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COMPLEX GANGLIOSIDES MODULATE THE INTEGRIN-MEDIATED ADHESION IN A RAT HEPATOMA CELL LINE

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In this study, we investigated whether complex gangliosides influence cell adhesion by modulating the activity of integrin receptors. Our experimental model was represented by CMH5123 cells, a line of neoplastic hepatocytes derived from the minimal deviation Morris hepatoma 5123c of the rat, which adhered to substrata coated with fetal calf serum (FCS) by an integrin-mediated mechanism, being vitronectin the specific serum protein which sustained cell adhesion. We found that ganglioside depletion, obtained by inhibiting complex ganglioside biosynthesis, was accompanied by a reduction of cell adhesiveness to FCS-coated substrata. Integrins appeared to mediate the effect of ganglioside depletion on cell adhesiveness. In fact, sensitivity to the integrin inhibitor GRGDSPC peptide was ten times higher in ganglioside-depleted cells compared to control cells. Moreover, growth of ganglioside-depleted CMH5123 cells in media supplemented with complex gangliosides restored the cell sensitivity to the integrin inhibitor to the same level as that found in control cells. Furthermore, ganglioside depletion of CMH5123 cells decreased the affinity of vitronectin receptors for vitronectin without modifying their number; affinity of vitronectin receptors was re-established in ganglioside-depleted cells by supplementing their growth media with complex gangliosides. In conclusion, these results support the participation of gangliosides to cell adhesion as modulators of integrin receptors. © 1993 Academic Press, Inc.

Several reports indicate that cell surface gangliosides are implicated in cell adhesiveness to extracellular matrix [1-13]. The recent discovery that adhesive proteins are recognized by a new class of cellular receptors, the integrins [14], raised the issue that gangliosides play a role in the adhesion process by modulating the function of these receptors. Indeed, disialogangliosides were

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Abbreviations: **DMEM**, Dulbecco's modified Eagle's minimal essential medium; **EDTA**, ethylene diamino tetraacetic acid; **EGTA**, ethylene bis (oxy-ethylenenitrilo) tetraacetic acid; **FCS**, fetal calf serum; **HPTLC**, high-performance thin layer chromatography; **PBS**, phosphate-buffered saline. Gangliosides are indicated according to the nomenclature of Svennerholm [34]: **GM3**, NeuAcα2→3Galβ1→4Glcβ1→1Cer; **GM2**, GalNAcβ1→4 [NeuAcα2→3]Galβ1→4Glcβ1→1Cer; **GM1**, Galβ1→3GalNAcβ1→4[NeuAcα2→3]Galβ1→4 Glcβ1→1Cer; **GD1a**, NeuAcα2→3Galβ1→3GalNAcβ1→4[NeuAcα2→3]Gal β1→4Glcβ1→1 Cer.

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shown to form a complex with integrins in melanoma cells [15], while anti-ganglioside antibodies prevented the integrin-dependent attachment of various cell types to adhesive proteins [16, 17]. Moreover, anti-ganglioside antibodies and specific inhibitors of the integrin function exerted a synergistic inhibitory effect on the adhesion of melanoma cells to fibronectin matrices [18]. Further evidence that gangliosides are involved in integrin function was provided by the observation [19] that cholera toxin B subunit, a ganglioside-specific ligand [20], stimulates neuritogenesis, a cell differentiation process dependent on integrins.

In order to define the modulatory role of gangliosides in integrin-mediated cell adhesion to extracellular matrix, we investigated whether the depletion of complex ganglioside in CMH5123 cells, a line of neoplastic hepatocytes derived from the Morris 5123c minimal deviation hepatoma of the rat [21], would change the sensitivity of CMH5123 cells to an integrin inhibitor, a RGDS-containing peptide [22]. Moreover, we explored whether ganglioside depletion affected the binding of soluble vitronectin to its receptors on the surface of CMH5123 cells. Depletion of complex gangliosides in CMH5123 cells was obtained by growth in the presence of EGTA+EDTA [13, 23].

MATERIALS AND METHODS

Cells and culture conditions. CMH5123 cells were maintained in a humidified atmosphere of 10% CO_2 in air at 37°C with DMEM (GIBCO, Grand Island, NY) supplemented with 10% FCS (Boehringer, Mannheim, Germany) and no antibiotics. Confluent cultures were subcultivated by trypsinization (0.025% trypsin in PBS). CMH5123 cells were routinely checked for Mycoplasma contamination by the fluorochrome stain procedure [24].

Inhibition of complex ganglioside biosynthesis. Synthesis of complex gangliosides of CMH5123 cells was inhibited by treatment with EGTA and EDTA [13, 23]. Cells were grown for several passages in media containing 1.45 mM EGTA+ 0.40 mM EDTA, as reported in a previous paper [13] (EGTA+EDTA-treated cells). In order to metabolically label the cellular gangliosides, cultures were exposed to media containing 1.0 mCi/ml of D-[6-^{3}H]galactose (sp. act. 31.5 Ci/mmol; Amersham International, England) for 48 h before harvesting [13]. Cells were detached by incubation in 0.5 mM EGTA in PBS and washed twice in PBS. Total lipids were extracted from the sonicated cell suspensions with 19 volumes of chloroform:methanol 2:1 [25]. Gangliosides were isolated from the other lipid components following Siakotos and Rouser's chromatographic system [26], and then fractionated into GM3, GM2, GM1, GD1a by thin layer chromatography on HPTLC plates (Merck, Darmstadt, Germany) with chloroform : methanol : 0.25% $CaCl_2$ (55:40:9). The radiolabelled gangliosides were revealed by fluorography and quantitatively transferred into scintillation vials for counting.

Incorporation of complex gangliosides into CMH5123 cells. EGTA+EDTA-treated and untreated CMH5123 cells were enriched in complex gangliosides by growth for 12 h in DMEM supplemented with 1% FCS and a 50 μM (ganglioside-glucose) mixture of ox brain gangliosides.

Measurement of cell adhesion to different substrata. In this study, substrata used for cell adhesion were FCS, fibronectin, vitronectin and the B subunit of the cholera toxin. Fibronectin and vitronectin solutions were diluted at various concentrations in DMEM, while FCS was used at 5% in DMEM. The cholera toxin B subunit was used at a concentration of 100 μg/ml in DMEM. The various protein solutions were layered on 96-well cluster dishes which were incubated in a humidified atmosphere of 10% CO_2 in air at 37 ° C for 1 h and then rinsed with PBS. In order to block any vacant adhesion sites, wells were re-inoculated with DMEM containing heat-denatured bovine serum albumin (BSA) (250 μg/ml), and then incubated in a humidified atmosphere of 10% CO_2 in air at 37° C for 1 h.

All cells used in this study (control and EGTA+EDTA-treated CMH5123 cells, either supplemented or not supplemented with complex gangliosides) were radiolabelled by a 24 h exposure to growth media containing [6-^{3}H]thymidine (0.1 μCi/ml, sp. act. 24 Ci/mmol, Amersham International, England). Radiolabelled cells were detached by using a 0.5 mM EGTA solution in PBS and suspended at $4x10^5$ cells/ml in DMEM containing bovine serum albumin (250 μg/ml). 100 μl aliquots of the cell suspensions were pipetted into the protein-coated wells of cluster dishes which were incubated in a humidified atmosphere of 10% CO_2 in air at 37° C for the indicated periods of time. After discarding the supernatant, each well was washed with two strokes of PBS in order to remove the non-adherent cells. The adherent cells were solubilized with 100 μl of 1% SDS in 0.1 N NaOH and, after neutralization with 3% acetic acid, quantitatively transferred into scintillation vials for counting. The radioactivity of the adherent cells in each well was calculated as percent of the radioactivity present in 100 μl aliquots of the original cell suspensions.

Measurement of cell adhesion in the presence of an integrin inhibitor. In order to ascertain whether adhesiveness of CMH5123 cells to protein-coated substrata was mediated by integrins, we measured cell adhesion in the presence of a GRGDSPC (Gly-Arg-Gly-Asp-Ser-Pro-Cys) peptide, which competes with adhesive proteins for the binding to integrins [22]. A GRGESP (Gly-Arg-Gly-Glu-Ser-Pro) peptide was used as an inactive control. Then, we measured cell adhesiveness to FCS-coated substrata of ganglioside-depleted CMH5123 cells in the presence of increasing concentrations of GRGDSPC peptide. Change of the sensitivity to the peptide in ganglioside-depleted CMH5123 cells was instrumental for determining whether gangliosides modulated integrin function.

Radioiodination of vitronectin. 50 μg of vitronectin were reacted with 1.0 mCi of NaI^{125} (Amersham, U.K.) at 4° C for 30 min in the presence of 10 μg of Iodogen (Pierce, U.S.A.). The radioiodinated vitronectin was freed from unreacted iodine by desalting on a Bio Gel P6 column (Bio Rad, U.S.A.) equilibrated with PBS containing 2% BSA.

Vitronectin binding assay. The binding of ^{125}I-vitronectin to CMH 5123 cells was estimated according to the procedure developed by Akiyama and Yamada for determining the binding of fibronectin to BHK cells [27]. Subconfluent cultures of control or EGTA+EDTA-treated CMH5123 cells, either supplemented or not supplemented with complex gangliosides, were detached by using 0.5 mM EGTA in PBS. Cells were washed twice and resuspended in DMEM-HEPES containing 2% BSA (binding medium). Aliquots of cell suspensions containing $1.25x10^4$ cells were incubated for 2 h at 4° C together with serial dilutions of ^{125}I-vitronectin (0.1 - 3.6 μg of protein /ml) in a final volume of 125 μl. An analogous set of ^{125}I-vitronectin dilutions containing a large excess (160 μg/ml) of unlabeled vitronectin was used to estimate the aspecific binding of radioiodinated vitronectin. At the end of the incubation period, cells were washed twice with 500 μl of binding medium, resuspended in 100 μl of binding medium and centrifuged through a layer of 500 μl of DMEM/HEPES containing 10% BSA at 16000 rpm for 1 min in a Haereus Biofuge equipped with a type 1400 fixed-angle rotor. The radioactivity bound to the cell pellet was quantitated in a Minaxi 5000 Series γ counter (Packard Instruments, U.S.A.). Binding data were analyzed by the Scatchard method by using a data handling software [28].

Reagents. The mixture of ox brain gangliosides was supplied by Fidia Research Laboratories (Abano Terme, Italy). Analysis of this mixture revealed the following composition: GM1, 21.3%; GD1a, 42.4%; GD1b, 16.1%; GT1, 20.2%. Fibronectin and vitronectin were isolated from human plasma according to Engvall and Ruoslahti [29] and to Yathogo et al. [30], respectively. The B subunit of the cholera toxin was supplied by Sigma (St. Louis, MO). GRGDSPC and GRGESP peptides were generously provided by Dr. Lloyd Culp, Department of Molecular Biology and Microbiology, Case Western Reserve University, Cleveland, Ohio.

RESULTS AND DISCUSSION

As shown in Table 1, approximately 80% of CMH5123 cells adhered to FCS-coated substrata. Cell adhesion to these substrata was completely abolished by GRGDSPC peptide, an effect indicating that adhesion of CMH5123 cells to FCS-coated substrata is mediated by

Table 1. Adhesion of CMH5123 cells to substrata coated with fetal calf serum or selected serum proteins and effect of integrin inhibitor GRGDSPC peptide [a]

	Adhesion medium[c]		
Substrate[b]	Control	GRGDSPC[d]	GRGESP[d]
Fetal calf serum	83.8 ± 12.3	3.6 ± 0.2	82.4 ± 11.3
Plasma vitronectin	82.8 ± 10.2	1.6 ± 0.3	88.3 ± 9.8
Plasma fibronectin	2.4 ± 0.7	N.D.[e]	N.D.
Bovine serum albumin	1.2 ± 0.2	N.D.	N.D.

[a] Cell adhesion was measured by percent of cells inoculated into each well of 96-well cluster dishes which remained adherent to substrata after 1h incubation in adhesion medium. Values are means ± S.D. (n=4).
[b] Substrate refers to a cell culture dish coated with plasma vitronectin, 20 μg/ml; plasma fibronectin, 40 μg/ml; or bovine serum albumin, 250 μg/ml in DMEM. Fetal calf serum was used at 5% in DMEM.
[c] Adhesion medium was DMEM containing 250 μg/ml of heat-inactivated bovine serum albumin.
[d] The integrin inhibitor GRGDSPC peptide or the non-inhibitory GRGESP peptide was added to adhesion medium at a concentration of 400 μg/ml.
[e] Not determined.

cellular integrins. The adhesion of CMH5123 cells to vitronectin was comparable to that sustained by FCS, and it was also prevented by GRGDSPC peptide. Adhesion to both FCS and vitronectin was not affected by the non-inhibitory GRGESP peptide. Neither fibronectin nor BSA, appeared to be suitable substrata for CMH5123 cells. These data, as a whole, indicate that adhesion of CMH5123 cells to FCS-coated substrata depends on vitronectin via an integrin-mediated mechanism.

As we reported in a previous paper [13], growth in the presence of EGTA+EDTA reduced the proportions of complex gangliosides in CMH5123 cells (Table 2). Ganglioside-depleted CMH5123 cells showed a reduced adhesiveness to FCS-coated substrata. Cell adhesiveness was partially restored in ganglioside-depleted CMH5123 cells after exposure to growth media supplemented with complex gangliosides (Table 3).

Figure 1 shows that a concentration of 100 μg/ml of GRGDSPC peptide reduced CMH5123 cell adhesion to FCS-coated substrata by 50%. A ten times lower concentration of GRGDSPC peptide sufficed to reduce the adhesiveness of ganglioside-depleted cells by the same proportion, indicating a higher sensitivity to integrin inhibitor. Supplementation with complex gangliosides restored the sensitivity of ganglioside-depleted cells to GRGDSPC peptide to the level found in control cells. Control CMH5123 cells also reduced their sensitivity to the integrin inhibitor after supplementation with complex gangliosides, perhaps because these cells, being typically poor in complex gangliosides [31], have an impaired integrin-dependent adhesion.

Table 2. Changes in ganglioside composition of CMH5123 cells grown in the presence of chelating agents [a]

	Growth medium[b]	
Ganglioside	Control	EGTA+EDTA
GM3	66.8 ± 3.85	90.4 ± 0.21
GM2	17.2 ± 2.65	4.8 ± 0.82
GM1	6.7 ± 0.85	2.0 ± 0.46
GD1a	9.0 ± 0.92	2.6 ± 0.74

[a] Values are percentages (means ± S.D. of 3 determinations) of the total ganglioside-associated radioactivity.
[b] Cells were grown for three successive passages in DMEM+10% FCS (control medium) or in DMEM+10% FCS containing EGTA+EDTA.

Our data show that gangliosides play a role in the adhesion of CMH5123 cells to FCS-coated substrata by influencing the interaction of cell surface integrins with vitronectin contained in FCS. The fact that the GRGDSPC peptide did not affect the adhesion of cells to a ganglioside-specific ligand, such as the cholera toxin B subunit (Figure 1), points to such a modulatory role for complex gangliosides, rather than to their direct involvement in the adhesion of CMH5123 cells to FCS-coated substrata.

Gangliosides might modulate the affinity of integrin receptors for adhesive proteins. In order to verify this possibility, we analyzed vitronectin binding to control or EGTA+EDTA-treated

Table 3. Change in the adhesiveness of ganglioside-depleted CMH5123 cells to FCS-coated substrata and effect of ganglioside supplementation [a]

Cells	% of adherent cells
Control	78.7 ± 6.0
Ganglioside-depleted [b]	23.3 ± 2.1
Ganglioside-depleted + gangliosides [c]	48.7 ± 3.5
Control + gangliosides [c]	85.3 ± 6.2

[a] Substrate refers to a tissue culture plastic dish coated with 5% FCS in DMEM. Cell adhesiveness was measured as described in Table 1. Values are means ± S.D. (n=4).
[b] Cells were depleted of gangliosides by growth in the presence of EGTA+EDTA for three successive passages.
[c] Cells were enriched with complex gangliosides by exposure for 12 h to media containing 50 μM gangliosides.

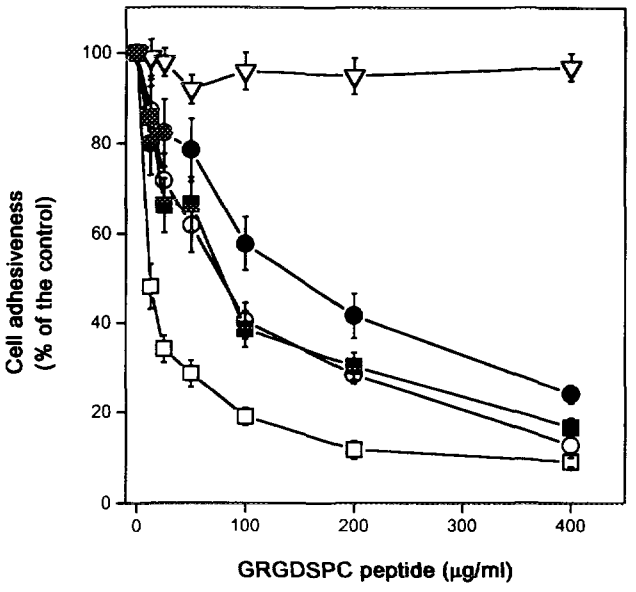

Figure 1. Increased sensitivity of ganglioside-depleted CMH5123 cells to the integrin inhibitor GRGDSPC peptide and effect of complex ganglioside supplementation. CMH5123 cells were depleted of gangliosides and enriched in complex gangliosides as reported in Table 3. Cells were inoculated into serum-coated 96 well cluster dishes and exposed for 1h to GRGDSPC peptide at the indicated concentrations in DMEM. Cell adhesiveness was assayed as described in Table 1 and reported as the percentage of CMH5123 cells which adhered to the substrate in the absence of peptide (control). Values are means ± S.E. (n=4). Cells grown in regular medium (O); cells grown in the presence of chelating agents (□); cells grown in the presence of chelating agents and supplemented with complex gangliosides (■); cells grown in regular medium and supplemented with complex gangliosides (●); cells grown in regular medium and inoculated into wells coated with cholera toxin B subunit (▽).

CMH5123 cells, either supplemented or not supplemented with complex gangliosides. Figure 2 shows the binding isotherms (A) of radioiodinated vitronectin to these four cell populations. When these data were analyzed by the Scatchard method (Figure 2, B), the experimental points could be fit to four straight lines, indicating that, independently of cell treatment, only a single class of vitronectin binding sites is present in CMH5123 cells. Since the binding of vitronectin to CMH5123 cells was inhibited up to 70 % by an excess (1mg/ml) of the peptide GRGDSPC (not shown), we can conclude that vitronectin binding sites represent the integrin receptors for vitronectin. Assuming an average molecular size for vitronectin of 70 kDa, the number of vitronectin receptors per cell and their apparent dissociation constants were calculated, respectively, from the *x*-intercepts and the slopes of the fitted lines (Table 4). Control CMH5123 cells contained about 200.000 vitronectin receptors per cell with an apparent K_d of 2.1×10^{-8} M. Thus, the affinity of vitronectin receptors of CMH5123 cells is two orders of magnitude higher than that measured for fibronectin receptors in BHK cells [27] and for vitronectin receptors in

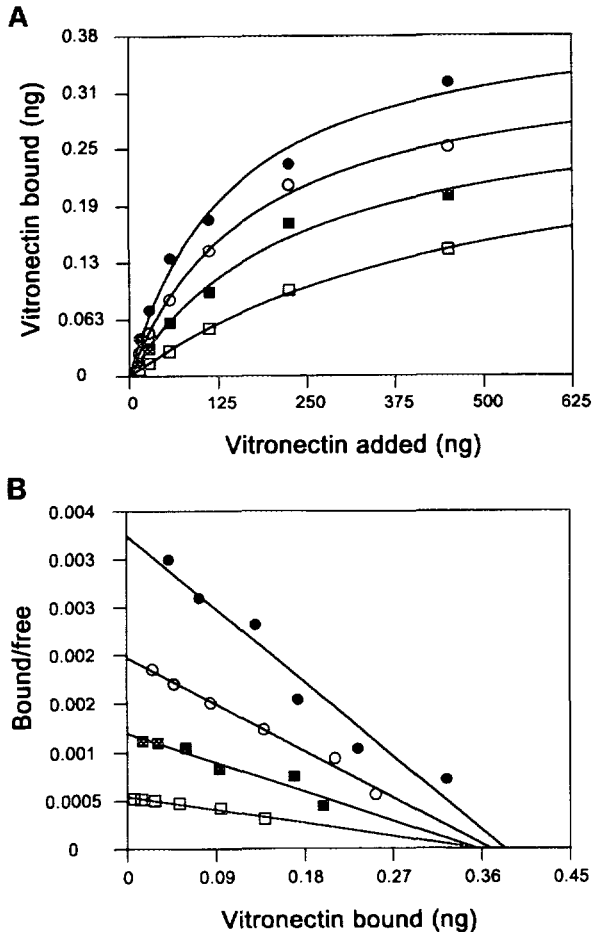

Figure 2. Changes in the characteristics of the binding of radiolabeled vitronectin in ganglioside-depleted CMH5123 cells and effect of complex ganglioside supplementation. CMH5123 cells were depleted of gangliosides and enriched in complex gangliosides as reported in Table 3. EGTA-detached cells were incubated with varying amounts of ^{125}I-vitronectin for 1 h at 4° C, either in the presence or in the absence of an excess of unlabeled vitronectin in order to determine the specific binding of radiolabeled vitronectin. Bound radiolabeled vitronectin was separated from unbound as described in Materials and Methods and the amounts of specifically bound radioactivity were measured. Panel A shows the specific binding of radiolabeled vitronectin to control cells (○), ganglioside-depleted cells (□), ganglioside-depleted cells enriched with complex ganliosides (■) and control cells enriched with complex gangliosides (●). Points represent the mean of triplicates. Panel B shows the Scatchard plots of the data.

human endothelial cells [32] or activated human platelets [33]. The number of vitronectin binding sites was not modified by treatment of CMH5123 cells with chelating agents or by supplementation with gangliosides. On the other hand, the dissociation constant of these binding sites was increased in EGTA+EDTA-treated cells compared to control cells, indicating that ganglioside depletion reduces the affinity of the integrin receptor for vitronectin. Supplementation with complex gangliosides reduced the apparent dissociation constant of vitronectin receptors of EGTA+EDTA-treated cells to a value close to that found in control cells. A reduction in the

Table 4. Changes in the characteristics of vitronectin receptors in ganglioside-depleted CMH5123 cells and effect of ganglioside supplementation [a]

Cells	Number of receptors (sites X 10^3 / cells)	Dissociation constant (nmol / L)
Control	212	2.14
Ganglioside-depleted[b]	204	7.53
Ganglioside-depleted + gangliosides[c]	204	3.38
Control + gangliosides[c]	220	1.39

[a] Receptor numbers and dissociation constants were calculated, respectively, from the *x*-intercepts and the slopes of the lines shown in Figure 2, B.

[b,c] As in Table 3.

dissociation constant of vitronectin receptors was also found in control cells after supplementation with complex gangliosides.

In conclusion, our results provide support to the knowledge that gangliosides participate in cell-to-matrix interactions by regulating an integrin-mediated mechanism.

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