Cancer Immunol Immunother (2007) 56:237–248
DOI 10.1007/s00262-006-0176-0

ORIGINAL ARTICLE

Low-dose cyclophosphamide modulates galectin-1 expression and function in an experimental rat lymphoma model

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Received: 6 March 2006 / Accepted: 20 April 2006 / Published online: 30 May 2006

Abstract In recent years, one of the most important insights into tumor immunity was provided by the identification of negative regulatory pathways and immune escape strategies that greatly influence the magnitude of antitumor responses. Galectin-1 (Gal-1), a member of a family of highly conserved β-galactoside-binding proteins, has been recently shown to contribute to tumor cell evasion of immune responses by modulating survival and differentiation of effector T cells. However, there is still scarce information about the regulation of Gal-1 expression and function in vivo. Here we show that administration of a single low-dose cyclophosphamide (Cy), which is capable of restraining metastasis in the rat lymphoma model L-TACB, can also influence Gal-1 expression in primary tumor, metastasis, and spleen cells and modulate the effects of this protein on T cell survival. A time-course study revealed a positive correlation between Gal-1 expression and tumor volume in primary tumor cells. Conversely, Gal-1 expression was significantly reduced in spleen cells and lymph node metastasis throughout the period studied. Interestingly, cyclophosphamide treatment was capable of restoring the basal levels of Gal-1 expression in primary tumors and spleens. In addition, this antimetastatic agent rendered spleen T cells from tumor-bearing animals resistant to Gal-1-induced cell death. Our results suggest that, in addition to other well-known functions of cyclophosphamide, this immunomodulatory agent may also modulate Gal-1 expression and function during tumor growth and metastasis with critical implications for tumor-immune escape and immunotherapy.

Keywords Galectin-1 · Immunomodulation · Cyclophosphamide · Lymphoma · Apoptosis

Introduction

Galectins, an evolutionarily conserved family of carbohydrate-binding proteins, have recently emerged as novel immunoregulatory agents [39, 40, 44]. Despite extensive sequence homology and similar carbohydrate specificity, various members of this protein family behave as amplifiers of the inflammatory response, whereas others activate homeostatic signals that serve to shut off immune effector functions [39]. Recently, it has become clear that galectin-1 (Gal-1), a member of this protein family, has the potential to inhibit T cell effector functions by promoting growth arrest and apoptosis of activated T cells [5, 19, 29, 32, 36, 37, 41], inducing partial T cell activation [10] and/or blocking Th1 and proinflammatory cytokine secretion [37, 38, 40, 49]. In vivo, therapeutic administration of Gal-1 suppresses Th1-mediated disease in experimental models of chronic inflammation by increasing T cell

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susceptibility to activation-induced cell death and skewing of the immune response towards a Th2 profile [4, 37, 49].

Expression of Gal-1 has been well documented in many different tumor types including astrocytoma, thyroid, bladder, and ovary carcinomas (reviewed in [12, 21, 44]). In general, such expression correlates with the aggressiveness of these tumors and the acquisition of metastatic phenotype [22]. We have recently demonstrated that tumors can overwhelm T cell effector functions through Gal-1-dependent mechanisms [48]. Blockade of the inhibitory effects of Gal-1 within tumor tissue resulted in reduced tumor mass and enhanced tumor rejection, stimulating the generation of a potent tumor-specific Th1-type response in syngeneic mice [48]. However, there is still scarce information available on how Gal-1 is regulated during tumor growth and metastasis in vivo and whether cytostatic or immunomodulatory agents may regulate Gal-1 expression in tumor cells.

To overcome the problems caused by the traditional chemotherapeutic treatment, a great effort is being made toward the search of new modalities of drug administration oriented to a more efficient and non-toxic antitumoral and/or antimetastatic therapy. We have previously demonstrated that a single low-dose cyclophosphamide, a treatment completely devoid of toxicity, inhibits the growth of spontaneous and experimental metastasis of the rat lymphoma L-TACB, while it does not affect primary tumor growth [24]. Such antimetastatic effect was mainly associated with the modulation of antitumor immune responses, which can indirectly influence tumor progression and metastasis [25–28].

As Gal-1 has been associated with the invasive and metastatic potential of several tumor cells [22], we investigated the participation of Gal-1 in cyclophosphamide-induced immunomodulation in the experimental L-TACB lymphoma model. We found that a single low-dose cyclophosphamide induces down-regulation of Gal-1 expression by lymphoma cells with simultaneous up-regulation of spleen Bcl-2 expression; this effect was associated with a decrease in spleen cell apoptosis [42].

In this study, we investigated the kinetics of Gal-1 expression in spleen, primary tumor, and metastatic cells of cyclophosphamide-treated and non-treated L-TACB-bearing rats. For comparison purposes, we also explored the regulated expression of this protein during tumor progression in animals challenged with sarcoma S-E100 and mammary adenocarcinoma M-234p cells. To gain insight into the potential mechanisms underlying this regulated expression, we explored, in the L-TACB tumor model, the effect of Gal-1 on T cell survival. Our present findings show that the increased Gal-1 expression during L-TACB progression is positively correlated with tumor volume and that cyclophosphamide restores the basal levels of Gal-1 in primary tumors and spleen cells. Conversely, the expression of Gal-1 was significantly reduced during the process of metastasis. In contrast, the different kinetics of Gal-1 expression in two other tumor models suggests that a functional dialogue mediated by Gal-1 might exist between the tumor and the immune system, depending on the immunogenicity of the tumor and its spatiotemporal requirements to overcome effector immune responses. Interestingly, cyclophosphamide treatment rendered spleen cells resistant to Gal-1-induced cell death. Thus, a single low-dose cyclophosphamide is sufficient to modulate Gal-1 expression and function in tumor, metastatic, and immune cells, an effect which might have critical implications for tumor-immune escape and cancer immunotherapy.

Materials and methods

Animals

Ten-to-twelve weeks old female inbred IIM e/Fm rats (from here onwards *e* rats) [6], female Fm-*m* rats (from here onwards *m* rats), and female BALB/c mice were housed and cared at the animal facilities of the School of Medical Sciences, National University of Rosario. Animals were fed commercial chow and water ad libitum and were maintained in a 12-h light/dark cycle. All the experiments were done during the first half of the light cycle and in accordance with animal care standards of the institution, which complies with the guidelines issued by the Canadian Council on Animal Care [7].

Tumors

L-TACB is a poorly differentiated B cell lymphoma that arose spontaneously in an inbred *e* rat [8] and metastasizes exclusively to regional lymph nodes when injected subcutaneously [25]. This tumor is maintained by serial subcutaneous trocar implantation of 1-mm^3 tumor fragments (approximately 10^6 cells) in syngeneic rats.

S-E100 is a fibrosarcoma that appeared spontaneously in an outbred population of IIM rats in 1955 and it is maintained by s.c. passages in rats of the allogeneic inbred line *m* with 100% lethality [13].

M-234p is a type B semi-differentiated mammary adenocarcinoma, according to Squartini's classification [51], arisen spontaneously in a BALB/c female mouse. This tumor is maintained in vivo in syngeneic mice.

Drugs

Cyclophosphamide (Cy) (Filaxis Lab., Argentina) was dissolved in sterile distilled water to a concentration of 20 mg/ml and injected at a dosage of 10 mg/kg of body weight.

Recombinant Gal-1 and antibodies

Recombinant Gal-1 (rGal-1) was produced and purified as previously described [20]. In brief, *Escherichia coli* BL21 (DE3) was transformed with expression plasmids constructed using pET expression systems (Novagen, Madison, WI) and production of rGal-1 was induced by the addition of 1 mM isopropyl-β-D-thiogalactoside. Soluble fractions were obtained for subsequent purification by affinity chromatography on an asialofetuin-agarose column and stored at −20°C in 4 mM β-mercaptoethanol to keep its sugar-binding activity under reducing conditions. Lipopolysaccharide content of the purified samples was tested using a Gel Clot Limulus Test (Cape Cod, Inc. East Falmouth, MA). The anti-human Gal-1 polyclonal Ab was prepared in rabbits as previously described [36, 37]. Consistent with the high conservation of Gal-1 throughout animal evolution, this antibody also cross-reacts with mouse and rat Gal-1, as has been previously described [36, 37, 42, 43, 56]. Horseradish-peroxidase-conjugated goat anti-rabbit IgG was purchased from BioRad, USA.

Time-course study of Gal-1 expression during tumor progression and metastasis

The kinetics of Gal-1 expression was studied in spleen, primary tumor, and lymph node metastasis of control and cyclophosphamide-treated L-TACB-bearing rats. The animals were challenged subcutaneously with the tumor, on day 0, and were distributed in six groups as shown in Fig. 1. Primary tumor, metastasis, and spleen were excised on the indicated days. A T-cell-enriched non-adherent population of spleen cells was obtained as described [43]. Protein homogenates prepared from each sample were used for Western blot analysis. The kinetics of Gal-1 expression in *m* rats bearing S-E100 and BALB/c mice bearing M-234p tumors was also studied. The experimental procedure was essentially the same as indicated in Fig. 1.

Protein extracts preparation

Protein extracts from spleen, primary tumors, and the respective lymph node metastases were prepared in lysis buffer [2 mM EDTA (Sigma) + 4 mM EGTA (Sigma) + 10 mM β-mercaptoethanol (Sigma) + 1 mM phenylmethylsulfonyl fluoride (Sigma) + 10 μg/ml aprotinin (Boerhinger, Mannheim, Germany) in 20 mM Tris–HCI, pH 7.5 at 4 °C]. Protein cell lysates were centrifuged at 2,500×*g* for 10 min at 4 °C. Protein concentration of each sample was determined using the micro-BCA kit (Pierce, Rockford, IL).

Western blot analysis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed in a Miniprotean II electrophoresis apparatus (BioRad, Richmond, CA). Briefly, lysates corresponding to spleen, primary tumors, or metastases (30 μg of protein per lane) were diluted in sample buffer and resolved on a 15% separating polyacrylamide slab gel. After electrophoresis, the separated proteins were electrotransferred onto nitrocellulose membranes (Hybond-C, Amersham-Pharmacia Biotech, Piscataway, NJ) and probed with

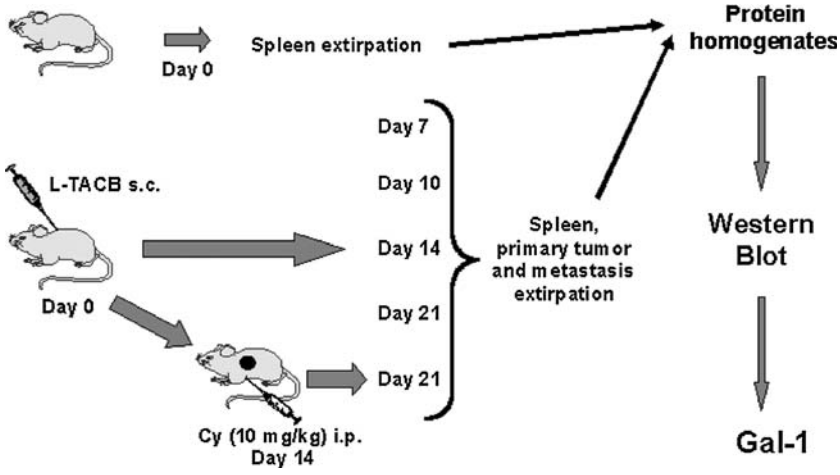

Fig. 1 Experimental model for the study of the kinetics of *Gal-1* expression in *L-TACB* tumor model

1:1,000 dilution of the anti-Gal-1 Ab, as described [48]. Blots were then incubated with a 1:3,000 dilution of a horseradish-peroxidase-conjugated goat anti-rabbit IgG, developed using enhanced chemoluminescence detection and finally exposed for 6 min to Kodak Biomax ML films. rGal-1 was used as a positive control for immunodetection. Equal loading in each lane of the gel was checked by Coomassie blue staining and Ponceau staining of nitrocellulose membranes. The films were scanned with the ScanJet 2400 (Hewlett Packard, Miami, FL, USA) and the bands were semiquantified. Results were expressed as relative densitometric values.

Immunohistochemistry

For immunohistochemical analysis of Gal-1 expression, excised L-TACB primary tumors, treated or non-treated with a low-dose cyclophosphamide and lymph node metastasis, were fixed immediately in 10% buffered neutral formalin and then were dehydrated and paraffin-embedded. Immunostaining was performed in 5-μm tissue sections placed on plus-charged glass slides. After deparaffinization in xylene and graded alcohols, endogenous peroxidase was quenched by immersing the sections in 0.03% hydrogen peroxide for 5 min. Non-specific binding was blocked with normal goat serum. Slides were washed for 5 min with Tris-buffered saline solution containing Tween 20 at pH 7.6 and incubated with rabbit anti-Gal-1 antibody (1:50), followed by incubation with 1:200 horseradish-peroxidase-conjugated goat anti-rabbit IgG antibody. These antibodies were the same as those used for Western blot analysis. Sections were then visualized with 3,3′-diaminobenzidine (Sigma) as a chromogen for 5 min and were counterstained with methyl green. Negative controls were performed avoiding the primary antibody. Slides were washed in tap water, dehydrated, and mounted with glass coverslips.

Effect of Gal-1 on a splenic T-cell-enriched subpopulation isolated from cyclophosphamide-treated L-TACB-bearing rats

Rats ($n = 8$ per experiment) were implanted s.c. with L-TACB by trocar on day 0. Half of the animals were i.p. inoculated with cyclophosphamide (10 mg/kg) on day 14 (Group I) and the remaining rats received no further treatment (Group II). The animals were sacrificed on day 21 and spleens were removed. Single-cell suspensions were prepared in RPMI 1640 culture medium (GibcoBRL, Life Technologies, Grand Island, NY, USA) by mechanical disruption; cells were washed twice and resuspended in RPMI 1640 culture medium supplemented with 10% FBS (Natocor, Córdoba, Argentina) at a concentration of 1×10^6 cells/ml. The viability of spleen cells prepared under this procedure was above 90%, as determined by a trypan blue exclusion test. A T-cell-enriched non-adherent population of spleen cells was obtained as described [43]. Each cell suspension was seeded in 96-well flat-bottomed microplates (Corning Costar) in quadruplicates and incubated 96 h at 37 °C and 5% CO_2 with 4 μg/ml rGal-1. Two identical plates were seeded for viability and apoptosis assays.

Viability

After incubation with rGal-1 (4 μg/ml), cell viability was determined with the MTS/PMS colorimetric assay. Briefly, 20 μl of the combined MTS (tetrazolium salt)/PMS (electron coupling agent) (20:1) was added into each well of the 96-well microplate containing the samples. The plates were incubated for 4 h in a humidified 5% CO_2 atmosphere. The conversion of MTS into the aqueous soluble formazan is accomplished by deshydrogenase enzymes found in metabolically active cells. The quantity of formazan product, as measured by the amount of absorbance at 490 nm, is directly proportional to the number of livings cells in culture.

Apoptosis

After 96-h incubation, cells were collected and stained with FITC-conjugated annexin-V (Apoptosis Detection Kit, Oncogene Research Products, Boston, MA). Quantification of early apoptotic cells was performed in a fluorescence microscope, counting 100 cells/sample.

Statistical analysis

Student's t test, χ^2, and Pearson correlation tests were performed using GraphPad Prism® version 3.03 (GraphPad Software, San Diego, CA). Differences between groups were considered significant when $p < 0.05$.

Results

Kinetics of Gal-1 expression during tumor growth in primary tumors, metastasis, and spleen cells

The kinetics of Gal-1 expression during tumor growth was studied in L-TACB primary tumor, spleen, and lymph node metastasis (Fig. 2a–c, respectively) of

tumor-bearing animals, treated or not with cyclophosphamide. The expression levels of Gal-1 in the primary tumor, 7 days after tumor challenge, was similar to those found on day 10. In contrast, Gal-1 expression significantly increased on days 14 and 21 with respect to day 7 (34%, $p < 0.01$ and 31%, $p < 0.05$, respectively), as we had previously demonstrated [42]. Interestingly, after cyclophosphamide treatment, Gal-1 expression was restored to basal levels obtained on day 7. Indeed, the expression levels of Gal-1 were significantly lower in primary tumors of treated rats on day 21 than those observed in non-treated animals on the same day ($p < 0.01$) (Fig. 2a). Conversely, spleen cells showed a decrease in Gal-1 expression throughout tumor progression when compared to the basal level obtained on day 0. Cyclophosphamide treatment induced, on day 21, an increase in Gal-1 expression, which reached the basal levels found on day 0. In fact, the expression of Gal-1 on day 21 in spleen T cells of cyclophosphamide-treated rats was significantly higher than that observed on the same day in non-treated animals ($p < 0.05$) (Fig. 2b). In contrast, Gal-1 expression was significantly lower in lymph node metastasis when compared to the respective primary tumors ($p < 0.001$) (Fig. 2c).

To confirm these results, we performed immunohistochemical analysis on tissue sections of primary tumors treated or not with cyclophosphamide and respective sections of a lymph node metastasis (Fig. 3). In agreement with the results observed by Western blot analysis, Gal-1 was expressed at a lower intensity in the tumor tissue of cyclophosphamide-treated rats (Fig. 3c), compared to the primary tumor of control rats (Fig. 3a). In contrast, Gal-1 was expressed at a lower intensity in metastatic tissue compared to primary tumors (Fig. 3b). As a control of immunoreactivity, the primary anti-Gal-1 antibody was omitted (insets). All tumor samples showed specific nuclear and cytoplasmic Gal-1 staining.

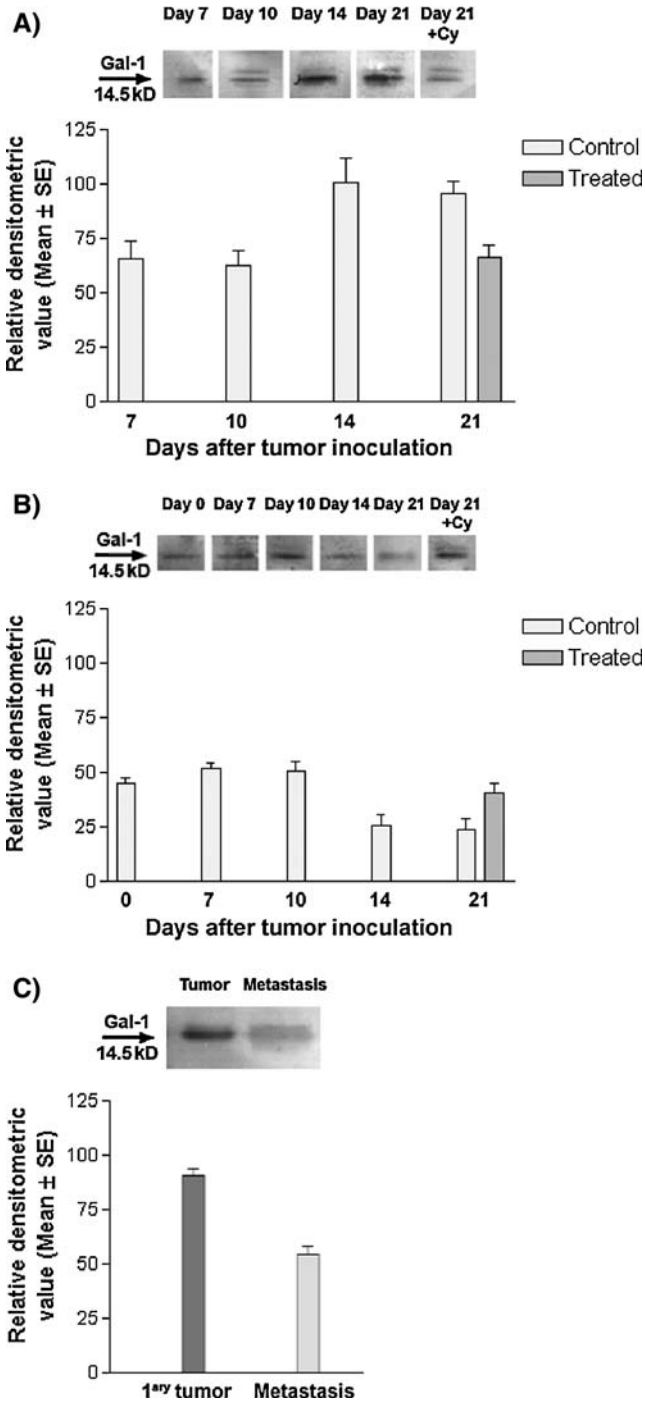

Fig. 2 Kinetics of *Gal-1* expression in *L-TACB* tumor model. Extracts corresponding to tumor cells (**a**), a splenic T-cell-enriched population (**b**), and metastatic cells (**c**) were prepared and processed for Western blot analysis. Briefly, samples were lysed in the presence of protease inhibitors and equal amounts of protein (30 μg) were subjected to *SDS–PAGE* on a 15% polyacrylamide slab gel and immunoblotted with a rabbit anti-Gal-1 polyclonal Ab (1:1,000). Blots were then incubated with a 1:3,000 dilution of a horseradish-peroxidase-conjugated goat anti-rabbit IgG and developed using enhanced chemiluminescence detection. Recombinant *Gal-1* was used as a positive control. The film image shows one representative out of four animals tested for each time point. Immunoreactive protein bands (indicated by the *arrow*) were semi-quantified by densitometry and expressed as relative densitometric values. Equal loading in each lane was checked by Coomassie Blue staining of the gels and Ponceau S staining of nitrocellulose membranes. Mean ($n = 4$) ± SEM of three independent experiments is shown

For comparison purposes and in order to evaluate whether the regulated expression of Gal-1 could be extrapolated to other tumor types, we also analyzed the kinetics of Gal-1 expression in the rat S-E100 fibrosarcoma. As shown in Fig. 4a, no significant changes in Gal-1 expression could be observed during tumor progression. However, on days 14 and 21 spleen cells from fibrosarcoma-bearing animals expressed significantly less Gal-1 than that observed on day 0 (Fig. 4b).

In addition, the M-234p adenocarcinoma showed a kinetics of Gal-1 expression which substantially differed from that observed both in L-TACB and S-E100. In fact, a significant decrease in Gal-1 expression was observed which paralleled tumor growth. When compared with the level observed on day 10, the decrease was statistically significant on day 30 ($p < 0.05$) and was marginally significant ($p = 0.07$) on day 40 (Fig. 5a). In addition, no changes in Gal-1 expression were observed in spleen cells corresponding to M-234p-bearing mice (Fig. 5b).

Correlation between tumor volume and Gal-1 expression

To statistically evaluate the quantitative changes observed in the levels of Gal-1 expression during tumor growth, a correlation between L-TACB volume and Gal-1 expression by primary tumor cells was determined using the Pearson test. As shown in Fig. 6, the levels of Gal-1 expression positively correlated with tumor volume ($r^2 = 0.12$; $p < 0.05$).

Effect of recombinant Gal-1 on a splenic T-cell-enriched population from cyclophosphamide-treated L-TACB-bearing rats

As Gal-1 has been shown to induce apoptosis of activated T cells [5, 19, 29, 32, 36, 37, 41] but not of other immune cells [11, 43, 56], we next investigated the viability of a splenic T-cell-enriched population from cyclophosphamide-treated L-TACB-bearing rats incubated or not with recombinant Gal-1. As shown in Fig. 7a, T-cell-enriched spleen cells from cyclophosphamide-treated tumor-bearing animals (Group III) demonstrated a higher viability than T-cell-enriched spleen cells from non-treated tumor-bearing rats (Group I) ($p < 0.05$). Interestingly, splenic T cells from cyclophosphamide-treated tumor-bearing animals incubated with recombinant Gal-1 (Group IV) showed significant increased viability when compared with splenic T cells from non-treated tumor-bearing animals incubated with recombinant Gal-1 (Group II) ($p < 0.01$). These observations suggest that cyclophosphamide treatment in vivo modulates T cell susceptibility to Gal-1. Furthermore, we could not find significant differences in spleen cells from cyclophosphamide-treated animals whether incubated (Group IV) or not (Group III) with recombinant Gal-1. In addition, the viability of spleen cells from non-treated tumor-bearing animals incubated with Gal-1 (Group II) was significantly lower than that of control spleen cells (Group I) ($p < 0.05$) in agreement with the ability of Gal-1 to negatively regulate T cell survival.

To correlate this finding with the levels of Gal-1-induced cell death, we also evaluated the levels of apoptosis using the Annexin V binding test. As shown in Fig. 7b, incubation of a splenic T-cell-enriched population with recombinant Gal-1 significantly increased the levels of apoptosis (Groups I vs. II: $p < 0.001$). Furthermore, administration of cyclophosphamide at day 14 did not change the levels of splenic T cell apoptosis from tumor-bearing rats (Group I vs. Group III). Interestingly, a significant decrease in the number of apoptotic cells was observed in the cyclophosphamide-treated group compared with the non-treated group when both groups of cells were incubated with recombinant Gal-1 (Group II vs. Group IV: $p < 0.01$). Moreover, the levels of apoptosis shown by splenic T cells from cyclophosphamide-treated L-TACB-bearing rats could not be modified by the incubation with recombinant Gal-1 (Group III vs. Group IV) (Fig. 7b). Thus, cyclophosphamide treatment confers T lymphocyte resistance to Gal-1-induced cell death, suggesting another molecular target for this immunomodulatory drug.

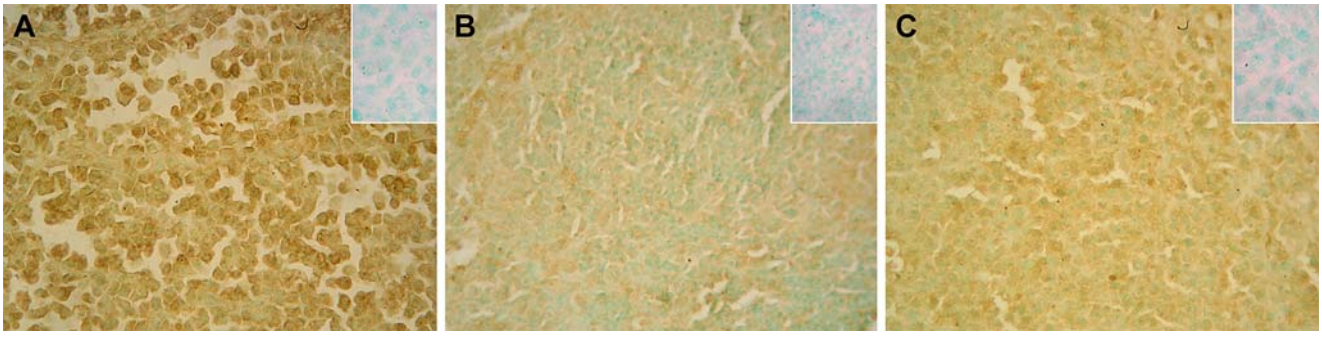

Fig. 3 Immunohistochemical analysis of *Gal-1* expression in sections of primary tumor (**a**), lymph node metastasis (**b**), and primary tumor from *Cy*-treated rats (**c**) (× 40). Tissue samples were obtained after 21 days of tumor challenge. The intensity of *Gal-1* expression was lower in the tumor of the treated animals (**c**) and in the metastatic tissue (**b**) when compared to the tumor of non-treated rats (**a**). Insets indicate negative controls including tumor sections exposed to secondary antibody in the absence of the anti-*Gal-1* antibody

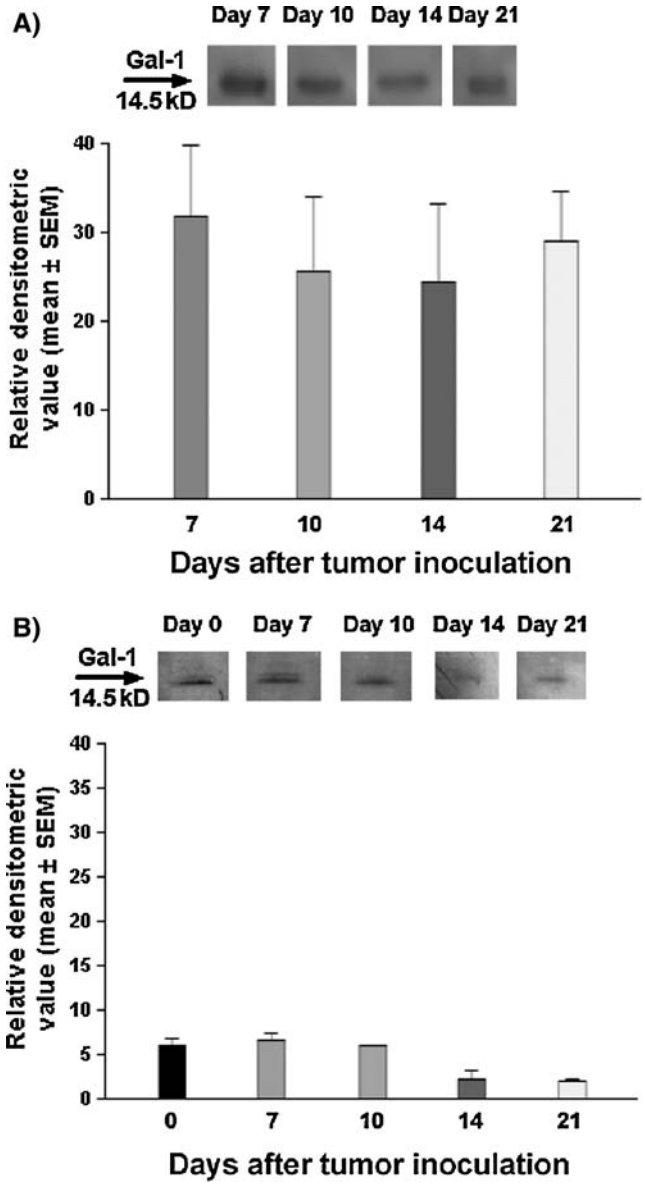

Fig. 4 Kinetics of *Gal-1* expression in S-E100 tumor model. Extracts corresponding to tumor cells (**a**) and a T-cell-enriched population purified from spleen cells (**b**) were prepared and processed for Western blot analysis. The procedure was the same as described in Fig. 2. The film image shows one sample out of four animals tested for each time-point. The immunoreactive protein bands (indicated by the *arrow*) were semi-quantified by densitometry and expressed as relative densitometric values. Equal loading in each lane was checked by Coomassie Blue staining of the gels and Ponceau S staining of nitrocellulose membranes. Mean ($n = 4$) ± S.E.M. of three independent experiments is shown

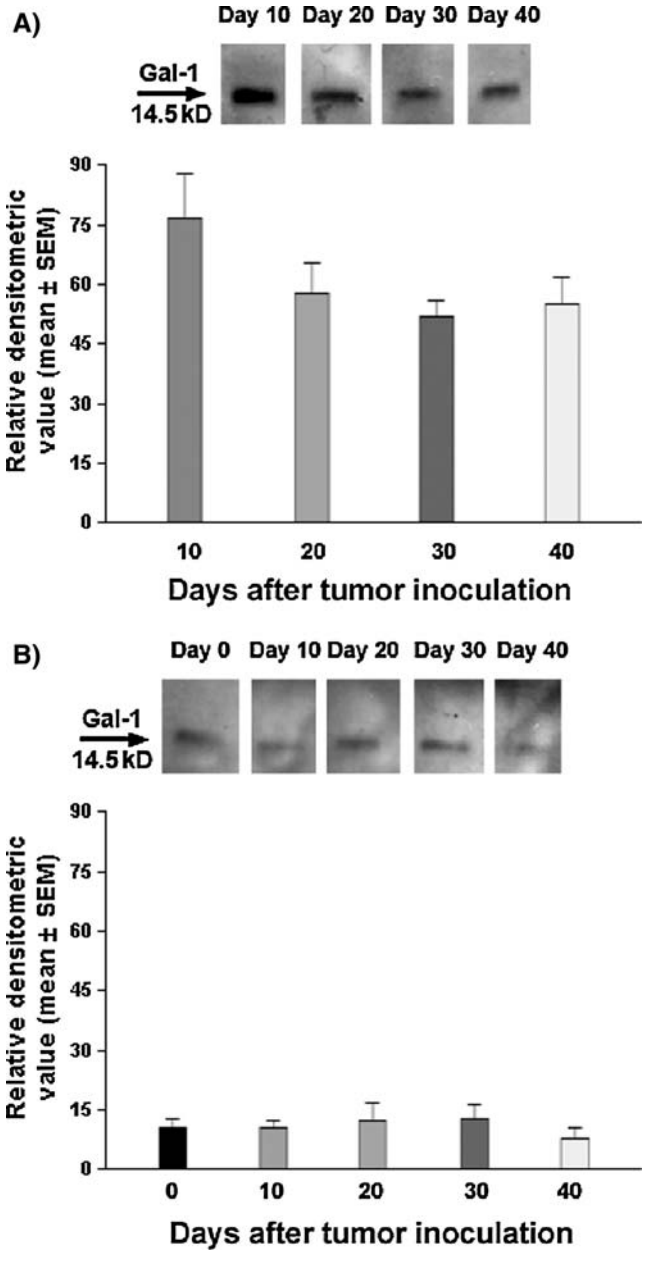

Fig. 5 Kinetics of *Gal-1* expression in M-234p tumor model. Extracts corresponding to tumor cells (**a**) and a splenic T-cell-enriched population (**b**) were prepared and processed for Western blot analysis. The procedure was the same as described in Fig. 2. The film image shows one representative sample for each time point studied. Immunoreactive protein bands were semi-quantified by densitometry and expressed as relative densitometric values. Equal loading in each lane was checked by Coomassie Blue staining of the gels and Ponceau S staining of nitrocellulose membranes. Mean ($n = 4$) ± S.E.M. of three independent experiments is shown

Discussion

Most of the immunotherapy strategies for cancer have focused on activating the immune system. However, growing evidence suggests that these therapies are thwarted by mechanisms used by tumors to suppress the antitumor immune response [14, 31]. Thus, stimulating the immune system may be ineffective in the face of local and systemic mechanisms of immune suppression. We have recently demonstrated that Gal-1, a carbohydrate-binding protein secreted by tumor and stromal cells, substantially contributes to the immunosuppressive

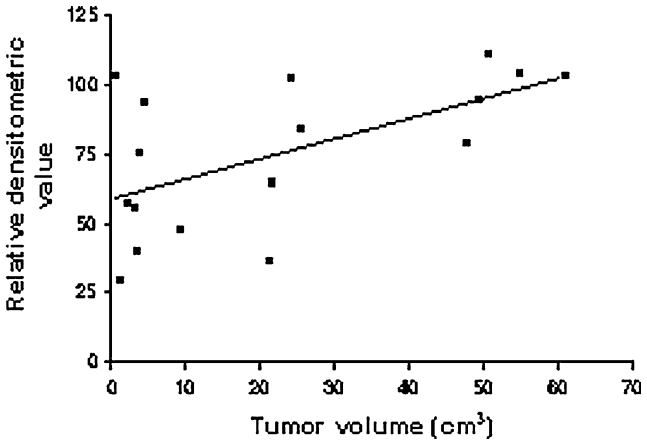

Fig. 6 Correlation between *L-TACB* tumor volume and *Gal-1* expression. Pearson correlation test ($r^2 = 0.12$; $p < 0.05$)

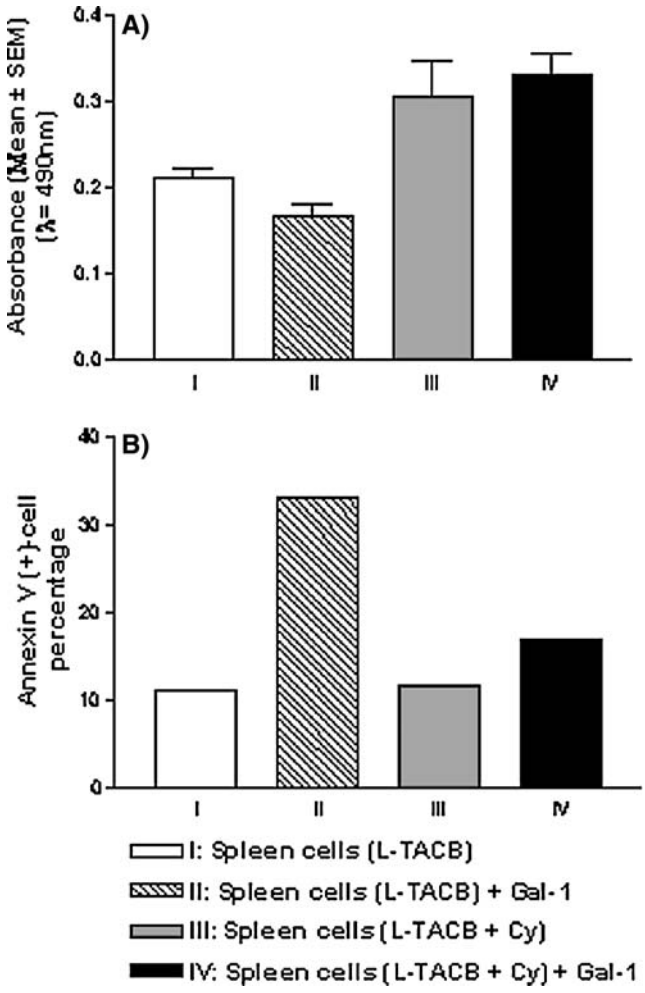

Fig. 7 Effect of *Gal-1* on a splenic T-cell-enriched population from *Cy*-treated *L-TACB*-bearing rats. **a** Cell viability measured by the colorimetric test *MTS/PMS* (absorbance at 490 nm: mean ± S.E.M.; $n = 4$). **b** Apoptosis detected by Annexin V test (% Annexin-V-positive cells)

activities of human and mouse cancer cells by regulating the survival and differentiation of effector T cells [48]. However, there is still scarce information about endogenous and exogenous stimuli which might control expression of this carbohydrate-binding protein in vivo.

In this study, we demonstrated that Gal-1 expression is differentially regulated during tumor growth in primary tumor cells, metastatic cells, and spleen cells. Furthermore, the kinetics of Gal-1 expression showed a different pattern according to the tumor type studied. In addition, expression of Gal-1 and its ability to modulate T cell survival are regulated following treatment with low doses of cyclophosphamide. During the growth of L-TACB rat lymphoma, the expression of Gal-1 in primary tumor cells increased significantly from days 7 and 10 to higher values on days 14 and 21. Similarly, recent experiments studying Gal-1 expression in primary tumor cells of a mouse lymphoma (L-DGE, a spontaneous tumor which appeared in a Balb/c mouse) confirmed the findings observed in L-TACB cells (Zacarías Fluck et al., personal communication). Interestingly, treatment of the animals with a single low-dose cyclophosphamide down-regulated Gal-1 expression which, on day 21, returned to the lower values observed on day 7 (Fig. 2a). Simultaneously, the same animals showed, on day 21, a significant decrease in Gal-1 expression by splenic T cells when compared to the basal levels observed on day 0. Like a mirror image of the situation occurring at the same time in primary tumor cells, cyclophosphamide treatment induced an up-regulation of Gal-1 expression in spleen cells, which returned to basal levels on day 21 (Fig. 2b).

Considering the natural history of lymphoma evolution: Which could be the physiopathological role of increased Gal-1 expression in primary tumors in contrast to decreased Gal-1 expression in spleen lymphoid cells? We have already proposed that Gal-1 would act as a counterattack mechanism to enable tumor cells to evade the antitumor T cell response [48]. In this context, Gal-1 would have an active role by protecting cancer cells from the immune attack. Yet, what would be the function of Gal-1 in spleen T cells? We might speculate that Gal-1 would induce apoptosis of lymphoma cells, because these belong to the lymphoid lineage. If that is the case, tumor pressure would lead to its own survival by driving splenic T cells to a lower Gal-1 expression.

An interesting finding of our study is that expression of Gal-1 in metastatic cells was lower than that observed in primary tumor cells at the same time point (Fig. 2c). Such behavior could be explained, at least, by two hypothetical mechanisms that are not mutually exclusive: (1) the progression to the metastatic phenotype

would have led to non-immunogenic cellular variants which, therefore, would have no need to escape from the immune response and will not require to overexpress Gal-1 and/or (2) several molecules involved in the complex process of generating the metastatic phenotype would compete with Gal-1 for providing a tumor escape mechanism.

Interestingly, the situation was completely different in two other tumor models. The expression of Gal-1 in the sarcoma S-E100 did not show any significant changes during tumor progression along the period studied (Fig. 4a). Following the same logic, if we take into account that S-E100 is a poorly immunogenic tumor (Bay et al., pers. comm.), we might speculate that there would be no need for the tumor to increase Gal-1 expression because the threat of an immune attack does not apparently exist. On the contrary, spleen cells from sarcoma-bearing animals showed a significant decrease in Gal-1 expression on days 14 and 21 when compared with that observed on day 0 (Fig. 4b). This behavior was similar to that observed in L-TACB tumor model. In contrast, in the mouse mammary adenocarcinoma M-234p results were unexpected, because tumor growth induced a clear reduction of Gal-1 expression, which was significant on day 30 and marginally significant on day 40 when compared with the levels observed on day 10 (Fig. 5a). If modulation of Gal-1 expression is explained in the context of the relationship between tumor growth and immune system, the observed behavior in this third model could be explained by the phenomenon of "tumor immunostimulation." Prehn and Lappé [33] found that intermediate levels of antitumor immune reactivity might often stimulate, rather than inhibit, the development of certain tumor types [34, 35]. Other data obtained in human breast cancer [52, 53] were in line with Prehn's postulation. Hence, if that was the case in our experimental M-234p tumor model, a reduction in Gal-1 expression would allow an intermediate immune response which, through the released cytokines and/or soluble growth factors, could stimulate tumor growth.

Taken together, the results obtained with the three tumor-models suggest that, hypothetically, Gal-1 could work as a potentiometer in a servomechanism. The tumor would be the electric motor that transmits a signal which is delivered after fleeting through the potentiometer (Gal-1) that adjusts it, in a continuous fashion, to the exact quantity regulated by the tumor and that finally reaches the executor arm that would be represented by cells involved in immune surveillance. Like in common servomechanisms, this system operates on the principle of negative feedback. When the tumor increases Gal-1 expression, there is a decrease of the antitumor immune response; on the contrary, when the tumor needs a mild immune response to grow, the tumor diminishes Gal-1 expression, thus allowing the immune response to proceed. Furthermore, when there is no sign from the tumor, no changes are induced in Gal-1 expression, and immune surveillance is not affected. This hypothesis should require further evaluation to enable us the unraveling of the mechanisms involved in such modulation. In addition, the decrease in Gal-1 expression by spleen cells of L-TACB-bearing and S-E100-bearing rats and the lack of changes in M-234p tumor-bearing mice (Figs. 2b, 4b, and 5b, respectively) awaits further investigation in order to explain its biological significance.

The demonstration of the existence of a positive and significant correlation between tumor volume and Gal-1 expression, during lymphoma growth, is a finding that warrants potential clinical implications (Fig. 6). This data correlates with recent findings showing an association between high Gal-1 expression and the aggressiveness of different tumor types [12, 21, 22].

It has been already demonstrated that Gal-1 is able to inhibit T cell effector functions by promoting growth arrest and apoptosis of activated T cells [5, 10, 19, 29, 32, 36, 37, 41]. Interestingly, previous studies report a biphasic modulation of cell growth and survival by recombinant human Gal-1 [1, 55]. Although low concentrations of this protein (in the order of 5–10 ng/ml) are mitogenic and increase the formation of granulocyte-macrophage colonies during hematopoiesis, high concentrations of this protein (in the order of 5–10 μg/ml) dramatically reduce cell growth and promote apoptosis [1, 55]. To investigate the functional significance of regulated Gal-1 expression by cyclophosphamide, we examined the effects of this carbohydrate-binding protein on T cell apoptosis by addition of recombinant Gal-1 (at a concentration of 4 μg/ml) which has been previously shown to promote cell death [37, 41, 43, 55]. As expected, we found that Gal-1 significantly reduced the viability of splenic T cells from tumor-bearing rats.

Probably one of the most intriguing findings of this study is the ability of cyclophosphamide to generate T cell resistance to Gal-1-induced cell death. Although the mechanisms involved in this resistance remain to be elucidated, one might speculate that cyclophosphamide would modulate the repertoire of specific glycans on T cells created by the regulated activity of glycosyltransferases, including the core two β-1,6-*N*-acetylglucosaminyltransferases (C2GnTs) and the α-2,6-sialyltransferase (ST6Gal I) [2, 16]. Controlled T cell surface glycosylation would in turn regulate Gal-1 binding and Gal-1-induced cell death, as has been previously demonstrated [2, 46, 47]. Alternatively, we

might also speculate that the immunostimulatory activity of cyclophosphamide would be sufficiently powerful to overcome the Gal-1 death pathway in vivo. These possibilities and the precise mechanism involved in this regulatory effect are currently under investigation in our laboratory.

Finally, by using specific inhibitors, we found that Gal-1 expression in L-TACB primary tumor cells, but not in metastatic cells, is partially dependent on activation of the NF-κB, mTOR, and p38-MAPK pathways (Zacarías Fluck et al., personal communication). This finding is worth to be discussed in terms of our previous observations about the mechanisms involved in the regulation of Gal-1 expression in activated T lymphocytes. We found that regulated expression of Gal-1 during T cell activation involves Lck and Fyn kinases and signaling through MEK1/ERK, p38 MAP kinase, and $p70^{S6}$ kinase [15]. We are currently studying the potential differences in the mechanisms that regulate Gal-1 expression in lymphoma cells vs. non-transformed lymphoid cells.

Research over the past decade has provided significant support for the role of Gal-1 in tumor growth and metastasis in vivo [44, 54], suggesting that selective inhibition of Gal-1 or other members of the galectin family might have profound implications for cancer therapy. Thus, it is predicted that inhibitors of these carbohydrate-binding proteins [3, 18, 30, 45, 50] will find their way into cancer clinical trials, leading to delays in tumor progression, and improvements in overall survival. In this regard, the modulation of Gal-1 expression in vivo will have clear therapeutic implications. However, there is still scarce information about endogenous or exogenous agents that regulate Gal-1 gene expression in tumor cells [9]. It has been shown that differentiating agents including sodium butyrate [17] and retinoic acid [23] can modulate Gal-1 content in tumor cells.

In this study, we provide novel evidence showing that treatment with low doses of cyclophosphamide, an immunomodulatory and antimetastatic agent, can differentially regulate Gal-1 expression in primary tumor, metastatic, and lymphoid cells. The increased understanding of the role of Gal-1 in cancer and inflammation and the mechanisms of action of this carbohydrate-binding protein at different steps of tumor progression would provide insights into how the regulation of Gal-1 expression and activity might be exploited for therapeutic purposes.

Acknowledgements We thank J. Hirabayashi and K.I. Kasai for providing the plasmid pET21a/hGal1, Dr. E. Roggero for his advice in the immunohistochemical pictures, and Ana Rossa for her technical assistance. This research was supported by a grant from the National University of Rosario (to O.G.S), grants from the National Agency for Promotion of Science and Technology (PICT 2003-05-13787 to G.A.R.), Sales Foundation (to G.A.R.), University of Buenos Aires (M091 to G.A.R.). G.A.R. is a member of the Scientific Career of CONICET. O.G.S. is a member of the Scientific Career of C.I.U.N.R.

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