Localization of Shed Human Tumor Gangliosides: Association with Serum Lipoproteins

Leonard A. Valentino and Stephan Ladisch

ABSTRACT

Tumor gangliosides are biologically active (immunosuppressive and tumor-enhancing) cell surface molecules which are shed into the circulation in vivo. However, the mechanism of transport of these molecules (i.e., in solution or bound to proteins or other lipids) is not known. To resolve this question we traced, by direct chemical detection, the serum localization of a specific human tumor ganglioside, GD2, shed by neuroblastoma cells. Sera from patients with tumors were separated into the lipoprotein fractions [very low-density lipoprotein, low-density lipoprotein (LDL), and high-density lipoprotein] and lipoprotein-depleted serum. All three lipoprotein fractions contained GD2. 75% of the total GD2 was present in the LDL fraction, while very low-density lipoprotein and high-density lipoprotein contained 21 and 6%, respectively. Significantly, lipoprotein-depleted serum, which would contain both albumin-bound and free gangliosides, was devoid of GD2. Thus, shed neuroblastoma tumor gangliosides are not, exclusively associated with the serum lipoprotein (and predominant LDL) fractions in vivo. These findings have implications for the immunological detection of these molecules and the development of approaches to their removal.

INTRODUCTION

Circulating tumor-derived gangliosides, sialic acid-containing glycosphingolipids which are shed from the membrane of tumor cells (2) into the extracellular milieu, have been shown to enhance tumor formation in mice (3) and have been linked to tumor progression and metastasis in humans (4). Although the possible mechanism leading to these observations remains undefined, one hypothesis suggests that shed tumor gangliosides may enhance the survival of tumor cells in vivo by abrogating host antitumor responses (2, 5). To have such an effect, the shed gangliosides should be transported to and then bind to host antitumor effector cells. While such binding is well documented (6, 7), the molecules or particles responsible for the immunological detection of these molecules and the development of approaches to their removal.

MATERIALS AND METHODS

Samples. Tumor ganglioside-containing (pathological) sera were obtained from children with disseminated neuroblastoma following an overnight fast. Sera from healthy adult males served as normal controls. Informed consent was obtained from all subjects.

Lipoprotein Purification. Four fractions were isolated from the sera by sequential preparative ultracentrifugation at 15°C using a type 70.1 Ti rotor (Beckman). Serum density was adjusted according to the method of Havel et al. (12). Serum samples were clarified by repeated centrifugation (1150 and 6350 x g), and 1% by volume of a solution of 1% EDTA-0.9% NaCl, pH 7.40, was added. The first fraction (d < 1.019) was isolated (113,000 x g for 22 h) following adjustment of the serum density to 1.019 using a solution containing 15.3 g NaCl and 35.4 g KBr. The second fraction (d = 1.019–1.063) was isolated (133,000 x g for 25 h) following readjustment of the density of the infranate to 1.063. The third fraction (d = 1.063–1.210) floated following ultracentrifugation (326,000 x g for 25 h) after the density of the infranate had been adjusted to 1.210 using solid KBr. This top layer was isolated leaving a fourth fraction (d > 1.210) at the bottom of the tube. To further purify the individual fractions, each ultracentrifugation step was repeated at the appropriate serum density. The fractions were exhaustively dialyzed against 0.01 M phosphate buffer, pH 7.4, containing 0.01% EDTA.

PAGE and Densitometric Scanning. The lipoprotein composition of the fractions was determined by a PAGE system (Lipo-Phor model 400; Quantimetrixics, Hawthorne, CA). Briefly, a 25-μl serum equivalent was loaded onto the premade gel containing 3% polyacrylamide and overlaid with a solution containing 3% acrylamide, 0.25% N,N-methylethanesulfonyl acid and 4.5% Sudan Black B. The tubes were completely filled with buffer [Tris (27%, w/v) and boric acid (73%, w/v), pH 7.4] and placed into the electrophoresis chamber after a 30-min photopolymerization period using a fluorescein preparation light. A 60-mA current (400 V) was applied for approximately 20–25 min. The identity and purity of each fraction were evaluated by comparison of the electrophoretic migration of the Sudan Black B-positive bands to the migration of known lipoproteins. Qualitative assessment of lipoprotein particle homogeneity within each fraction was obtained by densitometric scanning of the gels using a Shimadzu CS-930 integrating scanner.

Total Lipid Extraction. Whole serum, the LPDS, and the lipoprotein fractions were extracted with 8 volumes of chloroform-methanol (1:2) for 18 h at 4°C with magnetic stirring. The pellet was reextracted with 10 volumes of chloroform-methanol (1:1). The two extracts were combined, dried, and then redissolved in a small volume of chloroform-methanol (1:1). The samples were stored overnight at -20°C and then centrifuged (1000 x g) to remove insoluble glycoproteins, without loss of gangliosides. Then, the supernatant was dried under a stream of N2.

Ganglioside Purification. Gangliosides were purified from the total lipid extracts by diisopropylether/1-butanol partitioning (13, 14). Briefly, the dried total lipid extracts were resuspended in 2 ml diisopropylether-1-butanol (6:4) per ml original sample volume, with bath sonication and vortexing to achieve a fine suspension. One volume of saline solution (0.25% NaCl) was then added, and the sample was again sonicated and vortexed. After centrifugation (750 x g), the upper organic phase was removed and the ganglioside-containing aqueous phase repartitioned with fresh organic phase. The final aqueous phase was lyophilized to remove trace organic solvents, redissolved in a small amount of distilled water with sonication, and loaded onto a column containing Sephadex G50-150 equilibrated with distilled water (13). The eluate was monitored by UV detection at 220 nm. The gangliosides were collected in the void volume peak and lyophilized.

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3 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; HPTLC, high-performance thin-layer chromatography; LPIA, lipid-bound sialic acid; LPDS, lipoprotein-depleted serum; d, density; VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein. Gangliosides are abbreviated according to the nomenclature of Svennerholm (1).
LIPOPROTEIN ASSOCIATION OF SHED TUMOR GANGLIOSIDES

Quantitative Analysis, HPTLC, and Densitometry. The LBSA was determined by a modification of the method of Svennerholm (15), the protein content by the Biuret reaction (Boehringer Mannheim Diagnostics, Indianapolis, IN), and the cholesterol content by an enzymatic method (Boehringer Mannheim). HPTLC was used to visualize the total ganglioside pattern and particularly to quantitate the G_{12} ganglioside band (16). The dried total gangliosides were resuspended in chloroform-methanol (1:1) and spotted on prerun desiccated HPTLC plates (Merck, Darmstadt, Germany). The solvent system used was chloroform-methanol-0.25% CaCl_{2} (60:40:9). Gangliosides were visualized as purple bands by staining with resorcinol reagent, and G_{12} was quantitated by densitometric scanning (Shimadzu CS-930 integrating scanner). The area under the peak corresponding to the G_{12} band was compared to the areas of peaks of known quantities of standard gangliosides (16).

RESULTS

Isolation of the Major Serum Lipoprotein Complexes. Fractionation of serum by sequential preparative ultracentrifugation resulted in the isolation of the VLDL, LDL, and HDL fractions, at the following respective densities: <1.019, 1.019-1.063, 1.063-1.210. The d > 1.210 fraction, essentially devoid of lipoprotein, is LPDS. Efficiency of fractionation was assessed by PAGE. Each fraction isolated from patient serum was compared to both the corresponding normal control fraction isolated in parallel and to a standard reference lipoprotein (Sigma). This analysis confirmed the essentially complete fractionation of the serum lipoproteins at the appropriate densities and excluded cross-contamination between fractions (Fig. 1).

Localization of Total Circulating Gangliosides in Tumor-bearing Patient Serum. Chemical quantitation of the total LBSA content of each serum fraction confirmed the essentially complete (>99%) localization of the total circulating gangliosides of pathological (patient) sera to the lipoprotein fractions, with a mean of <0.1 nmol detectable/ml LPDS (Table 1). The total lipoprotein-associated ganglioside concentration was 10.2 ± 1.2 nmol LBSA/ml serum in the tumor-bearing patients and slightly lower in the normal sera (9.6 ± 0.4 nmol LBSA/ml), with similar overall apparent recovery rates (86 and 83%, respectively).

About four-fifths of the total serum gangliosides are associated with the LDL fraction in both the pathological and the normal sera, as confirmed by direct quantitation of the LBSA content of each fraction (Table 1; Fig. 2, open and hatched columns). The mean LDL-associated ganglioside concentration in the pathological and the normal sera was 8.3 and 8.0 nmol LBSA/ml serum, respectively. Each of the other lipoprotein fractions contained much less ganglioside: the mean ganglioside content associated with VLDL and HDL together was 1.9 and 1.6 nmol LBSA/ml (pathological and normal sera, respectively). Thus, in the tumor-bearing patient sera, 82% of the total gangliosides are associated with LDL, 12% with VLDL, and 6% with HDL (Fig. 2). The distribution in the normal sera was similar (LDL, 83%, VLDL, 11%, and HDL, 6%) and consistent with reported values (10) (Table 1). The lipoprotein-depleted serum component, which includes albumin, had only trace (possibly contaminating) amounts of gangliosides.

Because previous results suggested that even in patients with cancer it is the normal gangliosides which constitute the major portion of the total circulating gangliosides (16, 17), it is possible that a low concentration of tumor-derived gangliosides in a serum fraction might escape detection by direct biochemical quantitation. Therefore, we used a more sensitive technique, HPTLC (limit of detection of a specific ganglioside, 50 pmol/ml serum), to examine the total gangliosides of a tumor ganglioside-containing serum and a normal serum (Fig. 3). This experiment revealed that the ganglioside pattern of 1-ml equivalents of the major lipoprotein fractions of both the tumor-bearing (lanes 2-4) and normal (lanes 8-10) samples were qualitatively similar to those of the unfractionated sera (lanes 5 and 7, respectively). Importantly, only trace amounts of gangliosides were detected in the HPTLC pattern of 1 ml of the pathological (lane 1) or normal (lane 11) lipoprotein-depleted serum.

Shed Tumor-derived Gangliosides Are Associated with Serum Lipoproteins. The results of direct chemical quantitation and HPTLC analysis together show essentially complete lipoprotein association of the total circulating gangliosides in tumor-bearing patients. Because this includes those gangliosides directly derived from the tumor by shedding, the next question we addressed was that of specific localization of these tumor-derived gangliosides.
Table 1 Distribution of total circulating gangliosides among the serum lipoprotein fractions

<table>
<thead>
<tr>
<th>Serum fraction</th>
<th>Patient</th>
<th>Normal</th>
<th>Reported normal (Ref. 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total lipoprotein</td>
<td>10.2 ± 1.2*</td>
<td>9.6 ± 4.2</td>
<td>10.2</td>
</tr>
<tr>
<td>VLDL</td>
<td>1.3 ± 0.6 (12)*</td>
<td>1.0 ± 0.3 (11)</td>
<td>0.7 (7)</td>
</tr>
<tr>
<td>LDL</td>
<td>8.3 ± 1.0 (82)</td>
<td>8.0 ± 1.0 (83)</td>
<td>6.7 (66)</td>
</tr>
<tr>
<td>HDL</td>
<td>0.7 ± 0.4 (6)</td>
<td>0.6 ± 0.3 (6)</td>
<td>2.6 ± (25)</td>
</tr>
<tr>
<td>LPDS</td>
<td>ND*</td>
<td>ND</td>
<td>0.2 (2)</td>
</tr>
<tr>
<td>Total serum</td>
<td>11.4 ± 0.8</td>
<td>11.6 ± 0.5</td>
<td>10.5 ± 3.2</td>
</tr>
</tbody>
</table>

* pmol/mL by chemical quantitation, mean ± SD (n = 4).
* Numbers in parentheses, % of total lipoprotein-associated gangliosides.
* ND, not detected (sensitivity, 50 pmol/2-ml sample).

Fig. 2. Distribution of circulating gangliosides among the lipoprotein fractions of serum. Column, mean percentage (bar, ±SD) of the total gangliosides present in the indicated serum fraction. Open column, total normal circulating gangliosides; hatched column, total neuroblastoma patient circulating gangliosides; solid column, tumor-derived circulating GD2 gangliosides.

Fig. 3. HPTLC of the total circulating gangliosides purified from individual serum lipoprotein fractions isolated from pathological (neuroblastoma tumor ganglioside-containing) serum (lanes 1–5) and from normal serum (lanes 7–11). Right ordinate, migration of the major normal plasma gangliosides; lane 6, migration of the tumor-derived ganglioside, GD2. The ganglioside patterns of whole, unfractionated sera (lanes 5 and 7), the lipoprotein fractions (lanes 2–4 and 8–10), and LPDS (lanes 1 and 11) are shown.

Table 2 Distribution of tumor-derived GD2 ganglioside among the serum lipoprotein fractions

<table>
<thead>
<tr>
<th>Serum fraction</th>
<th>Tumor ganglioside-containing sera</th>
<th>Mean %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum fraction</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Total lipoprotein</td>
<td>228*</td>
<td>1000</td>
</tr>
<tr>
<td>VLDL</td>
<td>55 (24)*</td>
<td>250 (25)</td>
</tr>
<tr>
<td>LDL</td>
<td>133 (58)</td>
<td>700 (70)</td>
</tr>
<tr>
<td>HDL</td>
<td>40 (18)</td>
<td>50 (5)</td>
</tr>
<tr>
<td>Total nonlipoprotein (LPDS)</td>
<td>ND*</td>
<td>ND</td>
</tr>
<tr>
<td>Total serum</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* pmolGD2/mL by densitometric quantitation.
* Numbers in parentheses, % of total lipoprotein-associated GD2.
* ND, not detected (detection limit, 25 pmol/mL, 2-ml sample).

derived gangliosides (which make up only a minor fraction of the total gangliosides). We used the disialoganglioside GD2 as an easily detectable marker of such circulating tumor-derived gangliosides (16–18), because it is shed into the circulation in substantial quantities by human neuroblastoma (16, 17) but is virtually absent in the normal circulation (<2 pmol/ml [19]).

In the pathological (tumor ganglioside-containing) serum, GD2 is visible as a distinct band in the HPTLC patterns of the gangliosides isolated from VLDL and LDL fractions (Fig. 3, lanes 3 and 4). A faint band in the pattern of HDL, although not visible in the photograph, is visible on the original HPTLC plate (Fig. 3, lane 2) and was visualized clearly in a separate chromatogram intentionally overloaded to detect minor constituents. In contrast to its identification in each lipoprotein fraction, GD2 was not detected in the lipoprotein-depleted serum (Fig. 3, lane 1). Consistent with the previous findings, GD2 was not detected in unfractionated normal serum or in any of its components (Fig. 3, lanes 7–11).

These results were quantitatively confirmed in all samples by densitometric scanning of HPTLC plates. All of the GD2 contained in the unfractionated pathological sera was associated with the lipoprotein fractions, and none was detectable in the lipoprotein-depleted serum (Table 2). Thus, circulating gangliosides that are shed by a tumor (such as GD2 of human neuroblastoma) are associated with the lipoproteins.

LDL Fraction Is the Major Carrier for Pathological Circulating Tumor Gangliosides. The concentration of tumor-derived GD2 in the samples examined ranged from 228 to 1750 pmol LBSA/ml serum. The densitometric quantitation of GD2 showed that almost three-fourths (73%) of the GD2 was localized to the LDL fraction (Table 2, Fig. 2). Lesser amounts were associated with the VLDL (21%) and HDL (6%) fractions. While the absolute concentration of GD2 varied almost 10-fold among these four serum samples (probably reflecting different degrees of tumor burden), the relative distribution of GD2 among the lipoprotein fractions of the individual serum samples is similar. These results show that, independently of their total concentration in the circulation, gangliosides which have been shed by tumor cells, of which GD2 is an example in the case of neuroblastoma, are associated specifically with the lipoprotein fractions, predominantly with the LDL fraction.
DISCUSSION

Gangliosides, molecules synthesized by the cell and incorporated into the cell membrane, can be released, or shed, into the extracellular milieu. Such endogenously formed gangliosides can be the metabolic product either of normal cells (the presumed origin of normal circulating gangliosides) or of pathological cells, such as tumor cells. In the case of tumor cells, gangliosides are shed into the local environment of the tumor and then enter the circulation. According to our findings, these shed tumor gangliosides, of which the neuroblastoma-derived disialoganglioside GD2 is a representative example, are exclusively transported in the circulation in association with the major serum lipoprotein fractions. Furthermore, particles or complexes in the LDL fraction serve as the predominant carrier of tumor-derived gangliosides. Very little, if any, ganglioside of tumor origin is associated with albumin or other particles or complexes in the lipoprotein-depleted serum.

This in vivo lipoprotein association contrasts sharply with the findings when a purified exogenous ganglioside (GM1) was added in vitro to human or fetal calf serum (11). In this case, the ganglioside was found to be almost completely bound to nonlipoprotein proteins, mainly to albumin, in the form of ganglioside-albumin complexes. No GM1 was found in association with the albumin-free (lipoprotein-containing) fraction.

The observed difference between endogenous in vivo localization and exogenous in vitro (and possibly exogenous in vivo) localization of gangliosides suggests something about the possible link between the physiological processes of ganglioside shedding and ganglioside transport: i.e., if ganglioside transport were random and gangliosides associated nonselectively with potential carrier particles, one would predict that a substantial proportion of the circulating tumor gangliosides would be associated with albumin, since albumin is the most prevalent serum protein (making up nearly half of the total serum proteins) and binds to experimentally added gangliosides (11). Since shed tumor gangliosides are not bound to albumin, ganglioside binding is clearly selective, and therefore, tumor ganglioside transport is likely to be a directed process.

Our findings that shed tumor-derived gangliosides are associated with lipoproteins, together with previous studies tracing binding of exogenously added gangliosides mainly to albumin (11) and normal circulating gangliosides to lipoproteins (predominantly LDL) (8–10), begin to give a more complex picture of ganglioside transport than has been previously assumed. We propose that circulating gangliosides be classified into three operational categories which consider both their source and mode of transport. They are (a) normal, endogenously formed gangliosides normally produced by the cell; (b) pathological, endogenously formed gangliosides that are synthesized and shed by tumor (or other pathological) cells and are not normally present in the circulation; and (c) exogenous, experimentally added gangliosides that were not produced by, but rather added to, either the organism (in vivo) or cells in tissue culture (in vitro). Endogenous (normal and pathological) gangliosides have a mode of transport (i.e., association with serum lipoproteins) strikingly different from that of exogenous gangliosides, which associate with nonlipoprotein complexes.

Pathological gangliosides appear to have the same mode of transport as do normal gangliosides, i.e., in association with serum lipoproteins. However, the specific transport particles or complexes may be different. This conclusion is drawn from preliminary experiments in which an effort was made to critically analyze the composition of each highly purified lipoprotein fraction, despite the limitation of only small samples available for study. Densitometric scanning of the polyacrylamide gels following electrophoretic separation of the lipoprotein fractions from serum samples (shown in Fig. 1) demonstrated a surprising result. Although each fraction examined by PAGE and analyzed visually consisted of one major electrophoretic band, the more sensitive densitometric analysis of these gels demonstrated an apparent abnormality within each lipoprotein fraction of the tumor ganglioside-containing pathological sera. This abnormality was a heterogeneity within the electrophoretic band and, hence, of composition of the lipoprotein fraction. By densitometry, a seemingly broad, single electrophoretic band was found to actually be composed of two separate peaks with very close (but not identical) migration characteristics (Fig. 1). The more slowly migrating band accounted for 30% and the slightly faster migrating band accounted for 70% of the total lipoprotein in the fraction. The VLDL and HDL fractions, while less heterogeneous than the LDL fraction, nevertheless also demonstrated a small notch, visible in the densitometric tracings. This heterogeneity of the lipoprotein fractions is in contrast to the homogeneous normal lipoprotein patterns (Fig. 1); each fraction from the normal serum had one band by PAGE and one major peak by densitometry. Thus, the LDL and possibly the other lipoprotein fractions from tumor-bearing patients are heterogeneous in composition, compared to the corresponding normal serum lipoprotein fractions and reference lipoproteins. This observation may reflect the existence of a tumor-derived lipoprotein-like particle with flotation characteristics and electrophoretic mobility similar to (but distinct from) that of normal lipoproteins. Alternately, it could be a consequence of changes in normal lipoprotein metabolism caused by the presence (and metabolic consequences) of a tumor. It is tempting to speculate that, when adequate samples become available to permit separation of and ganglioside purification from the lipoprotein subfractions, tumor-associated lipoprotein subfractions will be shown to harbor the tumor-derived gangliosides.

The identity of specific particles within the lipoprotein fractions which are responsible for the transport of tumor-derived gangliosides is thus a very interesting unresolved question. Heterogeneity of lipoprotein particles in the circulation of tumor-bearing patients has also been found by subfractionation of the lipoprotein particles (reviewed in Ref. 20), and novel LDL particles have been directly identified in the circulation of rats with chemically induced (21) and transplanted tumors (22). A separate line of investigation further suggests that human tumor gangliosides might be associated with such tumor-derived proteolipid complexes, i.e., certain gangliosides and neutral glycosphingolipids have been found to be associated with unique proteolipid complexes in the sera of cancer patients (23–25). These observations suggest that tumor ganglioside transport may be linked to unique and possibly tumor-derived lipoprotein complexes (24, 26–29), but the presence and the molecular identity of such complexes in the serum of patients with neuroblastoma remains to be investigated.

The overall conclusion that gangliosides shed by human tumors circulate in association with the serum lipoprotein fractions has potential practical importance. In experimental work, presentation of gangliosides to effector cells in a form which is representative of their physiological state would be ideal. By this reasoning, simple injection of gangliosides into animals may not simulate the physiological state if these in-
jected molecules bind to albumin instead of associating with lipoproteins/proteolipids, as occurs naturally. These aspects of ganglioside transport may modulate ganglioside availability and, thereby, bioactivity in vivo. And, since they are associated with lipoproteins, the difficulty in detecting tumor-associated gangliosides in the circulation using immunological (monoclonal antibody) methods is likely explained by steric hindrance caused when the very small ganglioside molecule is associated with a very large lipoprotein complex. The sensitivity of immunological detection of shed tumor gangliosides may therefore be enhanced by a preliminary lipoprotein separation followed by separation of the gangliosides from the lipoproteins. Similarly, the localization of tumor gangliosides to the lipoprotein fractions of human serum in vivo implies that selective removal of lipoproteins could be effective in removing tumor-derived gangliosides from the circulation.

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