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CARRIER-IMMOBILIZED DERIVATIZED LYSOGANGLIOSIDE GM_1 IS A LIGAND FOR SPECIFIC BINDING SITES IN VARIOUS HUMAN TUMOR CELL TYPES AND PERIPHERAL BLOOD LYMPHOCYTES AND MONOCYTES

Sigrun Gabius[1], Klaus Kayser[2], Klaus P. Hellmann[2], Thomas Ciesiolka[2], Andrea Trittin[2] and Hans-J. Gabius[2,*]

[1]Abteilung Hämatologie-Onkologie, Medizinische Universitätsklinik, Robert-Koch-Str. 40, D-3400 Göttingen, FRG

[2]Max-Planck-Institut für experimentelle Medizin, Abteilung Chemie, Hermann-Rein-Str. 3, D-3400 Göttingen, FRG

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Summary. Biotinylation of ganglioside-protein conjugates, derived from selective N-acylation of the sphingoid amino group of deacylated ganglioside GM_1 or a ganglioside mixture, yielded probes to detect specific binding sites in fixed specimens. GM_1-containing neoligandoprotein significantly bound to tumor cells in sections of 15 out of 16 cases of human lung cancer, while the probe, derived from the mixture, was ineffective under these conditions. The same graduation of staining was under identical conditions observed with these two probes on fixed human tumor cells and on peripheral blood lymphocytes and monocytes. Attempts of biochemical isolation of proteins, responsible for this binding capacity, from tumor cell extracts in the presence of the abundant endogenous ligands led to protein bands with apparent molecular weights of 44,000, 68,000 and 72,000 with yields of 0.1 - 0.24 µg/mg protein, after the detergent extracts had been passed over a resin, exposing gangliosides of the markedly less efficient mixture, to exclude binding by non-specific ionic or hydrophobic interactions.

Cell surface constituents unquestionably mediate the pivotal steps in cellular adhesion and communication. Consequently, the analysis of determinants with supposed relevance for recognitive interactions will help to elucidate the molecular nature of the intriguingly complex interplay of events that establish cellular development. Viewed on the basis of their strategically favorable placement and the widely documented association of their expression with the cell type, stage of differentiation or malignant transformation, gangliosides have been considered to participate in cellular interactions and growth regulation (1-3). The ability of certain gangliosides to serve as ligands for bacterial toxins

*To whom correspondence should be addressed.

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has underscored their ability to be involved in molecular recognition (4). However, the biological consequences of the developmental regulation as well as the modes of defined interactions of gangliosides are poorly understood. Remarkably, embryonic neural cell adhesion is supported by gangliosides, adsorbed to an artificial surface (5). Further evidence for the presence of a putative ganglioside receptor has been provided in solid-phase assays with membrane proteins, using ganglioside derivatives, covalently linked to a carrier via their ceramide moieties (6). Since carrier-immobilized carbohydrate structures are increasingly exploited as ligands in search of respective binding sites in cells and in tissue sections (7-9), this development has prompted us to employ such a neoligandoprotein with a derivatized lysoganglioside as tool. Immobilized derivatized lysoganglioside GM_1 is compared in its efficiency to a lysoganglioside mixture under the same conditions glycohistochemically and glycocytologically. Additionally, preliminary data on the characteristics of proteins from detergent extracts that bind to this type of derivative are reported.

MATERIALS AND METHODS

Synthesis of the neoligandoproteins. Lysogangliosides from GM_1 (Fidia, Munich, FRG) and from a ganglioside mixture containing 40 % GD_{1a}, 21 % GM_1, 19 % GT_{1b} and 16 % GD_{1b} (Calbiochem, Frankfurt, FRG) were obtained, as described (6,10). They were processed for conjugation to periodate-treated bovine serum albumin of highest commercially available purity with bis(sulfosuccinimidyl) suberate, synthesized as described (11), as outlined recently (6). Analytical procedures for the products and biotinylation of the probes followed given protocols (6,9).

Glycohistochemical reactions. 5 - 6 µm sections of routinely fixed specimens from 16 cases with primary bronchus carcinoma were processed by rehydration, treatment with 0.1 % methanolic hydrogen peroxide to block endogenous peroxidase activity, treatment with 1 % bovine serum albumin solution to saturate unspecific protein-binding sites, incubation with 10 µg/ml biotinylated neoligandoprotein for 60 min at room temperature, thorough washing, incubation with ABC reagents (Camon, Wiesbaden, FRG), color development and hematoxylin counterstaining in the presence of rigorous inherent specificity controls, as described in detail elsewhere (9, 12).

Glycocytological reactions. The human colon adenocarcinoma cell line COLO205, the human breast carcinoma cell line DU4475 and the human erythroleukemia cell line HEL 92.1.7, obtained from the American Type Culture Collection (Rockville, USA), were grown in 90 % RPMI 1640 medium and 10 % fetal calf serum supplemented with 2 mM glutamine and antibiotics (Gibco/BRL, Eggenstein, FRG). The erythroleukemia cell line was also grown under conditions that support long term bone marrow cultures, namely in the additional presence of 12.5 % horse serum and 1 µM hydrocortisone. Peripheral blood

lymphocytes and monocytes were isolated by gradient centrifugation with Lymphoprep (Paesel, Frankfurt, FRG). The cells were carefully washed in Hank's balanced salt sodium containing 1 % periodate-treated bovine serum albumin to remove any serum components. Cytospin preparations of approximately 5 x 10^4 cells were dried, fixed at 4°C in 80 % acetone for 10 min and were washed carefully. Blocking protein-binding sites was followed by incubation with the derivatized lysoganglioside GM_1-containing neoligandoprotein at a concentration of 50 µg/ml for tumor cells and 25 µg/ml for blood cells or with the lysoganglioside mixture-containing neoligando-protein at a concentration of up to 200 µg/ml for 4 hr at 37°C in phosphate-buffered saline. Following three washes with phosphate-buffered saline, endogenous peroxidase activity was abolished by treatment with 0.23 % periodic acid (13). Visualization of the bound probes was achieved by the application of ABC reagents and a heavy metal-mediated enhancement of the standard diaminobenzidine/ H_2O_2 reaction (14). Counterstaining was performed with hemalaun. The slides were finally covered with Entellan. Control experiments were performed by excluding the unspecific binding of the labelled, but non-glycosylated carrier protein at a concentration of 200 µg/ml and by competitive inhibition studies, using the label-free neoligandoprotein as inhibitor. Omission of the labelled probe in the standard protocol excluded any binding of ABC reagents to the cells, because no deposition of the colored reaction product was visible.

Biochemical procedures. 40 µmol of the activated ganglioside-sulfosuccinimidyl esters were coupled to 5 ml amino group-exposing Affi-Gel 102 gel (Biorad, Munich, FRG). Carefully washed pellets of the erythroleukemia cells were homogenized in a Potter homogenizer with 6 volumes of 25 mM Tris-HCl buffer (pH 7.8) containing 1 % Triton X-100, 0.1 % sodium deoxycholate, 0.1 mM benzylsulfonyl fluoride, 1 mM dithiothreitol, 5 µg/ml leupeptin, antipapain, chymostatin and benzamidine hydrochloride, each, and 0.2 M KCl. The further processing and the involved analytical procedures had already been outlined in detail in the case of leukemia and lymphoma cells (15). Subsequent elutions from the resins were performed with 4 mM EDTA and 0.1 M NH_4OH (pH 11).

RESULTS

The synthesis resulted in an incorporation of approximately two GM_1 units per carrier molecule. As judged from the shift in gel electrophoretic mobility, the ganglioside:protein ratio was higher in the product, derived from coupling activated gangliosides of the mixture, as already reported (6). The GM_1-containing probe caused significant staining in nearly all of the fixed and paraffin-embedded sections from the lung tumors, as illustrated exemplarily in Fig. 1a. Each of the four cases of large cell anaplastic carcinoma, epidermoid carcinoma and adenocarcinoma as well as three out of four cases of small cell anaplastic carcinoma of the lung specifically bound this probe. However, under the same conditions no significant specific staining was noted with the probe, derived from the ganglioside mixture (Fig. 1b). Remarkably, this excluded media-

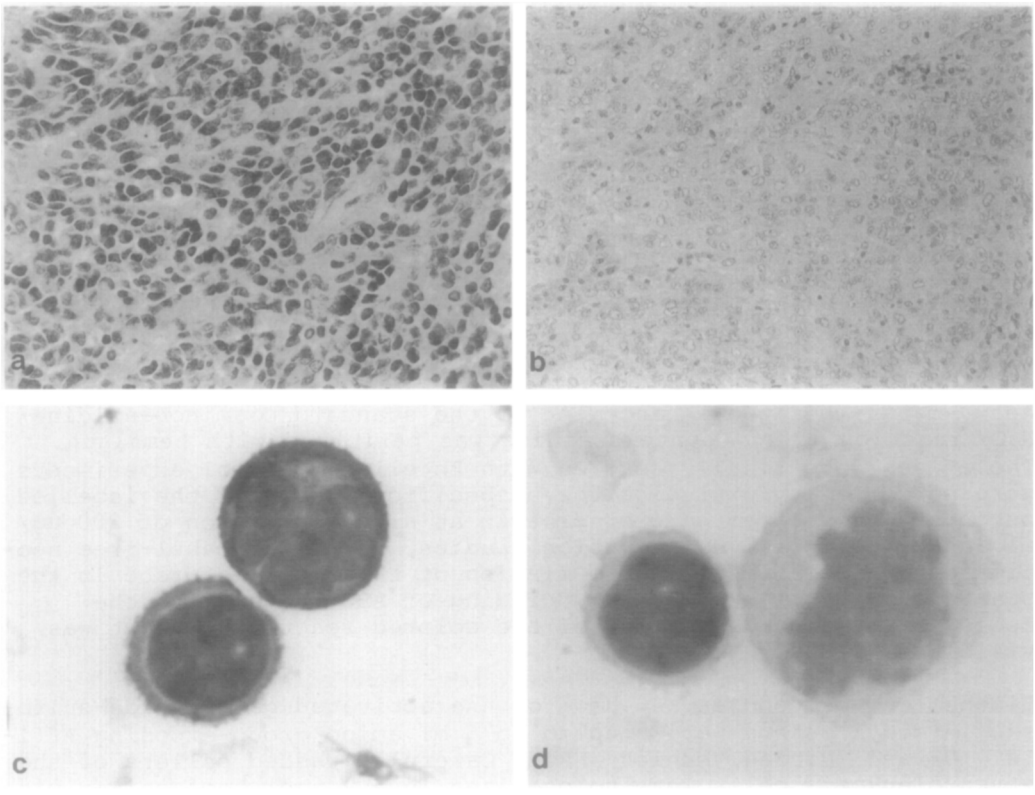

Fig. 1. Light micrographs of sections of a small cell anaplastic carcinoma (a) and a large cell anaplastic carcinoma of the human lung after incubation with 10 µg/ml biotinylated neoligandoprotein (GM_1-containing probe: a; ganglioside mixture-containing probe: b) as well as ABC reagents and hematoxylin counterstaining and of cytospin preparations of human peripheral blood lymphocytes and monocytes after subsequent incubation with 25 µg/ml biotinylated neoligandoprotein (GM_1-containing probe) in the absence (c) or presence (d) of an excess of competitive inhibitor, ABC reagents, the sensitive color development system and hemalaun counterstaining; original magnification x 400 (a), x 200 (b), x 800 (c,d).

tion of binding, governed by non-specific ionic or hydrophobic interactions.

When analyzing the binding capacity to acetone-fixed tumor cells of different histogenesis, the same pattern was disclosed. The GM_1-containing probe specifically bound to the various cell types invariably stronger than the other marker. This might reflect the low proportion of GM_1 in the mixture, with the other gangliosides not or only weakly exhibiting ligand properties. Besides tumor cells peripheral blood monocytes and lymphocytes expressed respective binding sites that can be blocked by an excess of unlabelled reagent (Fig. 1c,d). Strong membrane staining was seen for all the cell types. This result encouraged attempts to employ re-

sin-immobilized ganglioside derivatives, proven to be effective as ligands for the localization of specific binding sites in sections and cells, for the isolation of respective binding proteins despite the abundant presence of endogenous gangliosides. Since sufficient amounts of human cells were easier accessible from tumor cells than from blood cells, extracts of the human erythroleukemia cell line were passed over the resin, derivatized by coupling the activated ganglioside. To exclude any preferentially non-specific hydrophobic or ionic interactions in binding to the immobilized ganglioside derivative, the detergent extracts of the tumor cells, grown in the absence and presence of horse serum and hydrocortisone, were first passed over column resin that contained immobilized derivatives of the ganglioside mixture at a comparable density. Only rather small yields of protein were obtained from the detergent extracts of cells under these conditions, namely 0.1 - 0.24 µg/mg protein after subsequent elutions with chelating agent and 0.1 M NH_4OH (pH 11). GM_1-containing resin bound proteins at apparent molecular weights of 44,000, 68,000 and 72,000. These proteins were present, if detectable, in significantly reduced levels in the eluates from the ganglioside mixture-containing resin.

DISCUSSION

Conjugation of suitably activated lysogangliosides to a labelled carrier renders probes available for localizing respective binding sites, as illustrated in this study. The affinity of the resulting markers in comparison to the native ganglioside can be advantageously enhanced by increasing the ligand density (6,16,17). Application in parallel experiments of related probes, derived from the same synthetic pathway, will assess the degree of specificity. Notably, within a panel of liposomes containing a ganglioside mixture, certain purified gangliosides or phospholipids relatively highest uptake and retention in bone marrow has been shown for the GM_1-containing liposome preparation in biodistribution, pointing to the presence of an inherent, yet not clearly defined mechanism for specificity (18). It is not related to sialic acid-binding structures, known from macrophages or placenta (19-21). Nonetheless, the neoligandoprotein provides evidence for specific binding without addition of Ca^{2+}-ions in organic solvent-treated tissue sections and cells. Based on the relative inefficiency of the probe, derived from the ganglioside mixture, as also noted in biodistribution of ganglioside-bearing liposomes (18), a binding, governed by non-specific hydrophobic or ionic interactions, ap-

pears rather unlikely. This reduces the already emphasized number of ways, in which the ganglioside GM_1 can interact with targets on various cell types (6,22, 23). Besides proteins carbohydrate structures, too, deserve to be considered as possible receptor structures (24).

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