Advances in Brief

Human Tumor Gangliosides Inhibit Murine Immune Responses in Vivo

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Abstract

Gangliosides which are shed by tumor cells clearly inhibit cellular immune responses in vitro. However, the immunosuppressive activity of these molecules has been more difficult to ascertain in vivo. Here we have adapted a murine model to determine the effects of tumor gangliosides in an in vivo microenvironment, the lymph node draining the site of stimulation by allogeneic cells. In this model, allogeneic splenocytes (BALB/c) are s.c. injected into C3H mice. The cellular immune response in the draining popliteal lymph nodes 4 days later is evidenced as an increase in lymph node mass (2-fold), lymphocyte number (6-fold), and lymphocyte DNA synthesis (6-fold). Purified human neuroblastoma gangliosides (10 nmol) coinjected with the stimulating allogeneic cells significantly suppressed this in vivo immune response. The increase in the lymph node mass was reduced by 65% (0.66 versus 1.89 mg), the increase in lymphocyte number (4.0 x 10^8 cells/node) was almost completely inhibited (1.1 x 10^8 cells/node), and in vivo [³H]thymidine uptake by the lymphocytes recovered in vivo was reduced by 80%. In contrast to the inhibition by tumor gangliosides, liposomes of cholesterol:lecithin were not inhibitory. Thus, tumor gangliosides, specifically, modulate cellular immune responses in vivo, which may contribute to the observed enhancement of tumor formation by these molecules.

Introduction

Tumor formation is a complex, multiple-step process influenced by many factors. Local interactions between the tumor cell and the host are critical to tumor formation, and it is becoming recognized that the release of soluble factors by tumor cells into the local tumor microenvironment may dramatically alter the functions of host cells (1). One of such factors is cell surface gangliosides, an important class of tumor-derived molecules, which may act as intercellular signaling molecules. Gangliosides synthesized by tumor cells and expressed in their cell membranes are shed in substantial quantities into the tumor cell microenvironment (2–6). Subsequently, these bioactive molecules bind to host immunocytes in this same microenvironment and may suppress the cellular antitumor immune response (7). This hypothesis is supported by the well-documented immunosuppressive effects of gangliosides in vitro. Gangliosides inhibit several steps in the cellular immune response, including antigen processing/presentation (8), lymphocyte proliferation (7, 9–12), and cytotoxic effector function (13–15). However, it has been more difficult to pinpoint the immunoregulatory effect of tumor gangliosides in vivo, partly due to the lack of availability of highly purified tumor gangliosides and partly due to the lack of an appropriate model in which the small available quantities of gangliosides can be studied.

Certain previous studies have implied the biological activity of tumor gangliosides in vivo. In human neuroblastoma patients, for example, in which shed tumor gangliosides were chemically detected in the circulation in high concentrations (16), tumor progression is highly associated with the level of the circulating concentration of shed tumor gangliosides at the time of diagnosis (17). Also, the addition of tumor gangliosides to a tumor cell inoculum can enhance tumor formation in mice (18, 19), further suggesting biological and possibly immunoregulatory activity of tumor gangliosides in vivo.

Here, we have adapted a murine model to directly assess the effect of tumor gangliosides in vivo. The results show that human neuroblastoma tumor gangliosides have a significant suppressive effect on murine immune responses.

Materials and Methods

Ganglioside Preparation. Tumor gangliosides were purified from human neuroblastoma LAN-5 cells by a sequence of steps including extraction of cells with chloroform:methanol (1:1), partition of the total lipid extract in diisopropyl ether:1-butanol, and Sephadex G-50 gel filtration of the ganglioside-containing aqueous phase (20). Gangliosides were quantified by resorcinol assay (21) and analyzed by HPTLC (22).

Murine Model System. The murine model developed by Kroczek et al. (23) was adapted for the present study. In this system, footpad injection with the stimulating antigen (allogeneic cells) with or without tumor gangliosides was subsequently followed by the harvest of the popliteal lymph nodes and by the assessment of the cellular immune response. This was accomplished by assessing the node mass, lymphocyte number, and specific proliferative response. C3H (H-2b) and BALB/c (H-2d) were murine virus-free strains (Charles River, Wilmington, MA) and were used at 7–12 weeks of age in these experiments.

Preparation of Stimulator Cells. BALB/c spleen cells were removed aseptically and immediately placed in murine complete media (RPMI 1640 supplemented with 10% FBS, 1% MEM nonessential amino acids, sodium pyruvate, l-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, and 10 mM Hepes buffer) and then transferred to a 60 x 10-mm Petri dish. A sterile single-cell suspension was prepared by gently pressing the spleen onto a cell dissociation sieve. Mononuclear cells were isolated by Ficoll-Hypaque density gradient separation, followed by lysis of erythrocytes (ACK lysing buffer, pH 7.4). The cells were washed and resuspended in saline.

Injections. BALB/c splenocytes were γ-irradiated (2880 rads in 8 min), and then the cells immediately were injected s.c. into the left hind footpad of C3H mice (2.5 x 10^7/30 μl). When tumor gangliosides were injected together with the allogeneic cells, gangliosides were first resuspended in 0.9% NaCl and then mixed with the cells. Cyclosporin A-treated mice received allogeneic cells in the footpad and 4 daily i.p. injections (24 mg/kg/day x 4 days) of cyclosporin A (Sandoz Pharmaceutical Corp., East Hanover, NJ).

Isolation of Popliteal Lymph Nodes. Primed C3H mice were sacrificed by cervical dislocation on day 4, and the popliteal lymph nodes draining the left and right footpads were aseptically removed, weighed, and placed on ice in tubes containing culture medium. The nodes were then teased with a flat end of a 3-ml syringe to obtain a single-cell suspension, which was washed in complete murine media containing 0.1% 2-mercaptoethanol (GIBCO, Grand Island, NY). The cells were counted, and their viability was determined by trypan blue dye exclusion.

[³H]Thymidine Incorporation Assay. To measure the proliferative response of these lymph node cells caused by their in vivo exposure to the allogeneic cells, 2 x 10^6 lymph node cells in 100 μl complete medium were cultured for 18 h with 0.5 μCi [³H]thymidine immediately after their recovery.
from in vivo allostimulation as described above. At the end of the 18 h, the cells were harvested, and \[3H\]thymidine incorporation was quantified by \(\beta\)- scintillation counting (24).

**Statistical Analysis.** The difference between control group and treatment group was determined at the significance level of 0.05 using Student's \(t\) test (one-tailed, unpaired).

**Results and Discussion**

**Murine Model for Modulation of Cellular Immune Responses.** To test whether tumor gangliosides inhibit cellular immune responses in an in vivo microenvironment, we adapted a murine model of the class II-restricted allogeneic immune response developed by Kroczek et al. (23). In this system (Fig. 1), \(\gamma\)-irradiated BALB/c spleen cells were s.c. injected into C3H mice, and 4 days later, the cellular immune response in the draining popliteal lymph nodes was evaluated as the increase in lymph node mass, lymphocyte number, and lymphocyte DNA synthesis. As shown in Table 1, the footpad injection with syngeneic cells did not cause any changes in these parameters, excluding the possibility of any nonspecific responses of the node caused by the injection procedure. In contrast, the injection of allogeneic cells caused a 2-fold increase of popliteal lymph node mass compared with the node mass of naive mice (from 1.34 to 2.99 mg) and a 6-fold increase in both lymphocyte number (from \(6.4 \times 10^6\) to \(41.8 \times 10^6\) cells/node) and \(\gamma\)H]thymidine uptake (from 217 to 1376 cpm/2 \(\times 10^5\) cells). Systemically administered i.p. cyclosporin A (24 mg/kg/day X 4 days), a well-studied immunosuppressive molecule, suppresses this response (25).

**Inhibition of Lymph Node Mass Increase by Tumor Gangliosides.** Human neuroblastoma gangliosides markedly inhibit lymphoproliferative responses in vitro (12, 26). For example, 10 \(\mu\)g ganglioside G\(\text{D}_2\) (a major neuroblastoma ganglioside) caused 90% inhibition of lymphoproliferation induced by a specific soluble antigen, tetanus toxoid (26). In the present study, we have purified the gangliosides from human neuroblastoma LAN-5 cells (which contains G\(\text{D}_2\) as the major ganglioside) and assessed their in vivo immunosuppressive activity in this murine model.

In the first set of experiments, we measured the popliteal lymph node mass to obtain preliminary evidence of the inhibitory effect of tumor gangliosides on alloimmune responses. When mice were immunized with allogeneic cells admixed with tumor gangliosides, there was a marked reduction in the increase of popliteal lymph node mass (Fig. 2). The mean increase in lymph node mass in mice stimulated with allogeneic cells (control) is 1.66 mg. The coinjection of allogeneic cells and 5 nmol of human neuroblastoma gangliosides caused only a 0.84-mg increase in the lymph node mass. Thus, human tumor gangliosides markedly suppress the popliteal lymph node mass increase caused by allostimulation \((P < 0.01)\). This is comparable to the effect of systemically administered cyclosporin A (0.61-mg increase).

**Inhibition of Lymphoproliferation in Vivo by Tumor Gangliosides.** Next we assessed the effect of tumor gangliosides on the in vivo lymphoproliferative responses of the lymph node cells to the allogeneic stimuli. This was determined by quantifying both the total mononuclear cells recovered from the lymph nodes and the spontaneous proliferative response in vitro (Table 2). From mice stimulated with allogeneic cells, 4.04 \(\times 10^6\) cells/node were recovered, while 1.1 \(\times 10^6\) cells/node were recovered from mice treated with 10 nmol human neuroblastoma gangliosides, which is very close to 0.64 \(\times 10^6\) lymphocytes/node recovered from naive (unstimulated) mice. Thus, the increase in lymphocyte number normally associated with allogeneic stimulation was almost completely inhibited.

The spontaneous proliferative response by these recovered cells was measured by the \([3H]\)thymidine uptake (Table 2). Tumor gangliosides reduced the \([3H]\)thymidine uptake by 80%, from 7.2 \(\times 10^5\) to 1.5 \(\times 10^5\) cpm/2 \(\times 10^5\) cells.

These results clearly show that human neuroblastoma tumor gangliosides inhibit the murine allogeneic cellular immune response in vivo. A single dose of 10 nmol human neuroblastoma gangliosides had an immunosuppressive effect similar to that of 4 daily injections of systemically administered cyclosporin A (24 mg/kg/day).

**Lack of Systemic Toxicity of Gangliosides.** It has been shown previously that the inhibition of cellular immune responses by gangliosides is not due to a toxic effect on the responding cell population in vivo (9, 27). In the third set of experiments, we examined the potential systemic toxicity of gangliosides in vivo. When gangliosides...
were coinjected in the footpad of mice with the allogeneic cells, the injection site appeared normal, and no erythema was observed 24 h after the ganglioside injection. The mice behaved normally, and the ganglioside injection did not affect the body weight of mice. At the age of 10 weeks, the body weight was 24.0 ± 0.4 g (n = 5) before the ganglioside injection and 24.3 ± 0.3 g 4 days later. The control group (n = 5) weighed 24.2 ± 1.2 g before the injection versus 24.8 ± 1.1 g 4 days later. Thus, there was no short-term systemic toxicity associated with tumor ganglioside treatment.

Specificity of Inhibition of Immune Responses by Tumor Gangliosides. To determine whether lipid-cell interaction of a nonspecific nature causes inhibition of the in vivo allogeneic immune response, mice received either human gangliosides (5 nmol) or the liposomes (5 or 50 nmol) admixed with the allogeneic cells. As in the previous experiments, human neuroblastoma tumor gangliosides inhibited the allogeneic stimulation. In contrast, the liposomes had no inhibitory effect on either lymph node mass or lymph node lymphocyte number (Table 3), supporting a specific inhibitory effect on the murine immune response by gangliosides.

Gangliosides suppress major histocompatibility complex class I and class II genes in cultured astrocytes (28) and induce a selective and complete modulation of CD4 from the surface of T cells by endocytosis (29). These and other in vitro studies suggest that gangliosides have immunomodulatory properties in vivo. While the lack of a suppressive effect of brain gangliosides on humoral or cellular immunity in vivo was reported (30), one recent study shows that GM3 ganglioside prolonged in vivo allograft rejection (rat heart, Ref. 31).

The present study provides the first direct evidence of inhibition of the cellular immune response by tumor gangliosides in vivo. Tumor cells shed gangliosides both in vitro and in vivo (2–7). By the continuous shedding of these biologically active cell surface molecules, the tumor cells create a microenvironment containing a high concentration of tumor-derived gangliosides which may downregulate the host antitumor response by inhibiting the function of leukocytes which infiltrate tumors, thereby facilitating tumor progression. The murine model used in this study mimics the local microenvironment of tumor cells where tumors form and gangliosides are shed. The coinjection of tumor gangliosides (5–10 nmol) with allogeneic cells inhibits the murine allogeneic immune response in vivo. This is comparable to the effect of 4 daily doses of systemically administered cyclosporin A (24 mg/kg/day). These findings support the hypothesis that gangliosides released by tumor cells may function as intercellular signaling molecules to enhance tumor cell survival in the host. Clearly, however, further studies are needed to delineate the mechanism of how tumor gangliosides modulate the cellular immune responses in vivo.

Table 3 Specificity of inhibition of allogeneic immune responses by tumor gangliosides

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Popliteal lymph node mass increase (mg)</th>
<th>Lymphocytes × 10^10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.7 ± 0.4</td>
<td>2.03</td>
</tr>
<tr>
<td>Gangliosides (5 nmol)</td>
<td>0.7 ± 0.3</td>
<td>1.12</td>
</tr>
<tr>
<td>Liposomes</td>
<td>2.1 ± 0.3</td>
<td>2.34</td>
</tr>
<tr>
<td>50 nmol</td>
<td>1.7 ± 0.3</td>
<td>2.52</td>
</tr>
</tbody>
</table>

*α γ-Irradiated allogeneic spleenocytes (BALB/c; 2.5 × 10^8/30 μl) were s.c. injected into C57Bl/6 mice with or without 5 nmol human neuroblastoma tumor gangliosides (NBTG, 5 nmol). On day 4, the popliteal lymph node draining the left (stimulated) and the right footpad (unstimulated) were removed; then the node mass was measured, and the net increase of popliteal node mass was calculated. Points, the mean net increase in the lymph node mass; bars, SEM. The data are from two separate experiments (10 mice/group). Cyclosporin A (CSA) was used as a control. NBTG inhibited the increase in the lymph node mass caused by the allogeneic stimulation (P < 0.01).

*β The number of lymphocytes recovered/popliteal lymph node.

*γ Spontaneous lymphoproliferation was measured by cellular [3H]thymidine incorporation at the cell density of 2 × 10^5 cells/well. The data represent the mean ± SD cpm of three cultures.

Table 2 Tumor gangliosides inhibit murine cellular immune responses

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Lymph node mass, mg</th>
<th>Lymphocytes × 10^3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Cyclosporin A</td>
</tr>
<tr>
<td>Unstimulated</td>
<td>1.47 ± 0.20</td>
<td>1.21 ± 0.12</td>
</tr>
<tr>
<td>Stimulated</td>
<td>3.36 ± 1.15</td>
<td>2.12 ± 0.23</td>
</tr>
<tr>
<td>Net increase</td>
<td>1.89 ± 1.04</td>
<td>0.91 ± 0.24</td>
</tr>
<tr>
<td>[3H]Thymidine uptake, cpm × 10^3</td>
<td>7.2 ± 0.3</td>
<td>2.9 ± 0.2</td>
</tr>
</tbody>
</table>

*α γ-Irradiated allogeneic spleenocytes (BALB/c; 2.5 × 10^8/30 μl) were injected i.c. into the left hind footpad of C3H mice without (control) or with human neuroblastoma gangliosides (10 nmol). Cyclosporin A was systemically administered i.p. (24 mg/kg/day) × 4 days. On day 4, the popliteal lymph nodes draining the left (stimulated) and the right footpad (unstimulated) were removed, and the lymph node mass was measured. The data represent the mean ± SD of five measurements in each group. The difference in the net increase of popliteal nodes between control and ganglioside-treated groups is statistically significant (P < 0.05).

*β The number of lymphocytes recovered/popliteal lymph node.

*γ Spontaneous lymphoproliferation was measured by cellular [3H]thymidine incorporation at the cell density of 2 × 10^5 cells/well. The data represent the mean ± SD cpm of three cultures.


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