Differentiation in Murine Mastocytoma Induced by Macrophage Gangliosides

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Gangliosides, Tumor, Growth Arrest

In the membrane of mouse macrophages two gangliosides were detected which inhibit the division of murine mastocytoma P815 tumor cells. The two gangliosides were incorporated into the cytoplasmatic membrane of mastocytoma cells. The concentration necessary to achieve a complete inhibition of P815 tumor cell division is about 1 μM for both effective gangliosides. Macrophage ganglioside-induced inhibition of cell division is accompanied by morphological changes of the mastocytoma cells. While the cells are rounding, their diameter increases and serotonin and granules appear in the cytoplasm of the enlarged cells. Our findings suggest that macrophage gangliosides may differentiate mastocytoma cells into mast cells.

Introduction

Macrophages are effectors of the natural tumor immunity. They are able to recognize tumor cells and destroy them (Klein and Mantovani, 1993). Furthermore, it was shown that macrophages induce a nonlytic cytostatic effect on tumor cells (Mantovani *et al.,* 1992). The cytostatic effect is mediated by components of the macrophage membrane, which were demonstrated by us to be gangliosides (Ritter *et al.,* 1986). Gangliosides are amphiphilic molecules, their hydrophobic ceramide moiety is immersed in the outer leaflet of the plasma membranes. At this site gangliosides are of great importance for the mediation and modulation of intercellular signals that regulate cellular proliferation and differentiation (Hakomori, 1994). Whereas the brain type gangliosides GM3 and GM1 are known to reduce the growth of tumor cells (Igarashi *et al.,* 1989), a distinct inhibitory effect on cell division of the leukemia cell line HL-60 followed by differentiation has been described for the neolacto-series ganglioside LM1 (IV^3NeuAc-nLcOse$_4$Cer) (Nojiri *et al.,* 1988). Recently, we identified macrophage gangliosides causing inhibition of cell divison in murine mastocytoma and human Burkitt lymphoma. They were shown to be gangliosides of the neolacto-series as well: IV^3NeuAc-nLcOse$_4$Cer and II^4NeuAc-nLcOse$_4$Cer (Schaade *et al.*, 1999). In this study we investigated the incorporation of gangliosides and its effect on the differentiation of mastocytoma cells.

Abbreviations: FCS, fetal calf serum; HPTLC, high performance thin layer chromatography; IUDR, 5-Iododeoxyuridine; PBS, phosphate buffered saline.
Gangliosides were named according to Svennerholm's nomenclature (Svennerholm, 1994), glycolipid abbreviations are used according to the recommendations of the International Union of Pure and Applied Chemistry (IUPAC-IUB, 1977).

Materials and Methods

Macrophages

Macrophages were obtained from the ascites of Naval Medical Research Institute (NMRI) mice with 30–35 g body weight each (outbred Swiss mice, established at the National Institutes of Health and given to the NMRI, purchased from Charles River, Sulzfeld, Germany) five days after intraperitoneal injection of thioglycollate medium (Difco) as described (Ashikaga *et al.*, 1994).

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Target cells

DBA/2 mastocytoma P815 cells were routinely cultured in Dulbecco's Modified Eagle Medium (MEM/RPMI-1640 (1+1, v/v) as described by Ritter *et al.* (1990).

High performance thin layer chromatography (HPTLC)

For detection, identification, and quantification gangliosides were sprayed in 5 mm lines on silica gel 60 plates (10 × 10 cm; Merck) by a Linomat III applicator (Camag, Muttenz, Switzerland). Chromatographic separation was performed with chloroform/methanol/0.02% aqueous $CaCl_2$ (60+40+9, v/v/v) for 50 min at room temperature. The chromatogram was made visible by resorcinol-HCl spray and heating at 110 °C for 15 min (Svennerholm, 1957). After staining, the gangliosides were quantitatively analysed by direct densitometry (λ = 570 nm) with a TLC scanner II (Camag, Muttenz, Switzerland) equipped with the CATS evaluation software. GM1 was used as a standard for quantification.

Macrophage gangliosides

All solvents and reagents were of analytical grade (Merck, Darmstadt, Germany). Macrophage gangliosides were purified as decribed by Nagai and Iwamori (1985) applying an additional purification step by preparative HPTLC. Ganglioside solutions were sprayed on precleaned silica gel 60 HPTLC plates (Merck). After staining parts of the chromatograms with resorcinol-HCl reagent, the ganglioside bands were localized, scraped out and eluted from silica gel with chloroform/methanol as a solvent.

Marker and reference gangliosides

Marker gangliosides GM3, GM1, GD3, GD1a, GD1b, GT1b were extracted from bovine brain (Svennerholm and Fredman, 1980). For HPTLC the marker gangliosides were dissolved in chloroform/methanol (4+1, v/v) and adjusted to the concentration 1 mg/ml.

Preparation and incorporation of fluorescent gangliosides

Macrophage gangliosides were labeled with Lucifer yellow CH (Serva, Heidelberg, Germany) as described previously (Spiegel *et al.*, 1984). Gangliosides were separated by HPTLC and detected by ultraviolet light.

Fluorescent gangliosides were added to the suspended P815 cells in a concentration of 1 µM. After an incubation between 10 min and 2 h at 37 °C in a humidified incubator with an atmosphere containing 5% CO_2, the cells were washed twice in PBS (2 mM KH_2PO_4, 9 mM Na_2PO_4, 180 mM NaCl). The resuspended viable cells were examined with a fluorescence microscope (Zeiss, Göttingen, Germany).

Determination of cell division inhibition

P815 cells (10^4) were suspended in 100 µl FCS-free MEM/RPMI-1640 and placed in microtiter plates (Nunc, Denmark). The gangliosides were dissolved in 50 µl MEM/RPMI-1640 (1+1, v/v) without FCS and added to the cells. The structures of the gangliosides used in this study are shown in Table I. After addition of gangliosides incubation was continued at 37 °C for 4 h in a moist atmosphere containing 5% CO_2.

In order to evaluate the inhibition of cell division DNA synthesis was estimated by pulse labeling with ^{125}IUDR according the method of Boyle and Ormerod (1975). The percentage of inhibition of DNA synthesis was calculated as:

$$\% \text{ inhibition} = \left(1 - \frac{\text{counts of test sample}}{\text{counts of control sample}}\right) \times 100.$$

Table I. Structures of gangliosides used in this study.

Ganglioside	Structure
GM1	Galβ1→3GalNAcβ1→4(NeuAcα2→3)Galβ1→4Glcβ1→1Cer
GD3	NeuAcα2→8NeuAcα2→3Galβ1→4Glcβ1→1Cer
IV^{3}NeuAc-nLcOse$_4$Cer	NeuAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer
II^{4}NeuAc-nLcOse$_4$Cer	Galβ1→4GlcNAcβ1→3(NeuAcα2→4)Galβ1→4Glcβ1→1Cer

Each assay was done in triplicate.

Determination of cytotoxicity

Cells were prepared as described above. Cell death and cell lysis were measured by lactate dehydrogenase (LDH) activity released from the damaged cells into the supernatant using a colorimetric assay (Cytotoxicity Detection Kit, Boehringer Mannheim, Germany). The percentage of lytic cells was calculated as:

$$\% \text{ lytic cells} = \frac{\text{absorbance of test sample} - \text{absorbance of spontaneous LDH release}}{\text{absorbance of maximum LDH release} - \text{absorbance of spontaneous LDH release}} \times 100.$$

Maximum LDH release was obtained by addition of Triton X-100 2% instead of gangliosides. Each assay was done in triplicate.

Determination of differentiation

The differentiation of P815 cells induced by gangliosides was assessed morphologically and immunoserologically. The morphological evaluation of P815 cells was performed under a light microscope. The size of the cells and the appearance of granules in the cytoplasm were evaluated.

For immunoserological assessment of cell differentiation the serotonin synthesis in the P815 cells after the addition of gangliosides was investigated. For this purpose an aliquot of 10 µl was taken from the microwells and distributed on coverslips. After drying and fixation in ice-cold aceton, the cell smears were incubated with a rabbit anti-serotonin antibody (ICN Biochemicals, Meckenheim, Germany) for 1 h at 37 °C (dilution 1+9, v/v, in PBS). After washing in PBS at room temperature for 30 min, fluorescein-conjugated swine anti-rabbit immunoglobulin (DAKO, Hamburg, Germany), diluted 1+50 (v/v) was applied. Following incubation (1 h, 37 °C) the coverslips were washed for 30 min in PBS and examined with a fluorescence microscope.

Results

Presentation of macrophage and brain type gangliosides applied

Twenty-five different gangliosides can be isolated from the membrane of mouse macrophages. A cytostatic activity was found for the two gangliosides IV^3NeuAc-nLcOse$_4$Cer and II^4NeuAc-nLcOse$_4$Cer. In HPTLC, the purified cytostatically active ganglioside IV^3NeuAc-nLcOse$_4$Cer behaved identically to the brain type ganglioside GM1, whereas II^4NeuAc-nLcOse$_4$Cer migrated between the brain type gangliosides GM1 and GD3 (Fig. 1). Since the gangliosides GM1 and GD3 behaved similarly to the macrophage gangliosides in HPTLC, they were applied in the inhibiton tests, too.

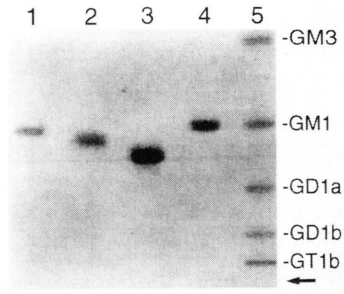

Fig. 1. HPTLC of purified macrophage and brain type gangliosides. Lane 1, IV^3NeuAc-nLcOse$_4$Cer; lane 2, II^4-NeuAc-nLcOse$_4$Cer; lane 3, brain ganglioside GD3; lane 4, brain ganglioside GM1; lane 5, marker gangliosides. Resorcinol-HCl staining. Arrow indicates origin.

Incorporation of the fluorescent macrophage gangliosides into mastocytoma cells

The uptake of the macrophage gangliosides into the cytoplasmatic membrane was time dependent. After a ten-min incubation period the surface fluorescence was already visible. The intensity increased with further incubation period and reached its maximum after 45 min. The gangliosides were stably integrated into the cytoplasmatic membrane and could not be removed by extensive washing (Fig. 2, see page ▶▶▶).

Effect of gangliosides on cell growth and differentiation of P815 cells

When P815 cells were cultured for 24 h with variant concentrations of macrophage or brain gangliosides, cell division was suppressed depending on the concentration. Cell growth was almost completely inhibited by 0.6 µM ganglioside II^4-

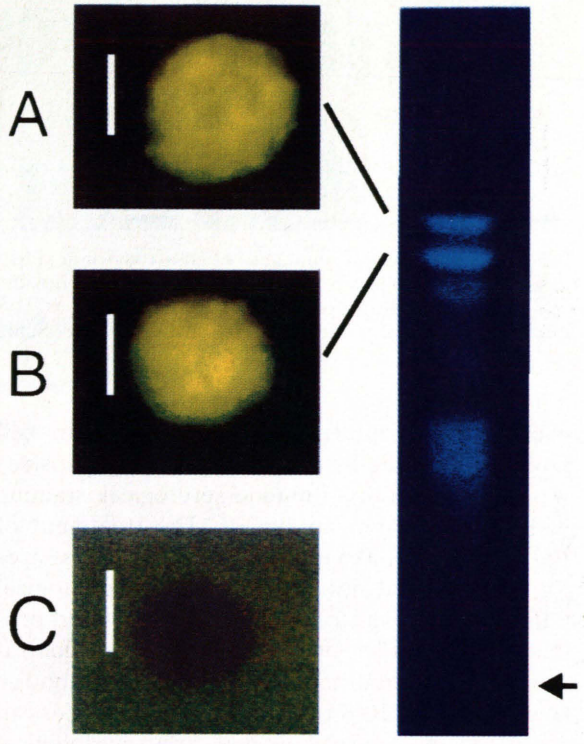

Fig. 2. Surface fluorescence of mastocytoma P815 cells after treatment with fluorescent gangliosides (1 μM) for 45 min. *(A)* Fluorescence pattern with lucifer yellow CH-labeled IV^{3}NeuAc-nLcOse$_4$Cer *(B)* Fluorescence pattern with lucifer yellow CH-labeled II^{4}NeuAc-nLcOse$_4$Cer. *(C)* The negative control shows the background fluorescence after incubation with 1 μM unconjugated lucifer yellow CH. Scale bar = 10 μm. The thin layer chromatogram shows the prepared macrophage gangliosides. Arrow indicates origin.

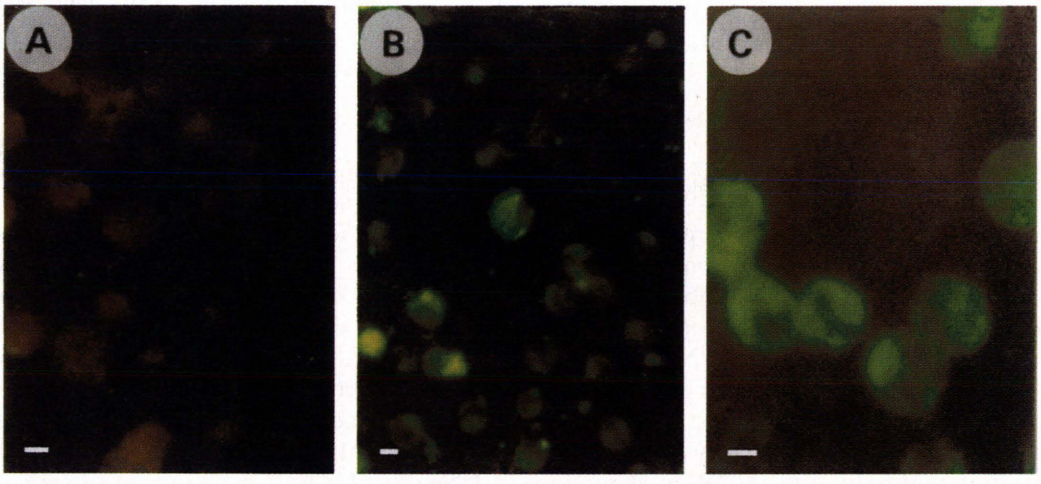

Fig. 5. Immunoserological detection of serotonin in mastocytoma P815 cells. *(A)* In the absence of the macrophage ganglioside. *(B)* 24 h after addition of 0.6 μM II^{4}NeuAc-nLcOse$_4$Cer. *(C)* 48 h after addition of 0.6 μM II^{4}NeuAc-nLcOse$_4$Cer. Scale bar = 10 μm.

NeuAc-nLcOse$_4$Cer. The same effect could be obtained by 1.2 μM IV^{3}NeuAc-nLcOse$_4$Cer. However, a complete inhibition of cell division could not be achieved by the brain type gangliosides GM1 and GD3. Only when applying the ganglioside GM1 in a concentration which was 15 times as high, a 50% inhibition of cell division compared to II^{4}NeuAc-nLcOse$_4$Cer was attainable. (Fig. 3A).

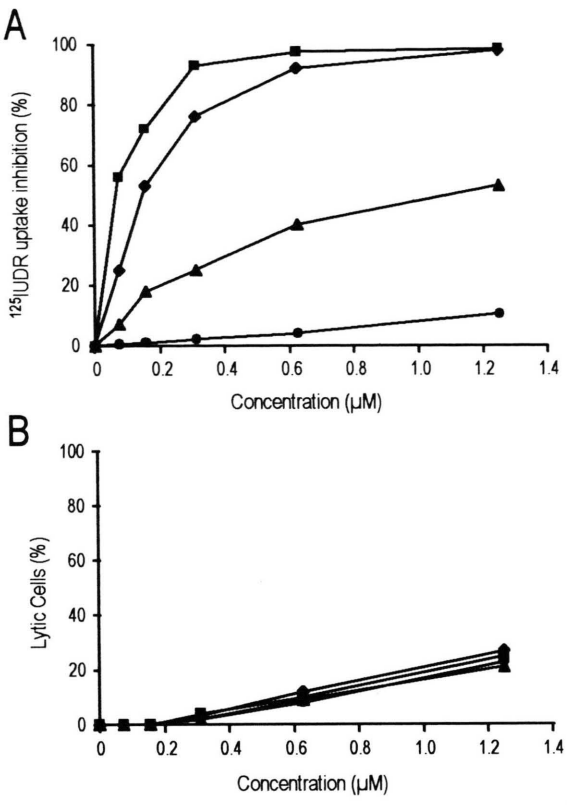

Fig. 3. Effect of the gangliosides IV^{3}NeuAc-nLcOse$_4$Cer, II^{4}NeuAc-nLcOse$_4$Cer, GM1 and GD3 on P815 mastocytoma cells. *(A)* Inhibition of DNA synthesis measured by ^{125}IUDR uptake. *(B)* Cytolysis measured by LDH release. ◆)=IV^{3}NeuAc-nLcOse$_4$Cer, ■)=II^{4}NeuAc-nLcOse$_4$Cer, ▲)=GM1, ●)=GD3. The incubation took 24 h.

All gangliosides used had comparable cytotoxic effects. The rate of lytic cells at a concentration of 0.6 μM II^{4}NeuAc-nLcOse$_4$Cer was about 10% and could therefore not be responsible for the effective inhibition of DNA synthesis. IV^{3}NeuAc-nLcOse$_4$Cer showed 27% cytotoxicity at a concentration that was necessary to achieve almost complete cytostasis. GM1 caused a cytotoxicity of 20% at a concentration that reduces ^{125}IUDR uptake to the half. Applying GD3, the reduced DNA-synthesis corresponds to the rate of necrotic cells (Fig. 3B).

During a 24 hours period of culture cells became adherent and acquired an elongated form. There was a formation of weakly refracting granules in the cytoplasm. In the following 24 hours the adherent cells rounded again and raised to a diameter of 28–30 μm. Now the cytoplasm was densely

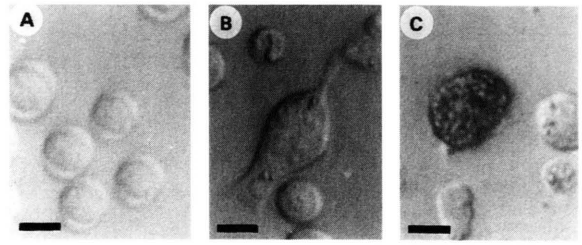

Fig. 4. Morphological changes of mastocytoma P815 cells. *(A)* In the absence of the macrophage ganglioside. *(B)* 24 h after addition of II^{4}NeuAc-nLcOse$_4$Cer. *(C)* 48 h after addition of 0.6 μM II^{4}NeuAc-nLcOse$_4$Cer. Scale bar = 15 μm.

packed with granules (Fig. 4). Changes in cell physiology caused by the macrophage gangliosides were evaluated by immuno-serological staining with anti-serotonin antibodies. The treatment of P815 cells with 0.6 μmol II^{4}NeuAc-nLcOse$_4$Cer for 48 hours did not only lead to morphological differentiation with cellular enlargement and production of granules, but also to a raised concentration of serotonin detectable by specific antibodies (Fig. 5 see page 1007). IV^{3}NeuAc-nLcOse$_4$Cer can cause the same morphological and physiological changes in P815 as II^{4}NeuAc-nLcOse$_4$Cer. In contrast, no significant changes in the cellular phenotype were observed in P815 cells treated with the brain gangliosides GM1 and GD3.

Discussion

When getting in contact with tumor cells macrophages are able to inhibit tumor cell division without previous sensibilization or activation (Mantovani *et al.*, 1992). The inhibitory activity is mediated by components of the macrophage membrane. They were identified as gangliosides by us (Ritter *et al.*, 1986). The structures of the two effective macrophage have recently been elucidated (Schaade *et al.*, 1999). An unbranched (IV^{3}NeuAc-nLcOse$_4$Cer) and a branched (II^{4}NeuAc-nLcOse$_4$Cer) neolacto-series monosialoganglioside were found.

In the mouse model using mastocytoma P815 cells as targets two gangliosides, IV^{3}NeuAc-nLcOse$_4$Cer and II^{4}NeuAc-nLcOse$_4$Cer, were incorporated tightly into the cytoplasmatic membrane of the target cells and showed an inhibitory effect on cell division, measurable by the inhibited uptake of ^{125}IUDR. Additional, changes in the P815 cells

were demonstrated by morphological and immunoserological methods. Cellular enlargement and the appearence of granules was shown. Serotonin was detected, a typical content of mast cell granules. Thus, a differentiation from mastocytoma to mast cells was demonstrated. Mast cells induced by macrophage gangliosides in vitro resemble those tissue mast cells described by Dunn and Potter that were transformed to the mastocytoma P815 by methylcholanthren treatment (Dunn and Potter, 1957).

The influence of gangliosides on cell growth and differentiation is thought to depend on the modulation of several tyrosine kinase receptors (Yates and Rampersaud, 1998). Incorporation of soluble gangliosides released by other cells into the cell membrane is known (Ladisch *et al.,* 1983) and seems to be a necessary prerequisite for their efficiency (Bremer *et al.*, 1984). After incorporation into the plasmamembrane of murine mastocytoma cells, the gangliosides IV^3NeuAc-nLcOse$_4$Cer and II^4NeuAc-nLcOse$_4$Cer induce an inhibition of cell division that results in differentiation.

Investigations with the ganglioside IV^3NeuAc-nLcOse$_4$Cer by other authors gave a hint to the pathway that may transduce the differentiating signal. Besides cell specific growth factors all animal cells essentially require insulin and transferrin. The lacto-series ganglioside IV^3NeuAc-nLcOse$_4$Cer inhibits the insulin receptor function (Nojiri *et al.,* 1991), arrests growth of HL-60 cells, and induces the differentiation to mature granulocytes (Nojiri *et al.,* 1988). The detailed mechanism is not known, however, the authors assume that the gangliosides surrounding the receptors play an important role in modulation of cell proliferation through direct or indirect interaction with receptor-associated tyrosine kinases. Whether gangliosides IV^3NeuAc-nLcOse$_4$Cer and II^4NeuAc-nLcOse$_4$Cer develop their differentiating activity on mastocytoma cells by modifying this signal pathway or another, will be an aim of future investigations.

The influence of gangliosides on growth factor receptors crucially depends on the conformation of the gangliosides (Nojiri *et al.,* 1991; Schlessinger 1988). IV^3NeuAc-nLcOse$_4$Cer and II^4NeuAc-nLcOse$_4$Cer possess identical molecular masses and identical components of their carbohydrate moiety, but isomeric carbohydrate conformations (Schaade *et al.,* 1999). The cytstatic activity of IV^3NeuAc-nLcOse$_4$Cer and II^4NeuAc-nLcOse$_4$-Cer differs quantitatively. Thus, it is conceivable that IV^3NeuAc-nLcOse$_4$Cer and II^4NeuAc-nLcOse$_4$Cer modulate different receptors in the cell membrane. However, the activity of both gangliosides leads to growth arrest and mast cell differentiation, suggesting a common inherent signal pathway.

The clarification of this signal pathway will help to enlighten the interaction between macrophages and neoplastic cells, as well as aspects of the malignant transformation itself.

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