Expression Pattern of Galectin-3 in Neural Tumor Cell Lines

Stephan Kuklinski, 1 Penka Pesheva, 2 Cornelia Heimann, 1 Stephanie Urschel, 1 Sergio Gloor, 3 Sebastian Graeber, 3 Volker Herzog, 3 Torsten Pietsch, 4 Otmar D. Wiestler, 4 and Rainer Probstmeier 1, 1

1 Department of Biochemistry, Institute for Animal Anatomy and Physiology, Bonn, Germany
2 Department of Nuclear Medicine, University of Bonn, Bonn, Germany
3 Department of Cell Biology, University of Bonn, Bonn, Germany
4 Department of Neuropathology, University of Bonn, Bonn, Germany
5 Laboratory of Biochemistry, Swiss Federal Institute of Technology, Zürich, Switzerland

Galectin-3 is a member of the galectin family of β-galactoside-specific animal lectins. Here we show that galectin-3 is constitutively expressed in 15 out of 16 glioma cell lines tested, but not by normal or reactive astrocytes, oligodendrocytes, glial O-2A progenitor cells and the oligodendrocyte precursor cell line Oli-neu. Galectin-3 is also expressed by one oligodendroglioma cell line, but not by primitive neuroectodermal tumor and 4 neuroblastoma cell lines tested so far. In all galectin-3 expressing cell lines, the lectin is predominantly, if not exclusively, localized intracellularly and carries an active carbohydrate recognition domain (shown for C6 rat glioma cells). Moreover, in contrast to primary astrocytes, glioma cells do not or only weakly adhere to substratum-bound galectin-3, probably reflecting an unusual glycosylation pattern. Our findings indicate that the expression of galectin-3 selectively correlates with glial cell transformation in the central nervous system and could thus serve as a marker for glial tumor cell lines and glial tumors. J. Neurosci. Res. 60:45–57, 2000. © 2000 Wiley-Liss, Inc.

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Galectin-3 is a member of the galectin family of animal lectins that specifically recognizes β-galactosidic carbohydrate structures (Leffler, 1997). Although galectin-3 displays properties of a typical cytoplasmic protein, it is found not only intracellularly, but also on the cell surface and in the extracellular space. As recently demonstrated, the extracellular expression results from an unusual secretion mechanism (Lindstedt et al., 1993; Mehul and Hughes, 1997). Due to its complex subcellular distribution, it is not possible to specify a precise function of galectin-3. Intracellularly, galectin-3 seems to be involved in splicing events (Dagher et al., 1995) and in the molecular control of apoptotic processes (Yang et al., 1996; Akahani et al., 1997b). On the cell surface and in the extracellular space, galectin-3 can modify cell–cell and cell–substrate interactions or exhibit cytokine-like properties that could play a role during inflammatory processes, such as the activation of mast cells or neutrophils, and the regulation of cytokine expression and function (Iriye et al., 1993; Krugluger et al., 1997; Cortegano et al., 1998).

Besides its expression in normal tissues and cells, galectin-3 has been identified in a number of tumors, such as breast (Castronovo et al., 1996; Marder and Hughes, 1996), colon (Inamura et al., 1991; Castronovo et al., 1992; Lotz et al., 1993), gastric, ovarian and thyroid (Xu et al., 1995) carcinomas, in lymphomas (Konstantinov et al., 1996), melanomas and fibrosarcomas (Raz et al., 1986). An increase in galectin-3 expression often correlates with increased malignancy and metastatic capacity of tumor cells (see, for example, Nangia-Makker et al., 1995 and references therein). In bladder transitional-cell- and ovarian carcinomas, however, no correlation between galectin-3 expression and pathological phenomena could be found (van den Brule et al., 1994; Cindolo et al., 1999). Inconsistent results have been described for colon carcinomas: either a decrease of galectin-3 expression in cancer tissues with respect to normal mucosa (Lotz et al., 1993; Castronovo et al., 1996; Sanjuan et al., 1997) or an increased lectin expression with transformation and metastasis formation (Shoepnner et al., 1995; Bressler et al., 1998).

In normal neural tissues, the expression of galectin-3 is restricted to subpopulations of dorsal root ganglion (DRG) neurons (Regan et al., 1986). Under traumatic conditions, the lectin becomes expressed by Schwann cells in the lesioned sciatic nerve (Reichert et al., 1994) and by brain microglial cells in vivo and in vitro (Reichert and Rothshenker, 1996; Pesheva et al., 1998b). Recently, Stephan Kuklinski and Penka Pesheva contributed equally to this study. Contract grant sponsor: Deutsche Forschungsgemeinschaft; Contract grant numbers: PR 278/2-1, PR 278/2-2.

*Correspondence to: Dr. Rainer Probstmeier, Department of Biochemistry, Institute of Animal Anatomy and Physiology, Rheinische Friedrich-Wilhelms-Universität Bonn, Katzenburgweg 9A, D-53115 Bonn.

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Galectin-3 has also been found to be expressed in glial tumors in vivo (Bresalier et al., 1997; Gordower et al., 1999). To gain more insights into the functional role of galectin-3 in neoplastic neural cells we have analyzed the expression pattern of the lectin in a number of neural tumor cell lines and compared it to that by cultured central nervous system (CNS) glial cells, i.e., astrocytes, oligodendrocytes and their precursors.

MATERIALS AND METHODS

Lectins and Antibodies

Recombinant galectin-3 was expressed from plasmid prCBP35s in *E. coli* strain JA221 and affinity purified on a lactose-agarose column as described (Agrwal et al., 1993; Probstmeier et al., 1995).

Polyclonal antibodies against recombinant galectin-3 were raised in rabbits (Pesheva et al., 1998b). The hybridoma cell line expressing a monoclonal antibody against galectin-3 (Mac-2) was obtained from the American Type Culture Collection. Monoclonal antibody against cyclic nucleotide phosphodieste-
as enzyme (CNPase) was from Roche Diagnostics. Polyclonal rabbit antibodies against glial fibrillary acid protein (GFAP) were from Serva. Secondary antibodies against rabbit and rat IgG coupled to horseradish peroxidase were from Promega and those coupled to cyanin3 or dichlorotriazinyl-amino-fluorescin (DTAF) were from Jackson Laboratories.

Cytokines/Growth Factors and Other Reagents

Ciliary neurotrophic factor (CNTF), epidermal growth factor (EGF), leukemia inhibitory factor (LIF), transforming growth factor-β (TGF-β) and tumor necrosis factor-α (TNF-α) were from Roche Diagnostics. Basic fibroblast growth factor (bFGF), granulocyte macrophage colony stimulating factor (GM-CSF), interleukin-1β (IL-1β), interleukin-6 (IL-6) and β-nerve growth factor (β-NGF) were purchased from Pepro-Tech. Lipopolysaccharides (LPS) were from Difco Laboratories. Collagen type I was from Serva and RNase A from Sigma.

Cell Cultures

The following cell lines were used. Rat C6 glioma cells (Benda et al., 1968) were cultured in Ham's F12 medium (Gibco) containing 10% fetal calf serum (FCS, from Sigma). Human astrocytoma cell lines A172 (Giard et al., 1973), A582, CCF-STTG1 (Barna et al., 1985), SK-MG-2, SK-MG-3, SK-MG-4, SK-MG-5, SK-MG-9, SK-MG-11, SK-MG-13, SK-MG-15 (Jennings et al., 1989), U87MG (Giard et al., 1973), U138MG (Rehentulla et al., 1988), U251MG (Osborn et al., 1981) and U343MG (Asai et al., 1994) were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% FCS. Human neuroblastoma MHH-NB-11 (Pietsch et al., 1988) and human oligodendrogloma cell line TC620 (Merrill and Matsushima, 1988) were cultured in DMEM containing 10% FCS, human SK-N-MC primitive neuroectodermal tumor cell line (Sano et al., 1990), SK-N-SH (Spranger et al., 1973) and SMS-KAN neuroblastoma (Reynolds et al., 1986) and mouse N2A neuroblastoma cells (Rathjen and Schachner, 1984) were maintained in RPMI 1640 medium (PAA Laboratories) containing 10% FCS. Murine oligodendrocyte precursor cell line Oli-neu (Fok-Seang et al., 1995; Jung et al., 1995) was cultured in SATO medium containing 1% FCS.

Mixed neural primary cell cultures were prepared from cerebella of six-day-old mice (Pesheva et al., 1989) and cultured on poly-l-lysine (PLL)-coated coverslips in Basal medium Eagle (BME) containing 10% horse serum (HS). Cultures of cerebral hemispheres derived from newborn mice or rats were cultured in DMEM containing 10% FCS. Primary astrocytes were obtained from mixed glial cell cultures derived from neonatal mouse brain after shaking off microglial cells (Pesheva et al., 1998b) and maintained in DMEM containing 10% FCS. O-2A progenitor cells were obtained from mixed cerebral glial cultures according to Pesheva et al. (1997) and cultured on PLL-coated coverslips in SATO medium.

In some experiments, cytokines/growth factors or LPS were added to the cultures for 24 to 72 hr at the following concentrations: CNTF, 10 ng/ml; EGF, 500 ng/ml; bFGF, 100 ng/ml; GM-CSF, 5 ng/ml; IL-1β, 10 U/ml; IL-6, 10 ng/ml; LIF, 20 ng/ml; LPS, 20 μg/ml; β-NGF, 100 ng/ml; TGF-β, 1 ng/ml; TNF-α, 200 U/ml. For incubations longer than 24 hr fresh factors were added daily.

Cell-Substratum Adhesion Assay

Galectin-3 or collagen type I were diluted in phosphate-buffered saline, pH 7.2 (PBS), spotted (3 μl/spot) onto plastic petri dishes (from Falcon) and incubated for 2 hr at room temperature in a humid atmosphere. Dishes were then washed once with PBS containing 10% heat-inactivated bovine serum albumin (BSA) and incubated for 1 hr at room temperature in the same solution. Dishes were subsequently washed three times with PBS and cell suspensions 

10^3 to 10^6 cells/ml in DMEM containing 1 mg/ml fatty acid-free BSA (Roche Diagnostics) were added to the substrata. After 4 hr of incubation at 37°C, nonadherent cells were removed by washing the dishes 3 to 5 times with DMEM and the number of cells adherent to galectin-3 and collagen type I substrata was monitored microscopically.

Immunocytochemistry

For extracellular staining, cells grown on PLL-coated coverslips were washed twice with PBS containing 0.1% BSA (PBS-BSA), fixed for 10 min in 4% paraformaldehyde (PA) in PBS and blocked for 15 min in Dulbecco's PBS (DPBS) containing 10% HS and 10% FCS. After washing twice in PBS-BSA, cells were incubated for 20 min with the first antibody (diluted in DPBS, 10% HS, 10% FCS) and washed three times in PBS-BSA. Cells were then incubated for 20 min with the secondary fluorochrome-conjugated antibody (diluted in DPBS, 10% HS, 10% FCS), washed again and embedded in PBS/glycerol (v/v; 1:1). For intracellular staining, cells were permeabilized for 2 min with methanol at −20°C or for 10 min with 0.1% Triton X-100 in DPBS at room temperature, fixed for 10 min with 4% PA and further treated as described above.

For the identification of the nuclear compartment by confocal microscopy, immunostained permeabilized C6 cells were treated for 15 min at room temperature with RNase (1 mg/ml in PBS), washed twice with PBS and incubated for 30
min at room temperature with propidium iodine (2 ng/ml in 10 mM Tris–HCl, 150 mM NaCl, pH 7.6).

Cell Extraction and Affinity Purification of Galectin-3

Cells grown in tissue culture dishes were washed three times with ice-cold PBS and solubilized for 60 min at 4°C in 100 mM NaH2PO4/Na2HPO4, 100 mM NaCl, 0.2 mM CaCl2, 0.2 mM MgCl2, 1% Triton X-100, pH 7.4 (buffer A) containing the protease inhibitors phenylmethylsulfonylfluoride (1 mM), and soybean trypsin inhibitor (15 μM). Solubilizes were centrifuged for 10 min at 10,000 × g and the protein concentration of the supernatant determined according to Bradford. 1976). For affinity purification of galectin-3, cell extracts (500 μl, protein content of 1.5 mg/ml) were incubated overnight at 4°C with 200 μl lactose–agarose (Sigma) or Sepharose 4B (Pharmacia). The gel slurry was washed three times with 1 ml of cold buffer A, three times with buffer A without Triton X-100 (buffer B), and three times with buffer B containing 100 mM sucrose. Proteins bound were eluted with 2 × 400 μl buffer B containing 100 mM lactose.

Western Blot Analysis

Western blot analysis was carried out essentially as described (Towbin et al., 1979). Briefly, proteins separated by SDS-PAGE using 10% slab minigels (Laemmli, 1970) were transferred for 45 min at 150 mA onto nitrocellulose filters. To visualize transferred proteins, filters were incubated with a 0.2% solution of Ponceau S in 3% trichloroacetic acid, subsequently washed in water and destained in PBS. Filters were blocked for 1 hr in PBS, 3% milk powder and incubated for 3 hr at 37°C with the first antibody diluted in PBS, 1% milk powder. Filters were then washed three times (10 min each) in PBS, 0.2% Tween 20 and incubated for 1 hr at room temperature with secondary antibodies diluted in PBS, 1% milk powder. After three washing steps, antibody binding was visualized using the enhanced chemiluminescent detection system (Pierce).

Biosynthetic Labeling of Cell Cultures and Immunoprecipitation

C6 cell cultures of about three quarter confluence were washed three times with methionine-free minimal essential medium and after 30 min of incubation in the same medium (preincubation period), 150 μCi 35S-methionine were added for 3 or 24 hr (pulse period). Cells were washed twice with RPMI containing 10% HS and 5% FCS and cultured for further 4 hr in the same medium (chase period). The culture supernatant was cleared by centrifugation (10 min at 10,000 × g) and used for further analysis. Cell monolayers were washed three times with RPMI containing 1% HS and lysed for 30 min on ice in Tris-buffered saline (50 mM Tris–HCl, 100 mM NaCl, pH 7.2; TBS) containing 1% Triton X-100, iodoacetamide (0.8 mM), phenylmethylsulfonylfluoride (1 mM), and soybean trypsin inhibitor (15 μM). Cell lysates were centrifuged for 10 min at 10,000 × g and the supernatants were used for further analysis; 250 μl of each supernatant were incubated overnight at 4°C with 75 μl of Sepharose 4B coupled with polyclonal galectin-3 antibodies (2 mg/ml gel). Beads were washed three times with lysis buffer and bound material eluted with SDS-sample buffer at 95°C. For autoradiography, eluted proteins were separated by SDS-PAGE using 12% slab gels. Gels were fixed in isopropanol/ acetic acid/H2O (25:10:65), amplified for 1 hr in 1 M sodium salicylate and exposed to XAR-5 films (Kodak).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was purified from cultured cells according to Chomczynski and Sacchi (1987) and 3 μg of RNA were used for oligo-dT–primed single-stranded cDNA synthesis with superscript reverse transcriptase (SuperScript PremiAlication Set, Gibco) in 20 μl of reaction volume. After hydrolysis of the RNA with RNase H, 4 μl of single-stranded cDNA were amplified in a total reaction volume of 20 μl using the rat galectin-3–specific primers 5’-CCCTTTTGTGTCGCCGACT-GGA-3’ (upstream primer) and 5’-CCCCAGTTGGCCTGAT-TTCCCTGA-3’ (downstream primer) or the human galectin-3 specific primers 5’-CCCCGTGGCCCGACTTATTATG-3’ (upstream primer) and 5’-CCAGAAATCCCGTTGGCTGAT-TT-3’ (downstream primer) in 10 mM Tris–HCl, pH 9.0, 50 mM KCl, 1.5 mM MgCl2, 0.1% Triton X-100, 0.2 μM dNTP and 0.26 μM primer each. Both of these amplifications led to 390 bp products. As control, the rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific primers 5’-TG-LAAGTTCTGGTCAAGCAAGTTTGGGC-3’ (upstream primer) and 5’-CATGTGGGCCATGAGGTCCACC-3’ (downstream primer) yielding a 950 bp product were used with 2 μl of template cDNA. PCR products were amplified through 35 cycles of 45 sec at 94°C, 30 sec at 50°C and 30 sec at 72°C (for galectin-3) and through 35 cycles of 45 sec at 94°C, 45 sec at 50°C and 90 sec at 72°C (for GAPDH; Pesheva et al., 1997).

RESULTS

Galectin-3 Is Not Expressed in Normal Macrogial Cells In Vivo and In Vitro

As previously demonstrated, with the exception of axonal processes of DRG neurons projecting into the spinal cord (Regan et al., 1986), galectin-3 is not expressed by neuronal and glial cells (astrocytes or oligodendrocytes) in the embryonic, early postnatal and adult mouse or rat brain (Regan et al., 1986; Reichert and Rotshenker, 1996; Pesheva and Probstmeier, unpublished observations). It has recently been demonstrated, however, that galectin-3 is expressed by activated microglial cells after injury in vivo and in vitro (Reichert and Rotshenker, 1996; Pesheva et al., 1998b). To examine if galectin-3 becomes expressed by macroglial cells in vitro, i.e., under conditions that partially lead to cell activation, we analyzed mixed neural cell cultures derived from newborn mouse or rat cerebral hemispheres and from six–day-old mouse cerebella maintained in vitro for 3 to 7 days by indirect double-immunofluorescence using galectin-3-, CNPase– (for oligodendrocytes) and GFAP–specific (for astrocytes) antibodies (Fig. 1, for mouse cerebellar cultures). Under these experimental conditions, neither astrocytes (Fig. 1A,B) nor oligodendrocytes (Fig. 1D,E) expressed galectin-3. In agreement with previous results (Pesheva et al., 1998b), microglial cells were the only cell type expressing galectin-3 (Fig. 1B,E), as shown by double-immunofluorescence using antibodies that recognize macrophage/microglia–specific antigens (not shown).
Comparable results were obtained for mouse and rat cerebral cultures maintained in vitro for four days without further additives and for further three days in the presence of CNTF, EGF, bFGF, GM-CSF, IL-1β, IL-6, LPS, NGF, TGF-β or TNF-α, cytokines/growth factors known to be associated with CNS injury and inflammation (not shown). Galectin-3 was also not expressed by O-2A progenitor cells derived from newborn mouse brain (not shown).

Characterization and Subcellular Distribution of Galectin-3 Expressed by C6 Glioma Cells

Galectin-3 is expressed by a number of tumor cells (Akahani et al., 1997a). To evaluate if this also holds for neoplastic cells of neural origin, we analyzed the expression of the lectin in different neural tumor cell lines by using the rat C6 glioma cell line as a first experimental paradigm. We first analyzed the expression of galectin-3 by C6 glioma cells grown on PLL-coated coverslips by indirect immunocytochemistry. In nonpermeabilized C6 cells, no galectin-3-specific cell surface staining was detectable (Fig. 2A). By contrast, all permeabilized cells exhibited a marked cytoplasmic immunostaining and in about 40% of the cells also a nuclear staining could be recognized. When permeabilized C6 cells were analyzed with GFAP-specific antibodies, the immunostaining was restricted to the cytoplasm (Fig. 2C). Omission of the first antibody resulted in a complete lack of immunoreaction product (Fig. 2D).

To prove the nuclear localization of galectin-3, we next performed confocal microscopic studies. For this purpose...
purpose, permeabilized C6 cells were double-labeled with galectin-3-specific antibodies using DTAF-conjugated secondary antibodies and propidium iodine. As shown in the selected plane given in Figure 3, galectin-3 immuno-reactivity (Fig. 3A) colocalized with the propidium iodine staining (Fig. 3B) demonstrating a nuclear localization of the protein.

To exclude the possibility that the lack of extracellularly detectable galectin-3 might be due to an extensive secretion of the lectin into the culture medium, we further analyzed its expression using biosynthetically labeled cells. Therefore, C6 cells were biosynthetically labeled for 3 or 24 hr with $^{35}$S-methionine and subsequently cultured for further 4 hr in a serum-containing medium. Galectin-3 was immunoprecipitated out of the cell lysates prepared from these cells using polyclonal galectin-3-specific antibodies coupled to Sepharose 4B and analyzed by autoradiography (Fig. 3D, for 24 hr of $^{35}$S-methionine labeling). For each time point galectin-3 was detectable only in immunoprecipitates of cell lysates (Fig. 3D, lane 1), but not in those of culture supernatants (Fig. 3D, lane 3).

When lysates from C6 cells were analyzed by Western blot, a single band of an apparent MW of 29 kD was recognized by polyclonal antibodies to galectin-3 (Fig. 4, lane 3). These antibodies recognize specifically the N-terminal collagenous-like half of the lectin (Pesheva et
al., 1998b) and after treatment of cell lysates with collagenase, that leads to a degradation of this part of the molecule (Agrwal et al., 1993), the galectin-3-specific band was no longer detectable (Fig. 4, lane 1). After treatment of cell lysates in the absence of collagenase performed in parallel, the 29 kD band of intact galectin-3 was still detectable in those lysates (Fig. 4, lane 2).

We were further interested in the question if the carbohydrate recognition domain of C6 cell-derived galectin-3 is present in a functionally active form. To evaluate the lectin activity of galectin-3, cell lysates were incubated overnight at 4°C with lactose agarose. After this treatment, galectin-3 was no longer detectable in the lysate (Fig. 4, lane 4). Galectin-3 bound to the lactose agarose could be eluted with lactose- (Fig. 4, lane 5), but not with sucrose-containing buffers (Fig. 4, lane 6).

The constitutive expression of galectin-3 by C6 cells could be the result of an autocrine mechanism (Zaheer et al., 1995). To analyze if the expression of galectin-3 is subject to regulation by CNTF, IL-1β, LIF or NGF (C6 cells express the corresponding receptor molecules as well as CNTF, LIF and NGF, at least at the mRNA level; Zaheer et al., 1995), C6 cells were cultured for 1 to 3 days in the presence of cytokines/growth factors and the level of galectin-3 expression was analyzed by Western blot of cell lysates. None of the growth factors tested had a significant effect on galectin-3 expression by C6 cells (not shown).

Taken together, these data demonstrate that in C6 glioma cells, galectin-3 is predominantly if not exclusively expressed in intracellular compartments in a functionally active form.
Galectin-3 expression in different neural cell lines was determined by Western blot analysis (WB) using monoclonal or polyclonal galectin-3-specific antibodies and by RT-PCR. *Strong to mediate expression; +/- weak expression; ~ no expression; NT, not tested.

Expression Pattern of Galectin-3 In Other Neural Tumor Cell Lines

In the following studies, we analyzed the expression pattern of galectin-3 in a representative collection of neural tumor cell lines consisting of 15 human glioblastoma cell lines, the human oligodendroglioma cell line TC620, the murine oligodendrocyte precursor cell line Oli-neu, three human (MHH-NB-11, SMS-KAN, SK-N-SH) and one mouse neuroblastoma (N2A) cell lines and one primitive neuroectodermal tumor cell line (SK-N-MC) (Table I).

In a first set of experiments we analyzed the expression of galectin-3 using Western blot and RT-PCR techniques (Table I). Using galectin-3-specific polyclonal antibodies, galectin-3 was detectable in 14 out of 15 glioblastoma cell lines by Western blot analysis (Fig. 5; Table I). Comparable results were obtained with the galectin-3-specific monoclonal antibody (recognizing human and mouse, but not rat galectin-3) with the exception of the SK-MG-15 cell line, for which no immunoreactivity was detectable (Fig. 5, Table I). If the latter results reflect alterations in the primary structure of galectin-3 in this cell line is yet a matter of consideration. In addition, galectin-3 was detectable by both monoclonal and polyclonal galectin-3 antibodies in the human oligodendroglioma cell line TC620, but not in the oligodendrocyte precursor cell line Oli-neu, four neuroblastoma and one neuroectodermal tumor cell lines (Fig. 5; Table I).

We next analyzed the subcellular expression pattern of galectin-3 by indirect immunofluorescence. As shown for C6 cells (Fig. 2), galectin-3 was amply detectable in the intracellular compartment of all neural tumor cells expressing the lectin. In some cell populations of the cell lines tested, a nuclear staining was also detectable (Fig. 6, for A172 and U87MG cells). In none of the cell lines, a cell surface expression of galectin-3 was detectable (Fig. 6 for A172 and U87MG cells). In contrast to glial tumor cells, none of the neuroblastoma and neuroectodermal tumor cell lines expressed detectable amounts of galectin-3 (Fig. 6M-O, for N2A, SK-N-SH and SK-N-MC cells). In two of the human glioma cell lines (A172 and U87MG), we finally examined the effect of different cytokines/growth factors on the regulation of galectin-3 expression. As for rat C6 glioma cells, the expression level of galectin-3 was not altered in the presence of different cytokines/growth factors (EGF, bFGF, GM-CSF, IL-1β, IL-6, NGF, TGF-β and TNF-α) or LPS (not shown).

Adhesion of Glioma Cells to Galectin-3 Substrata

We have previously shown that a number of neural cells adhere to galectin-3 substrate in vitro by a β-galactoside-mediated mechanism (Pesheva et al., 1998a). Applying the same cell-substratum adhesion assay, we first examined the adhesion of neoplastic glial cells (i.e., C6 glioma) and primary astrocytes to substratum-bound galectin-3 (Fig. 7). When C6 cells were allowed to adhere for 4 hr to galectin-3 or collagen type I substrata, cells strongly adhered to collagen type I (Fig. 7B), but not to galectin-3 substrata (Fig. 7A). Comparable results were obtained after 24 hr of incubation (not shown). By contrast, primary astrocytes derived from early postnatal mouse brain, efficiently adhered to both substrata after 4 hr of incubation (Fig. 7C,D).

To study if this behavior is a more general phenomenon for glioblastoma cells, we next analyzed the adhesion of A172, CCF-STTG1, SK-MG-2, -3, -5, -9, -13, U87MG and TC620 cell lines to galectin-3 (derived from four different preparations of the recombinant lectin) and collagen type I substrata and compared it to the adhesion pattern of primary astrocytes and C6 cells. Although all glioblastoma cells tested adhered to galectin-3 substrata to varying degrees (dependent on the cell line and the galectin-3 preparation used), the number of adherent cells was always between 0 (only for C6 cells) and 30% of the number of primary astrocytes adhering to galectin-3 substrata. In contrast, the number of primary astrocytes or glioblastoma cells adhering to collagen type I substrata was almost identical (not shown), suggesting that the glycostructures recognized by galectin-3 display certain variations among the different glioblastoma cell lines and are expressed at much lower levels than those on primary astrocytes.

DISCUSSION

In the present study we provide evidence that galectin-3 is expressed by high-grade astrocytoma and oligodendroglioma cell lines, but not by neuroblastoma,
primitive neuroectodermal and oligodendrocyte precursor cell lines or primary O-2A progenitor cells. The expression of the lectin can not be induced in cultured astrocytes or oligodendrocytes maintained in serum-containing media supplemented with a variety of cytokines/growth factors. It is noteworthy in this context that extended studies of ours now further give evidence for the lack of galectin-3 expression by reactive macroglial cells also under pathological conditions in vivo, such as experimental ischemia or facial nerve axotomy in the rat (Walther, Kuklinski, Pesheva and Probstmeier, unpublished observations). Together, these observations argue for an association of galectin-3 expression with a transformed glial phenotype.

Galectin-3 as a Marker Molecule for Glial Tumor Cells

There are yet contradictory data on the in vivo expression of galectin-3 in glial tumors. Bresalier et al. (1997) have reported a lack of galectin-3 expression in grade II, but mediate to high levels of expression, though by a varying number of tumor cells (between 10 and 90%), in all grade III and IV astrocytomas analyzed. In contrast, Gordower et al. (1999) have recently shown a decrease in galectin-3 expression from grade II to grade IV astrocytic tumors accompanied by an increasing heterogeneity in the expression level of the lectin and the appearance of tumor cell clones expressing large amounts of the lectin in high grade astrocytomas.

As it is assumed that glial tumors are of monoclonal origin (see, for example, Kleihues et al., 1995), galectin-3 expressing and non-expressing tumor cells most likely represent different cellular phenotypes whose molecular profile depends on the particular tissue microenvironment. Alternatively, this pattern could reflect secondary clonal tumor evolution. Thus, the dominant presence of galectin-3 in established glioma cell lines in vitro would reflect the presence of an optimal environment for the expression of galectin-3 under the culture conditions used. The only glioma cell line tested that does not express the lectin (SK-MG-5) has been derived from a patient with the diagnosis of anaplastic astrocytoma (grade III astrocytoma). At present we have no evidence for the cellular/molecular basis of this altered expression. In contrast to the findings of Bresalier et al. (1997) for the lack of galectin-3 expression in four oligodendrogliomas analyzed, the oligodendroglioma cell line TC620 tested by us expresses...
Figure 6. (Legend on following page.)
high levels of the lectin. Thus, further studies are needed to allow a more precise statement about the application of galectin-3 as a marker for this kind of glial tumors.

Regulation of Galectin-3 Expression in Glioma Cells

The regulation of galectin-3 expression by glioma cells is yet a matter of investigation. In principle, two main possibilities could be considered. 1) Autocrine mechanisms governed by cytokines/growth factors secreted by glioma cells. Although stimulation of glioma cells with various cytokines/growth factors does not lead to a further increase in the level of galectin-3 expression, we can not exclude the possibility that in this cell type, the lectin level of expression has already reached a maximal value. (2) Changes in the intrinsic machinery of gene expression leading to a constitutive expression of the lectin. For example, the constitutive expression of galectin-1 can be regulated by the methylation level of the promoter region (Benvenuto et al., 1996). By treating glioma cell lines (i.e., SK-MG-4, U251MG, U343MG and TC620) with the demethylating agent 5′-azacytidine, however, we were not able to observe changes in the expression level of galectin-3 (Kuklinski and Probstmeier, unpublished observations).

Biological Function of Galectin-3 in Glioma Cells

It has been speculated that one function of galectin-3 in tumor cells expressing the molecule at the cell surface is to mediate cell-cell or cell-matrix interactions (Akahani et al., 1997a). As all cell lines investigated in our present study express galectin-3 only intracellularly, these speculations cannot be extended to CNS-derived glial tumor cells.
In glioma cells, galectin-3 is present predominantly in the cytoplasm and, only in some cell lines and to a varying degree, also in the nucleus. Dependent on each particular subcellular location, distinct functions could be hypothesized. It has been reported that galectin-3 is involved in pre-mRNA splicing events either as an intrinsic part of the splicing machinery or as a “control” molecule (reviewed by Patterson et al., 1997). In the cytoplasm of glioma cells, galectin-3 could be involved in apoptotic processes. For T cells, it has been demonstrated that fas-induced apoptosis is prevented by overexpression of galectin-3 (Yang et al., 1996). In these cells, galectin-3 seems to interact directly or indirectly with bcl-2, a member of a protein family involved in the initiation or prevention of apoptosis (Yang et al., 1996). Such kind of interactions could be essential for the unlimited growth of glioma and other tumor cells.

It is very unlikely that galectin-3 serves as a key molecule leading to cell transformation per se, as the lectin is expressed in a number of non-malignant cells, such as DRG neurons (Regan et al., 1986) or activated microglial cells (Pesheva et al., 1998b). On the other hand, in non-tumorigenic carcinoma or weakly metastatic sarcoma cell lines, a tumorigenic phenotype (either de novo or with increased potential for metastasis formation) can be induced by an ectopic expression of galectin-3 (Raz et al., 1990; Nangia-Makker et al., 1995). As in these cases, however, cell lines have been used that can be propagated in culture for an unlimited period of time, i.e., they show growth properties different from those of their normal counterparts, the findings can be interpreted in the way that galectin-3 could favor the development of a malignant cellular phenotype when normal growth properties have already been disturbed.

The contradictory reports on galectin-3 expression in astrocytic tumors of different grade of malignancy, together with the observation that only a certain percentage of tumor cells express the lectin in vivo (Bresalier et al., 1997; Gordower et al., 1999), surely make possible hypotheses on the role of galectin-3 in such tumors a complicated matter.

**β-Galactosidic Carbohydrate Structures Expressed by Glioma Cells**

In contrast to a number of neural cell types (Pesheva et al., 1998a) and primary astrocytes (the present study), glioma cells do not or only weakly adhere to a galectin-3 substratum. As in the same assay neuroblastoma or PC12 cells are capable of adhering to galectin-3 (Pesheva et al., 1998a), this property of glioma cells is not shared by all neural tumor cells and could reflect an inadequate glycosylation pattern, i.e., the presence of low levels of β-galactosidic carbohydrate structures or inadequate terminal sialic acid residues. For instance, it has been demonstrated that (i) a terminal α2 → 3-linked sialic acid favors and (ii) a terminal α2 → 6-linked sialic acid or substitution of N-acetylglucosamine with fucose inhibits β-galactoside-specific binding of galectins (Hughes, 1992).

The main point addressed here, however, is the presence of galectin-3 in most of the CNS-derived glial tumor cell lines. Beside the possibility that galectin-3 expression may be useful as a marker in glial tumor diagnosis, further analysis of the lectin contribution to the malignant potential of glial tumors may, hopefully, lead to the development of reliable therapeutic tools.

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