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Research Article

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Gangliosides Shed by Tumor Cells Enhance Tumor Formation in Mice

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Abstract

The role of tumor cell membrane gangliosides in tumor formation was probed using a series of cloned murine AKR lymphoma cell lines. Tumor formation was directly related to high expression and shedding of membrane gangliosides. In vivo, as little as 1 pmol of purified total gangliosides of highly tumorigenic cells, injected intradermally with poorly tumorigenic cells (which lacked and did not shed gangliosides), markedly increased the tumorigenicity of these cells in syngeneic normal mice. Thus, gangliosides shed by tumor cells are a previously unrecognized, extremely potent enhancer of tumor formation in vivo.

Introduction

Tumor formation is a complex process, influenced by multiple factors. Among these, transformation and tumorigenicity are two phenotypic characteristics of cell lines that are under separate genetic control (1). Transformation indicates that a cell line is "immortalized"; capable of proliferating independently and indefinitely in vitro. In contrast, tumorigenicity demands that transformed cells proliferate in vivo, resist elimination by the syngeneic normal host, and form progressively growing tumors. It is highly likely that local tumor cell-host interactions influence the initial steps of tumor formation in vivo. Relatively little experimental attention has been given, however, to the shedding (2, 3) of membrane molecules such as gangliosides into the immediate extracellular environment of the tumor cell, and to the possible effects of these shed molecules on tumorigenicity.

Gangliosides are complex glycosphingolipids found primarily on the external membrane of cells, and some gangliosides are markers of development and oncogenesis (4–7). Our interest in this class of molecules, with respect to tumor formation, stems from the fact that tumor-derived gangliosides are potent inhibitors of the cellular immune response in vitro (8, 9), and are shed by tumor cells (8, 10). We hypothesized that quantitative aspects of the shedding of these biologically active molecules might modulate tumor formation, possibly by abrogating host anti-tumor immune responses (8). Some correlations between tumor cell ganglioside content and tumor formation (11–16) support this possibility, but to date there has been no direct proof that tumor cell gangliosides influence tumorigenicity (6).

To test this hypothesis, we have studied a murine lymphoma from which several cell lines of markedly different tumorigenic potential had been cloned. Our experiments correlate ganglioside shedding and tumorigenicity, and show that very small quantities of gangliosides synthesized and shed by tumor cells can markedly augment the tumorigenicity of poorly tumorigenic, ganglioside-deficient tumor cells. These findings suggest that tumor-derived gangliosides play an important physiologic role in the enhancement of tumor formation.

Methods

Tumor cell lines. The cell lines studied are all clones of spontaneous lymphoma 12 (SL12), a heterogeneous cell line isolated from a thymic lymphoma arising in an AKR mouse (17). These cell lines, SL12.1, SL12.3, and SL12.4, were chosen for the present study because of their previously determined markedly different tumorigenic potentials. Surface markers and other characteristics of the three clones (which were kindly provided by C. MacLeod) have been described elsewhere (17). All cell lines were free of infection by mycoplasma. The three cell lines had very similar doubling times and saturation densities in vitro (12–15 h and 5 $\times$ 10^6 cells/ml, respectively), suggesting that the differences in tumorigenicity among these three lines were not due to different intrinsic proliferative characteristics, but related to differences in the physiology of tumor formation.

Ganglioside purification. Gangliosides were purified from the total lipid extract (8) of 0.5–2 $\times$ 10^9 cells for each determination of total ganglioside content. The gangliosides were purified by partitioning the dried total lipid extract in diisopropyl ether/1-butanol/50 mM aqueous NaCl, 6/4/5 (vol/vol/vol) (18). The resulting (ganglioside-containing) aqueous phase was further purified by Sephadex G-50 gel filtration, as described (18), to remove salts and other low molecular weight contaminants. The purified total gangliosides thus obtained were quantitated as nmol lipid-bound sialic acid (LBSA)[1] by the modified resorcinol method (19).

Thin-layer chromatography. Thin-layer chromatographic (TLC) analysis of gangliosides was performed as previously described (8), using 10 $\times$ 10 cm precoated silica gel 60 high-performance TLC (HPTLC) plates (Merck, Darmstadt, West Germany), which were preactivated by heating to 90°C for 45 min. The plates were developed in chloroform/methanol/0.25% aqueous $CaCl_2 \cdot 2H_2O$, 60:40:9 and stained with resorcinol, or were exposed to Eastman Kodak Co. (Rochester, NY) XRP x-ray film at −80° to visualize radiolabeled gangliosides. Unlabeled or radiolabeled (20) standard brain gangliosides, identified by the nomenclature of Svennerholm (21), were spotted on each plate to identify the relative migration of the lymphoma cell gangliosides.

Assessment of ganglioside shedding. To measure the shedding of

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1. *Abbreviations used in this paper:* HPTLC, high-performance thin layer chromatography; LBSA, lipid bound sialic acid.

tumor cell gangliosides, cells were cultured in vitro (10^6 cells/ml complete medium) for 24 h with 0.5 μCi D-[1-^{14}C]glucosamine-HCl (sp act, 54.2 mCi/mmol) and 0.5 μCi D-[1-^{14}C]galactose (sp act, 56.5 mCi/mmol; New England Nuclear, Boston, MA) per ml culture medium (22). The cells were then washed to remove free label, and an aliquot taken to determine the specific activity of the radiolabeled gangliosides. The remaining cells were resuspended in fresh medium and cultured for an additional 24 h to determine the disappearance from the cells, and the appearance in the culture supernatant, of ganglioside-associated radioactivity. Gangliosides were purified from the cells and their culture supernatant as described above, with the addition of 20 nmol unlabeled purified brain gangliosides to each sample as carrier molecules for the purification procedure. Ganglioside-associated radioactivity was quantitated by β-scintillation counting.

Tumor formation. To assess the role of gangliosides in modulating tumor formation, we used the highly tumorigenic SL12.3 cells and the poorly tumorigenic ganglioside-deficient SL12.4 cells. Tumor cells were injected intradermally to optimize the intercellular and cellular-extracellular environmental interactions that might in turn modulate the process of tumor formation. In syngeneic mice the tumors were 3–5 mm in diameter within 2 wk, and all tumors grew progressively with time. Intradermal injection of between 2 and 3×10^3 SL12.3 cells yielded tumors in 50% of the mice. In contrast, 10^5 ganglioside-deficient SL12.4 cells were needed to give the same tumor incidence.

Results

Ganglioside expression. Total ganglioside content of the three clones cultured in vitro varied more than 100-fold (0.5 to 71 nmol LBSA/10^9 cells, Table I). There was a direct correlation between the concentration of cell membrane gangliosides and the previously determined ability of the cells to form tumors in vivo (Table I). Clone SL12.3, with the highest total ganglioside content, required injection of the lowest number of cells (10^3) to yield a 50% tumor incidence. In contrast, clone SL12.4, virtually deficient in gangliosides, required a 1,000-fold higher number of cells to yield the same tumor incidence. Clone SL12.1 was intermediate both in ganglioside content and in tumorigenicity. Thus, striking differences in tumorigenicity correlated directly with striking differences in ganglioside content.

Ganglioside shedding. Shedding of significant quantities of gangliosides by highly tumorigenic cells into their extracellular environment is predicted by the hypothesis that tumor cell gangliosides interfere with host responses to tumor. We quantitated this shedding process and found the highly tumorigenic SL12.3 clone to shed 18 pmol gangliosides/10^6 cells per 24 h. The rate was dramatically less (0.2 pmol/10^6 cells per 24 h) in the poorly tumorigenic SL12.4 clone (Table II). Like ganglioside content, ganglioside shedding thus differed markedly in these two clones, and tumorigenicity correlated with the ganglioside shedding rate.

The cellular and shed gangliosides of clone SL12.3 are shown in Fig. 1. The resorcinol-stained (Fig. 1 *a*) and the radiolabeled (Fig. 1 *b*) cellular ganglioside patterns were quantitatively and qualitatively nearly identical. (The minor ganglioside band below G_{Dla} in Figs. 1 *b* and *c* [autoradiogram] can also be detected by the less sensitive resorcinol stain if the chromatogram is overloaded [not shown].) Thus, the radiolabeled sugars were incorporated into all ganglioside species and in proportion to the quantity of each ganglioside present in the cells. The same radiolabeled cellular gangliosides (Fig. 1 *b*) were also recovered from the culture supernatant (Fig. 1 *c*), confirming their shedding by the SL12.3 cells, as established quantitatively in Table II. In contrast, the poorly tumorigenic (ganglioside deficient) SL12.4 cells contained and shed insufficient quantities of gangliosides to be detectable by HPTLC.

Table I. Ganglioside Expression and Tumorigenicity

Cell line	Ganglioside content*	Tumorigenicity‡
12.1	8±2	10^4
12.3	71±9	10^3
12.4	0.5±0.4	10^6

* nmol LBSA/10^9 cells; mean±SEM of three separate determinations.
‡ Number of cells injected intravenously to yield 50% tumor incidence in syngeneic, normal 4–6-wk-old AKR mice (from ref. 17).

Table II. Shedding of Gangliosides by Highly and Poorly Tumorigenic Cells

Cell line	Tumorigenicity	Ganglioside content	
		Cells*	Culture supernatant‡
SL12.3	High	70	18
SL12.4	Low	1	0.2

Cells were metabolically radiolabeled in vitro, washed, and the specific activity of the radiolabeled gangliosides was determined using an aliquot of the cells. Then, remainder of the cells were cultured for an additional 24 h in fresh complete medium, after which the radiolabeled gangliosides were purified from the cells and culture supernatant.
* pmol/10^6 cells.
‡ pmol shed/10^6 cells/24 h.

Enhancement of tumorigenicity by gangliosides. The purified total gangliosides of the highly tumorigenic SL12.3 cells were used to directly test the influence of ganglioside expression and shedding on tumorigenicity. These gangliosides are shown in Fig. 1 *a* and are recognized by their characteristic purple staining with resorcinol reagent (19). If shed gangliosides "protect" tumor cells from host destruction, then the co-injection of these gangliosides with the poorly tumorigenic, ganglioside-poor SL12.4 cells should promote tumor formation by SL12.4 cells. To test this, 0.1 pmol to 10 pmol purified total gangliosides from SL12.3 cells were injected intradermally together with 5×10^4 SL12.4 cells. This number of cells, when injected alone, yielded a tumor incidence, at 2 wk, of 8% (1 of 12 syngeneic normal mice in two separate experiments, Table III). In contrast, the tumor incidence increased to 83% when as little as 1 pmol of the gangliosides was injected together with the SL12.4 cells (Table III), showing that gangliosides shed by tumor cells are highly potent in enhancing tumor growth in vivo.

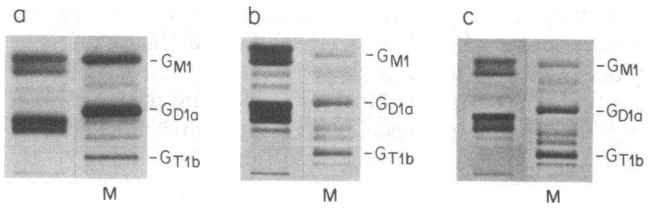

Figure 1. Synthesis and shedding of gangliosides by SL12.3 murine lymphoma cells. TLC analysis of SL12.3 cellular gangliosides is shown. (Lane *a*) cellular gangliosides stained with resorcinol; all bands are resorcinol positive. (Lanes *b* and *c*) 2×10^3 cpm radiolabeled cellular and supernatant gangliosides, respectively, visualized by autoradiography of the HPTLC plates. Unlabeled or radiolabeled standard brain gangliosides were spotted on each HPTLC plate to identify the relative migration of the SL12.3 lymphoma gangliosides, and are shown in the right lane of each pair, marked *M*.

Table III. Tumor Cell Gangliosides Enhance Tumor Formation

Gangliosides added	Tumor incidence (Mice with tumors/total)		
	Expt 1	Expt 2	Total
pmol			
None (control)	0/6	1/6	1/12
0.1	ND	2/6	2/6
0.3	ND	2/6	2/6
1.0	5/6	5/6	10/12
10.0	5/6	5/6	10/12

Purified gangliosides isolated from the highly tumorigenic SL12.3 cells were dried, sonicated in buffered saline, and mixed with 5×10^4 viable SL12.4 cells immediately before intradermal injection in a shaved site in the intrascapular area in 50 μl buffered saline. Tumor incidence was assessed 2 wk postinjection. 1 and 10 pmol added ganglioside caused a highly significant ($P = 0.001$) increase in tumor formation.
ND, not determined.

Table IV. Synergism in Tumor Formation by Cloned Lymphoma Cell Lines

No. of cells injected		Tumor incidence (Mice with tumors/total)		
Clone SL12.3	Clone SL12.4	Expt 1	Expt 2	Total
0	1×10^4	0/6	0/6	0/12
5×10^2	0	2/6	1/6	3/12
1×10^3	0	0/6	1/6	1/12
3×10^3	0	2/6	5/6	7/12
5×10^2	1×10^4	5/6	4/6	9/12
1×10^3	1×10^4	2/6	6/6	8/12
3×10^3	1×10^4	5/6	6/6	11/12

Tumor cells were injected intradermally as in Table III. Tumor incidence reflects final values. The combined tumor incidence of all mice receiving both SL12.3 and SL12.4 cells (28/36) was significantly greater than the tumor incidence in mice receiving only SL12.3 cells (11/36, $P < 0.0005$).

Immunological rejection of SL12.4 cells. Results of two sets of experiments suggest that the poor tumorigenicity of the ganglioside-deficient SL12.4 cell line, reversible by the addition of gangliosides, results from susceptibility of these cells to host immune responses. First, as we previously reported (17), all of 10 mice immunosuppressed by 400 rad total body irradiation developed tumors after the i.v. injection of 1×10^6 SL12.4 cells versus only a 50% tumor incidence in unirradiated (control) mice ($P < 0.05$). Second, intraperitoneal pretreatment of mice with 150 mg silica gel, which inhibits macrophage tumoricidal activity (23), increased the incidence of tumor formation by 1×10^6 SL12.4 cells (injected intravenously 48 h later) to 90% (18 of 20 mice) from 43% (13 of 30 mice) in the control group ($P < 0.005$). These results indirectly suggest that the mechanism of the protective action of SL12.3 cell gangliosides could be to block host anti-tumor immune responses.

Synergism in tumor formation by highly and poorly tumorigenic cells. A final series of experiments show that the SL12.3 cells which shed gangliosides exerted a tumor-protective activity for the poorly tumorigenic ganglioside–deficient SL12.4 clone. These experiments tested for synergy in tumor formation by highly and poorly tumorigenic cells. The reason for this is that a small number of the protective SL12.3 cells are injected together with a nontumorigenic number (1×10^4) of SL12.4 cells. If the biologically important difference between the two cell lines is the shedding of gangliosides, gangliosides shed by the SL12.3 cells might protect the SL12.4 cells from host destruction, rendering all of the total of slightly more than 10^4 cells tumorigenic. The results (Table IV) support this view: The inclusion of the small number of SL12.3 cells with the nontumorigenic number (1×10^4) of SL12.4 cells resulted in the formation of progressively growing tumors in 28 of 36 syngeneic normal mice injected, versus 11 of 36 mice receiving only the SL12.3 cells (78% vs. 31%, $P < 0.0005$).

Discussion

This study demonstrates that the in vivo administration of purified total gangliosides shed by tumor cells enhances tumor formation. Using two distinct strategies to provide tumor-derived gangliosides to the local environment of the poorly tumorigenic cells that are deficient in gangliosides, tumor formation was markedly increased. These experiments simulate the natural biologic process of shedding and therefore suggest in vivo significance and potential biologic relevance of ganglioside expression and shedding by tumor cells.

An alternate explanation for the association between ganglioside shedding and high tumorigenicity would be that characteristics of ganglioside metabolism are a manifestation, rather than a cause, of the tumorigenic phenotype. However, the ganglioside addition experiments and the experiments testing synergy in tumor formation suggest that shed molecules, and specifically gangliosides, enhance tumor formation. This conclusion now requires confirmation in other tumor systems.

Consideration of quantitative aspects of ganglioside shedding further supports the conclusion that shed membrane gangliosides enhance the tumorigenicity of transformed cells. As little as 1 pmol SL12.3 cell gangliosides produced optimal effects. This clone contains 3.6 pmol gangliosides/5×10^4 cells, of which 0.9 pmol/24 h are shed (calculated from Tables I and II). We therefore injected only as much gangliosides as the equivalent number of highly tumorigenic cells shed in one day. Tumor-promoting activity thus resulted from the presence of biologically relevant quantities of ganglioside.

Tumor formation by the poorly tumorigenic SL12.4 cells was also enhanced when immunosuppressed rather than immunologically intact mice were used as hosts. We postulate that the addition of gangliosides protected the SL12.4 cells from host immune destruction. Whether tumor gangliosides block immunological surveillance (24) remains to be proven, and to be distinguished from a possible effect of added ganglioside on the ganglioside-deficient cells themselves. It seems possible, however, for the following reasons: (*a*) Gangliosides inhibit the in vitro function of lymphocytes (8, 9, 25–28), monocytes (29), and natural killer cells (30). (*b*) All of these cells are active in immunological surveillance in its current, broader definition (31, 32). (*c*) The amount of ganglioside shed by SL12.3 cells (18 pmol/10^6 cells per 24 h, Table II) could yield, in the local environment of the tumor, the nanomolar ganglioside concentrations previously shown to be immunosuppressive in vitro (8, 9, 25–30).

The enhancement of tumor formation caused by gangliosides emphasizes the influence of shed molecules on tumorigenicity. Minute quantities of shed molecules such as gangliosides may be a crucial element in determining whether a transformed cell

will form a tumor in a normal syngeneic host and may constitute a mechanism contributing to tumor progression. Our findings may be directly relevant to human cancer since it has already been shown that one aggressive human tumor, neuroblastoma, sheds significant quantities of gangliosides in vivo (33, 34) and that these gangliosides are immunosuppressive in vitro (manuscript in preparation). Understanding exactly how tumor-derived molecules affect the extracellular environment of tumor cells may explain how tumor cells are (at the crucial initial stages of tumor formation) able to escape host defense mechanisms. Separation of the individual gangliosides making up the total tumor cell ganglioside complement (used in the present study), determination of their molecular structures, and testing of their tumor-modulating activity is now necessary. This will help to answer the question of whether specific molecular details of ganglioside structure, as well as quantitative differences in ganglioside expression and shedding, are important factors in tumor formation. Finally, engineering an approach to counteract or eliminate the shed molecules holds the promise of improving the host response to tumor.

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