Functional Evidence that Cell Surface Galectin-3 Mediates Homotypic Cell Adhesion

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Abstract

Galectin-3 (Gal-3) is a β-galactoside-binding protein with Mr ~30,000. Cell surface Gal-3 is postulated to be involved in homotypic aggregation of tumor cells in the circulation during metastasis through attachment to a complementary serum glycoprotein(s), which serves as a cross-linking bridge between adjacent cells. To test this hypothesis a recombinant strain of baculovirus encoding Gal-3 was used to infect Sf9 insect cells, which lack endogenous Gal-3. Immunoblotting and indirect immunofluorescence studies revealed that the infection with recombinant virus conferred Gal-3 expression on Sf9 cells, and the Gal-3 was localized on the cell surface as well as in the cytoplasm. Sf9 cells infected with recombinant virus underwent homotypic aggregation in the presence of exogenous glycophorin (i.e., asialofetuin), whereas control cells uninfected or infected with wild-type virus did not. Lactose and Fab' fragments of anti-Gal-3 antibodies markedly inhibited the cell-cell aggregation. Moreover, cosuspension of SIV cells infected with the recombinant virus with uninfected cells in the presence of asialofetuin resulted in a preferential cosuspension of SIV cells infected with the recombinant virus with uninfected cells. Lactose and Fab' fragments of anti-Gal-3 antibodies markedly inhibited the cell-cell aggregation. Moreover, cosuspension of SIV cells infected with the recombinant virus with uninfected cells in the presence of asialofetuin resulted in a preferential cell-cell adhesion of the Gal-3-expressing cells. These results directly demonstrate the ability of cell surface Gal-3 molecules to mediate homotypic cell adhesion by bridging through branched, soluble complementary glycoconjugates.

Introduction

An increasing number of vertebrate lectins have been identified and characterized in a diversity of tissues and cells and were implicated in cell-cell and cell-matrix interactions by combining with complementary glycoconjugates, and they played a key role in the control of various normal and pathological processes (1, 2). Galectin is a growing family of β-galactoside-binding proteins, and membership in the galectin family requires the fulfillment of two criteria: (a) affinity for β-galactosides; and (b) significant sequence homology in the carbohydrate-binding site (2). Gal-3, formerly known as CBP-35, Mac-2, eBP, L-29, L-34, and L-31 and other names, is isolated as a monomer with Mr ~30,000 (2-6). It consists of two distinct structural domains: (a) an amino-terminal domain containing a repetitive sequence rich in glycine, tyrosine, and proline; and (b) a carboxy-terminal domain with a globular structure encompassing the carbohydrate-binding site (2-6). Galectin-3 is presumed to be involved in the multiple biological processes through interaction with specific ligands, including cell growth, adhesion, differentiation, inflammation, transformation, and metastasis (2, 7). Direct evidence to support any particular function proposed for Gal-3 has not yet been reported. However, we described previously the presence of Gal-3 in various human and murine tumor cells and proposed that interaction of cell surface Gal-3 with a complementary serum glycoprotein(s) promotes homotypic aggregation of tumor cells in the circulation, thereby playing an important role in the pathogenesis of metastasis (7).

Materials and Methods

Cells and Culture Conditions. Spodoptera frugiperda cell line Sf9 cells were grown in complete TNM-FH [Grace's insect medium (Invitrogen, San Diego, CA) supplemented with 10% (v/v) fetal bovine serum and 10 μg/ml gentamicin] at 27°C in a humidified incubator. Suspension cultures >100 ml were adjusted to 0.1% pluronic surfactant F-68 (Sigma Chemical Co., St. Louis, MO) to protect the cells from hydrodynamic stress.

Plasmid Construction and Generation of Recombinant Baculovirus. The cDNA encoding human Gal-3 was subcloned into BamHI site of the pBlueBac III baculovirus transfer vector (Invitrogen) to generate pBlueBac III/hGal-3. The cDNA was digested with EcoRI. A 0.9-kb fragment containing the entire coding region was blunt ended with a Klenow fragment of DNA polymerase I and ligated into pBlueBac III, which had been linearized with BamHI and blunt ended with Klenow. Recombinant plasmid pBlueBac III/ hGal-3 was isolated by CsCl gradient ultracentrifugation, and the proper orientation of the cDNA insert with respect to the polyhedrin promoter in pBlueBac III was confirmed by restriction enzyme analysis and DNA sequence analysis. pBlueBac III/hGal-3 was cotransfected along with linearized wild-type AcMNPV baculovirus DNA (Invitrogen) into Sf9 cells with the use of cationic liposome (Invitrogen). Four days after transfection, the supernatant was used to infect Sf9 cells with an overlay of 1% SeaPlaque agarose (FMC BioProducts, Rockland, ME) containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Boehringer Mannheim, Indianapolis, IN), a substrate for β-galactosidase, which is present in the pBlueBacIII vector. After ~7–10 days, viral clones of recombinant baculovirus giving rise to blue plaques were plaque

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4 The abbreviations used are: Gal-3, galectin-3; AcMNPV, Autographa californica nuclear polyhedrosis virus; NDS, normal donkey serum.

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purified, propagated by passage in Sf9 cells infected at low multiplicity of infection (0.1–0.5 plaque-forming unit/cell), and titred by plaque assay.

Expression of Recombinant Protein. Sf9 cells were infected at high multiplicity of infection (5 plaque-forming units/cell) and were grown in complete TNM-FH. All the experiments described below were performed with the use of the cells at 60-h postinfection.

Immunoblot Analysis. Aliquots of Sf9 cells with or without viral infection were boiled in SDS sample buffer, resolved by 10% SDS-PAGE, and electrotransferred to Immobilon P filter (Millipore, Bedford, MA). The filter was blocked overnight at 4°C with Ca²⁺ and Mg²⁺ -free PBS (pH 7.4) containing 5% nonfat dried milk and 0.1% sodium azide, and probed for 1 h with rat anti-Gal-3 mAb M3/38, which had been produced by a hybridoma TIB-166 (American Type Culture Collection, Rockville, MD). After washing for 1 h, the filter was treated with 0.2 μCi/ml 125I-sheep anti-rat IgG (ICN Biomedicals, Irvine, CA) for 1.5 h in PBS containing 5% nonfat dried milk, 0.1% sodium azide, and 0.1% Tween-20 and further washed for 1 h. The blot was then subjected to autoradiography at ~70°C.

Indirect Immunofluorescence. Cells grown on glass coverslips were washed twice with PBS containing 1 mM CaCl₂ and 0.1% sodium azide (PBS) followed by fixation and permeabilization with precooled (~70°C) 100% methanol at ~20°C for 30 min. The cells were washed briefly with PBS, blocked with 2% NDS in PBS at 4°C for 1 h, then incubated with rat anti-Gal-3 antibody M3/38 in PBS/NDS at 4°C for 1 h, washed 3 times for 5 min with PBS², labeled with β-phycoerythrin-conjugated F(ab')₂ fragment of donkey anti-rat IgG (Jackson Immunoresearch, Westgrove, PA) in PBS/NDS at 4°C for 1 h, and finally washed 3 times for 5 min with PBS. The samples were mounted in 90% glycerol and observed under a fluorescent light microscope. Alternatively, the cells were processed as described above except that they were fixed with 2% paraformaldehyde in PBS at 4°C for 30 min after labeling. The latter procedure is to detect Gal-3 on the cell surface, whereas the former procedure is for the detection of cytoplasmic Gal-3. Controls receiving either no primary antibody or a nonspecific rat IgG exhibited no background binding.

Asialofetuin-induced Homotypic Aggregation. Sf9 cells were harvested at 60 h postinfection, and single cell suspensions were made at 1 × 10⁶ cell/ml in Grace’s insect medium containing 150 μg/ml DNase I. One-ml aliquots of the cell suspension were placed in siliconized glass tubes in the presence or absence of 25 μg/ml asialofetuin and agitated at 95 rpm at 27°C for up to 1 h. The aggregation was then terminated by fixing the cells with 1% formaldehyde. The number of single cells in these aliquots was counted with the use of a hemocytometer. The extent of aggregation was calculated according to the following equation:

\[ 1 - \left( \frac{N_t}{N_c} \right) \times 100, \]

where \( N_t \) and \( N_c \) represent the number of single cells in the presence of the tested compounds and that in the control (Grace’s insect medium/DNase I),

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**Fig. 1.** Expression of Gal-3 gene product in Sf9 cells. A, immunoblot analysis of cell lysates from control Sf9 cells (Lane a), cells infected with recombinant virus (Lane b), and cells infected with wild-type virus (Lane c). Each lane contained lysate corresponding to 1 × 10⁶ cells. Rat anti-Gal-3 M3/38 was used. Migrations of standard proteins are shown at extreme left. B, immunofluorescence staining of recombinant virus-infected (Panels a and b) and control (Panels c and d) Sf9 cells. Fixed and permeabilized (Panels a and c) or intact (Panels b and d) cells were incubated with rat anti-Gal-3 M3/38 and visualized with β-phycoerythrin-conjugated F(ab')₂ fragment of donkey anti-rat IgG. X 600.
respectively. Asialofetuin was prepared from fetuin (Sigma) by mild acid hydrolysis as reported previously (8).

In a subset of assays, 100 μg/ml Fab' fragments of rabbit IgG antibody directed against a synthetic peptide of the carbohydrate-binding domain of Gal-3 (10, 11), 100 μg/ml Fab' fragments of normal rabbit IgG, 50 mM lactose, or 50 mM sucrose were added in addition to asialofetuin. Triplicate cell suspensions of SF9 cells expressing Gal-3 were agitated as above for 1 h. For the preparation of Fab' fragments, IgG fraction was purified from rabbit antisera with the use of an IgG purification kit (Pierce Chemical Co., Rockford, IL) followed by fragmentation with papain with the use of a Fab preparation kit (Pierce).

Fluorescent labeling of cells with the use of Dil was performed according to the manufacturer's instruction (Molecular Probes, Eugene, OK). SF9 cells were incubated with 20 μM Dil for 15 min at room temperature and then washed twice with Grace’s insect medium. Dil-labeled SF9 cells expressing Gal-3 were mixed with an equal number of unlabeled control cells, and aggregation assays were performed in the presence of 25 μg/ml asialofetuin as described above. Aliquots were spotted onto microscope slides, and Dil-labeled cells were observed for epifluorescence.

Results

Cotransfection of SF9 cells with linearized wild-type AcMNPV baculovirus DNA and pBlueBacIII/hGal-3 produced both wild-type baculovirus and recombinant baculovirus through frequent homologous recombination between AcMNPV DNA and the transfer vector. Infection of SF9 cells with the mixture of the viruses resulted in the generation of recombinant blue plaques with the occlusion body-negative phenotype, which accounted for as much as 30% of the plaques generated. Viral clones of recombinant baculovirus were plaque purified and used for recombinant protein expression. The cells were collected at 60-h postinfection, and expression of recombinant Gal-3 was analyzed by immunoblotting with the use of the rat antismouse Mac-2 M3/38 mAb. Fig. 1A depicts the expression of the human Gal-3 gene product in SF9 cells. Immunoreactive protein with an apparent molecular mass of 31 kDa exhibiting the same electrophoretic mobility as native human Gal-3 was detected in cells infected with recombinant virus, whereas no immunoreactive protein was detectable in infected or wild-type virus-infected cells even after prolonged exposure. To determine subcellular distribution of the recombinant Gal-3 protein, indirect immunofluorescence studies were performed on SF9 cells infected with recombinant virus. Recombinant Gal-3 was diffusely distributed throughout the cytoplasm with little or no nuclear localization in permeabilized cells (Fig. 1B, panel a). When intact viable cells were immunofluorescently stained, an intense cell surface labeling in the form of microclusters was observed (Fig. 1B, panel b), similar to the distribution pattern observed in mammalian cells (7). Because all of the staining procedures used here were carried out at 4°C in the presence of 0.1% sodium azide in an attempt to minimize internalization of the antigen-antibody complex by endocytosis, these results indicate that recombinant Gal-3 localized on the cell surface as well as in the cytoplasm, which was similar to the subcellular distribution pattern of endogenous Gal-3 in vertebrate cells (7). Moreover, a similar cell surface-labeling pattern was obtained when the cell surface staining was performed in the presence of 100 mM lactose, a disaccharide competitive inhibitor for carbohydrate-binding by Gal-3 (data not shown). Therefore, most likely the antigenicity detected on the cell surface was not due to recombinant Gal-3 released by the cells, which might have subsequently combined with cell surface complementary glycoconjugates. In turn, it is likely that the carbohydrate-binding site of the SF9-expressed recombinant Gal-3 protein was not occupied for cell surface anchoring but rather exposed on the external surface. This supposition was further substantiated by indirect immunofluorescence studies using a rabbit polyclonal antibody directed against a synthetic peptide of the carbohydrate-binding domain of Gal-3, which showed a high degree of antigenic recognition on the cell surface (data not shown) similar to the mAb-labeling pattern. It is important to note that the epitope recognized by the anti-Gal-3 M3/38 mAb is at the amino-terminal domain of the molecule, which is not involved in carbohydrate binding. In sharp contrast, no labeling was observed when uninfected or wild-type virus-infected cells were stained (see Fig. 1B), corroborating the results of the above immunoblotting analysis.

Next, we proceeded to determine whether cell-cell aggregation could be induced in these recombinant cells by asialofetuin, in a manner analogous to that described for malignant cells (8). Fig. 2A shows the extent of asialofetuin-induced homotypic aggregation as a function of time. The cells infected with recombinant virus rapidly formed multicell aggregates, whereas the uninfected or the wild-type virus-infected cells were refractory to the exogenous glycoprotein molecules and remained as single cells in suspension. The size of the aggregates varied from doublets up to large aggregates comprising >100 cells (Fig. 2B). The ability of the recombinant virus-infected cells to undergo this homotypic aggregation could be markedly impaired with lactose, the competitive sugar, but not with sucrose, a control disaccharide (Fig. 3). In addition, monovalent Fab' fragments of rabbit IgG antibody directed against a synthetic peptide of the carbohydrate-binding domain of Gal-3 significantly inhibited homo-
or sucrose (50 mM). The extent of aggregation was determined as described in "Materials and Methods." Columns, mean; bars, SD. Asterisks, statistically significant differences from control: *P < 0.001, t-test.

typic aggregation, whereas Fab' fragments of nonspecific rabbit IgG had no effect (Fig. 3). These results demonstrate that cell surface Gal-3 molecules interact with asialofetuin, which acts as a crosslinking bridge between adjacent cells, resulting in the formation of cell aggregates. The kinetics of aggregation was not affected by the addition of sodium citrate or EDTA, which are chelating reagents (data not shown), reiterating that Gal-3 functions independently of divalent cations.

The above data did not, however, exclude the possibility that the Gal-3 molecules on one recombinant virus-infected cell mediated aggregation via interaction with some ubiquitous complementary glycoconjugates on an adjacent cell. Thus, to address this question, recombinant virus-infected Sf9 cells that had been labeled with Dil stain were mixed at a 1:1 ratio with unlabeled control Sf9 cells and were allowed to interact in the presence of asialofetuin. The resulting aggregates comprised predominantly of labeled cells (Fig. 4), whereas the unlabeled cells accounted for ~80% of the remaining single cells. Thus, it may be concluded that the interaction of Gal-3 with exogenous soluble glycoprotein rather than a cell surface complementary glycoconjugate must be primarily responsible for this aggregation.

Discussion

Metastasis is a complex cascade consisting of numerous sequential steps, which is the most life-threatening aspect of malignant diseases. Tumor cell embolization is thought to be a crucial step in the metastatic cascade, because tumor emboli, which often derive from homotypic and heterotypic aggregation of tumor cells, may be responsible in part, for tumor cell arrest followed by extravasation at secondary sites (for review see Ref. 7). A strong correlation has been demonstrated between the propensity of tumor cells to undergo homotypic aggregation in vitro and their metastatic potential in vivo. For example, lung colonization after i.v. inoculation of B16 melanoma cell aggregates is much higher than that of single cell suspensions (12). B16 melanoma variant subline B16-F10, which has been selected in vitro for reduced tendency to undergo homotypic aggregation in the presence of asialofetuin, exhibits decreased lung colonization in syngeneic mice (14). Therefore, elucidation of molecular mechanism by which tumor cells form aggregates may provide a clue for the control of metastasis, provided that tumor embolization itself cannot be sufficient to explain organ specificities preferentially displayed by various types of tumor cells.

In the present study we have shown that cell surface Gal-3 is involved in asialofetuin-induced homotypic aggregation. Given the knowledge that highly metastatic cells express cell surface Gal-3 to a higher extent than their non- or weakly metastatic counterparts (7), it is plausible to envision that cell surface Gal-3 plays a significant role in metastasis by promoting tumor cell embolization in the circulation. The property of tumor cells to undergo homotypic aggregation induced by asialofetuin directly correlates with that induced by syngeneic serum (14). Accordingly, it is conceivable to seek a glycoprotein(s) in the serum which should participate in the process of Gal-3-mediated aggregation in vivo. One possible candidate for such a molecule is the Mac-2-binding protein, which has been recently identified as a secreted ligand for Gal-3 and exists in the serum (15–17). It is intriguing that an elevated serum level of the L3 antigen, which is identical to the Mac-2-binding protein, has been shown to reflect the progression of various malignant diseases in addition to some benign diseases (18). In human cancers, a correlation exists between Gal-3 levels in colon cells and tissues and the stage of tumor progression (19–21). Similarly, the tissue levels of Gal-3 are higher in certain primary gastric cancers and so is their metastases compared with adjacent normal mucosa (22). In human breast carcinoma cells it was suggested that estradiol and progesterone might act as coordinators regulating specific genes, including Gal-3, leading to the acquisition of the metastatic phenotype (23).

Fig. 3. Inhibition of asialofetuin-induced homotypic aggregation by lactose and anti-Gal-3 antibody. Sf9 cells infected with recombinant virus were agitated at 27°C for 1 h in the presence of asialofetuin with or without added Fab' fragments of rabbit anti-Gal-3 antibody (100 µg/ml), Fab' fragments of normal rabbit IgG (100 µg/ml), lactose (50 mM), or sucrose (50 mM). The extent of aggregation was determined as described in "Materials and Methods." Columns, mean; bars, SD. Asterisks, statistically significant differences from control: *P < 0.001, t-test.

Fig. 4. Aggregation in heterogeneous cell populations. Dil-labeled Sf9 cells expressing Gal-3 were mixed at a 1:1 ratio with unlabeled control cells, and the cells were allowed to aggregate in the presence of asialofetuin with agitation at 27°C for 1 h. A. Panel a, phase contrast photomicrograph, whereas Panel b shows a photograph of the same field with the use of an epifluorescence microscope to visualize Dil. × 100. B, quantitative distribution of labeled cells in aggregates.
In conclusion, we have provided direct evidence that cell surface Gal-3 is a cell-cell adhesion molecule that mediates homotypic cell-cell adhesion through interaction with a complementary glycoprotein by utilizing the baculovirus-infected Sf9 insect cell system. We believe that the data presented here should give insight into the biological function of Gal-3 in vivo, which remains largely unknown.

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References

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