·论著(译文)·

Effect of Gangliosides on the Adhesion of Neuroblastom Cells to Collagen

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Abstract: Objective To study the effect of endogeneous ganglioside (GS) on the adhesion of LA-N5 neuroblastoma cells to collagen. **Methods** LA-N5 cells were cultured in the presence of D-threo-1-phenyl-2-decanolamino-3-morphinoline-1-propanol (D-PDMP), an inhibitor of glucosylceramide synthase. The adhesion of the LA-N5 cells to immobilized collagen was tested. **Results** After 6 days, endogenous GS was reduced by 98%. No change in the cell morphology, viability, proliferation rate or percentage of apoptotic cells was observed. The adhesion to collagen of cells exposed to D-PDMP was reduced by 65% compared to the control LA-N5 cells: OD_{570} 0.07 ±0.01 vs 0.21 ±0.030 ($P < 0.01$). When GS-depleted cells were pre-incubated in a conditioned medium collected from the control cells, the adhesion to collagen was restored and was comparable to that of the control cells ($P > 0.05$). Similarly, the pre-incubation of GS-depleted cells with purified tumor GS GD_2, the most abundant GS in LA-N5 cells, restored adhesion. **Conclusions** Endogenous tumor GS regulates neuroblastoma cell adhesion to collagen, suggesting that it may play a role in tumor cell migration, invasion and metastasis.

Key words: Gangliosides; Neuroblastoma; Adhesion

[CLC number] R-73-3; R730.246 **[Document code]** A **[Article ID]** 1008-8830(2001)04-0455-06

Adhesion is a requisite function for successful tumor metastasis. Rapid changes in the ability of tumor cells to attach to extra-cellular matrix (ECM) proteins are necessary for adhesion, invasion and migration. The factors that regulate these changes in tumor cell adhesion are poorly characterized. Ganglioside, a sialic acid-containing glycosphingolipid embedded in the outer leaflet of the cell membrane, is a candidate molecule for this activity. Our study has shown that platelet adhesion to collagen is increased by neuroblastoma tumor gangliosides[1]. The experiments described in this report examined the role of endogenous cellular gangliosides in cell adhesion. We chose to study a neuroblastoma cell line (LA-N5) that avidly adheres to collagen and contains very high concentrations of endogenous cellular gangliosides. We examined the effect of pharmacological inhibition of endogenous ganglioside biosynthesis on neuroblastoma tumor cell adhesion to immobilized type I collagen.

1 Material and Methods

1.1 Materials and reagents

The LA-N5 neuroblastoma cells were a gift from Susan Cohn, Northwestern University, Chicago, IL. The rat tail type I collagen was purchased from Sigma and used as a substrate for the adhesion experiments. The BCA Protein Assay Reagent kit was from Pierce. The CYQUANT cell proliferation assay kit was purchased from Clontech. The D-threo-1-phenyl-2-decanolamino-3-morphinoline-1-propanol (D-PDMP) was from Matreya.

1.2 Ganglioside isolation and biochemical analysis

Gangliosides were isolated from neuroblastoma tumor cells as was previously described[2]. Briefly, total lipids were extracted twice with 10 volumes of chloroform-methanol. The extracts were combined, dried under a stream of N2, re-dissolved in a small volume of chloroform-methanol, and stored overnight

[Received] February 6, 2001; [Revised] May 15, 2001
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at - 20 ℃. Insoluble glycoproteins were removed by centrifugation and the supernatant was dried under a stream of N_2. Gangliosides were isolated by partitioning the dried total lipid extracts in disopropyl ether/1-butanol/water[3], followed by Sephadex G-50 gel exclusion chromatography to remove traces of salts and other low molecular weight contaminants, and further purified by normal phase high pressure liquid chromatography. The total ganglioside fraction was collected, lyophilized and re-purified by gel exclusion chromatography to remove salts. Gangliosides were quantified as nmol lipid bound sialic acid (LBSA) as was previously described, separated by high performance thin layer chromatography and visualized as purple bands following staining with resorcinol reagent[4].

1.3 Cell culture and depletion of cellular gangliosides

The LA-N5 cells were cultured in RPMI 1640 supplemented with penicillin G (50 units/ml), streptomycin (50 μg/ml), 2 mM glutamine and 10 % heat-inactivated fetal bovine serum (Gibco). Cultures were maintained at 37 ℃ in a humidified atmosphere with 5 % CO_2. To inhibit ganglioside biosynthesis and deplete neuroblastoma cells of endogenous cellular gangliosides, the LA-N5 cells were cultured in the presence of 10 μm D-PDMP.

1.4 Tumor cell adhesion

For adhesion assays, the cultured cells were collected by a short exposure to cell dissociation solution (Sigma), pelleted by centrifugation and re-suspended in RPMI containing 0.1 % BSA. Fifteen thousand cells in 100 μL medium were added to the wells of non-tissue culture-treated plates (Falcon # 3915, Becton Dickinson) which had been coated with type I rat tail collagen (4 μg/well/100 μl) overnight and then blocked with 0.1 % BSA. Following the incubation with collagen, the wells were washed with RPMI to remove unbound cells and the number of residual adherent cells was determined using the BCA assay[5]. Each experiment included determinations of background cell adhesion to plastic and to albumin-coated wells.

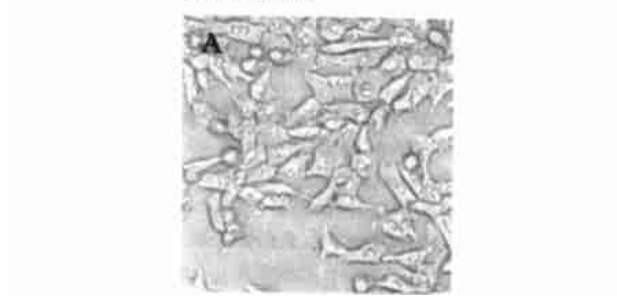
1.5 Apoptosis Assay

DNA was isolated with a DNA-RNA isolation kit (United States Biochemicals). Briefly, cells were lysed and then incubated with the buffers provided. The DNA was precipitated with sodium acetate and isopropyl alcohol at 40 ℃ and collected by centrifugation (12,000 g, 5 min). The dried pellets were dissolved in Tris-EDTA buffer, and incubated with 100 μg/ml DNAase-free RNAase at 40 ℃ for 1 h. Five microgram samples were separated by electrophoresis (1 % agarose gel containing 0.5 μg/ml ethidium bromide, 80～90 V for 1～2 hr) and the DNA was visualized under ultraviolet light.

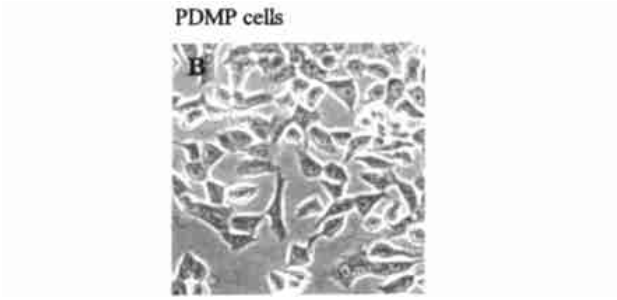
2 Results

2.1 Effect of ganglioside depletion on cell growth, morphology and apoptosis

Li[6] demonstrated that following a six-day exposure to D-PDMP, ganglioside biosynthesis of LA-N5 neuroblastoma cells was completely inhibited and the cellular ganglioside content was reduced to less than 10 % of the pre-exposure amount. We employed a similar strategy to examine the effect of ganglioside depletion. Following 6 days in culture with D-PDMP, the ganglioside content of neuroblastoma cells was reduced by 98 % to 0.76 nmol/10^8 cells compared with 43.69 nmol/10^8 for the control cells exposed to ethanol vehicle. The viability and morphology of ganglioside-depleted cells (Figure 1A) were identical to the control cells (Figure 1B). D-PDMP did not affect cell spreading on the plastic surface or neurite extension. The proliferation rate of cells cultured with D-PDMP was identical to that of the control cells at both high (7.5×10^6) and low (1×10^6) seeding concentrations (Figure 1C). There was no evidence for increased apoptosis in cells exposed to D-PDMP (Figure 1D, Lane 1) compared with the cells cultured with ethanol vehicle (Figure 1D, Lane 2).



1A



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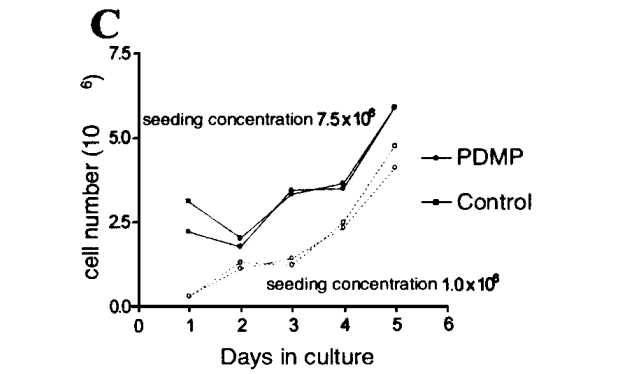

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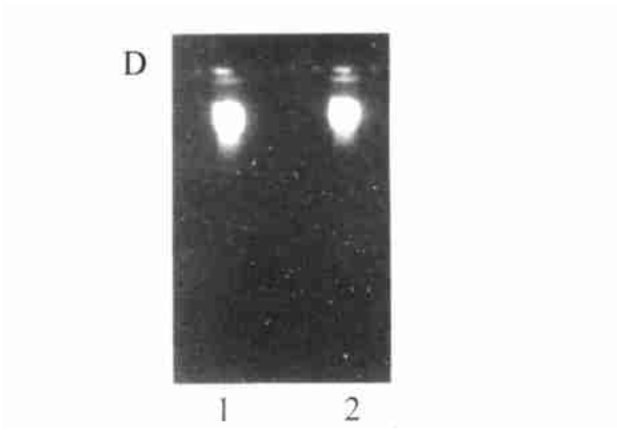

1D

Figure 1 Effect of ganglioside depletion on LA-N5 neuroblastomna cells.

Panels A and B, photomicrographs (200x) of LA-N5 cells in culture for 6 days with ethanol (< 0.05 %) vehicle control (A) or in the presence of 10 μM PDMP (B). Panel C, cell proliferation measurments made using the CyQuant assay on cells cultured with 10 μM PDMP (λ) or ethanol (ν) on days 1～5 after on cells seeded at high (7.5×10^6 cells/ 75 cm^2) or low (1.0×10^6 cells/ 75 cm^2) density.

2.2 Effect of ganglioside depletion on LA-N5 cell adhesion to ECM proteins

The effect of D-PDMP on neuroblastoma cell adhesion to fibronectin, Matrigel™ and collagen was examined. The inhibition of ganglioside biosynthesis and depletion of endogenous cellular gangliosides resulted in decreased tumor cell adhesion to all 3 substrates (Figure 2). When fibronectin was used as the substrate, culturing cells in D-PDMP resulted in a 26 % decrease in cell adhesion compared with the control cells: OD_{570} 0.17 ±0.01 vs 0.23 ±0.01 ($P < 0.05$). When Matrigel™, a form of reconstituted basement membrane consisting primarily of laminin, was used as the substrate, culturing cells in D-PDMP resulted in a 31 % reduction in the number of adherent cells compared with the control cells: OD_{570} 0.22 ±0.01 vs 0.32 ±0.01($P < 0.05$). A similar effect of ganglioside depletion on collagen adhesion was demonstrated with a 37 % reduction in the number of adherent cells: OD_{570} 0.18 ±0.01 vs. 0.28 ±0.02 ($P < 0.05$). Background adhesion to plastic was minimal (OD_{570} 0.01 ±0.002) as was adhesion to albumin-coated wells.

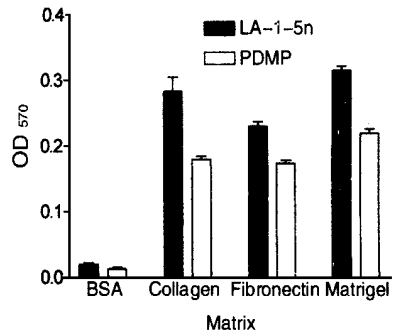

Figure 2 Effect of ganglioside depletion on LA-N5 cell adhesion to extracellular matrix proteins

2.3 Ganglioside depletion reduces the collagen adherent phenotype of LA-N5 neuroblastoma cells

Further study was conducted on the role of endogenous cellular endogenous cellular gangliosides in adhesion of neuroblastoma cells to collagen, the most abundant protein in the extracellular matrix. Culturing LA-N5 cells in D-PDMP for 6 days decreased the number of cells adherent to collagen-coated wells over the entire range of incubation duration tested (Figure 3). After only 3 minutes in contact with the collagen-coated surface, the adhesion of D-PDMP-cultured cells was reduced by 67 % compared to the control LA-N5 cells: OD_{570} 0.07 ±0.01 vs. 0.21 ±0.03($P < 0.001$) and similar to the background attachment to albumin (0.06 ±0.01). The reduction in collagen adhesion observed with ganglioside-depleted cells persisted at all time points despite a plateau in adhesion

after 15 minutes (Figure 3).

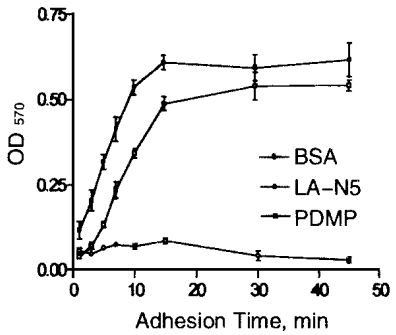

Figure 3 Effect of ganglioside depletion on the time course of the collagen adhesion of LA-N5 cells

2.4 Pre-incubation of ganglioside-depleted cells with a conditioned medium restores adhesion to collagen

To determine if the reduction in collagen adhesion following culturing cells for 6 days in D-PDMP was reversible, ganglioside-depleted cells were pre-incubated for 2 hours with a conditioned medium recovered from the control LA-N5 cells. The conditioned medium partially restored collagen adhesion (Figure 4). The effect was observed after ganglioside-depleted cells were in contact with the collagen matrix for 10 minutes: OD_{570} 0.31 ±0.02 vs. 0.27 ±0.03 vs. 0.34 ±0.02, for ganglioside-depleted cells pre-incubated with a conditioned medium vs. ganglioside-depleted cells pre-incubated with a fresh medium vs. control LA-N5 cells, respectively. After 30 minutes in contact with the collagen matrix, the adhesion of ganglioside-depleted cells pre-incubated with the conditioned medium was 92% of the controls: OD_{570} 0.45 ±0.02 vs. 0.49 ±0.035 ($P > 0.05$).

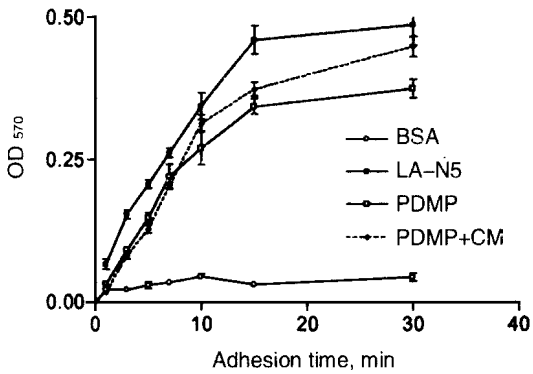

Figure 4 Conditioned medium partially restores collagen adhesion of ganglioside depleted cells.

2.5 Purified tumor gangliosides restore the collagen adherent phenotype of ganglioside-depleted cells

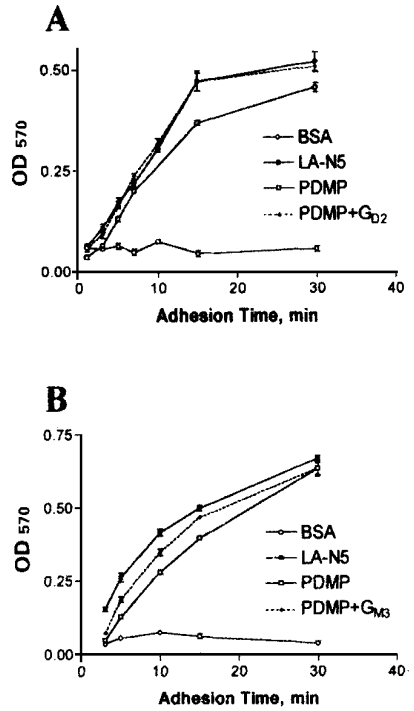

Figure 5 Exogenous gangliosides restore the collagen adhesion of PDMP-cultured LA-N5 cells.

Since gangliosides are shed into the culture medium and are able to insert into the cell membrane, we next tested the ability of purified gangliosides to restore the collagen adhesion of ganglioside-depleted cells. Cells cultured in D-PDMP for 6 days were pre-incubated with gangliosides isolated from LA-N5 cells. The di-sialoganglioside, G_{D2}, is a major tumor ganglioside of LA-N5 neuroblastoma cells, accounting for nearly 60% of the total lipid-bound sialic (LBSA) in these cells. Pre-incubation of ganglioside-depleted cells with 1 μM G_{D2} for 5 minutes completely restored collagen adhesion (Figure 5A). The ability of exogenous G_{D2} to restore the collagen adhesion of ganglioside-depleted cells was tested over a range of cell-collagen matrix incubation times. Within 3 minutes, there was a partial restoration of collagen adhesion by exogenous GD_2 and by 5 minutes there was no significant difference between ganglioside-depleted cells pre-incubated with G_{D2} and the control LA-N5 cells: OD_{570} 0.0.17 ±0.01 vs. 0.16 ±0.01 ($P > 0.05$). In contrast, the mono-sialioganglioside, G_{M3}, is only found in trace amounts (<2% of the total LBSA) in

LA-N5 cells. Therefore, we tested the effect of G_{M3} on collagen adhesion of ganglioside-depleted cells to determine the specificity of the restorative effect of G_{D2}. Pre-incubation of ganglioside-depleted cells with G_{M3} for 5 minutes partially (72 %) restored collagen adhesion compared with the control LA-N5 cells (OD_{570} 0.19 ±0.009 vs. 0.262 ±0.015) and was significantly greater than the adhesion of ganglioside-depleted cells (OD_{570} 0.13 ±0.006, $P < 0.01$) (Figure 5B). Since pre-incubation of ganglioside-depleted cells with G_{M3} has never completely restored the collagen adherent phenotype of LA-N5 cells, there appears to be some degree of specificity for the tumor ganglioside, G_{D2}.

3 Discussion

We hypothesized that high concentrations of membrane gangliosides were necessary for neuroblastoma cell adhesion to collagen and that pharmacological depletion of membrane gangliosides would abrogate cell adhesion to collagen. The data reported here support both hypotheses. Despite near complete depletion of cellular gangliosides following 6 days of exposure to D-PDMP, the cell morphology, viability, proliferation rate and percentage of apoptotic cells were not different from those of the control LA-N5 cells. Adhesion to collagen of cells exposed to D-PDMP was reduced to one-third of the control LA-N5 cells. The adherent phenotype could be restored by pre-incubating ganglioside-depleted cells with a conditioned medium (into which gangliosides are shed) and with purified tumor G_{D2} ganglioside. Taken together, these data demonstrate that endogenous tumor gangliosides regulate neuroblastoma cell adhesion to collagen and suggest that they may play a role in tumor cell metastasis.

Direct evidence for the effect of gangliosides on tumor formation and metastasis was obtained in experimental animals. For example, using a spontaneous lymphoma cell line, Ladisch demonstrated that tumor formation and progression increased ten-fold when nude mice were injected with tumor-derived gangliosides and tumor cells in contrast with tumor cells alone: 83 % with gangliosides vs. 8 % without gangkiosides[7]. Neuroblastoma, an aggressive tumor of the sympathetic nervous system, is characterized by substantial shedding of the gangliosides from the cell surface. Once in the blood, tumor gangliosides are transported in association with lipoproteins, primarily with the LDL fraction[8]. To examine the relationship of shed tumor gangliosides, expressed as the circulating G_{D2} concentration, and patient outcome, 74 children with advanced stage (Ⅲ and Ⅳ) neuroblastoma were examined[9]. Progression-free survival (PFS) was inversely related to the circulating G_{D2} concentration at the time of diagnosis. The quartile of patients having the highest circulating G_{D2} levels had a strikingly different outcome from the quartile of patients with the lowest G_{D2} levels. The median PFS was shorter (9 vs. 28 months) and the long-term survival rate lower (2 year PFS of 24 % vs. 70 %). The mechanism that underlie these tumor-promoting effects are still unknown.

We have shown that shed neuroblastoma tumor gangliosides promote platelet activation and aggregation. The interaction of tumor cells with platelets and the formation of tumor cell emboli may be one mechanism for the tumor promoting effect of gangliosides[1]. Another mechanism might be a direct effect on tumor cell adhesion. Gangliosides influence the adhesion of cells to extracellular substrates[10]. This includes adhesion specifically mediated by integrin molecules[11]. Barletta et al demonstrated that the depletion of cellular gangliosides reduced cell adhesion. This effect was ascribed to an alteration in the binding characteristics of integrin $\alpha_v\beta_3$ vitronectin receptor. Interestingly, the replenishment of cellular gangliosides restored the functional activity of the integrin. We have demonstrated that tumor gangliosides influence the function of the collagen-binding platelet integrin $\alpha_2\beta_1$. Experiments to examine the role of gangliosides on tumor neuroblastoma cell integrins are in progress.

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(本文编辑:孙晓玲)