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Essential roles of integrin-mediated signaling for the enhancement of malignant properties of melanomas based on the expression of GD3

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ABSTRACT

We reported that ganglioside GD3 enhances cell proliferation and invasion of melanomas causing stronger tyrosine-phosphorylation of p130Cas and paxillin after stimulation with fetal calf serum. Besides signals via growth factor/receptor, adhesion signals via integrin might be also enhanced by GD3. Here, roles of integrin-mediated signaling in the cell proliferation and invasion, and in the activation of adaptor molecules were examined, showing that integrin was also important for the cell growth and invasion. p130Cas and paxillin underwent stronger tyrosine-phosphorylation in GD3+ cells than in GD3− cells during the adhesion in the absence of serum. On the other hand, no proteins underwent tyrosine phosphorylation in GD3+ and GD3− cells in a suspension state when stimulated with fetal calf serum. These results suggested that integrin-mediated signaling is essential in the effects of GD3 on the malignant properties of melanomas. Co-localization of GD3 and integrin at the focal adhesion supported these results.
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Although a number of studies on the roles of gangliosides in the regulation of cell proliferation have been performed [1], regulatory mechanisms are not well understood. In particular, ganglioside GD3 is widely expressed in melanomas [2,3], so that anti-GD3 monoclonal antibodies (mAbs) have been used in therapeutic trials in melanoma patients [4]. To investigate roles of GD3, we have generated GD3-over-expressing transfectant cells (GD3+) from a GD3− mutant of SK-MEL-28 named N1 [5] by using GD3 synthase cDNA [6], and we have analyzed the phenotypic changes in GD3+ transfectant cells. Consequently, it was demonstrated that some adaptor molecules, i.e. p130Cas and paxillin, undergo stronger tyrosine phosphorylation after treatment with fetal calf serum (FCS) in GD3+ cells, and they are actually involved in the increased cell proliferation and invasion with GD3 expression [7]. In addition to the signals via growth factors/receptors, there should be another signals derived from interaction between integrins and extracellular matrix (ECM). Molecules reported to be involved in the signaling via growth factor/receptor were supposed to be also involved in integrin-mediated signaling [8]. In fact, it was reported that integrins play key roles in regulating tumor growth and metastasis as well as angiogenesis [9] through signals transduced upon integrin ligation to ECM, and integrin-mediated signals consisting of FAK, ILK and ERK are activated in melanomas [10].

In this study, we tried to clarify whether GD3 is implicated in integrin-mediated cell adhesion and consequent signaling involved in the malignant properties of melanomas. Consequently, it was demonstrated that integrins are involved in cell growth and invasion, and similar activation of p130Cas and paxillin during cell adhesion to those detected in FCS-treated cells was observed. Surprisingly, no proteins underwent tyrosine phosphorylation in GD3+ and GD3− cells in a suspension state when stimulated with FCS, suggesting that integrin-mediated signaling is essential in the effects of GD3 on the malignant properties of melanomas.

Materials and methods

Cell cultures. G5 and G11 were GD3+ transfectant cells generated from a GD3− mutant of SK-MEL-28 N1 [5] by using GD3 synthase cDNA [6]. V4 and V9 were GD3− vector control cells. These cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 7.5% FCS at 37 °C in a humidified atmosphere containing 5% CO_2.

Antibodies. Anti-rabbit IgG conjugated with horseradish peroxidase (HRP) was purchased from Cell Signaling Technology (Beverly, MA). Anti-mouse IgG conjugated with HRP was from Amersham Pharmacia. Anti-phosphotyrosine mAb (PY20), rabbit anti-p130Cas, mouse anti-integrin β1 and PE-streptavidin were from

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BD transduction (San Jose, CA). Monoclonal anti-human CD29 (β1 integrin)-biotin antibody was from Ancell (Bayport, MN). Protein A-FITC is from INC immunoBiologicals (Lisle, IL). Anti-integrin isoform mAbs were from DAKO corp. (Carpinteria, CA)(α2, α4, α5) and Santa Cruz Biotech. (Santa Cruz, CA) (α3).

Reagents. Fibronectin (FN), collagen (CL) type I (CL-I) and CL type IV (CL-IV) from human placenta were purchased from Chemicon Int. (Temecula, CA). Laminin (LN) from human placenta and poly-L-lysine (PLL) were from Sigma. siRNA for integrin β1 is from Invitrogen. The RNA sequence of ITGI was 5′-AUAAUGUUCCU ACUGCUGACUUAGG-3′ (sense) and 5′-CCUAAGUCAGCAGUAGGAA CAUUAU-3′ (anti-sense).

Flow cytometry. Cell surface expression of integrin β1 was analyzed by flow cytometry (Becton Dickinson) as described [11].

Adhesion activity of cells to ECM-coated plates. Cultured cells were detached by treatment with 0.05% trypsin/EDTA in PBS and resuspended at 3×10^5/ml in 0.02% BSA in DMEM. A 100 μl each of the cell suspension was added to 96-well plates coated with ECM (BioCoat™, Becton Dickinson). At the time points indicated, PBS was added to the wells and washed 3 times with PBS, then 0.02% BSA in DMEM was added. After washing with PBS, 50 μl of 0.1% crystal violet in 20% MeOH/water was added. Then, 1% Triton X-100 in water was added and incubated for 2 h at room temperature. Absorption at 590 nm was determined to obtain relative cell numbers.

In vitro invasion assay. Invasion assay was performed with a Boyden chamber as described [11]. Matrigel (Becton Dickinson) was diluted with ice-cold PBS (100 μg/ml), and 0.6 ml was added to each Falcon 3093 filter™ and left to be polymerized overnight. The membrane was reconstituted with serum-free medium. The lower chamber (6-well plate, Falcon 3502™) was filled with culture medium without serum. About 7×10^4 cells were added to serum-free medium in the upper chamber.

BrdU assay. Cells grown on a microatch plate (terasaki palate, Greiner bio-one, Frickenhausen, Germany) were incubated in the presence of BrdU for 14 h according to the instruction for a cell proliferation kit (Amersham Pharmacia Biosciences). Then, cells were fixed with acid–ethanol for 30 min. The cells were immunostained with anti-BrdU antibody and Alexa Fluor 546-conjugated secondary antibody (Molecular Probes, Invitrogen). The BrdU-positive cells were counted with a fluorescence microscopy (BX51, Olympus, Tokyo).

Inhibition of integrin β1 expression by small interfering RNAs (siRNAs). Human melanoma cells were plated at 70–80% confluency in 6- or 10-cm cell culture dishes (CELLSTAR, Greiner bio-one) and were cultured overnight. They were transfected with anti-integrin β1 siRNAs (100 nM) or control (fluorescein-labeled luciferase GL2 duplex, Dharmacon Research, Lafayette, CO) in Optimem I medium (Invitrogen) with Lipofectamine 2000™ reagent (Invitrogen) following the manufacturer's instruction. Four hours later, the Optimem I medium was replaced by regular culture medium. Knockdown of integrin β1 was assessed at 72 h after the transfection with immunoblotting. For invasion assay, cells were collected and replated at 2 days after the transfection.

Coating of plates with ECM proteins. Petri dishes (Greiner Bio-one) or glass base dishes (IWAKI, Funabashi, Japan) were coated with ECM in PBS (5 μg/mL) overnight at 4 °C. Then, plates were blocked with serum-free MEM containing 1% heat-inactivated BSA (MEM-BSA) (10 min at 60 °C). Plates coated with 0.01% poly-L-lysine (PLL, SIGMA) were also blocked with MEM-BSA.

Integrin-mediated adhesion to ECM. Cells were starved for 12–16 h in serum-free DMEM, and harvested with trypsin/EDTA in PBS or 0.02% EDTA in PBS. Trypsin activity was inhibited by adding soybean trypsin inhibitor (Invitrogen) at 100 μg/mL in PBS. To reduce basal phosphorylation of signaling molecules, cells were rotated for 1 h at 37 °C. Cell suspensions (4×10^5) were added on a 6-cm dish precoated with CL-I (CHEMICON) or CL-IV.

Preparation of cell lysate. Cells were lysed with cell lysis buffer (20 mM Tris–HCl, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mg/ml leupeptin) (Cell Signaling, Danvers, MA) added with Protease Inhibitor Cocktail™ (Calbiochem, San Diego, CA) and 1 mM PMSF. Insoluble materials were removed by centrifugation at 4 °C at 10,000g for 10 min.

Western immunoblotting. Cell lysates were separated by SDS–PAGE using 10–12% gels. The separated proteins were transferred onto an Immunobilon-P™ membrane (Millipore Corp. Bedford, MA). Blots were blocked with BSA in PBS containing 0.05% Tween 20. The membrane was first probed with primary antibodies reactive with p130Cas or paxillin. After being washed, the blots were incubated with goat anti-mouse IgGs conjugated with HRP (1:1000). Bound conjugates on the membrane were visualized with an enhanced chemiluminescence detection system (PerkinElmer, Boston, MA).

Immunocytostaining. Cells were fixed in paraformaldehyde (4% in PBS for 10 min) and then incubated with 0.1% Triton X-100 in PBS for 10 min. After being washed with PBS, nonspecific immunoreactivity was blocked with 2.5% BSA in PBS for 30 min at room temperature. Cells were incubated with anti-human CD29 (β1 integrin)-biotin or R24mAb in PBS containing 0.5% BSA for 60 min, then with PE-streptavidin or Protein A-FITC in PBS containing 0.5% BSA for 30 min. The resulting staining patterns were imaged using a confocal microscopy (OLYMPUS, FLUOVIEW FV500, Olympus, Tokyo).

Results

Adhesion activity of cells to ECMs

To examine whether GD3 is involved in the cell adhesion, two each of GD3+ and GD3− cell lines were used in the adhesion assay. Fig. 1 showed representative results of the adhesion assay. GD3+ cells showed stronger adhesion to CL-I and CL-IV than GD3− cells (Fig. 1A and B). To LN, GD3 also showed stronger adhesion than GD3− cells, although binding intensity was largely low (Fig. 1B). Intensity of adhesion to FN was almost equivalent between GD3+ and GD3− cells (Fig. 1D). These results were repeatable in any combination between GD3+ and GD3− lines. Therefore, it was concluded that GD3 expression enhanced adhesion activity to some ECMs such as CL-I and CL-IV.

Expression of integrins on GD3+ cells and control cells

Expression levels of integrins were analyzed by flow cytometry (Fig. 2A). Expression levels of integrin α2β1 and those of integrin α3β1 were higher than those of integrin α4β1 and integrin α5β1 in any cell lines. There were no differences in the expression levels of individual integrins among cell lines examined (data not shown). Integrin β1 levels in cell lysates prepared from GD3+ cells and control cells were analyzed by immunoblotting with an anti-integrin β1, showing almost equivalent intensity of bands among all cell lines examined (data not shown).

Effects of knockdown of integrin β1 on cell growth and invasion

To investigate whether GD3 is really implicated in integrin-mediated cell adhesion, cell growth and invasion were analyzed after knockdown of integrin β1 with small interfering RNAs (siRNAs). We first tested the suppression of integrin β1 protein with siRNAs in SK-MEL-28-N1 by immunoblotting (Fig. 2B). Transfec-

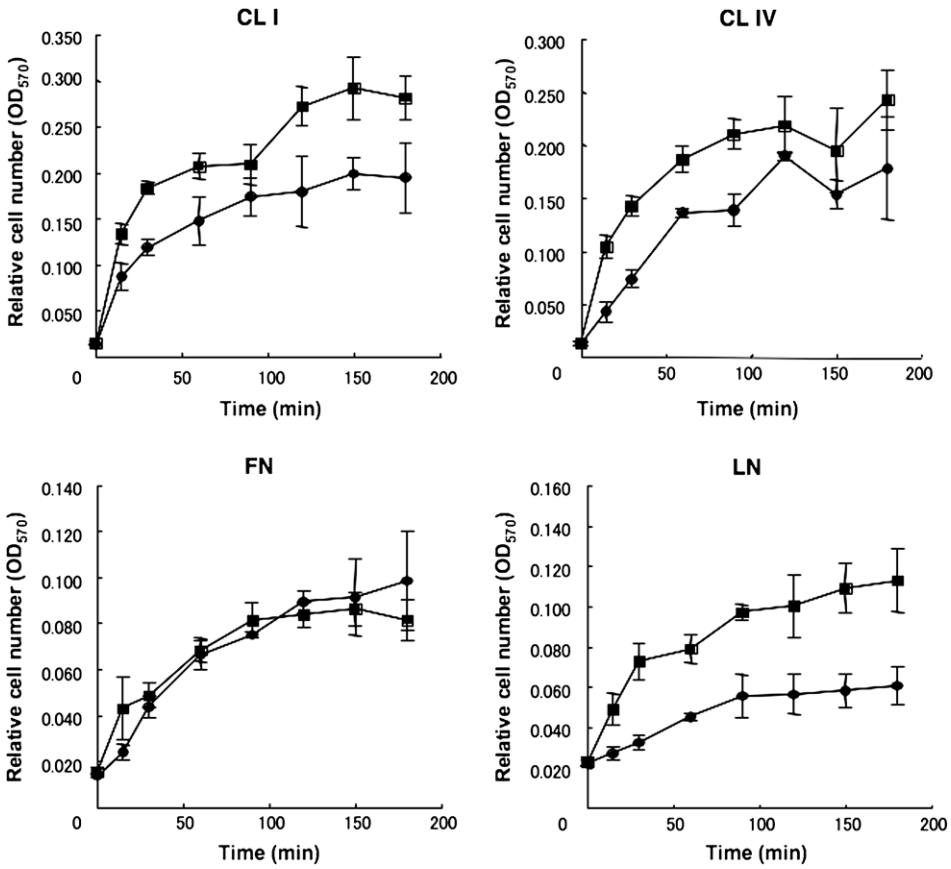

Fig. 1. Binding of GD3+ and GD3− cell lines to various ECMs. Ninety six-well plates coated with CL-I, Cl-IV, LN or FN were used for cell binding assay. Cells were prepared as described and applied to the plates. Attached cells at individual time points were determined by absorption at 590 nm and are plotted. Triplicated samples were analyzed. Closed circle, GD3− cells; closed square, GD3+ cells.

tion efficiency of siRNAs as analyzed with fluorescein-labeled luciferase was >80–85% constantly. Among effective siRNAs, the most effective one (ITG1) was selected for the following experiments. The transfection with the control siRNA had no effect on integrin β1 levels. Flow cytometry revealed reduced expression of integrin β1 on the cell surface transfected with ITG1 (data not shown).

To analyze the effects of knockdown of integrin β1 on BrdU uptake, siRNA ITG1 was transfected into the GD3+ cells and the vector controls, and then the ratio of BrdU-positive cells was calculated. Although GD3+ cells showed a significantly higher ratio of BrdU uptake than the controls, cell growth of GD3+ cells was suppressed more clearly by ITG1 than that of the controls (Fig. 2C). Consequently, it is suggested that integrin β1 plays a key role in the increased cell proliferation under GD3 expression. Then, invasion assay was performed with the Boyden chamber method. Both GD3+ and GD3− cell lines showed marked suppression of invasion activity after knockdown, while basal levels of invasion were markedly different between GD3+ and GD3− lines (Fig. 2D). These results suggested that the enhanced invasion activity with GD3 expression might be dependent on integrin β1.

Increased phosphorylation of p130Cas and paxillin in GD3+ cells with adhesion to CL-I

To examine whether GD3 expression enhances cell growth signals upon adhesion to ECM, cells were plated on dishes precoated with CL-I under serum-free condition, and incubated at 37 °C. After incubation, cell lysates were prepared for immunoblotting. We analyzed phosphorylation of p130Cas and paxillin during adhesion to CL-I (Fig. 3A). Phosphorylation levels of p130Cas and paxillin as detected by PY20 were increased in GD3+ cells than in GD3− cells after adhering to CL-I. The strongest phosphorylation of p130Cas was observed in GD3+ cells at 10 min of incubation and that of paxillin was observed at 30 min of incubation. When the same study was performed for CL-IV, phosphorylation levels of these molecules were much lower and no distinct differences were observed between GD3+ and GD3− cells (date not shown). These results suggested that integrin-mediated signals were enhanced with GD3 expression, leading to the activation of adaptor molecules as observed after FCS treatment in the adherent melanoma cells.

p130Cas and paxillin were not phosphorylated after stimulation with FCS in non-adherent melanoma cells

We previously reported that p130Cas and paxillin were strongly phosphorylated in GD3+ transfectant cells after stimulation with FCS. Here, it was examined which is main pathway of phosphorylation of p130Cas and paxillin between the growth factor receptor- and integrin-mediated signaling. We examined the tyrosine phosphorylation of p130Cas and paxillin after stimulation with FCS in non-adherent cells using PY20 (Fig. 3B). Consequently, p130Cas and paxillin did not undergo phosphorylation at all both in GD3+ cells and GD3− cells. This result implied that FCS stimulation via the growth factor receptor could be effective only when cells were receiving adhesion signals via integrin-ECM interaction.

Integrin β1 clustered with GD3 at focal adhesion during cell adhesion

We analyzed intracellular localization of integrin β1 and GD3 by immunocytostaining. Cells were fixed with 4% paraformaldehyde,

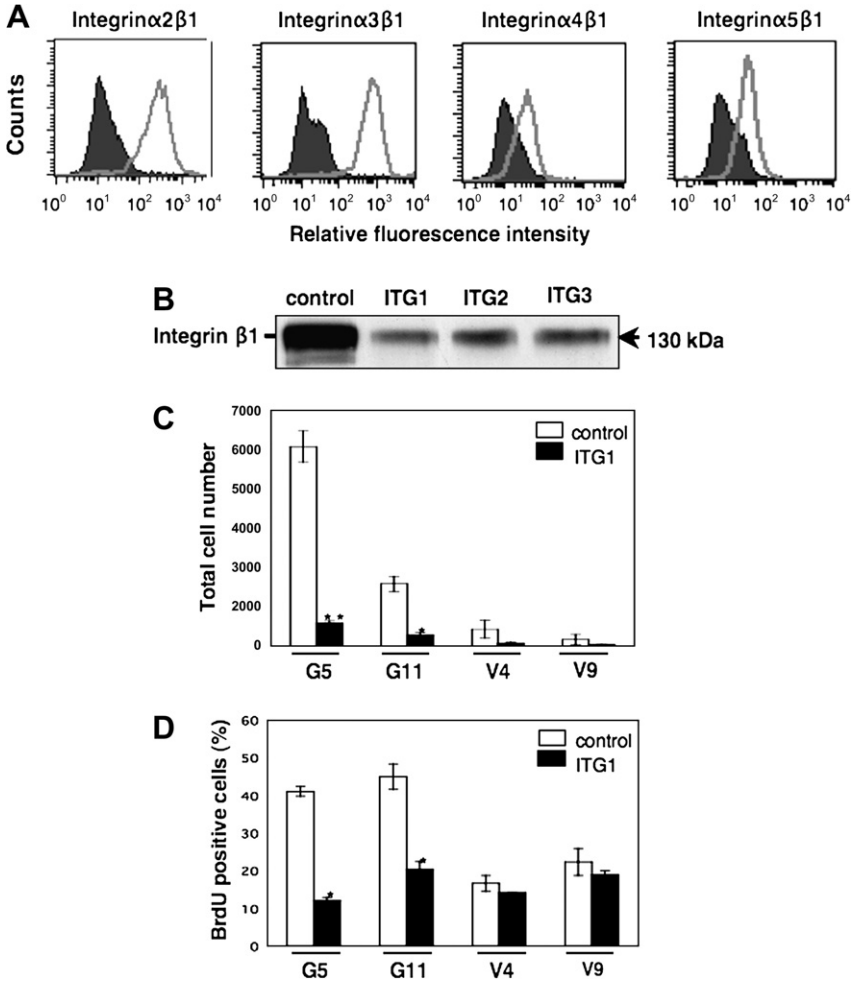

Fig. 2. Integrins were important in the cell growth and invasion. (A) Expression of integrins was examined by flow cytometry. Controls were prepared with the second antibody alone. Results about integrin α2 β1, α3β1, α4β1, and α5β1 are shown. (B) Knockdown of integrins with siRNAs. Three siRNAs (ITG1, ITG2, ITG3) were examined their activity to suppress integrinβ1 levels in immunoblotting. Anti-integrin β1 antibody (CD29) was used. (C) Effects of integrin knockdown with ITG1 siRNA on the cell growth as examined by BrdU uptake. (D) Effects of knockdown on the cell invasion was investigated after 2 days of transfection. Cell numbers in the lower surface of the filter were counted and presented. $^{*}P < 0.05$: $^{**}P < 0.01$.

then were stained with mAbR24 and biotin-anti-integrin β1. GD3 and integrins co-localized as punctate pattern at focal adhesion after 15 min of incubation, when cells just adhered to ECM (Fig. 4). On the other hand, integrin β1 and GD3 were uniformly merged on cell membrane at time 0. These staining patterns suggested that integrin β1 formed a molecular cluster with GD3 upon the cell adhesion, so that adhesion intensity might be enhanced in the presence of GD3.

Discussion

GD3 is highly expressed in melanomas, and it might be associated with malignant properties such as cell growth [5], invasion and cell adhesion to ECM [12]. In the past study, we identified molecular mechanisms for the enhanced malignant properties related with GD3 expression, i.e. stronger phosphorylation of p130Cas and paxillin in GD3+ cells after FCS treatment [7]. Then, it was questioned whether these molecules were also involved in integrin-mediated signaling as in the signaling via growth factor/receptors, and whether GD3 is implicated in integrin-mediated cell adhesion.

Integrins are cell surface receptors consisting of two subunit α and β. They interact with ECMs and mediate various intracellular signaling [13]. It is reported that integrin β1 is important in melanoma cell migration and migration-associated matrix reorganization [14]. In GD3+ cells treated with siRNA for integirn β1, cell proliferation and invasion were markedly suppressed than in control cells (Fig. 2). These results suggested that GD3 might be involved in the increased proliferation and invasion via enhancement of integrin signaling. On the other hand, roles of glycosphingolipids in the regulation of cell adhesion have been recognized for long time [15], but the mechanisms for this regulation remains unclear. Several studies on the modulation of integrins by gangliosides have been reported [16–18].

Our results suggested that strong cell adhesion was induced by GD3 expression. In addition, there are reports that integrin β1-α1, -α2, -α3 and αv interacts with CL-I and CL-IV which exist in basement membrane [19]. In fact, we detected stronger tyrosine phosphorylation of p130Cas and paxillin in GD3+ cells adhering to CL-I (Fig. 3).

p130Cas is now considered to be a significant adaptor molecule in a variety of biological processes, including cell adhesion [20], migration [21], growth factor stimulation [22], and cytokine receptor engagement [23]. Paxillin is a multidomain protein that primarily localizes to cell adhesion forming a linkage structure between the ECM and the actin cytokeleton, and are also important sites for signal transduction [24]. Strong phosphorylation of these molecules during the adhesion of GD3+ cells to CL-I suggests that

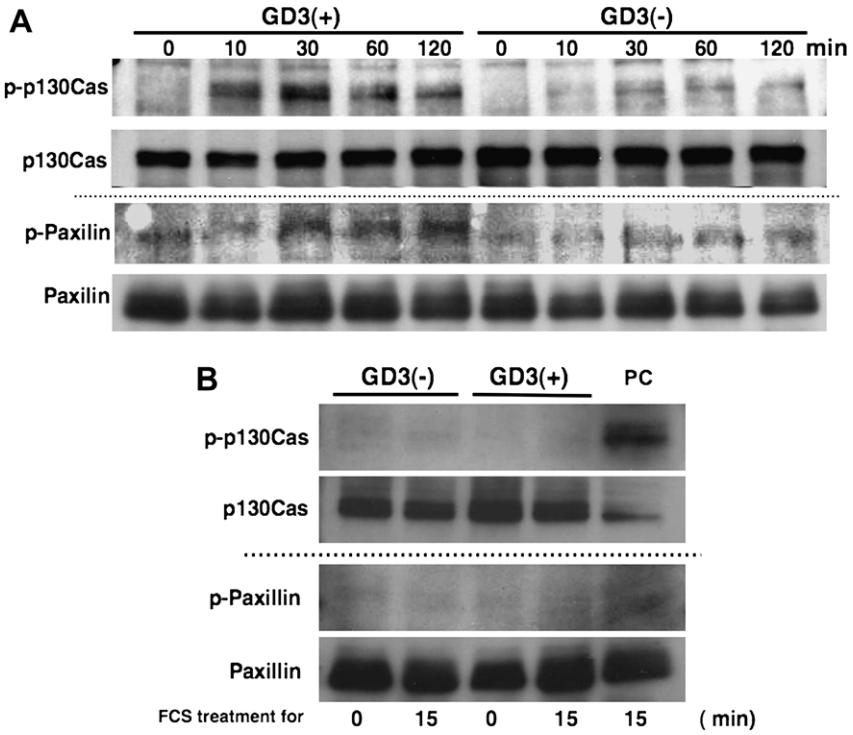

Fig. 3. Tyrosine phosphorylation of p130Cas and paxillin. (A) Adhesion signals were examined by immunoblotting with PY20. GD3+ and GD3− cells were prepared as described and were applied to collagen type I-coated dishes. Cell lysates from cells under adhesion at the individual time points were used for immunoblotting. Total p130Cas and paxillin levels were also examined using rabbit anti-p130Cas or mouse anti-paxilin antibodies. (B) Tyrosine-phosphorylation of proteins in suspension cells with FCS treatment was examined. Cells detached with 0.02% EDTA in PBS were rotated for 37 °C for 1 h, then FCS was added at a concentration of 7.5%. Cells were lysed after 15 min incubation. Immunoblotting was performed with PY20 as in A. PC is a control, a lysate from cells treated with FCS as attached state.

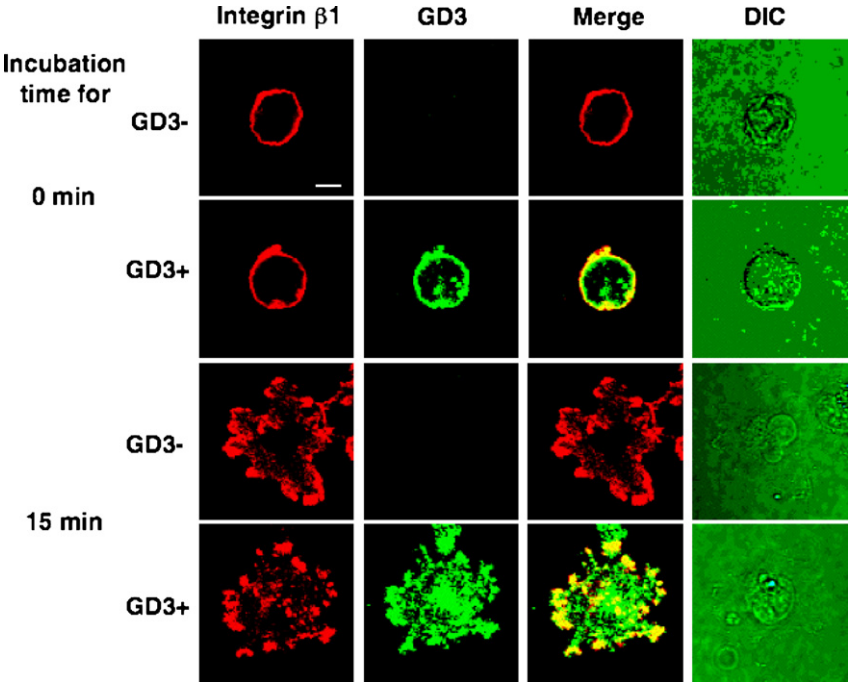

Fig. 4. Co-localization of GD3 and integrins. GD3+ and GD3− cells were added in plates pre-coated with CL-I after rotation for 1 hr at 37 °C. At time 0 or 15 min of incubation, cells were fixed with 4% paraformaldehyde and treated with 0.1% Triton X-100 in PBS. Simultaneous staining was performed using anti-GD3 mAbR24 and anti-integrin β1 antibody combined with FITC-Protein A and PE-streptavidin, respectively. Staining patterns with single reagents, merged images, and DIC are presented. Scale bars indicate 10 μm.

GD3 expression in melanoma cells enhances malignant properties via adhesion signaling. We also performed the same study about CL-IV. No distinct differences were observed between GD3+ and GD3− cells. Phosphorylation level of p130Cas and paxillin were

much lower than that with CL-I. This result suggested that integrin-mediated signaling is selectively enhanced depending on the kind of ECM in the presence of GD3.

Our results strongly suggested that adhesion signals via the interaction of integrins and ECMs were essential in the activation of adaptor molecules even when cells were stimulated with FCS, and CL-I was particularly important for the signaling under GD3 expression.

In the Immunocytostaining, GD3 and integrins co-localized at the focal adhesion during cell adhesion with punctate pattern, while they co-localized uniformly on cell membrane before cell adhesion. Gangliosides are known to exist in clusters and form microdomains with cholesterol and sphingomyelin on the cell surface [25]. These microdomains are referred as lipid rafts, and have been considered to be involved in the signal transduction. In conclusion, GD3 may assemble integrins in the lipid raft and cause the formation of cluster, so that adhesion activity and integrin-mediated signaling are enhanced. Hopefully, these results would lead to define key molecules to regulate the critical nature of melanomas and to develop novel therapeutic application for melanoma patients.

Acknowledgments

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