

Galectin-1 expression in human glioma cells: Modulation by ionizing radiation and effects on tumor cell proliferation and migration

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Abstract. Galectins are evolutionarily conserved β -galactoside-binding lectins which recognize specific glycoconjugates on the cell surface and the extracellular matrix. Accumulating evidence indicates that these proteins are involved in a variety of physiological and pathological processes including tumor growth and metastasis. Up-regulated expression of galectin-1 is a hallmark of a variety of malignant tumors. Here, we examined the expression of galectin-1 in glioma cell lines, the influence of ionizing irradiation and the intracellular and extracellular effects of this protein on tumor cell proliferation and migration. Galectin-1 was detected in both A172 and U118 glioma cells by immunoblot analysis. Ionizing irradiation induced a statistically significant up-regulation in glioma cell lines. RNA-interference-mediated silencing resulted in a significant suppression of the proliferation of the A172 cells, while the addition of recombinant galectin-1 had no effect. On the other hand, the migratory capacity of both cell lines was reduced after galectin-1 down-regulation, and up-regulated by the addition of exogenous galectin-1. Our results provide evidence of a role for galectin-1 in the regulation of glioma cell proliferation and migration. While an intracellular mechanism seemed to prevail in galectin-1-mediated regulation of tumor cell proliferation, the control of cell migration was exerted by both intracellular and extracellular mechanisms. In addition, this protein was up-regulated by ionizing radiation, indicating that the blockade of this protein should be performed before radiotherapy to avoid any undesired stimulating effects. Given the multifactorial role of

galectin-1 in the regulation of tumor escape and metastasis, we conclude that targeting galectin-1 may have therapeutic benefits in the treatment of malignant glioma.

Introduction

Despite considerable therapeutic efforts, the median survival of all patients suffering from glioblastoma (the most malignant form of gliomas) still does not exceed ~12 months (1) in unselected patients and 14-17 months (2,3) for patients treated with a combined modality treatment. Reasons for the poor response to antineoplastic therapy include a marked primary resistance against apoptosis-inducing stimuli (4), a high potential of tumor cells for migration and invasion (5), and escape from the immune response (6). Although some mechanisms have been elucidated within the past years, there are presumably other causes leading to the aggressive behavior of malignant gliomas.

Galectins constitute a family of β -galactoside-binding proteins which are found in the nucleus, the cytoplasm, and the extracellular space in a wide variety of cell types under physiological and pathological conditions (7). Up-regulated expression of galectins and a correlation with the malignant potential was found in a number of neoplasms, including thyroid carcinoma (8), uterine adenocarcinoma (9) and malignant glioma (10-12). Galectins participate in many physiological and pathological processes such as cell growth regulation, embryogenesis, inflammation, migration and invasion and apoptosis (7). In addition, recent evidence indicates that galectin-1 plays a key role in tumor cell evasion of immune responses (13).

Expression of galectin-1 has been described in human gliomas *in vivo* with a higher expression of galectin-1 mRNA in higher grades of malignancy (10). The suppression of galectin-1 using an antisense strategy inhibited the growth of 9L rat gliomas (10) and resulted in the prolonged survival of nude mice with intracranially implanted U87 or U373 human glioma cells (14). Moreover, low levels of galectin-1, detected by immunohistochemistry, were associated with a significantly longer survival of patients with malignant astrocytomas or glioblastomas (11). No significant effect of

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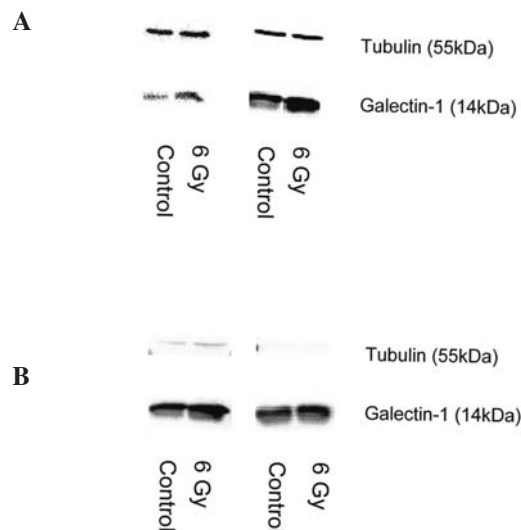


Figure 1. (A) Galectin-1 expression was up-regulated in A172 cells 4 h after irradiation with 6 Gy as compared with untreated controls (immunoblot). (B) Galectin-1 expression was up-regulated also in U118 cells 4 h after irradiation with 6 Gy as compared with untreated controls (immunoblot).

exogenous galectin-1 was observed in the growth of U87 cells (14). Conversely, exogenous administration of galectin-1 promoted the migration of U87 glioma cells (15). This effect seemed to result from the modulation of extracellular matrix (ECM) components and may contribute to the aggressiveness of human gliomas. In view of its numerous effects within the tumor microenvironment, it is tempting to speculate that galectin-1 plays a role in human gliomas that is more complex than is known to date.

Here, we examined the expression of galectin-1 in A172 and U118 human glioma cell lines by Western blot analysis and the effect of ionizing irradiation on this expression. In addition, we investigated the influence of galectin-1 on the proliferation and migration of glioma cells using two different approaches: a) exogenous addition of recombinant galectin-1 and b) down-regulation using RNA-interference.

Materials and methods

Cell lines and reagents. The human glioma cell lines A172 and U118, kindly provided by Dr N. de Tribolet (Lausanne, Switzerland), were cultured in 75-cm³ Falcon plastic flasks using DMEM supplemented with 1% glutamine (Gibco Life Technologies, Paisley, UK), 10% FCS (Biocrom KG, Berlin, Germany) and penicillin (100 IU/ml)/streptomycin (100 µg/ml). All cell lines were routinely tested for mycoplasma contamination by DAPI staining (Sigma, Deisenhofen, Germany). Where indicated, the cells were irradiated while adherent with a single dose of ~6 Gy. Cell lysates were obtained 4 h after irradiation. For immunoblot analysis, a polyclonal anti-galectin-1 antibody was used as previously described (16). Recombinant galectin-1 was obtained as previously described (16).

Immunoblot analysis. Galectin-1 protein levels were analyzed by immunoblotting using 10 µg of total protein lysate per lane on a 15% acrylamide gel (Bio-Rad, Munich, Germany). After

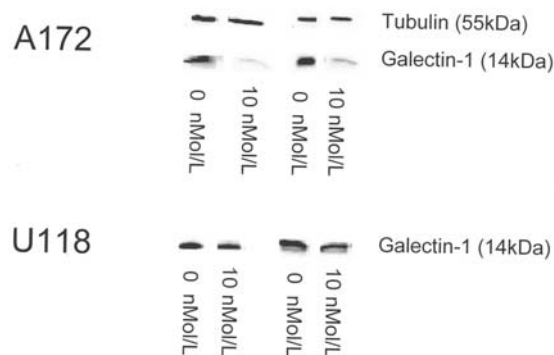


Figure 2. Expression of galectin-1 was down-regulated with RNA-interference on average by 30% (A172) and 20% (U118).

transfer to nitrocellulose (Bio-Rad, Hercules, CA), blots were blocked in PBS containing 5% skim milk and 0.05% Tween-20 and incubated overnight at 4°C with anti-galectin-1 antibody (1:7000 in 5% skim milk). The visualization of protein bands was accomplished using horseradish peroxidase-coupled anti-rabbit IgG secondary antibody (Sigma) and enhanced chemiluminescence (ECL) (Amersham). The staining intensity of the protein bands was quantified using the Fluor-S MultiImager detection system (Bio-Rad, Hercules, CA), and the intensity of each protein band, relative to the tubulin control was indicated.

siRNA preparation and transfection. Galectin-1 sense 5'-UC CAGGUUUGAGAUUCAGG-3', and antisense 5'-CCUG AAUCUCAACCUGGA-3' were designed. The siRNA yield of the preparation was quantified by spectrophotometry. According to different proliferation rates, the cells were seeded in 5000 µl medium at 150,000 (A172) and 200,000 (U118) cells per well in 24-well plates. After an incubation period of 24 h, the medium was completely removed. Transfection of siRNA was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. One µl Lipofectamine 2000 was diluted in 50 µl of serum-free medium at room temperature and further mixed with 6 or 12 pmol of siRNA in 50 µl of serum-free medium. Incubation was prolonged for 20 min at room temperature, and the mixture was applied to culture wells that had been supplied beforehand with 500 µl serum-free medium, resulting in a final siRNA concentration of 10 or 20 nM respectively. Protein lysates were generated and the level of galectin-1 expression was assessed by immunoblot analysis.

Proliferation assay. Proliferation was measured with the WST-1 assay (Roche Diagnostics, Mannheim, Germany). Approximately 5x10³ cells per well were seeded in a 96-well plate in 100 µl DMEM. After 24 h, the medium was exchanged and 0, 1, 2 or 4 µg/ml recombinant galectin-1 was added to the serum-free medium. After 48 h, 10 µl of the WST-1 reagent was added and the cells were incubated for 2 or 4 h. Absorption was measured with a microplate reader at 450 nm. The measured values were compared with controls.

Migration assay. To assess the ability of galectin-1 to regulate tumor cell migration, transwell devices with 8-µm

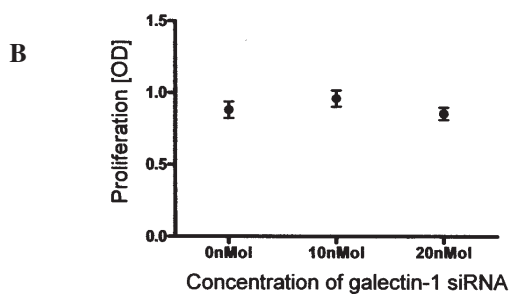
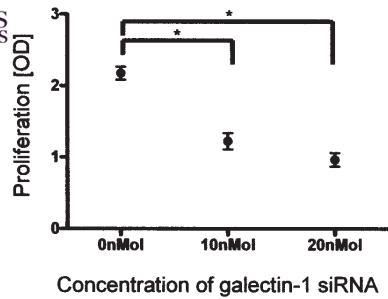


Figure 3. (A) WST-1 assay. Proliferation of A172 cells after galectin-1 down-regulation with 0, 10 and 20 nMol galectin-1 siRNA. The difference of proliferating cells was statistically significant ($p < 0.01$). OD, optical density. (B) WST-1 assay. Proliferation of U118 cells after galectin-1 down-regulation with 0, 10 and 20 nMol anti-galectin-1 siRNA. OD, optical density.

pores and a proliferation area of 0.3 cm were introduced into 24-well plates. Approximately 50,000 cells per well were seeded in 500 μ l serum-free medium. After 24 h, the transwell devices were obtained from the 24-well plate, cells were removed from the upper surface with a cell scraper and cells that had migrated to the lower surface were counted under a microscope. To test the effect of recombinant galectin-1 in cell migration 1, 2 or 4 μ g/ml was added to the serum-free culture medium. When transfection with anti-galectin-1 siRNA was conducted before the seeding, the cells did not adhere to the plastic surface. Therefore, for the migration assays, cells were transfected with 10 or 20 nMol siRNA after being allowed to adhere and results were compared with transfection of 0 nMol.

Evaluation of results and statistical analysis. Expression levels in the immunoblots were assessed by comparison of the chemiluminescence with the Quantity One program (Bio-Rad, Hercules, CA). The influence of different concentrations of recombinant galectin-1 or anti-galectin-1 siRNA on cell proliferation and migration was compared with the one-way ANOVA and Dunnett's post-test.

Results

Galectin-1 expression was induced by ionizing radiation. To determine whether galectin-1 is modulated by ionizing radiations, we first assessed the levels of galectin-1 in A172 and U118 glioma cell lines. As shown by immunoblot analysis,

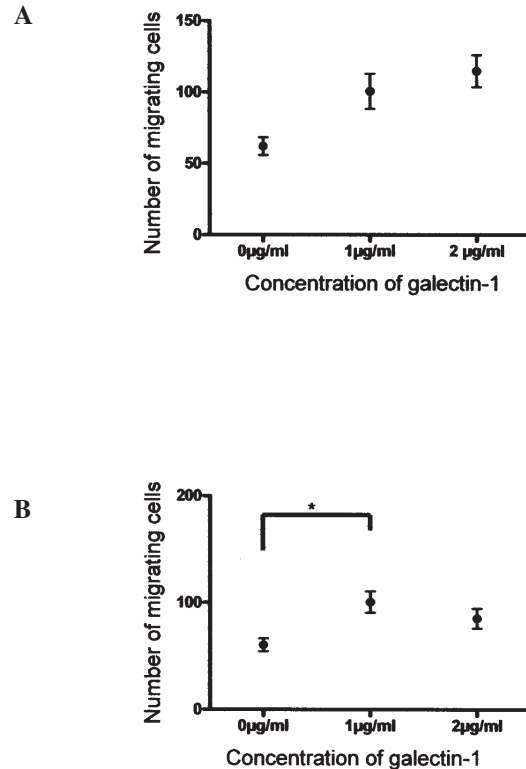


Figure 4. (A) Number of migrating A172 cells after external application of 0, 1 and 2 μ g/ml recombinant galectin-1. The difference between 0 and 1 or 2 μ g/ml, respectively, was statistically significant ($p < 0.05$). (B) Number of migrating U118 cells after external application of 0, 1 and 2 μ g/ml recombinant galectin-1. The difference between 0 and 1 μ g/ml was statistically significant ($p < 0.05$).

galectin-1 was expressed at different levels in these cell lines (Fig. 1). In addition, ionizing radiation induced a significant up-regulation of galectin-1 in both cell lines 4 h after irradiation with 6 Gy: band intensity increased by up to 40% in A172 cells (mean 24%) and by up to 20% in U118 cells (mean 13%) as compared with untreated controls (Fig. 1).

Efficiency of RNA-interference. In order to modulate galectin-1 expression for functional assays, we transfected siRNA into glioma cell lines as described above. As shown in Fig. 2, galectin-1 was markedly down-regulated after 24 h. In both cell lines, staining intensity was reduced by up to 40% (mean 20%, Fig. 2). By contrast, control transfection with an anti-EGFP-siRNA resulted in 0-5% down-regulation of galectin-1 (not shown).

Effects of recombinant galectin-1 on the proliferation of human glioma cells. Next, we examined the role of exogenous and endogenous galectin-1 on the proliferation of human glioma cells. Interestingly, the addition of exogenous recombinant galectin-1 caused no statistically significant change in the proliferative capacity of both cell lines ($p > 0.05$, not shown).

In contrast, a marked anti-proliferative effect was observed in A172 cells following the down-regulation of galectin-1 by siRNA. Remarkably, cell proliferation was reduced by ~50% ($p < 0.01$). In U118 cells, however, down-regulation of intracellular galectin-1 had no effect (Fig. 3). Thus, galectin-1 may have different effects on cell pro-

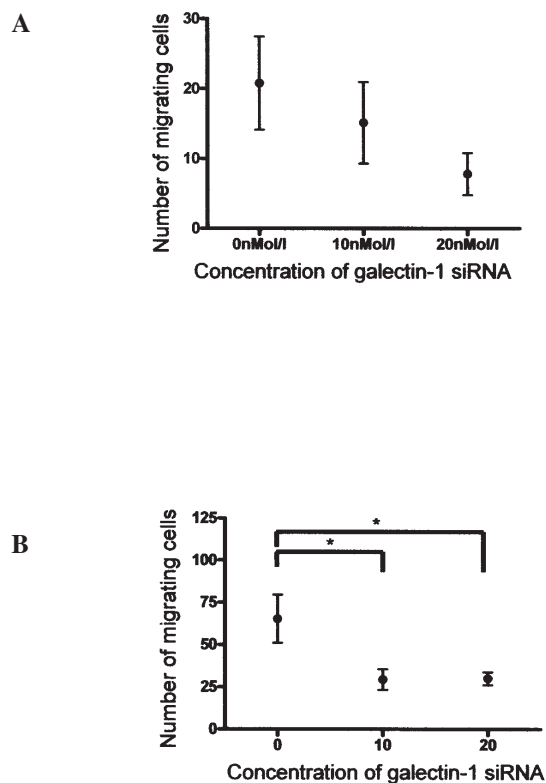


Figure 5. (A) The number of migrating A172 cells following transfection with 0, 10 and 20 nM galectin-1 siRNA. The difference was not significant. (B) The number of migrating U118 cells after 0, 10 and 20 nMol anti-galectin-1 siRNA. The difference between 0 and 10 or 20 nMol, respectively, was statistically significant ($p < 0.05$).

liferation depending on whether it acts extracellularly or intracellularly. The effect on cell proliferation may also depend on the susceptibility of different tumor cell types but this susceptibility relies on the balance of different glycosyl-transferases creating glycan ligand on the cell surface. The association of different glycan structures with the two different glioma cell lines studied remains to be ascertained.

Effect of recombinant galectin-1 on tumor cell migration. Finally, we investigated whether intracellular or extracellular galectin-1 might affect the migration of human glioma cell lines. When exogenous galectin-1 was added to cell cultures, a significant effect was observed on cell migration of human glioma cell lines. Regarding A172 cells, an increase of ~100% migrating cells was seen after the addition of 2 μ g/ml galectin-1 ($p < 0.05$). On the other hand, a bell-shaped effect was observed with U118 cells which migrated significantly better only with 1 μ g/ml ($p < 0.05$, Fig. 4). Surprisingly, migration assays could not be performed when cells were transfected before the seeding into the wells. Under this condition, cells remained in suspension and were not able to adhere to the plastic surface. Therefore, we decided to transfect glioma cells with siRNA following adherence of these cells to the migration devices. Using this experimental design, the number of migrating cells dropped by ~50% in U118 cells following transfection with 10 and 20 nM/l siRNA ($p < 0.05$). In contrast, only a slight effect was seen in A172 cells at both concentrations ($p > 0.05$, Fig. 5).

Discussion

In this study, we reported the expression of galectin-1 in immortalized human glioma cell lines. Expression levels of this protein were significantly up-regulated by ionizing irradiation, an effect which might have functional implications in the regulation of galectin-1 expression following radiotherapy. Furthermore, while only slight or mild effects on cell proliferation and migration were observed after the exogenous addition of recombinant galectin-1, we found a marked inhibition of cell proliferation and migration following the silencing of galectin-1 mRNA.

Expression of galectin-1 in glioma cells was previously reported (10,11,17). A higher expression of galectin-1 mRNA was found in higher grades of malignancy (10), indicating a tight correlation between galectin-1 and the aggressiveness of these tumors. The introduction of a galectin-1 antisense construct in 9L rat glioma cells resulted in remarkable changes in morphology and cell growth properties. The authors concluded that lower levels of galectin-1 might arrest the growth of 9L glioma cells. Our results in the A172, but not in the U118 cell line are in line with these observations. Interestingly, exogenous administration of recombinant galectin-1 produced only mild effects on cell growth regulation. One possible explanation is that the expression of galectin-1 reached significantly high levels and that incorporation to cell cultures of an excess of this protein could hardly cause an additional effect. On the other hand, a second possibility is that the most relevant effect exerted by galectin-1 would be mainly intracellular, so that exogenous administration of this carbohydrate-binding protein will not be efficient in modulating tumor growth.

Pioneer studies carried out by Rorive *et al* (11) and Camby *et al* (15) have analyzed the effects of galectins on cell migration and invasion in human gliomas. In experimental glioblastoma, a higher level of positivity of galectins-1, -3 and -8 was found in the invasive stroma compared to the parenchyma or other sections of the tumor. Both galectins-1 and -3 promoted tumor cell migration, as evaluated by computer-assisted microscopy after modulation of the galectin-1 content of the plastic support. Further analysis of the molecular basis showed that the influence of galectins on cell migration and invasion results from modifications of the organization of the cytoskeleton through small GTPases such as RhoA (17). The relevance of galectin-1 expression on glioma growth *in vivo* was demonstrated by a longer survival of nude mice xenografted with U87 or U373 cells transfected with galectin-1 antisense (14). Moreover, patients whose tumors had a lower immunohistochemical expression of galectin-1 survived significantly longer than patients whose tumors expressed higher levels of this protein (11). Our finding of a markedly reduced migration of U118 cells following down-regulation of galectin-1 underlines the impact of this β -galactoside-binding protein on migration of human glioma cells, which was more efficiently influenced by siRNA than by an exogenous addition of the recombinant protein, suggesting a major role for galectin-1 in regulating cell migration. Interestingly, galectin-1 down-regulation did not have the same effect on both cell lines and interfered with proliferation only in A172, whereas it affected proliferation



sation in both cell lines. Regarding the addition of s galectin-1, one might speculate that each glioma cell line may express different glycosyltransferases, compromising the susceptibility of these cell lines to galectin-1. However, in the case of intracellular galectin-1, disruption of this protein by siRNA might have different consequences depending on the differentiation state of the cell or its cell cycle progression status. In any case, longer survival associated with lower galectin-1 expression levels in experimental and human gliomas may not only result from a lower migratory phenotype of these cells, but also from a reduced proliferative activity.

In addition, our findings of ionizing irradiation up-regulating galectin-1 expression may be of particular relevance for the treatment of glioma patients, since external beam irradiation has been one of the most important elements of malignant glioma therapy for many years. In this regard, it has been shown that ionizing irradiation enhanced the migratory capacity of glioma cells (18). This may in part be explained by our findings that galectin-1 expression is induced by irradiation and can promote the migration of human glioma cells.

In conclusion, the data presented here support previous reports that galectin-1 has not only an impact on migration, but also on the proliferation of human glioma cells. This seems to be primarily an intracellular effect. In light of these findings, galectin-1 seems to be an interesting target to treat malignant gliomas by interference with proliferation and migration. Since ionizing irradiation induces the expression of galectin-1, down-regulation of this molecule would be feasible before radiotherapy in order to avoid any undesired side effects of this standard therapy. Taken together, these observations in conjunction with the novel role of galectin-1 in modulating angiogenesis (19) and tumor cell evasion of immune responses (13,20), suggest that the blockade of galectin-1 may contribute in overcoming several steps of tumor metastasis, including cell proliferation, migration, angiogenesis and tumor-immune escape. Thus, it is predicted that inhibitors of galectin-1 (21-23) will find their way into cancer clinical trials, aiming for delays in tumor progression and improvements in overall survival.

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