Ganglioside GD3 induces convergence and synergism of adhesion and hepatocyte growth factor/Met signals in melanomas

Keiko Furukawa,[1,2] Mariko Kambe,[1] Maiko Miyata,[1,2] Yuki Ohkawa,[2] Orie Tajima[1,2] and Koichi Furukawa[2]

[1]Department of Biomedical Sciences, Chubu University College of Life and Health Sciences, Kasugai; [2]Department of Biochemistry II, Nagoya University Graduate School of Medicine, Nagoya, Japan

Ganglioside GD3 is highly expressed in human melanomas and enhances malignant properties of melanomas, such as cell proliferation and invasion activity. In this study, we analyzed the effects of GD3 expression on cell signals triggered by hepatocyte growth factor (HGF)/Met interaction and by adhesion to collagen type I (CL-I). Although stimulation of melanoma N1 cells (GD3+ and GD3−) with either HGF or adhesion to CL-I did not show marked differences in the phosphorylation levels of Akt at Ser473 and Thr308 between two types of cells, simultaneous treatment resulted in definite and markedly increased activation of Akt in GD3+ cells. Similar increases were also shown in Erk1/2 phosphorylation levels with the costimulation in GD3+ cells. When resistance to induced apoptosis by H_2O_2 was examined, only GD3+ cells treated with both HGF and adhesion to CL-I showed clearly low percentages of dead cells compared with GD3− cells or GD3+ cells treated with either one of the stimulants. Cell growth measured by 5-ethynyl-2′ deoxyuridine uptake also showed synergistic effects in GD3+ cells. These results suggested that GD3 plays a crucial role in the convergence of multiple signals, leading to the synergistic effects of those signals on malignant properties of melanomas.

Although cancer cells generally obtain the ability to autonomously proliferate based on genetic changes in the key molecules involved in the regulation of cell growth, they are also supported by extrinsic signaling derived from growth factors and cell adhesion.[1] It has been reported that multiple signals from environmental factors such as tumor niche is essential for the survival and proliferation for melanoma cells.[2,3] Their importance increases particularly in cancer progression[4] and cancer metastasis.[5] Recently, a number of studies have been reported to indicate that multiple signaling pathways are present in melanoma cells, leading to the activation of common essential molecular systems involved in cell growth, such as Erks and Akt. Thus, blocking one of those pathways with molecular targeting drugs failed to suppress melanoma cell growth,[6] indicating the prominent roles of particular signals, such as HGF/Met-mediated signaling.[7–9]

Gangliosides are expressed in neuroectoderm-derived human cancers such as malignant melanomas and neuroblastomas,[10] and have been used as cancer-associated markers and/or targets of antibody therapies.[11,12] Recently, there have been increasing reports implicating gangliosides in the malignant properties of various cancer cells.[13–15] Since we isolated ganglioside synthase cDNAs such as GM2/GD2 synthase,[16] GD3 synthase,[17] GM1 synthase[18] and GM3 synthase,[19] the roles of gangliosides in cancer cells have been analyzed based on the genetic manipulation of those genes.[20]

In particular, disialyl gangliosides have been recognized as functional cell surface molecules in the enhancement of cell proliferation, migration, and metastasis.[13] Ganglioside GD3 is expressed in the majority of human melanoma tissues and cell lines,[21] enhanced cell proliferation and invasion,[15] and cell adhesion to ECMs.[22] Consequently, GD3 appears to converge two kinds of cell signals, that is, integrin-mediated adhesion signals and growth factor/receptor-mediated signals. Either one of them could not transduce sufficient signaling without the other.[22,23] In these studies, we have used serum stimulation as a representative growth signal with no definite identification of particular growth factors.

In this study, we used the hepatocyte growth factor (HGF)/Met system as a representative growth factor/receptor system. However, HGF/Met-mediated signaling was not simply enhanced by GD3 expression. Only when HGF was used for stimulation together with adhesion to ECM, definitely increased signaling as well as correspondingly increased tumor phenotypes could be observed in only GD3-expressing cells, indicating that GD3 plays a key role in the synergism of the two major signals.

Materials and Methods

Cell lines. GD3-expressing N1 cells (GD3+ cells) were established from a GD3-deficient mutant of human melanoma cell line SK-MEL-28, N1 (N1)[15] by transfection of human GD3

synthase cDNA.[22] N1 cells transfected with a vector alone were established as controls (GD3−) cells. These cells were maintained in DMEM supplemented with 7.5% FCS at 37°C in a humidified atmosphere containing 5% CO2.

Antibodies. Anti-Met, anti-phospho-Met (Tyr1234/1235), anti-phospho-Met (Tyr1349), anti-Akt, anti-phospho-Akt (Ser473), anti-phospho-Akt (Thr308), anti-Erk, anti-phospho-Erk, and anti-β-actin antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-GD3 mAb R24 was provided by K.O. Lloyd (Memorial Sloan-Kettering Cancer Center, New York, NY, USA).

Reagents. Hepatocyte growth factor was purchased from Sigma-Aldrich (St. Louis, MO, USA). Collagen type I (CL-I)-coated culture dishes and 96-well plates were purchased from BD Biosciences (Bedford, MD, USA).

Integrin-mediated adhesion to CL-I. Cells were starved for 14–16 h in serum-free DMEM, and harvested with 0.5 mM EDTA in PBS. Then, cells were rotated for 1 h at 37°C to reduce basal phosphorylation levels of signaling molecules and cell suspensions were added to 6-cm or 3.5-cm dishes or 96-well plates precoated with CL-I. Cells were lysed after incubation at 37°C, and lysates were used for Western immunoblotting.

Preparation of cell lysates. Cells were lysed with cell lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na2 EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM leupeptin) (Cell Signaling Technology) added with 1 mM PMSF. Insoluble materials were removed by centrifugation at 10 000 *g* for 10 min at 4°C. The amounts of protein of cell lysates were measured using Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA).

Western immunoblotting. Same amounts of proteins in cell lysates were separated by SDS-PAGE using 10–15% gels, and the separated proteins were transferred onto an Immunobilon-P membrane (Millipore, Billerica, MA, USA). Blots were blocked with 3% BSA in PBS containing 0.05% Tween-20 or 5% non-fat dry milk in TBS containing 0.1% Tween-20. The membrane was first probed with primary antibodies. After being washed, the blots were incubated with goat anti-rabbit IgG or goat anti-mouse IgG conjugated with HRP (1:2000) (Cell Signaling Technology). After washing, bound conjugates on the membrane were visualized with an Enhanced Chemiluminescence detection system (PerkinElmer Life Sciences, Waltham, MA, USA), or ImmunoStar LD (Wako Pure Chemical Industries, Osaka, Japan). Chemiluminescence was detected by the luminescent image analyzer LAS-3000 (Fujifilm, Tokyo, Japan) and the intensity of the chemiluminescence was analyzed using a software, Multi Gauge version 3.0 (Fujifilm).
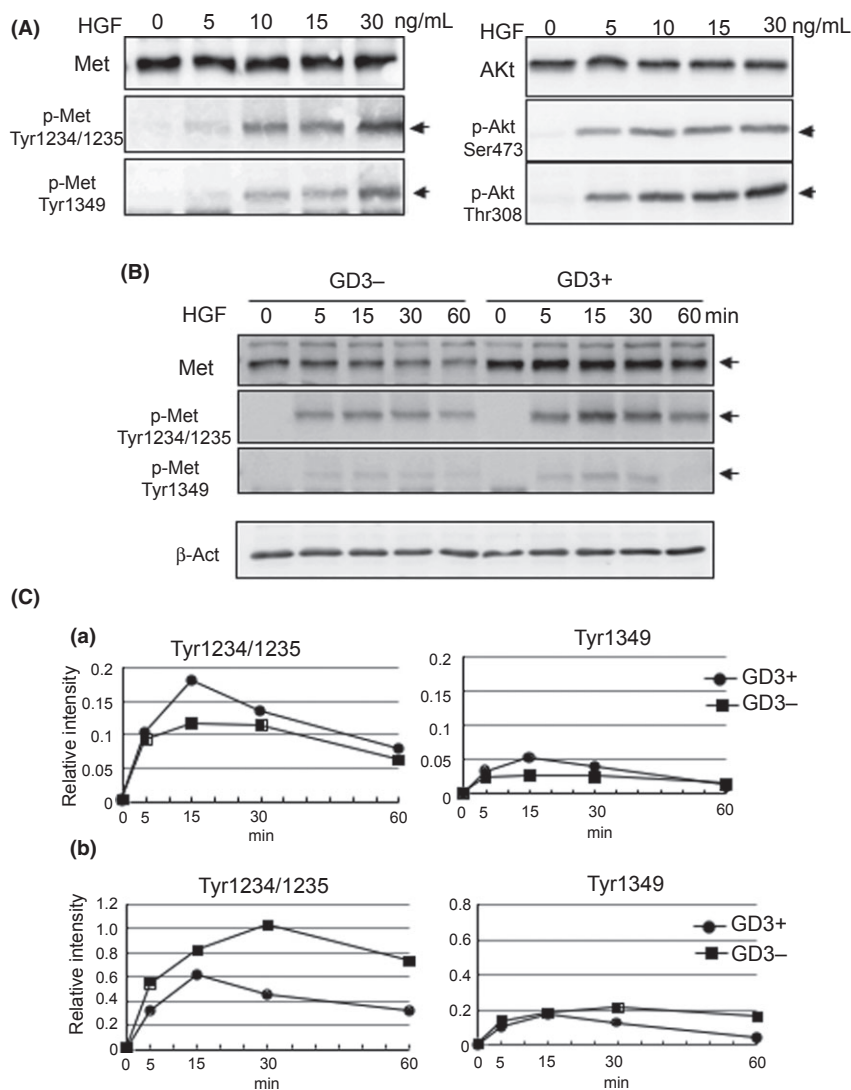

Fig. 1. Phosphorylation of Met and Akt in ganglioside GD3-expressing SK-MEL-28 N1 (GD3+) melanoma cells. (A) Phosphorylation of Met after treatment with hepatocyte growth factor (HGF) was analyzed (left). GD3+ cells were cultured in serum-free medium for 14–16 h and treated with 0, 5, 10, 15, or 30 ng/mL HGF for 15 min, then cells were lysed. The cell lysates were used for immunoblotting with an anti-Met antibody, or an anti-phospho-Met antibody. The detection of bands was carried out with ECL. Phosphorylation of Akt after treatment with HGF was analyzed (right). GD3+ cells were cultured in serum-free medium for 14–16 h and treated with 0, 5, 10, 15, or 30 ng/mL HGF for 15 min, then cells were lysed. The cell lysates were used for immunoblotting with an anti-Akt antibody, or anti-phospho-Akt antibodies. The detection of bands was carried out with ECL. (B) Tyrosine phosphorylation levels of Met after treatment with HGF in GD3+ cells and GD3− cells were analyzed. Met and phosphorylated Met (Tyr1234/1235 and Tyr1349) were analyzed at the time points indicated after HGF stimulation (30 ng/mL) in GD3+ and GD3− cells (1.5 × 10^6 cells/6-cm dish). After HGF stimulation, cells were lysed and the lysates were used for immunoblotting with an anti-Met antibody or an anti-phospho-Met antibody. The bands were detected with ECL. (C) Bands in (B) were scanned and the band intensities were measured and presented after correction with human β-actin bands (a) or with total Met bands (b).
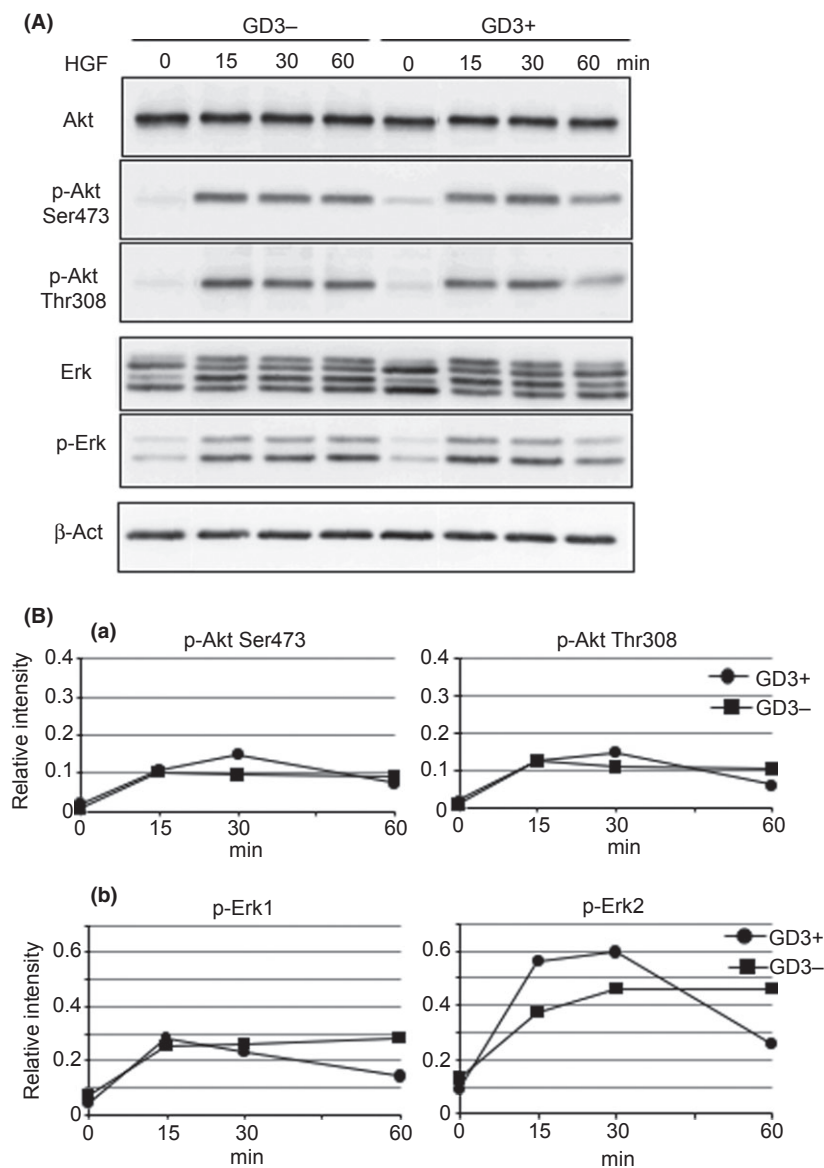
Fig. 2. Phosphorylation levels of Akt and Erks after treatment with hepatocyte growth factor (HGF) in ganglioside GD3+ N1 cells and GD3– N1 cells. (A) Akt and phosphorylated Akt (Ser473 and Thr308) were analyzed at the time points indicated after HGF stimulation (10 ng/mL) in GD3+ and GD3– cells (1.0×10^6 cells/6-cm dish). Erks and phosphorylated Erks were also analyzed after HGF stimulation. After HGF stimulation, cells were lysed and the lysates were used for immunoblotting with an anti-Met antibody or an anti-phospho-Met antibody. The bands were detected with ECL. (B) Bands in (A) were scanned and the band intensities of the phosphorylated Akt (a) and the phosphorylated Erks (b) were measured and presented after correction with those of total Akt or Erks, respectively. Human β-actin bands indicate equal loading of samples.

Knockdown of GD3 synthase. Knockdown of GD3 synthase was carried using SK-MEL-28 as described previously,[24] and stable silenced lines were obtained by the selection with DMEM containing puromycin (0.4 μg/mL) (Sigma) and 7.5% FCS.

Suppression of phosphorylation levels of signaling molecules by anti-GD3 mAb R24. Cells were starved for 14–16 h in serum-free DMEM, and harvested with 0.5 mM EDTA in PBS. Then, cells were rotated for 40 min at 37°C to reduce basal phosphorylation levels of signaling molecules and cell suspensions were collected. These cells (1.5×10^6 cells/150 μL DMEM) were incubated with or without purified mAb R24 (50 μg/mL) for 30 min at 4°C. After incubation with or without mAb R24, the cells were suspended and plated in CL-I-coated plates with 10 ng/mL HGF. Cells were lysed after incubation at 37°C, and lysates were used for Western immunoblotting.

Detection of apoptotic cells. Cells were serum-starved for 14–16 h, and harvested with 0.5 mM EDTA in PBS. To reduce basal phosphorylation levels of signaling molecules, cells were rotated for 1 h at 37°C. The cells were suspended in a tube with 10 ng/mL HGF and 5 μM CellEvent Caspase-3/7 Green Detection Reagent (Life Technologies, Carlsbad, CA, USA) or

the cell suspensions were plated in CL-I-coated 96-well plates, and were added with or without 10 ng/mL HGF and 5 μM CellEvent Caspase-3/7 Green Detection Reagent. After 30 min incubation at 37°C, the cells were treated with 0, 1000, or 1500 μM H_2O_2 for 2 h or 4.5 h. The apoptotic cells were fluoresced bright green, and were detected by the fluorescence microscope with NIBA filter.

Uptake of 5-ethynyl-2′ deoxyuridine (EdU). Measurement of cell ability to proliferate was carried out using Click-It EdU Imaging Kits (Life Technologies, Carlsbad, CA, USA). Cells were serum-starved for 14–16 h, and harvested with 0.5 mM EDTA in PBS. To reduce basal phosphorylation levels of signaling molecules, cells were rotated for 1 h at 37°C. The cell suspensions in tubes were added with 10 ng/mL HGF and 10 μM EdU, or the cell suspensions were plated in CL-I-coated plates, and were added with or without 10 ng/mL HGF and 10 μM EdU. Then cell proliferation was assayed with EdU uptake after incubation for 4 or 21 h according to the instructions for Click-iT EdU Imaging Kits. The EdU positive cells were observed using the fluorescence microscope with NIBA filter.
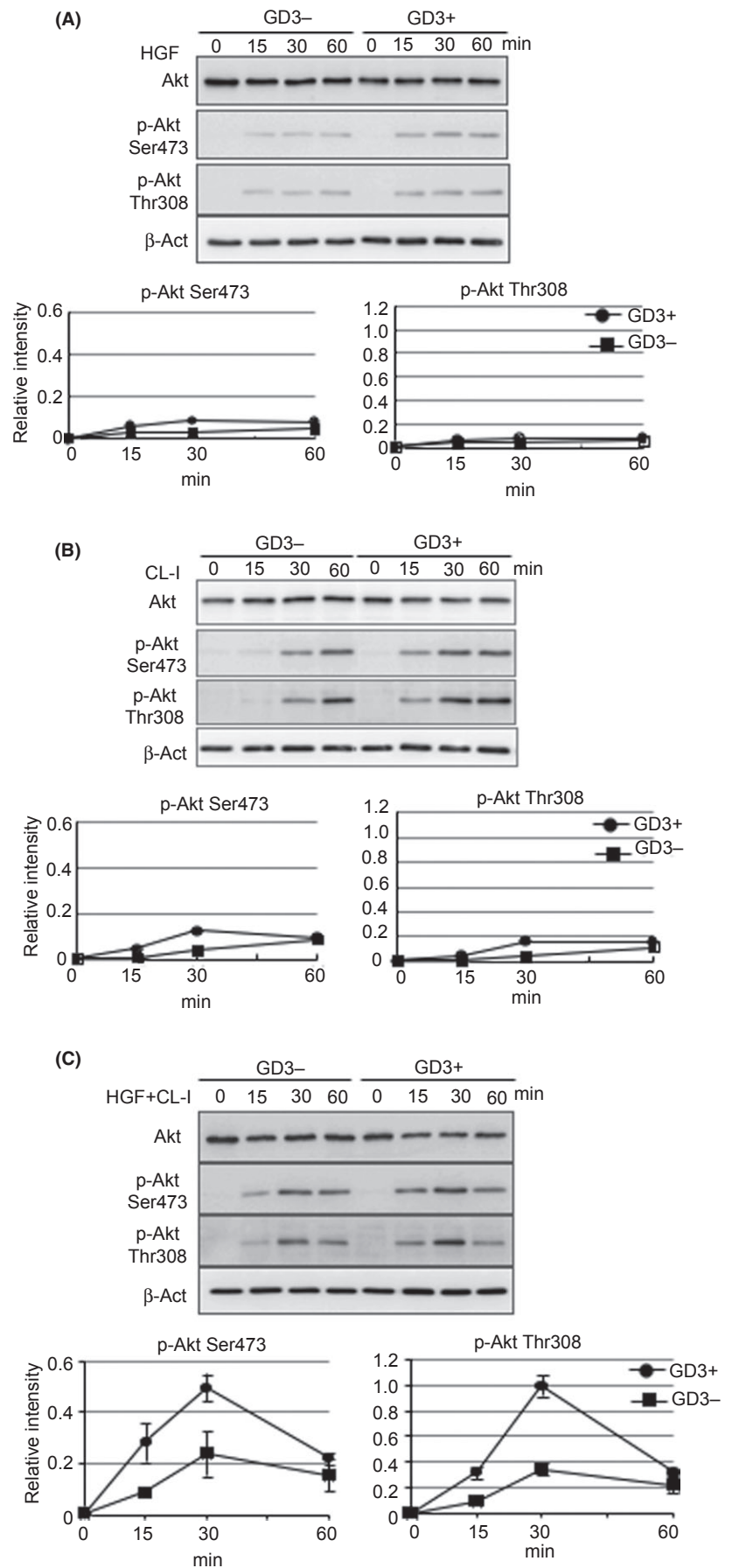
Fig. 3. Synergistic enhancement of Akt phosphorylation in ganglioside GD3+ N1 melanoma cells by costimulation with hepatocyte growth factor (HGF) and adhesion to collagen type I (CL-I). Phosphorylation levels of Akt after stimulation with HGF, adhesion stimulation with CL-I, or both HGF and CL-I were compared between GD3+ cells and GD3− cells. The cells were harvested and rotated for 1 h at 37°C after serum starvation for 14–16 h, then used for each experiment. (A) Akt and phosphorylated Akt (Ser473 and Thr308) were analyzed at the time points indicated after adding HGF (10 ng/mL) in GD3+ and GD3− cell suspensions (1.0×10^6 cells/5 mL). After HGF stimulation, cells were lysed and the lysates were used for immunoblotting with an anti-Akt or an anti-phospho-Akt antibodies. The bands were detected with ECL. The bands were scanned and the relative intensities of phosphorylated bands were presented after correction with intensities of total Akt. (B) Akt and phosphorylated Akt (Ser473 and Thr308) were analyzed at the time points indicated after CL-I adhesion stimulation in GD3+ and GD3− cells (0.4×10^6 cells/6-cm dish). The relative intensities of phosphorylated bands were measured as in (A). (C) Akt and phosphorylated Akt (Ser473 and Thr308) were analyzed at the time points indicated after simultaneous stimulation with HGF (10 ng/mL) and CL-I adhesion in GD3+ and GD3− cells (1.0×10^6 cells/6-cm dish). The relative intensities of phosphorylated bands are presented as in (A). β-actin bands indicate equal loading of samples.
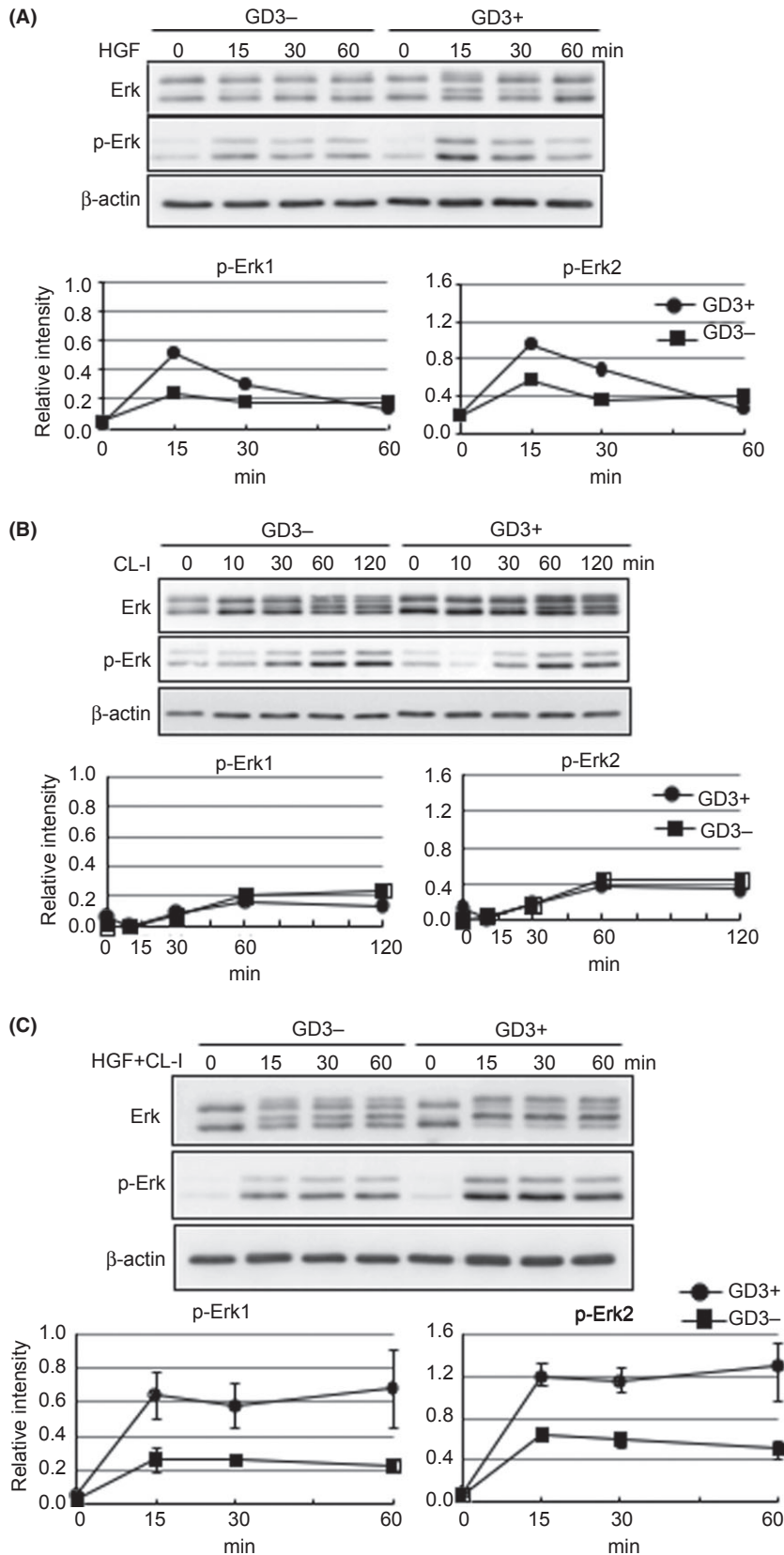
Fig. 4. Synergistic increase of phosphorylation levels of Erks by simultaneous stimulation with hepatocyte growth factor (HGF) and adhesion to collagen type I (CL-I) in melanoma cells. Phosphorylation levels of Erks after growth factor stimulation with HGF, adhesion stimulation with CL-I, or both in GD3+ cells and GD3− cells were examined. The cells were harvested and rotated for 1 h at 37°C after serum starvation for 14–16 h, then used for each experiment. (A) Total Erk1/2 and phosphorylated Erk1/2 were analyzed at the time points indicated after adding HGF (10 ng/mL) in GD3+ and GD3− cell suspensions (1.0×10^6 cells/ 5 mL). After HGF stimulation, cells were lysed and the lysates were used for immunoblotting with anti-Erks or anti-phospho-Erks antibodies. The bands were detected with ECL and scanned. The relative intensities of phosphorylated bands are presented after correction with intensities of total Erks. (B) Total Erk1/2 and phosphorylated Erk1/2 were analyzed at the time points indicated after CL-I adhesion stimulation in GD3+ and GD3− cells (0.4×10^6 cells/6-cm dish). The relative intensities of phosphorylated bands are presented as in (A). (C) Total Erk1/2 and phosphorylated Erk1/2 were analyzed at the time points indicated after simultaneous stimulation with HGF (10 ng/mL) and CL-I adhesion in GD3+ and GD3− cells (1.0×10^6 cells/6-cm dish). The relative intensities of phosphorylated bands are presented as in (A). β-actin bands indicate equal loading of samples.

Statistical analysis. Statistical significance of data was determined using Student's *t*-test.

Results

Hepatocyte growth factor receptor, Met, and Akt phosphorylation increased depending on added HGF concentration irrespective of GD3 expression in human melanoma cells. It is well known that HGF binding to Met triggers activation of Akt, so we analyzed phosphorylation of Met and Akt after stimulation with HGF in GD3+ cells. The phosphorylation of Met and Akt was detected by immunoblotting with phosphorylation site-specific antibodies for Met (p-Met Tyr1234/1235, Tyr1349) or Akt (p-Akt Ser473, Thr308) as well as anti-Met or anti-Akt antibodies. Phosphorylation of Met and Akt after 15 min of stimulation with HGF increased depending on HGF concentration (Fig. 1A). We analyzed the time-course of Met phosphorylation during stimulation with HGF. The peak of phosphorylation was found at 15 min, as shown in Figure 1(B). When phosphorylation of Met (p-Met Tyr1234

/1235, Tyr1349) after HGF treatment was compared between GD3+ cells and GD3− cells, relative intensities of phosphorylated Met bands corrected by β-actin or total Met bands showed no clear differences between GD3+ cells and GD3− cells (Fig. 1C). These results suggested that phosphorylation of Tyr1234/1235 and Tyr1349 in Met is not affected by GD3 expression in the human melanoma cell line, SK-MEL-28 N1.

Phosphorylation of Akt and Erks with HGF stimulation alone was not affected by GD3 expression in human melanoma cells. We analyzed phosphorylation of Akt (p-Ser473, p-Thr308) and Erks (phosphorylation of Erk1/2) after HGF treatment in GD3+ cells and GD3− cells (Fig. 2A). Relative intensities of phosphorylation of Akt and Erks corrected by total Akt and Erk, respectively, showed no clear differences between GD3+ cells and GD3− cells (Fig. 2B). These results suggested that phosphorylation of Akt and Erks with HGF stimulation alone is not affected by GD3 expression in the human melanoma cell line SK-MEL-28 N1.

We examined mRNA levels and protein levels of Met in several clones of GD3+ and GD3− cells. No differences in
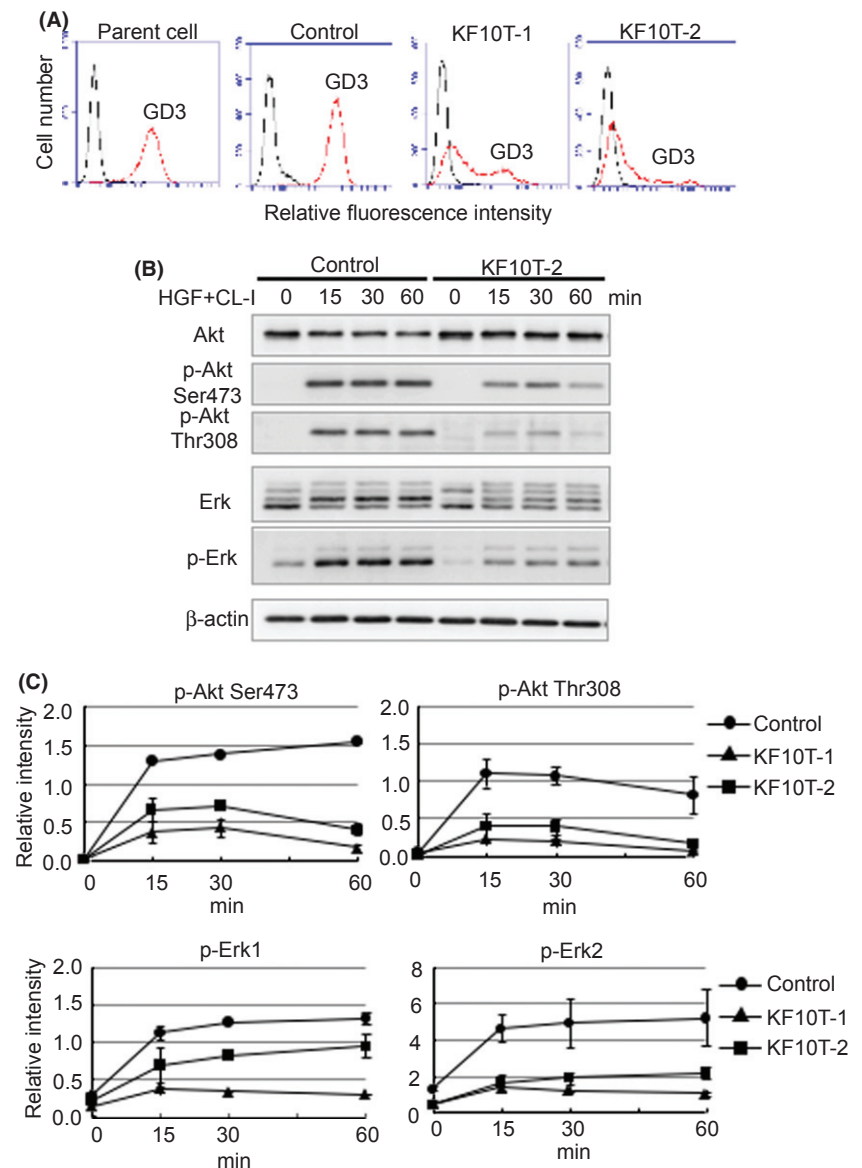

Fig. 5. Reduction of Akt phosphorylation levels by costimulation with hepatocyte growth factor (HGF) and adhesion to collagen type I (CL-I) in ganglioside GD3 synthase knockdown cells. Phosphorylation levels of Akt and Erks after stimulation with HGF and CL-I were compared between GD3 synthase knockdown cells and control cells. The cells were harvested and rotated for 1 h at 37°C after serum starvation for 14–16 h, then used for each experiment. (A) GD3 expression in SK-MEL-28 (parent cell), control cells (vector alone), and GD3 synthase knockdown cells (KF10T-1 and KF10T-2). GD3 was detected with mAb R24 by flow cytometry. (B) Phosphorylation levels of Akt and Erks were analyzed with Western immunoblotting. (C) Bands of Western immunoblotting were scanned and the band intensities of the phosphorylated Akt and Erks were measured and presented after correction with those of total proteins. β-actin bands indicate equal loading of samples.

mRNA levels of Met were found between them (data not shown), whereas Met protein was approximately 1.5-fold higher in GD3+ cells than in GD3− cells. Mechanisms for the differences in protein levels of Met remain to be investigated.

In this study, when cells were treated with HGF at 30 ng /mL, phosphorylation of Met was almost 2-fold stronger in GD3+ cells than in GD3− cells at 15 min after stimulation (Fig. 1C(a)), though no differences were detected at other time points during the observation. Then, after treatment with HGF at 10 ng/mL, phosphorylation levels of Akt and Erks were almost same between GD3+ and GD3− cells (Fig. 2), suggesting that induced signaling intensity was equivalent when treated by a relatively low amount of HGF regardless of Met levels. Therefore, we carried out experiments from this point on using HGF at 10 ng/mL.

Phosphorylation of Akt was synergistically enhanced by costimulation with HGF and adhesion to CL-I in GD3+ N1 cells. We

analyzed phosphorylation of Akt (p-Ser473, p-Thr308) by either HGF stimulation or adhesion to CL-I, or both of them at the same time in GD3+ cells and GD3− cells. The cells were harvested after starving for 14–16 h in serum-free DMEM and rotated for 1 h at 37°C to reduce basal phosphorylation levels of Akt. After HGF stimulation, phosphorylation of Ser473 in Akt was slightly higher in GD3+ cells, but phosphorylation of Thr308 in Akt was not different between GD3+ and GD3− cells (Fig. 3A). Phosphorylation of Ser473 and Thr308 of Akt was higher in GD3+ cells than in GD3− cells during adhesion to CL-I, corresponding with our previous report,[22] while the phosphorylation levels were low (Fig. 3B). Then, we analyzed phosphorylation of Akt during costimulation of GD3+ and GD3− cells with HGF and adhesion to CL-I. Consequently, phosphorylation levels of Ser473 and Thr308 of Akt were definitely higher in GD3+ cells than in GD3− cells (Fig. 3C). These results suggested that phosphorylation of Akt was synergistically
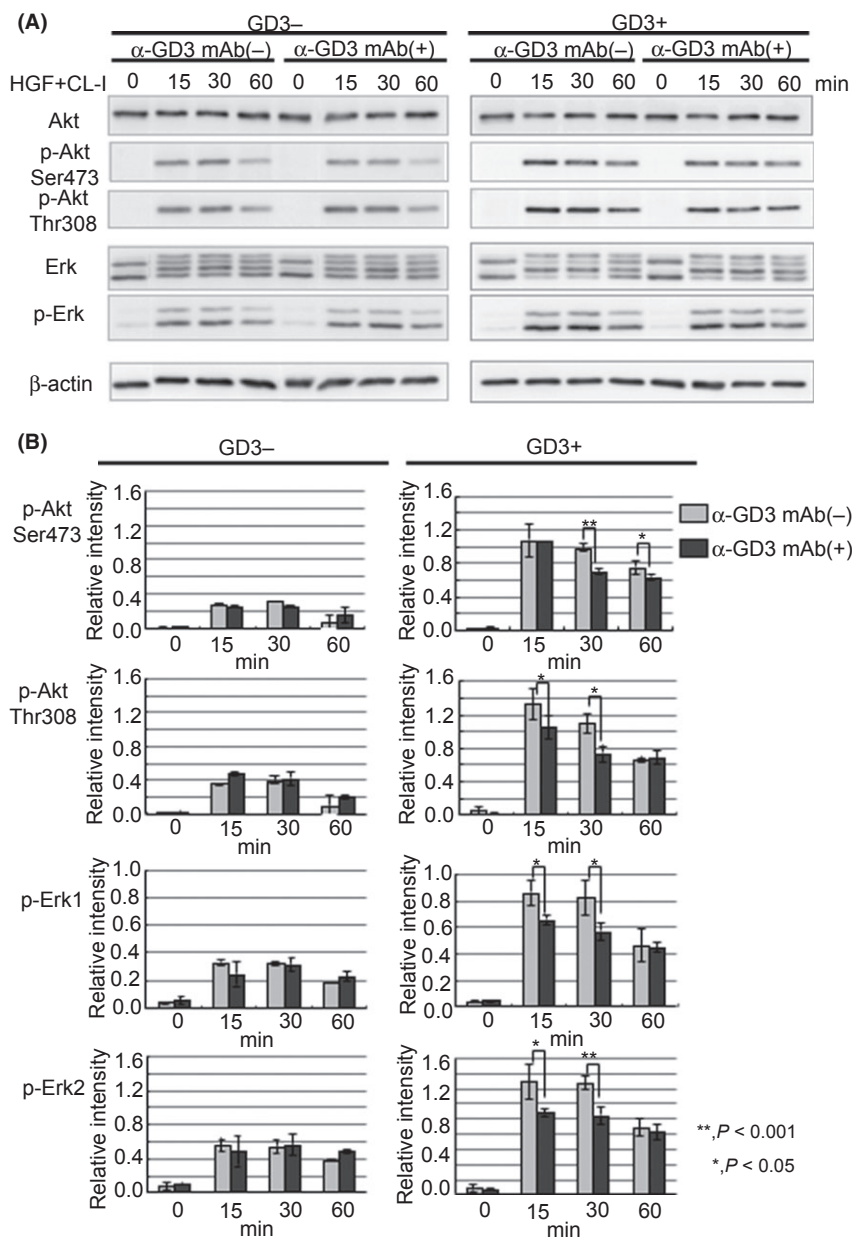

Fig. 6. Reduction of Akt and Erks phosphorylation levels by costimulation with hepatocyte growth factor (HGF) and adhesion to collagen type I (CL-I) using an anti-GD3 mAb. (A) Phosphorylation levels of Akt and Erks by costimulation with HGF and adhesion to CL-I (3.0 × 10^5 cells/3.5-cm dish) after treatment with an anti-GD3 mAb R24 were analyzed with Western immunoblotting. (B) Bands of GD3+ cells and GD3− cells in (A) were scanned and the band intensities of the phosphorylated Akt and Erks were measured and presented after correction with those of total proteins. Black bars, samples with mAb R24 treatment; gray bars, samples without mAb R24 treatment. β-actin bands indicate equal loading of samples.

enhanced by costimulation with HGF and adhesion to CL-I in GD3+ cells.

Phosphorylation of Erks was synergistically enhanced by costimulation with HGF and adhesion to CL-I in GD3+ N1 cells. We then analyzed phosphorylation of Erks by either HGF stimulation or adhesion to CL-I, or both of them at the same time, in GD3+ and GD3− cells. After starving for 14–16 h in serum-free DMEM, the cells were harvested and rotated for 1 h at 37°C to reduce basal phosphorylation levels of Erks. Phosphorylation levels of Erks by HGF stimulation were slightly higher in GD3+ cells than in GD3− cells (Fig. 4A). As for adhesion stimulation to CL-I, phosphorylation levels of Erks were not different between GD3+ cells and GD3− cells (Fig. 4B). We then costimulated GD3+ and GD3− cells with HGF and adhesion, and analyzed phosphorylation levels of Erks. In this case, phosphorylation levels of Erks were remarkably increased in GD3+ cells compared with GD3− cells (Fig. 4C). Among four bands in total

Erks detected after the stimulation, new bands above original Erk1/2, representing phosphorylated forms of Erk1/2, were more prominent in GD3+ cells than in GD3− cells after costimulation. These results suggested that phosphorylation of Erks was synergistically enhanced by costimulation with HGF and adhesion to CL-I in GD3+ cells.

GD3 synthase knockdown in SK-MEL-28 resulted in reduction of phosphorylation levels of Akt with costimulation of HGF and adhesion to CL-I. To confirm that GD3 is involved in the enhanced phosphorylation of Akt and Erks, we also analyzed the effect of knockdown of the GD3 synthase gene on the phosphorylation of Akt and Erks with HGF and adhesion to CL-I using the human melanoma cell line, SK-MEL-28. GD3 synthase knockdown cells (KF10T-1 and KF10T-2) were established by transfection with the shRNAi expression plasmid to SK-MEL-28 that strongly expresses GD3 (Fig. 5A).[24] In GD3 synthase-silenced SK-MEL-28 (KF10T-1 and KF10T-2),
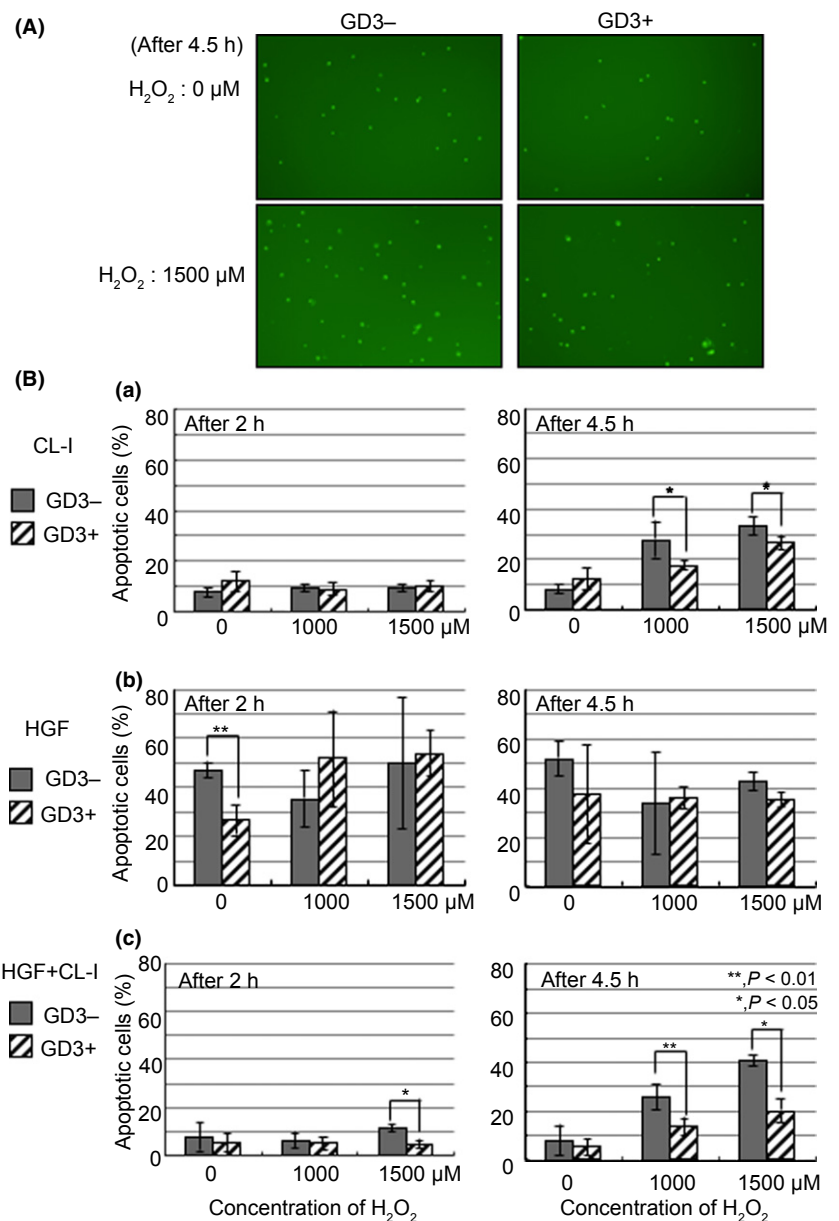

Fig. 7. Increased resistance to apoptosis induced by H_2O_2 by simultaneous stimulation with hepatocyte growth factor (HGF) and collagen type I (CL-I) in ganglioside GD3+ N1 cells. (A) After incubation for 16 h in serum-free medium, GD3+ cells and GD3− cells were collected. These cells were suspended in FCS-free medium and rotated for 1 h at 37°C. Cell suspensions were plated in CL-I-coated 96-well plates (2.0×10^3 cells/100 μL/well), and added with 10 ng/mL HGF and 5 μM CellEvent Caspase-3/7 Green Detection Reagent. After 30 min incubation at 37°C, the cells were treated with 0 or 1500 μM H_2O_2 for 4.5 h and apoptotic cells (identified by bright green nuclei caused by activated caspase-3/7) were counted. (B) Cells were harvested and rotated for 1 h at 37°C after serum starvation for 14–16 h. Then cell suspensions were plated in CL-I-coated 96-well plates ($2.0–3.0 \times 10^3$ cells/100 μL/well) (a), or cultured with HGF (10 ng/mL) in non-coated 1.5 mL tubes (5.0×10^5 cells/1.0 mL) (b), or plated in CL-I-coated 96-well plates ($2.0–3.0 \times 10^3$ cells/100 μL/well) with simultaneous addition of HGF (10 ng/mL) (c). After 30 min incubation, these cells were treated with different concentrations of H_2O_2 for 2 or 4.5 h, and apoptotic cells with activated caspase-3/7 showed bright green nuclei. The percentage of apoptotic cells was calculated by the number of cells labeled with bright green in the total number of cells. The percentage of dead cells at the starting point just after seeding cells to plates was subtracted as a baseline from the percentage of apoptotic cells at each time point.

phosphorylation levels of Akt and Erks were reduced compared with control cells (Fig. 5B).

Phosphorylation levels of Akt and Erks with costimulation of HGF and adhesion to CL-I were reduced by treatment with anti-GD3 mAb in GD3+ cells. To examine whether GD3 is involved in the enhanced phosphorylation of Akt and Erks when cells are costimulated with HGF and adhesion to CL-I, we examined the inhibitory effects of an anti-GD3 mAb R24 on GD3+ and GD3− N1 cells. Phosphorylation levels of Akt and Erks were diminished in GD3+ cells but not in GD3− cells after treatment with mAb R24 (Fig. 6). These results, together with the results of GD3 synthase knockdown, indicate that GD3 is involved in the enhanced phosphorylation of the signaling molecules.

GD3 expression confers resistance to apoptosis. As the phosphorylation of Akt was synergistically enhanced by costimulation with HGF and adhesion to CL-I in GD3+ cells, we examined the role of enhanced Akt activation caused by the combined signals in apoptosis. We treated GD3+ and GD3− cells with H_2O_2 after stimulation with either adhesion to CL-I or HGF treatment or costimulation with HGF and CL-I at the same time in GD3+ cells and GD3− cells. After adhesion stimulation to CL-I, the percentage of apoptotic cells in total cells was lower in GD3+ cells than in GD3− cells where cells were treated with 1000 or 1500 μM H_2O_2 for 4.5 h (Fig. 7B(a)). When cells were stimulated only by HGF, and subsequently treated with 1000 or 1500 μM H_2O_2 for 2–4.5 h, the percentage of apoptotic cells in total cells was 40–50% in both GD3+ and GD3− cells (Fig. 7B(b)). After costimulation with HGF and CL-I, the percentage of apoptotic cells in total cells was significantly lower in GD3+ cells than in GD3− cells when cells were treated with 1500 μM H_2O_2 for 2 h, and 1000 and 1500 μM H_2O_2 for 4.5 h (Fig. 7B(c)). Compared with single stimulation using either HGF or adhesion to CL-I, costimulation with both was more effective in GD3+ cells, as the percentage of apoptotic cells in GD3+ cells was less than half that in GD3− cells after treatment with 1500 μM H_2O_2 for 2 h and 4.5 h (Fig. 7B(a,c)). The apoptotic cells (indicated by bright green nuclei) at 4.5 h of H_2O_2 treatment are shown in Figure 7(A). These results suggested that GD3 expression confers resistance to apoptosis with costimulation with HGF and adhesion to CL-I.

Expression of GD3 promotes cell proliferation with costimulation with HGF and adhesion to CL-I. To examine the implications of enhanced Erk activation in cell proliferation, we carried out the EdU uptake assay. The percentage of EdU positive cells was higher in GD3+ cells than in GD3− cells after 21 h of costimulation with HGF and adhesion to CL-I (Fig. 8A). The percentage of EdU positive cells was also higher in GD3+ cells than in GD3− cells after 21 h of stimulation with adhesion to CL-I alone, whereas HGF treatment showed no significant differences in the ratios of EdU positive cells between GD3+ and GD3− cells (Fig. 8B). When cells were costimulated by HGF and adhesion to CL-I, the percentage of positive cells in GD3+ cells was clearly higher than that in GD3− cells, and also than that of GD3+ cells stimulated by CL-I alone; no clear difference was found in GD3− cells between the CL-I-treated and CL-I/HGF-treated groups (Fig. 8B). These results suggested that GD3 expression promotes cell proliferation with costimulation using HGF and adhesion to CL-I.

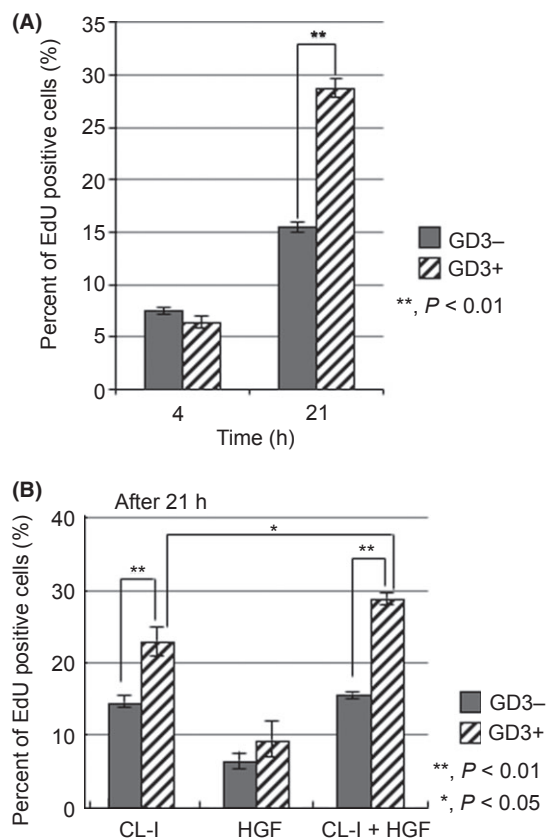These results are summarized in a schema in Figure 9.

Fig. 8. Ganglioside GD3+ N1 cells showed markedly increased cell proliferation when simultaneously stimulated by hepatocyte growth factor (HGF) and collagen type I (CL-I). (A) After incubation for 16 h in serum-free medium, GD3+ cells and GD3− cells were collected. These cells were suspended in FCS-free medium and rotated for 1 h at 37°C and cell suspensions were plated in CL-I-coated dishes (3.0 × 10^5 cells /3.5-cm dish) with 10 ng/mL HGF and 10 μM 5-ethynyl-2 deoxyuridine (EdU). Cell proliferation was assayed with EdU uptake after incubation for 4 or 21 h. The percentages of EdU positive cells were calculated by the number of cells labeled with green fluorescence in the total number of cells. (B) After incubation for 16 h in serum-free medium, GD3+ and GD3− cells were collected. These cells were suspended in FCS-free medium and rotated for 1 h at 37°C and cell suspensions were plated in CL-I-coated dishes (3.0 × 10^5 cells/3.5-cm dish) and cultured with EdU (10 μM), or cultured with HGF (10 ng/mL) in non-coated 1.5 mL tubes (1.0 × 10^6 cells/1.0 mL) and EdU (10 μM), or plated in CL-I-coated dishes (3.0 × 10^5 cells/3.5-cm dish) with HGF (10 ng/mL) and EdU (10 μM). Cell proliferation was assayed with EdU uptake after incubation for 21 h. The percentages of EdU positive cells were calculated by the number of cells labeled with green fluorescence in the total number of cells.

Discussion

Ganglioside GD3 is highly and specifically expressed in melanomas.[21,25] To investigate the roles of GD3, we established a set of melanoma cell lines with or without GD3 expression using a GD3-deficient mutant, N1.[26] Re-expression of GD3 with GD3 synthase cDNA resulted in the establishment of cell lines, which enabled us to identify phenotypic changes due to GD3 expression, and to investigate the molecular mechanisms of the enhanced malignant properties under GD3 expression.[15]

From past data, induced gangliosides by GD3 synthase cDNA in N1 cells were almost exclusively GD3,[15,27] and sometimes a very faint GD2 could be seen in thin-layer chromatography or flow cytometry. No other components could be
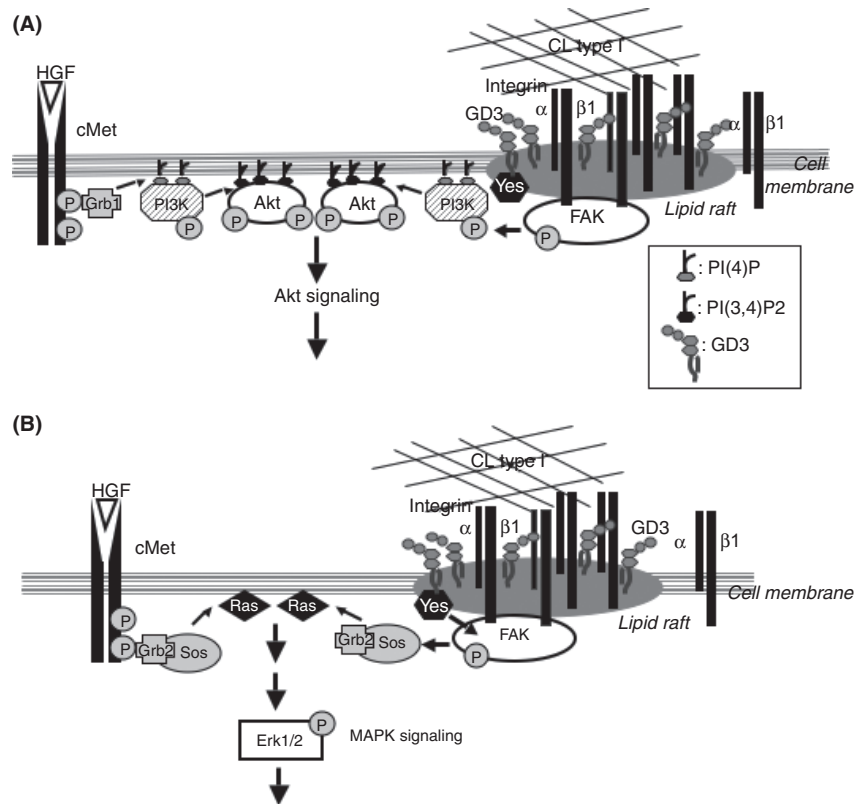
Fig. 9. Convergence of signaling molecules in the vicinity of membranes and synergistic enhancement of the signaling and phenotypes in ganglioside GD3+ N1 cells. (A) Phosphatidylinositol-3-OH kinase (PI3K)−Akt exists downstream of the collagen type I (CL-I)/integrin-mediated signaling and hepatocyte growth factor (HGF)/Met-mediated signaling pathway. After adhesion of cells to CL-I, focal adhesion kinase (FAK) is phosphorylated (P), leading to activation of PI3K and subsequent Akt phosphorylation. After binding of hepatocyte growth factor (HGF), Met is phosphorylated and growth factor receptor bound protein-1 (Grb-1) can bind to phosphorylated Met, then PI3K is activated resulting in Akt phosphorylation. (B) Ras-Erks exist downstream of the CL-I/integrin-mediated signaling pathway. After adhesion of cells to CL-I, FAK is phosphorylated and an Src family kinase, Yes, binds phosphorylated FAK, then sequential activation of the signaling pathway takes place. After binding of HGF, Met is phosphorylated and Grb2−Sos can bind to phosphorylated Met, resulting in the activation of Ras, then of Erks. CL type I, collagen type I; PI(3,4)P2, phosphatidylinositol 3,4-bisphosphate; PI(4)P, phosphatidylinositol 4-monophosphate.

seen. Therefore, the effects shown in the GD3 synthase transfectant cells should be due to GD3 itself. We have reported that GD3 is involved in malignant properties, such as cell growth and invasion[15] and cell adhesion to the ECM.[22]

Strong phosphorylation of FAK, p130Cas, paxillin, Akt, and Erks after treatment with FCS was observed[15,27] in GD3+ cells. Increased phosphorylation of FAK and paxillin was also observed in GD3+ cells compared with those in GD3− cells during adhesion to CL-I.[22] Although increased phosphorylation of Akt was also observed during adhesion to CL-I, the relative intensity in the phosphorylation of Akt was weaker than that with FCS stimulation, and the relative intensity of bands of phosphorylated Erk1/2 during adhesion to CL-I was not different between GD3+ cells and GD3− cells.

In this study, we showed the synergistic effects of adhesion- and growth factor/receptor-mediated stimulations on the activation of Akt and Erks in GD3+ cells. Furthermore, the suppressive effects of combined stimulation with HGF and adhesion to CL-I on induced apoptosis and the enhancing effects on cell growth were also observed. Although we have used FCS to examine growth factor signals in melanoma cells,[15,24,27] we focused on HGF as a growth factor in this study because HGF receptor Met was overexpressed in melanomas[28] and overexpression of Met in human melanoma tissues was correlated with a poor clinical outcome of patients.[29] Upregulation of Met expression enabled HGF to protect melanoma cells from apoptosis.[30] Moreover, HGF upregulated the expression of CD44v6,[31] and induced the expression of fibronectin[32] in melanoma cells.

We first examined the effect of HGF on enhancement of signaling in GD3+ cells because HGF activates the MAPK and Akt signaling pathways in human melanoma cells, as mentioned above. Although the phosphorylation levels of Met and Akt were increased depending on the concentration of added

HGF, the phosphorylation levels of Met were not affected by GD3 expression in N1 cells. These results were quite different from those of breast cancer cell lines, in which GD2 expression induced cell growth due to constitutive activation of Met regardless of the presence of HGF.[33] They showed increased phosphorylation of Akt and Erks. In contrast, constitutive Met activation without HGF was not detected in either GD3-expressing N1 cells or GD2-expressing N1 cells (data not shown). The phosphorylation levels of Akt and Erks with HGF were also not affected by GD3 expression in a melanoma cell line.

We then tried to elucidate whether HGF stimulation could enhance phosphorylation levels of Akt or Erks during cell adhesion to CL-I more strongly in GD3+ cells than in GD3− cells. Phosphorylation levels of Ser473 and Thr308 in Akt were higher in GD3+ cells than in GD3− cells during the adhesion to CL-I, while the phosphorylation levels were very low (Fig. 3B). Surprisingly, phosphorylation levels of Akt were increased remarkably and definitely in GD3+ cells compared with those in GD3− cells during costimulation with HGF and adhesion to CL-I (Fig. 3C). Phosphorylation levels of Erks showed similar results (Fig. 4). These results suggested that phosphorylation of Akt and Erks was synergistically enhanced by costimulation with HGF and adhesion to CL-I in GD3+ N1 cells.

As strong phosphorylation of Akt and Erks in GD3+ cells after costimulation with HGF and adhesion to CL-I was observed, we questioned whether this costimulation affects tumor phenotype, such as resistance to apoptosis and/or promotion of cell proliferation in GD3+ cells. To examine the effects of costimulation with HGF and adhesion to CL-I on apoptosis, we treated GD3+ and GD3− cells with H_2O_2 after stimulation with either adhesion to CL-I or HGF treatment, or both. In the case of simultaneous stimulation with HGF and

CL-I, the percentage of apoptotic cells in total cells was significantly lower in GD3+ cells than in GD3− cells (Fig. 7B). We also examined the effects of simultaneous stimulation with HGF and CL-I on cell proliferation. The percentage of EdU positive cells was approximately two fold higher in GD3+ cells than in GD3− cells after 21 h of costimulation (Fig. 8B).

These results suggested that GD3 expression enhanced activation of Akt and Erks mainly when cells were costimulated with HGF and adhesion to CL-I, based on the convergence of two signaling pathways. Consequently, converged signals might bring about increased resistance to apoptosis and promotion of cell proliferation by synergistic effects of adhesion- and HGF/Met-mediated signaling.

Hepatocyte growth factor appeared to play a key role in the regulation of interaction between melanoma and its microenvironment. The growth- and metastasis-promoting effects of the microenvironment have been documented,[4,5] and clustered fibroblasts in tumor cell-conditioned medium produced a large amount of HGF.[34] A recent report has shown that stromal cell secretion of HGF in the tumor microenvironment resulted in activation of HGF receptor Met, and of MAPK and phosphatidylinositol-3-OH kinase–Akt signaling pathways in human melanoma cells. It led to immediate resistance to RAF inhibition treatment in BRAF-mutant melanomas,[6,35] and the immunohistochemistry experiments revealed stromal cell expression of HGF in patients with BRAF-mutant melanoma.[6,35] Of course, increased secretion of HGF from melanoma cells themselves might also be involved in the formation of the tumor microenvironment and progression of malignancy.[36]

Our results indicated that GD3 expression in melanomas enhances the MAPK and Akt signaling pathways by costimulation with HGF and adhesion to CL-I, and eventually enhances the resistance to apoptosis and/or cell proliferation. Together with the reports described above,[4–6,35] it is supposed that GD3 expression enhances cell adhesion to the environmental connective tissues and raises the sensitivity to HGF supplied from the tumor microenvironment, resulting in the convergence of multiple extrinsic signals in tumor tissues. These effects represent the driving source of GD3 expression for the development of micrometastases in the microenvironment of a secondary organ after the detachment of primary tumor cells, and confer high metastatic properties of melanomas.

Next question may be if GD3 itself is involved in the convergence of multiple extrinsic signals. As we previously reported,[15,27] only GD3 is highly and constantly synthesized in GD3 synthase cDNA transfectant cells. Moreover, growth suppression of a melanoma cell line with anti-GD3-specific mAb R24 was reported by us.[37] The fact that treatment of melanoma cells with mAb R24 could reduce the phosphorylation levels caused by costimulation of HGF and adhesion to CL-I suggests that GD3 itself can induce such synergistic effects, as shown in this study.

We reported previously that integrins were partially localized in membrane lipid rafts of GD3+ cells under cell suspension, resulting in the rapid activation of signal molecules on cell adhesion to the ECM.[22] In this study, we showed that HGF/Met stimulation enhances phosphorylation levels of Akt and Erks in GD3+ cells during cell adhesion to CL-I. Downstream molecules of the CL-I/integrin-mediated signaling and HGF/Met-mediated signaling pathways are common, as shown in Figure 9. These common signal molecules may be shared and converged in the vicinity of cell membranes, leading to synergistic enhancement of the signaling. What kinds of molecules are physically associated with GD3 on the membrane, and how the convergence of multiple signals are brought about under GD3 remain to be investigated.

Disclosure Statement

The authors have no conflict of interest.

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