Ganglioside GT1b inhibits keratinocyte attachment to and migration on a fibronectin matrix by binding to αβ1 and preventing αβ1 interaction with fibronectin. The role of gangliosides in triggering keratinocyte apoptosis, however, is unknown. Addition of GT1b to keratinocyte-derived SCC12 cells, grown in serum-free medium but exposed to fibronectin, suppressed Bad phosphorylation, activated caspase-9, and inhibited cyclin D and E expression, resulting in cell cycle arrest at G1, phase and initiation of apoptosis. The mechanism of GT1b activation of caspase-9 involved inhibition of β1 integrin serine/threonine phosphorylation and decreased phosphorylation of both integrin-linked kinase and protein kinase B/Akt at its Ser-473 site, leading to cytochrome c release from mitochondria. Consistently, blockade of GT1b function with anti-GT1b antibody specifically activated the Ser-473 site of Akt, markedly suppressing apoptosis. The ganglioside-induced inhibition of Akt phosphorylation was GT1b-specific and was not observed when cells were treated with other keratinocyte gangliosides, including GD3. These studies suggest that the modulation of keratinocyte cell cycle and survival by GT1b is mediated by its direct interaction with αβ1 and resultant inhibition of the integrin/integrin-linked kinase/protein kinase B/Akt signaling pathway.

Keratinocytes and keratinocyte-derived cells are anchorage-dependent cells that undergo apoptosis when they lose adherence from an extracellular matrix (“anoikis”) (1). Survival of these cells is protected by signaling from soluble factors in serum and from cell interactions with the extracellular matrix. In the absence of serum, keratinocytes are able to survive in these cells is protected by signaling from soluble factors in serum and from cell interactions with the extracellular matrix. In the absence of serum, keratinocytes are able to survive in

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The abbreviations used are: FN, fibronectin; ILK, integrin-linked kinase; PKB, protein kinase B; cdk, cyclin-dependent kinase; GST, glutathione-S-transferase; PI, propidium iodide; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; PAGE, polyacrylamide gel electrophoresis; MBP, myelin basic protein; PVDF, polyvinylidene difluoride; PBS, phosphate buffered saline; GSK, glycogen synthetase kinase; PI3K, phosphatidylinositol 3-kinase; GD3, Neu-ac2-8Neu-ac2-3Gal β1-4Glc β1-Cer; GM3, GalNAcβ1-4Gal β1-4Glc β1-Cer; GM2, GalNAcβ1-4Neu-ac2-3Gal β1-4Glc β1-Cer; GT1b, Neu-ac2-3Gal β1-4Glc β1-Cer; and spreading. Integrin αβ1 allows the specific interaction of keratinocytes with FN and is up-regulated at the advancing keratinocyte border during wound healing (3). The presence of β1 integrin on the keratinocyte has been shown to promote survival (4) through up-regulating expression of Bcl-2 (5), an anti-apoptotic protein that binds to mitochondria and inhibits the release of mitochondrial cytochrome c into the cytosol (6), a critical step in the intrinsic pathway for activation of apoptosis.

Anchorage-dependent cell survival is modulated by a cascade that involves phosphorylation of ILK, an intracellular serine/threonine protein kinase capable of interacting with the integrin β1 cytoplasmic domain via its carboxyl terminus (7); this interaction of ILK with β1 integrin is required for localizing ILK to focal adhesion plaques (8), but the role of the ILK/β1 integrin interaction in signaling is unclear. Overexpression of ILK in epithelial cells has also been shown to encourage anchorage-independent cell growth, cell cycle progression (9), and tumorigenicity in nude mice (10). Activation (or overexpression) of ILK activates protein kinase B (PKB/Akt) (11–14). PKB/Akt activity is regulated via its phosphorylation on two highly conserved sites: the activation loop of threonine (Thr-308) in the kinase core and a hydrophobic phosphorylation site on the carboxyl terminus at serine (Ser-473) (13, 15). While the Thr-308 site is phosphorylated by phosphoinositide-dependent kinase (PDK)-1, ILK (and also PDK-1 in the presence of protein kinase C-related kinase-2 peptide (15, 16)) activates the hydrophobic site of PKB/Akt at Ser-473 (11, 12). PKB/Akt, in turn, is able to phosphorylate and thus inactivate the proapoptotic factor Bad (17, 18), leading to inactivation of caspase-9 (19) and protection from apoptosis. Conversely, inhibition of phosphorylation of the ILK/PKB/Akt cascade provides a signal for apoptosis.

Gangliosides, ubiquitous membrane glycosphingolipids, modulate cell proliferation, adhesion, migration, and differentiation probably through their effects on transmembrane signaling (20–29). The cell membranes of keratinocytes and the keratinocyte-derived SCC12 cell line contain four gangliosides: GM3, the most prevalent, 9-O-acetyl GD3, GD3, and GT1b (29). GT1b, the most highly sialylated of the ganglioside gangliosides, inhibits keratinocyte proliferation (23) and is a potent trigger of keratinocyte differentiation at low calcium concentration (24). Recent studies have shown that ganglioside GT1b is able to inhibit keratinocyte attachment and migration on a FN matrix (25, 26) by the direct interaction of ganglioside GT1b with the α5 subunit of αβ1 integrin (29), a process that requires glycosylation of the α5β1.

Given known regulation of α5β1 with ILK, we hypothesized that the GT1b/α5β1 interaction could modulate ILK phosphorylation and thereby trigger keratinocyte apoptosis via inhibition of PKB/Akt phosphorylation. Using pharmacological
addition of gangliosides and specific blockade of ganglioside function with anti-ganglioside antibodies, we investigated the ability of gangliosides to trigger keratinocyte apoptosis in the presence of FN and the mechanism of the induction of this apoptosis. Our studies show that ganglioside GT1b is a potent stimulant of apoptosis when keratinocyte-derived cells are exposed to FN via a mechanism that involves inhibition of Ser/Thr phosphorylation of ILK and ultimately inhibition of phosphorylation of PKB/Akt at its Ser-473 site; consistently, blockade of GT1b function by anti-GT1b antibodies suppresses the induction of cell apoptosis, increasing phosphorylation of PKB/Akt at the Ser-473 but not at the Thr-308 phosphorylation site. Both gangliosides GT1b and GD3 trigger cytochrome c release from mitochondria and keratinocyte apoptosis. However, GD3-induced apoptosis is not modulated by the ILK/PKB/Akt signaling pathway.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—Antibodies directed against GT1b and GM3 were obtained from Seikagaku Corp. (Tokyo, Japan). Antibodies directed against PKB/Akt-phospho-(ser-473, PT-20), integrin β1, cyclin D1, D3, and D2 and with 10% FBS were purchased from Transduction Laboratories (Lexington, KY). Anti-cyclin-dependent kinase (cdk4 and anti-cytochrome c antibodies were purchased from Pharmingen (San Diego, CA). Antibodies directly against ILK, caspase-9, cdk2, Ser-p-112 Bad, Ser-p-136 Bad, and inactivated glutathione-S-transferase (GST)–PKB/Akt were purchased from Upstate Biotechnology (Lake Placid, NY). Antibody against PKB/Akt-p-Thr-308, the PKB/Akt Kinase Assay Kit, and the soluble cell-binding fragment of FN were purchased from New England Biolabs (Beverly, MA). Antibodies directed against Bads were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Fluorescein isothiocyanate-Annexin V and propidium iodide (PI) were purchased from Molecular Probes (Eugene, OR). Anti-phosphoerin and anti-phosphothreonine antibodies were purchased from Calbiochem (La Jolla, CA). Anti-BrdUrd antibody was purchased from Roche (Indianapolis, IN). Gangliosides, antibodies directed against 9-O-acetyl GD3 or GD3, and other chemical reagents were purchased from Sigma unless indicated.

**Cells and Cell Culture**—SCC12F2 (SCC12; courtesy of Dr. James Rheinwald, Boston, MA), a human keratinocyte-derived cell line, was used for studies because of its close resemblance to normal human keratinocytes in its biologic behavior, its ganglioside content, and its use for studies because of its close resemblance to normal human keratinocytes. The SCC12F2 cell line was derived from the skin of a 12-week-old fetus and was maintained in a serum-free, growth factor-free medium containing 10% FBS (no FN), serum-free DMEM/F12 with 1.5 mg/ml soluble cell-binding fragment of FN, or serum-free DMEM/F12 with 10 mg/ml soluble cell-binding fragment of FN containing 10% FBS and FN. Genomic DNA was isolated and purified using a DNeasy Tissue Kit per manufacturer’s instructions (Qiagen, Carlsbad, CA). The DNA concentration was measured at A<sub>260-280</sub> 5 μg of total genomic DNA was loaded onto a 1.5% agarose gel, electrophoresed, viewed under ultraviolet light, and photographed.

**Immunoprecipitations**—Cells were treated without or with gangliosides GT1b (1 μg to 10 μg), GD3 (1 μg to 10 μg), GM3 (1–200 μg), or GM2 (50–200 μg) for 48 h or with anti-ganglioside antibodies for 30 min. Cells were harvested and lysed (0.5 ml of buffer/10<sup>6</sup> cells) in either cold immunoprecipitation buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, pH 8.0, 0.2 mM Na<sub>2</sub>VO<sub>4</sub>, 0.5 mM phenylmethylsulfonyl fluoride, and 2 mM benzamidine) or 2% sodium dodecyl sulfate (SDS) buffer (1% SDS, 10 mM Na<sub>2</sub>VO<sub>4</sub>, 10 mM Tris, pH 7.4) to denature as appropriate for the protein to be immunoprecipitated. Total protein from the cell lysates (500 μg) was mixed with 5 μg of anti-integrin β1, -ILK, -PKB/Akt, -cdk2, -cdk4, -Bad, -caspase-9, -cytochrome c antibody, and the total reaction volume was adjusted to 1 ml in the appropriate buffer. After incubation for 2 h at 4°C with continuous rotation, 10 μl of 50% protein A-agarose was added into each reaction, vortexed, and incubated with agitation for 30 min at 4°C. If monoclonal antibodies were used, 5 μg of rabbit anti-mouse IgG antibody was also applied to each sample for an additional 30 min of incubation before adding the protein A-agarose. After incubation, protein A-agarose beads were washed three times, and the precipitated proteins were released from the beads by boiling in 30 μl of Laemmli buffer (30 μl for 10 min prior to loading onto SDS-polyacrylamide gel electrophoresis (PAGE) mini-gels.

**Immunoblotting**—Immunoblotting was carried out as described (29) using appropriate antibodies and enhanced chemiluminescence (ECL) detection system (Amer sham Pharmacia Biotech). The following antibodies were used to detect separated proteins by Western blotting: anti-PKB/Akt, anti-PKB/Akt-p-Thr-308, anti-PKB/Akt-p-Ser-473, anti-PKB/Akt-p-Ser-473 Thr phosphorylation of ILK and ultimately inhibition of phosphorylation of PKB/Akt at its Ser-473 site; consistently, blockade of GT1b function by anti-GT1b antibodies suppresses the induction of cell apoptosis, increasing phosphorylation of PKB/Akt at the Ser-473 but not at the Thr-308 phosphorylation site. Both gangliosides GT1b and GD3 trigger cytochrome c release from mitochondria and keratinocyte apoptosis. However, GD3-induced apoptosis is not modulated by the ILK/PKB/Akt signaling pathway.

**Ganglioside-induced Apoptosis** — Killing activity was determined in cell extracts by immunoprecipitation as described previously (11) after treatment with gangliosides for 48 h or with anti-ganglioside antibodies for 30 min. Myelin basic protein (MBP) and GST-PKB/Akt were used as substrates for PKB/Akt activity in separate experiments, and the phosphorylated proteins stimulated by the immunoprecipitated ILK were electro-phoresed on 12% SDS-PAGE gels. When MBP was used as a substrate, (γ-32p)ATP was used as the phosphatc donor in the kinase assay and the phosphorylated MBP was detected by autoradiography. When the ILK was treated with 32P-labeled GST-PKB/Akt antibody prebound to Protein A-agarose beads, and kinase assays were carried out according to manufacturer’s instructions with the PKB/Akt Kinase Assay Kit using GSK-3 fusion protein as the substrate for PKB/Akt. Phosphorylated proteins were electrophoresed on 12% SDS-PAGE mini-gels and electrotransferred onto PVDF membrane to detect phosphorylated GSK-3 using the p-GSK-3 antibody. Equivalent amounts of PKB/Akt were immunoprecipitated by anti-PKB/Akt antibody and subjected to Western blotting with anti-GD3, anti-GM3, or anti-9-O-acetyl GD3 antibodies as described (11).

**Cell Cycle Assays**—To monitor whether gangliosides GT1b or GD3 can affect the progress of the cell cycle, cells were pretreated for 48 h with GT1b, GD3, or GM2. Cells were washed, then synchronized in the G<sub>0</sub> phase by both serum and growth factor starvation for 18 h without further ganglioside exposure. Cells were then trypsinized, detached, and plated onto 96-well plates (10<sup>5</sup> cells/well) precoated with or without 5 μg/ml anti-cytokeratin antibody or 10 μg/ml poly-l-lysine. The cells were grown in serum-free, growth factor-free medium containing 10 μg/ml BrdUrd for 24 h, and BrdUrd incorporation was stopped by washing with cold phosphate buffered saline (PBS). In other studies, the effects of anti-ganglioside antibodies were assessed. After the 18-h incubation in serum-free, growth factor-free medium to synchronize cells, anti-GT1b, anti-GM3, or anti-9-O-acetylGD3 antibodies were added for...
30 min prior to trypsinization and replating. Cells were stained with anti-BrdUrd monoclonal antibody, and incorporation was detected with alkaline phosphatase-conjugated anti-mouse IgG (31). Absorbance was read at A_{500nm} as indicated by the manufacturer’s guidelines (Roche).

To determine the effect of increased content of gangliosides through supplementation on cyclin expression, SCC12 cells were grown as described above in 25-cm² flasks until 80% confluent. Cells were then treated with or without 1 μM GT1b, 20 μM GD3, or 200 μM GM2 for 48 h followed by culture in medium devoid of both FN and serum for an additional 18 h. In other experiments, cells were treated with anti-GT1b, anti-GM3, or anti-9-O-acetylGD3 antibodies for 30 min after growth in the serum-free and FN-free medium for 18 h. Cdk2 or cdk4 was immunoprecipitated from equal amounts of cell lysate. The immunoprecipitates were applied onto 12% SDS-PAGE mini-gels, and the protein was transferred to PVDF membrane. Equal loading of cdk4 and cdk2 was determined with anti-cdk4 or anti-cdk2 antibody. The blots were then washed and reprobed with anti-cyclin D1 or anti-cyclin E antibody.

Isolation of Cytosolic and Mitochondrial Fractions, Determination of Cytochrome c Content, and Statistical Analysis—Cells were treated with or without gangliosides as above, trypsinized, and harvested at 600 × g for 10 min at 4°C. The cytosol and mitochondrial fractions were isolated as described previously (32). Mitochondrial and cytosolic fractions were separated on a 12% SDS-PAGE mini-gel and electroblotted onto nitrocellulose membranes. Cytochrome c was detected by immunoblots with anti-cytochrome c antibody and an ECL kit. All data were analyzed statistically by Student’s t test, with p < 0.05 considered significant.

RESULTS

Ganglioside GT1b, but Not GD3, Induces Cell Apoptosis in a FN-dependent Manner—Keratinocytes and SCC12 cells become apoptotic when withdrawn from both serum and FN; in contrast, exposure to either serum or to FN provides a prosurvival signal and prevents apoptosis. To explore the influence of gangliosides on cell survival and apoptosis in the presence of FN, keratinocyte-derived SCC12 cells were either allowed to grow on culture plates with or without precoated 5 μg/cm² cell-binding fragment of FN, or cells were suspended in serum-free DMEM/F12 medium containing 10 μg/ml soluble cell-binding fragment of FN. Immunofluorescent annexin V-staining assays showed that ganglioside GT1b (1 μM) triggered the apoptosis of cells grown in contact with FN from <5% of the cells to 58.2% of the cells (Figs. 1, A–I). Ganglioside GD3 (1 μM) caused the SCC12 cells to become apoptotic, despite exposure to either FN (from 5% of the cells without GD3 to 59.5% of the cells with GD3, Fig. 1I) or to serum without FN (from 4% of the cells without GD3 to 26.4% of the cells with GD3, data not shown). Gangliosides GM2 and GM3 at concentrations as high as 200 μM had no effect on induction of cell apoptosis when cells were suspended in the presence of the soluble cell-binding fragment of FN. Similarly, when cells were plated on FN-coated wells, 1 μM GT1b and 1 μM GD3, but not other gangliosides, also increased apoptosis to more than 50% of cells (data not shown). No ganglioside caused cell necrosis; less than 1% of cells in the same fields in which healthy and apoptotic cells were counted stained with PI (Figs. 1, E–H). Pharmacological addition to the SCC12 cells of gangliosides GT1b and GD3, but not GM2 nor GM3, induced collapse and fragmentation of the chromatin as shown by DNA laddering when cells were grown in serum-free medium in the presence of soluble cell-binding fragment of FN (Fig. 2). Addition of concentrations of GD3 greater than 20 μM led to keratinocyte toxicity; however, as in the studies with annexin V staining and DNA laddering, keratinocytes treated with 1–20 μM GD3 became apoptotic in the presence of either serum or FN.

GT1b Modulates Integrin β1 Phosphorylation and Induces SCC12 Cell Apoptosis through Inhibition of ILK and PKB/Akt Phosphorylation—Addition of supplemental of GT1b, but not GD3, GM3, or GM2 inhibited the phosphorylation of integrin β1 threonine and serine sites. Consistently, blockade of GT1b

<Fig. 1. Ganglioside GT1b induces the apoptosis of SCC12 cells in a FN-dependent manner. SCC12 cells were grown in suspension in serum-free medium containing 10 μg/ml soluble cell-binding fragment of FN with (A–C) or without (D) either ganglioside GT1b at 1 μM (A), GM2 at 200 μM (B), or GM3 at 200 μM (C) for 24 h. Cell growth was stopped by washing with cold PBS, then with annexin V-binding buffer. Apoptotic cells were detected by fluorescein-conjugated annexin V and visualized by immunofluorescence. Staining with propidium iodide was performed to identify dead cells (E–H). The identical fields seen in A–D are showed with PI staining in E–H. Approximately equal numbers of cells were noted in each field of A–D. The percentage of apoptotic cells after treatment with gangliosides GT1b 1 μM, GD3 1 μM, GM3 200 μM, or GM2 200 μM was determined as 100 × apoptotic cells/total healthy (weak, interrupted staining) + apoptotic (bright, continuous staining) cells and expressed as mean ± S.D. I, cells were viewed at 200× magnification with at least five high power fields counted. All experiments were performed in triplicate in at least three separate experiments. Bar = 25 μm. ***, p < .001.>
function by anti-GT1b antibody, but not other anti-ganglioside antibodies, increased integrin β₁ phosphorylation (data not shown). The kinase activity of ILK was inhibited by as little as 1 nM GT1b in a dose-dependent manner in the presence of FN, as detected by phosphorylation of MBP (Fig. 4A, top row); treatment with 1 μM GT1b resulted in maximal inhibition of ILK activity. Using the GST fusion protein GST-PKB/Akt as the substrate to test in vitro ILK kinase activity, as little as 100 nM GT1b similarly inhibited PKB/Akt Ser-473 phosphorylation, with maximal inhibition by 1 μM GT1b (Fig. 4A, third row). GT1b had no detectable effect on Thr-308 phosphorylation (Fig. 4A, lowest row). ILK activity was not inhibited by gangliosides GD3, GM3, or GM2. Blockade of GT1b function with anti-GT1b antibody stimulated ILK serine/threonine phosphorylation, while treatment with GT1b inhibited it. In contrast treatment with GD3, GM3, or GM2 or antibodies directed against GM3, GD3, or 9-O-acetylGD3 had no effect on ILK phosphorylation (Fig. 4B). Expression of ILK was not altered by treatment with gangliosides or with anti-ganglioside antibody (data not shown).

**Inhibition of ILK Activity by GT1b Suppresses PKB/Akt Ser-473 Phosphorylation and PKB/Akt Activity**—Using serine phosphorylation of glycogen synthase kinase 3 (GSK-3) as a measure of PKB/Akt activity at Ser-473 (11, 12), as little as 1 nM GT1b inhibited the PKB/Akt kinase activity, and maximal inhibition was achieved by 1 μM GT1b (Fig. 5A). Addition of GM3 (Fig. 5A, 4th row), GD3, (Fig. 5A, 5th row) or GM2 (not shown) did not affect PKB/Akt phosphorylation. Ganglioside GT1b inhibited PKB/Akt phosphorylation at Ser-473 by 3.8-fold, without affecting Thr-308 phosphorylation (Fig. 5B, lane 2), with inhibition noted with as little as 1 nm GT1b (data not shown). Consistently, blockade of GT1b with anti-GT1b antibody increased Ser-473 phosphorylation 5.1-fold (Fig. 5B, lane 8), yet had no effect on Thr-308 phosphorylation. Incubation of cells with GD3 (10 μM), GM3, or GD2 at concentrations as high as 200 μM or blockade of GM3, GD3, or 9-O-acetylGD3 function did not decrease PKB/Akt phosphorylation (Fig. 5B). Treatment of cells with gangliosides or anti-ganglioside antibodies did not change the expression of PKB/Akt (data not shown).

**Inhibition of ILK by GT1b Induces G₁ Phase Cell Cycle Arrest—**GT1b (1 μM) markedly decreased BrdUrd incorporation, while pharmacological addition of ganglioside GD3 or GM2 had no effect. In contrast, BrdUrd incorporation of SCC12 cells in serum-free medium plated on a FN matrix, but not on poly-L-lysine, was significantly increased by treatment with anti-GT1b antibodies (Fig. 6A), but not by blocking with anti-9-O-acetyl GD3 or anti-GM3 antibodies. Our previous studies have shown that cyclin D1 is the predominant cyclin D expressed in the SCC12 cell; we have not been able to detect cyclins D2 or D3 (data not shown). Pharmacological addition of ganglioside GT1b dramatically decreased the expression of cyclin D1 and E to almost the level of G₀ phase cells; the addition of GD3 or GM2 or blocking of GM3 or 9-O-acetyl GD3 with antibodies had no effect on the expression of cyclin D1 or cyclin E (Fig. 6B).

**GT1b Induces Apoptosis by Dephosphorylation of Bad and Activation of Caspase-9 through Inhibition of PKB/Akt Ser-473 Activity**—Previous studies have revealed that PKB/Akt inhibits cell apoptosis by phosphorylating and inactivating apoptotic factors, such as Bad and caspase-9 (17). Blocking of GT1b function by anti-GT1b antibody (Fig. 7A, lane 5) significantly increased Bad phosphorylation at both Ser-112 and Ser-136 sites; pharmacological addition of GT1b decreased the phosphorylation at both sites (Fig. 7A, lane 2). Expression of Bad was not altered. Pharmacological addition of GT1b suppressed caspase-9 serine kinase phosphorylation and promoted the conversion of procaspase-9 to the 35-kDa activated form of caspase-9 (Fig. 7B, lanes 2 and 3), while blocking of GT1b or GD3 with antibodies increased caspase-9 serine kinase phosphorylation, which prevented procaspase-9 activation (Fig. 7B, lanes 5 and 6).

Gangliosides GT1b and GD3 Both Trigger Cytochrome c Release from Mitochondria—Both GD3 and GT1b triggered cytochrome c release from the mitochondria of SCC12 cells, leading to decreased mitochondrial cytochrome c (Fig. 8A) and increased cytosolic cytochrome c (Fig. 8B). GM2 at concentrations as high as 200 μM did not lead to release of cytochrome c and
Gangliosides are a diverse series of sialic acid-containing glycosphingolipids present on the outer leaflet of the plasma membrane of most vertebrate cells (33, 34). The structural diversity of the ganglioside oligosaccharide chains is determined by biosynthetic glycosyltransferases residing in the Golgi apparatus. In keratinocytes and SCC12 cells, the GM3 is converted to gangliosides of the b-series, particularly GD3 and GT1b (35). Although the role of ceramide in inducing apoptosis, including in keratinocytes, has attracted great attention in recent years (for review see Ref. 36), the role of gangliosides in modulation of cell apoptosis has received scant attention. The few reports suggest that gangliosides induce cell apoptosis in a manner that is cell-specific and ganglioside-specific. For example, GM3 causes apoptosis, decreases BrdUrd incorporation, and up-regulates the cell inhibitor p27kip1 in proliferating astrocytes, while GD3 and GM1 do not (37); similarly, GM3 has been shown to induce apoptosis of high-grade human glioblastoma multiform tumors and rat gliosarcomas (38). In contrast GD3 causes apoptosis of hepatocytes (39) and hematopoietic (40) cells, while neither GM3 nor GT1b has any effect. Gangliosides GM1 and GM2 of the a-series have been shown to protect cortical neurons (41–43) and rat heart fibroblasts (44) from apoptosis, but increased membrane content of GD2, a ganglio-

FIG. 4. GT1b inhibits activation of ILK serine/threonine phosphorylation. SCC12 cells were grown on 6-well plates to 80% confluence in DMEM/F12 containing 10% FBS then treated with gangliosides or anti-ganglioside antibodies as described for Fig. 3. Non-denatured ILK was immunoprecipitated with α-ILK antibody from cells (A). To determine the effect of GT1b on ILK activity in vitro, MBP and GST-PKB/Akt recombinant protein were used as substrates for the activated ILK. The ILK immunoprecipitates were mixed with MBP in the presence of [γ-32P]ATP. The reaction products were separated on a 12% SDS-PAGE mini-gel, and the phosphorylated MBP was detected on Kodak X-Omat film (A, top row). The ILK immunoprecipitates were also reacted with recombinant GST-PKB/Akt, the product was applied to a 10% SDS-PAGE mini-gel, and protein was transferred to PVDF membrane. Equal loading of recombinant GST-PKB/Akt protein was verified by Western blotting with α-PKB/Akt antibody and ECL detection (A, second row). Phosphorylation of the Ser-473 site was detected with specific α-PKB/Akt-ser-p-473 antibody (A, third row) and of the Thr-308 site with α-PKB/Akt-thr-p-308 antibody (A, bottom row). The effect of GT1b and α-ganglioside antibodies on ILK phosphorylation itself in SCC12 cells was detected by Western blotting. Equal loading of immunoprecipitated ILK protein was verified using α-ILK antibody (B, top row), and the effect on phosphorylation of Thr (B, middle row) and Ser (B, bottom row) was examined with α-phosphothreonine and α-phosphoserine antibodies, respectively.

FIG. 5. GT1b inhibits phosphorylation of PKB/Akt at its Ser-473 phosphorylation site. SCC12 cells were grown on 6-well plates to 80% confluence in DMEM/F12 containing 10% FBS and then treated with gangliosides or anti-ganglioside antibodies as described for Fig. 3. PKB/Akt activity and phosphorylation were measured using non-denatured immunoprecipitated PKB/Akt with α-PKB/Akt antibody (A, B). The effect of GT1b, GM3, or GD3 on kinase activity of PKB/Akt was determined using GSK-3 as a substrate (A). Immunoprecipitated PKB/Akt was mixed with the GSK-3 fusion protein (1 μg/assay). The amount of loaded GSK-3 was detected by immunoblotting with anti-GSK-3 antibody and chemiluminescence (A, top row, treatment with GT1b; second row, treatment with GM3; fifth row, treatment with GD3); the effect on serine phosphorylation of the GSK-3 was determined by reprobing the membranes with anti-GSK-3-p-Ser-21/9 antibody (A, second row, treatment with GT1b; fourth row, treatment with GM3; bottom row, treatment with GD3). The effect of ganglioside or anti-ganglioside antibodies on PKB/Akt phosphorylation at Thr-308 (B, middle row) or Ser-473 (B, bottom row) sites was determined by immunoblotting of PKB/Akt immunoprecipitates applied to a 10% SDS-PAGE mini-gel. Equal loading of immunoprecipitated PKB/Akt was confirmed by immunoblotting with α-PKB/Akt antibody (B, top row).

DISCUSSION

Gangliosides are a diverse series of sialic acid-containing glycosphingolipids present on the outer leaflet of the plasma membrane of most vertebrate cells (33, 34). The structural diversity of the ganglioside oligosaccharide chains is determined by biosynthetic glycosyltransferases residing in the Golgi apparatus. In keratinocytes and SCC12 cells, the GM3 is converted to gangliosides of the b-series, particularly GD3 and GT1b (35). Although the role of ceramide in inducing apoptosis, including in keratinocytes, has attracted great attention in recent years (for review see Ref. 36), the role of gangliosides in modulation of cell apoptosis has received scant attention. The
side of the b-series pathway, increases proliferation of small cell lung cancer cell lines, while anti-GD2 antibodies induce apoptosis (45). We have now demonstrated that GT1b causes apoptosis of a keratinocyte-derived squamous carcinoma cell line in the presence of FN, an effect not seen with gangliosides GM3 or GM2 for 48 h in the presence of 10 μg/ml soluble cell-binding fragment of FN. Mitochondrial (A) and cytosolic (B) fractions were isolated, and cytochrome c was immunoprecipitated with anti-cytochrome c monoclonal antibody. Immunoprecipitated cytochrome c was applied to a 12% SDS-PAGE mini-gel and electrophoresed. Bands were transferred onto nitrocellulose membrane, and cytochrome c was detected by probing the membrane with 50 ng/ml anti-cytochrome c monoclonal antibody, followed by detection with enhanced chemiluminescence.

The molecular mechanism by which gangliosides protect

FIG. 6. GT1b, but not GD3, induces G1 phase cell cycle arrest by decreasing the expression of cyclins D and E. Cells were treated with ganglioside for 48 h in the presence of the soluble cell-binding fragment of FN and then synchronized in G1 phase by deprivation of both serum and FN during the last 18 h of treatment in the absence of ganglioside. Alternatively, cells were synchronized in G0 phase by deprivation of serum and FN for 18 h and then incubated with anti-ganglioside antibody for 30 min. After treatment, cells in G0 phase were trypsinized, detached, and plated onto 96-well plates precoated with or without 5 μg/cm² FN or 10 μg/cm² poly-L-lysine. Cells were grown in serum-free medium containing 10 μg/ml BrdUrd for an additional 24 h. BrdUrd incorporation was stopped by washing with cold PBS. Cells were stained with α-BrdUrd monoclonal antibody, and incorporation detected with alkaline phosphatase-conjugated α-mouse IgG (A). * p < .05; *** p < .001. To determine the effect of gangliosides on cyclins (B), SCC12 cells were grown as described above in 25 cm² flasks until 80% confluent. Cells were treated with gangliosides for 48 h and then cultured in both serum-free and FN-free medium for 18 h. To test the effects of blocking of ganglioside function, cells were incubated with anti-ganglioside antibody for 30 min after the 18 h growth in the serum-free, FN-free medium. Cells were subsequently incubated for an additional 18 h in serum-free DMEM/F12 medium containing 10 μg/ml soluble cell-binding fragment of FN to stimulate cell growth. Cdk2 or cdk4 was immunoprecipitated from equal amounts of cell lysate. The immunoprecipitates were applied onto 12% SDS-PAGE mini-gels, and the protein was transferred to PVDF membrane. Equal loading of cdk4 and cdk2 was determined with α-cdk4 or α-cdk2 antibody, and the blots were then washed and reprobed with α-cyclin D1 and α-cyclin E antibody to determine changes in cyclin expression.

FIG. 7. GT1b stimulates cell apoptosis by inactivation of Bad and activation of procaspase-9. SCC12 cells, grown in serum-free medium in the presence of FN, were treated with or without gangliosides GT1b, GD3, or GM2 for 24 h prior to lysis or with anti-GT1b, anti-GD3, or anti-9-O-acetylgD3 antibodies for 30 min, then without antibody for 16 h prior to cell lysis. Equal amount of cell lysates were incubated with either α-Bad or α-caspase-9 antibody to immunoprecipitate Bad and caspase-9. The immunoprecipitates were applied to a 12% SDS-PAGE mini-gel and transferred to PVDF membrane. The Bad immunoprecipitates were probed with α-Bad antibody (A, top row), and phosphorylation of Bad was detected by α-Bad-p-Ser-112 (A, middle row) or α-Bad-p-Ser-136 (A, bottom row) antibody. The caspase-9 immunoprecipitates were probed with anti-caspase-9 antibody (B, upper row), and the phosphorylation of caspase-9 was determined by α-serine kinase antibody (B, lower row).

FIG. 8. Both ganglioside GT1b and GD3 induce cytochrome c release from mitochondria. Cells were pretreated with or without ganglioside GD3, GT1b, or GM2 for 48 h in the presence of 10 μg/ml soluble cell-binding fragment of FN. Mitochondrial (A) and cytosolic (B) fractions were isolated, and cytochrome c was immunoprecipitated with anti-cytochrome c monoclonal antibody. Immunoprecipitated cytochrome c was applied to a 12% SDS-PAGE mini-gel and electrophoresed. Bands were transferred onto nitrocellulose membrane, and cytochrome c was detected by probing the membrane with 50 ng/ml anti-cytochrome c monoclonal antibody, followed by detection with enhanced chemiluminescence.
from or stimulate apoptosis in specific cells, however, is still largely unknown. The ability of GD3 to cause cytochrome c release from mitochondria with activation of downstream caspase-9 has been well described (39, 40, 48–50), but the upstream events that lead to enhanced mitochondrial permeability and subsequent cytochrome c release by GD3 are not understood. Recent evidence points to a direct effect of GD3 on mitochondria at the level of the permeability transition pore complex (50). The anti-apoptotic effect of GM1 on neurons is thought to result, at least in part, from stimulation of Trk tyrosine autophosphorylation and of Trk-associated protein kinase activity (42). We have now shown that the molecular mechanism for the induction of apoptosis by GT1b, but not ganglioside GD3, involves inhibition of ILK and PKB/Akt signaling with resultant cytochrome c release from mitochondria, stimulation of Bad, and generation of caspase-9. Consistent with this observation, blockade of GT1b function increases serine/threonine phosphorylation of ILK and of the Ser-473 site of PKB/Akt, which inhibits cells from becoming apoptotic.

Cell adhesion to the extracellular matrix activates signaling pathways that encourage cell cycle progression through regulation of cyclin expression, activation of cyclin D-cdk4 and cyclin E-cdk2 kinases, and phosphorylation of retinoblastoma protein (51). Activation of these cyclins and kinases promotes cell cycle progression from G1 to the S phase. We have demonstrated that GT1b, but not GD3 or other gangliosides, significantly inhibits BrdUrd incorporation, and arrests cell cycling at the G1 phase, through inhibiting the expression of both cyclin D1 and cyclin E.

PI3K is well known to be upstream of ILK, and the phosphorylation of PI3K results in phosphorylation of both ILK and PKB/Akt (12). In previous studies, we have shown that GT1b is able to bind to the EGFR, just as GM3, and inhibits phosphorylation of both the EGFR and downstream PI3K (28); treatment of SCC12 cells with anti-GT1b antibody, in contrast, increases PI3K phosphorylation. Thus, the increase in PI3K phosphorylation might be expected to trigger ILK phosphorylation, leading to the anti-apoptotic stimulus and blockade of PI3K phosphorylation to block ILK phosphorylation, preventing cell survival. However, when the function of PIK is blocked by LY294002, anti-GT1b antibody is still able to protect SCC12 cells from undergoing apoptosis and to stimulate the phosphorylation of both ILK and PKB/Akt at Ser-473; furthermore, treatment with LY294002 profoundly blocks phosphorylation at the Thr-308 site of PKB/Akt, but has no effect on Ser-473 phosphorylation in the SCC12 cells when grown in the presence of FN without anti-ganglioside antibody. Ganglioside GM3, which strongly inhibits PI3K phosphorylation (28), does not trigger apoptosis and anti-GM3 antibody does not activate ILK or PKB/Akt phosphorylation at the Ser-473 site. These data provide evidence against modulation of PI3K function as the mechanism of ILK activation or suppression induced by GT1b or anti-GT1b antibodies, respectively.

Although ILK is known to interact directly with the cytoplasmic region of β1, the role of this interaction in signal transduction through the ILK/PKB/Akt pathway is unclear. Given the parallel alterations in serine/threonine phosphorylation of β1 integrin and ILK after exposure to GT1b or blockade of GT1b function and the known relationship between β1 and ILK, we suspect that β1 activation itself is able to trigger the phosphorylation of ILK and that modulation of β1 activity by GT1b is critical for the inhibition by GT1b of ILK and PKB/Akt activation. Our previous studies have shown that GT1b specifically inhibits the attachment of keratinocytes and the keratinocyte-derived SCC12 cells to FN through the ability of GT1b to bind strongly to the glycosylated N-terminal region of α5 and thereby abrogate the αβ/FN interaction (29). These studies also demonstrated that GT1b itself binds poorly to the β1 integrin subunit of α5β1, regardless of β1 integrin glycosylation (29). In view of the strong interaction between α5 integrin and GT1b, but not between GT1b and β1, we propose that binding of GT1b to α5 leads to a conformational change in the α5β1 complex. As a result, the serine/threonine phosphorylation of β1 integrin is inhibited, leading to inhibition of ILK serine/threonine phosphorylation, inhibition of phosphorylation of Ser-473 on PKB/Akt, and ultimately to apoptosis. As we have shown, GD3 is also able to trigger apoptosis of SCC12 cells through release of mitochondrial cytochrome c. However, the pro-apoptotic activity of GD3 does not involve inhibition of PKB/Akt phosphorylation in contrast to the mechanism of GT1b-induced apoptosis. Similarly, GD3 is unable to decrease BrdUrd incorporation or to decrease the expression of cyclins D1 and E. GD3, just as GT1b, is able to bind to α5, but the binding to α5 is significantly weaker than that of GT1b as is the ability of GD3 to inhibit cell binding to FN (29). In slot blot studies, the ratio of binding of GT1b:GD3 to affinity-purified α5β1 from SCC12 cells is 3:1:1, and to insect recombinant α5 it is 5:2:1 (29). In vitro competitive slot blot assays in which 200 μM each of gangliosides GM3, GM2, GT1b, and GD3 are incubated with the integrin or integrin subunit show that only GT1b and GD3 bind with a GT1b:GD3 binding ratio of 6:9:1 to affinity-purified α5β1 from SCC12 cells and 7:8:1 to the recombinant α5 subunit. Based on the inability of GD3 to influence PKB/Akt signaling, we propose that the weaker interaction of GD3 with α5 is unable to alter the conformation of the α5β1 integrin sufficiently to cause alterations in β1 phosphorylation and suppress downstream signaling components. Thus, in contrast to the mechanism by which changes in membrane content of GT1b affect keratinocyte survival, the possible interactions at the cell membrane of GD3 that lead to GD3-induced apoptosis remain unclear.

Neoplastic cells commonly demonstrate anchorage independence, resulting in resistance to apoptosis and accelerated cell cycle progression (52). Transformation to carcinoma or epithelial hyperplasia when PTEN phosphatase is mutated or inactivated has been attributed to constitutive activation of PKB/Akt via enhanced PI3K-induced phosphorylation of Ser-473 (11). Inhibition of ILK and PKB/Akt activity in cancer cells with PTEN mutations has been proposed as a means to treat these neoplasia. The demonstration that ganglioside GT1b is able to promote cell apoptosis and interrupt uncontrolled cell cycling through its inhibition of ILK and PKB/Akt stimulation suggests a phosphoinositide-independent approach to controlling neoplastic growth.

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Inhibition of Integrin-linked Kinase/Protein Kinase B/Akt Signaling: MECHANISM FOR GANGLIOSIDE-INDUCED APOPTOSIS
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