produce their antitumoral effects remain to be elucidated, these compounds have a potential use as coadjuvants in chemotherapy [5].

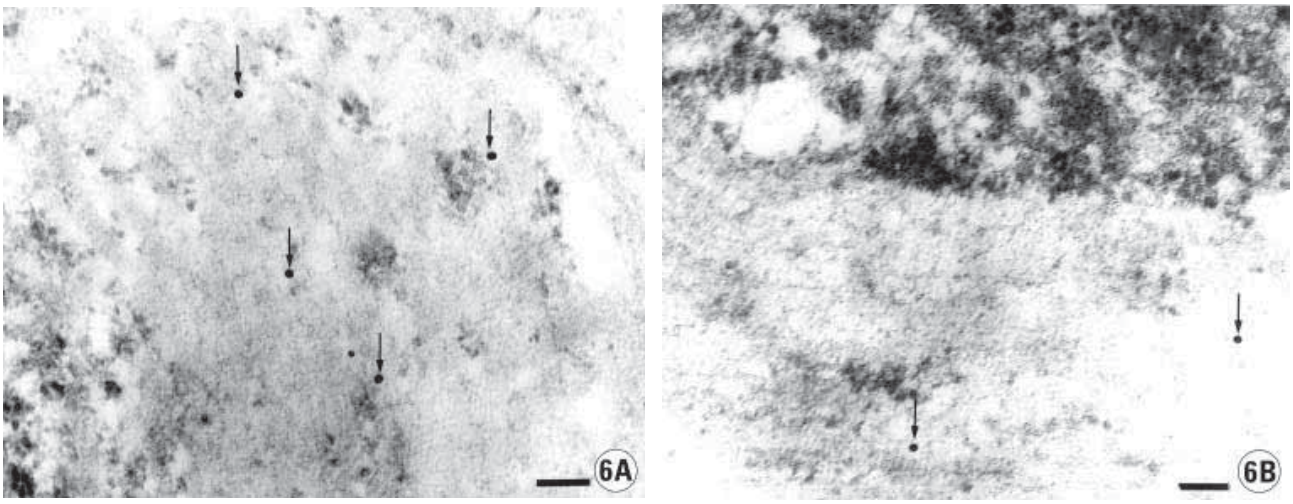

Figure 6. Electron micrographs of S-180. Fibronectin was labeled more in control mice (**A**) than in treated mice (**B**). Arrows = gold particle. Bars: 100 nm.

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