Anti-GD2 ganglioside antibodies could be a promising, novel therapeutic approach to the eradication of human small cell lung cancers, as anti-GD2 monoclonal antibodies (mAbs) induce apoptosis of small cell lung cancer cells in culture. In this study, we analyzed the mechanisms for the apoptosis of these cells by anti-GD2 mAbs and elucidated the mechanisms by which apoptosis signals were transduced via reduction in the phosphorylation levels of focal adhesion kinase (FAK) and the activation of a MAPK family member, p38, upon the antibody binding. Knock down of FAK resulted in apoptosis and p38 activation. The inhibition of p38 activity blocked antibody-induced apoptosis, indicating that p38 is involved in this process. Immunoprecipitation-immunoblotting analysis of immune precipitates with anti-FAK or anti-integrin antibodies using an anti-GD2 mAb revealed that GD2 could be precipitated with integrin and/or FAK. These results suggested that GD2, integrin, and FAK form a huge molecular complex across the plasma membrane. Taken together with the fact that GD2+ cells showed marked detachment from the plate during apoptosis, GD2+ small cell lung cancer cells seemed to undergo anoikis through the conformational changes of integrin molecules and subsequent FAK dephosphorylation.

In many of these studies, no clear implication of individual gangliosides have been elucidated, except that GD2 in small cell lung cancer (SCLC) was shown to induce increased cell growth and invasion using the transfectant cells with GD3 synthesize cDNA (7). Furthermore, it was shown that anti-GD2 monoclonal antibodies (mAbs) could suppress the cell proliferation of GD2+ SCLC cells and also induce apoptosis with caspase activation. These results indicated that antibody therapy might be a promising approach to overcoming the disastrous disease SCLC.

As for the tumor cell apoptosis with anti-ganglioside antibodies, a few studies have been reported. Hanai and co-workers (8) report apoptosis induction of melanoma cells with anti-GM2 mAb in multicellular heterospheroids. On the other hand, in neuronal tissues, anti-ganglioside antibodies often cause tissue damage mainly in the motor neuron system and trigger serious motor neuron paralysis, such as Guillain–Barré syndrome (9). These facts suggest that gangliosides on malignant tumor cells can be good targets of antibody therapy with mAbs or their modified forms, although not many examples of apoptosis induction with anti-ganglioside mAbs have been reported so far.

To clarify the possibility of therapeutic application of anti-ganglioside mAbs for malignant tumors, molecular mechanisms for the apoptosis of SCLC cells with anti-GD2 ganglioside were studied. The most intriguing issue in understanding the apoptosis signals triggered by an antibody binding to gangliosides express on the cell surface membrane is how the events outside of the cells can induce the sequential activation of molecules involved in the apoptotic pathway inside of the cells. Here we have demonstrated a whole feature of the signaling pathway for apoptosis triggered by anti-GD2 mAbs leading to apoptotic cell death.

**MATERIALS AND METHODS**

**Cell Lines**—A GD3 synthesize gene transfectant cell line, D-18, and a vector control cell line, C-2, were generated from SK-LC-17 in our laboratory (7) and were maintained in RPMI 1640 medium containing 10% fetal calf serum and G418 (250 μg/ml) in a humidified 5% CO₂ atmosphere at 37 °C. Other SCLC cell lines, NCI-417, ACC-LC-171, and ACC-LC-96 were as described previously (7).

**Antibody Purification**—The anti-GD2 monoclonal antibody (mAb) 220–51 (mouse IgG3), which was generated in our laboratory (10), was purified using a protein G affinity column, and the concentration of the protein was determined by Lowry’s method (11).
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Flow Cytometry—Cell surface expression of ganglioside GD2 was analyzed by flow cytometry (FACSscan, BD Biosciences) using an anti-GD2 mAb, 220–51. Cells were incubated with mAbs for 60 min on ice and then washed with FITC-conjugated anti-mouse IgG (ICN/Cappel, Durham, NC). Control cells for flow cytometry were prepared using the second antibody alone.

MTT Assay—For cell proliferation assay, transfected cells and control cells (1 × 10⁶ cells/well) were plated in 24-well plates (CellStar, Carrollton, TX) in serum-containing medium and cultured for 4 days. For cell growth inhibition assay, transfected cells and control cells (5 × 10⁴ cells/well) were seeded in 24-well plates (CellStar) in serum-containing medium and treated with anti-GD2 mAb 220–51 diluted to the indicated concentrations for 24 h. Freshly prepared medium containing antibodies was used for medium exchange everyday. To quantify the cell proliferation, MTT (Sigma) was added to each well (0.5 mg/ml). After incubation for 4 h at 37 °C, the supernatants were aspirated, and 100 μl of 10% trypsin containing 0.1% Nonidet P-40 and 4 mM HCl were added. The color reaction was quantitated using an automatic plate reader, Immuno-Mini NJ-2300 (Nihon InterMed, Tokyo, Japan) at 590 nm with a reference filter of 620 nm, as reported previously (12). MTT assays were carried out in triplicate. To analyze the growth suppression effects of mAb 220–51 in the transfected cells, (1 × 10⁵ cells/well) were prepared in 24-well plates in serum-containing medium and then incubated with 20–80 μg/ml of mAb 220–51 for 24 h and then counted with trypan blue.

Analysis of Apoptosis—Cells (1 × 10⁶ cells) were plated in 60-mm tissue culture plates (CellStar) in serum-containing medium and then treated with 60 μg/ml mAb 220–51 for 2 h at 37 °C. After the treatment, the cells were harvested with trypsin/EDTA, resuspended in the binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, and 0.5 mM CaCl₂), and cultured with FITC-conjugated Annexin V (50 μg/ml) (Annexin V-FITC kit; Roche Applied Science), according to the manufacturer’s protocol. The numbers of apoptotic cells were monitored by flow cytometry.

DNA Fragmentation Assay—Cells (1 × 10⁶ cells) were treated with 60 μg/ml mAb 220–51. After 42 h, the cells were harvested and lysed in 100 μl of a lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM EDTA, and 0.5% Triton X-100) at 4 °C. After centrifugation, the supernatants were collected, and 6 μl of RNase (10 mg/ml) and 6 μl of proteinase K (10 mg/ml) were added. After incubation for 1 h at 37 °C, the fragmented DNA was precipitated in 2-propanol (360 μl) and electrophoresed at 50 V for 1.6 h on a 2% agarose gel containing 0.2 μg/ml ethidium bromide in TAE buffer (40 mM Tris acetate, 1 mM EDTA). The ethidium bromide-positive RNAs were digested with RNase A and T1. The DNA fragments were separated by agarose gel electrophoresis for analysis.

RESULTS

GD2 Expression in the Transfected Cells and SCLC Cell Lines—It was known that the GD3 synthase gene was highly expressed in the SCLC cell lines (7). The GM2/GD2 synthase gene was broadly expressed in the majority of lung cancer cell lines examined, whereas SCLC lines showed slightly higher expression levels. The transfection of GD3 synthase cDNA can, therefore, induce GD2 expression, as shown in Fig. 1A. D-18 was generated by the transfection of GD3 synthase cDNA into SK-LC-17. GD2 expression was also detected in three SCLC cell lines at slightly lower levels than D-18 (Fig. 1B).

Suppression of Cell Growth by Anti-GD2 Antibodies—The effects of anti-GD2 mAbs on cell growth were then examined by adding antibodies to the culture medium. The increased cell growth after GD3 synthase gene transfection was strongly suppressed in the presence of an anti-GD2 mAb 220–51. The suppression effects were dependent on the concentration of the added antibody and were significant even at 10 μg/ml (Fig. 2A).

Apoptosis Induction by Anti-GD2 mAbs—To examine the induction of apoptosis during the growth suppression of SCLC cell lines with anti-GD2 mAb, double staining of cells with Annexin V (FITC) and propidium iodide was performed. A transfected line treated with anti-GD2 mAb was 38.7% positive for Annexin V binding at 2 h after the addition of the mAb (Fig. 2B), indicating the induction of apoptotic cell death. The vector control line C-2 showed no staining for both reagents. Three SCLC cell lines expressing GD2 treated with anti-GD2 mAbs were also positive for Annexin V and/or propidium iodide (Fig. 2B), indicating that the native GD2-expressing cells are also sensitive to apoptosis induction. To confirm the DNA degradation in the anti-GD2 mAb-treated cells, cytoplasmic DNA was prepared from 1 × 10⁶ cells and used for agarose gel electrophoresis. Only D-18 cells treated with the mAb showed definitive DNA ladder formation (Fig. 2C), suggesting that apoptosis was really induced by the anti-GD2 mAb. The transfected cells treated with anti-GD2 mAb showed marked shrink-
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Fig. 1. Expression of GD2 in the transfectant cell and SCLC cell lines. GD2 expression was analyzed with flow cytometry, as described under “Materials and Methods.” Results of a transfectant line (D-18) and a control (C-2) were shown. Results of three SCLC cell lines are also shown as labeled in the figure.

Phosphorylation of MAPks, ERK1/2, and p38 after Addition of the Antibody—For the antibody stimulation experiments, cells (8 × 10⁵) were plated in 60-mm tissue culture plates in serum-containing medium and then treated with 60 μg/ml mAb 220–51 for the appropriate times in the individual experiments. Phosphorylation of ERK1/2 was reduced from 240 min after the addition of the antibody in GD2-expressing D-18 (but not in the vector control cells) (Fig. 3A). Phosphorylation of p38 was induced from 120 min after the addition of the antibody in GD2-expressing D-18 (but not in the vector control cells) (Fig. 3B). Phosphorylation of p38 was also examined in a native GD2-expressant line, ACC-LC-171, and a very similar activation pattern of p38 was observed (Fig. 3C).

Phosphorylation of FAK after Addition of the Antibody—Cells (8 × 10⁵) were plated in 60-mm tissue culture plates in serum-containing medium and then treated with 60 μg/ml of mAb 220–51 for the appropriate times in the individual experiments. The lysates were incubated with anti-FAK (1 μg IgG) and immunoprecipitated and then used for immunoblotting with anti-phosphoryrosine antibody (PY20) or anti-FAK antibody. Phosphorylation of FAK was reduced along with the time of the antibody treatment from 10 min in GD2-expressing D-18, whereas no reduction in the FAK phosphorylation level was observed in vector control cells (Fig. 4A). In the case of a native GD2-expressant line, ACC-LC-171, a similar or even faster reduction of FAK phosphorylation was observed after the antibody treatment (Fig. 4B).

GD2, FAK, and Integrin Formed a Complex in the Transfectant Cells—To analyze the association of GD2 with integrin or FAK, immunoprecipitates with anti-integrin or those with anti-FAK were used for immunoblotting with anti-GD2 mAb. First of all, integrin was co-precipitated with FAK, as shown in Fig. 5A. Therefore, it was shown that GD2 was co-precipitated with FAK and/or integrin (Fig. 5B). This was also the case in the native GD2-expressant lines (Fig. 5C). Taken together, GD2 seemed to form a complex with integrin and FAK molecules across the plasma membrane.

Suppression of FAK Expression Resulted in Apoptosis—Five kinds of siRNAs were prepared based on the cDNA sequence and used to suppress FAK expression. The effects were examined with immunoblotting of anti-phospho-FAK as well as anti-FAK antibodies (Fig. 6A). More than 60% of suppression in FAK expression was achieved in siRNA1-treated cells. Bands of tyrosine-phosphorylated FAK also reduced in intensity in parallel with total FAK bands. Therefore, this was used for the apoptosis induction experiment. After siRNA transfection, cells were used for an apoptosis assay by FACS. After the transfection of FAK siRNA, expression of FAK was definitely reduced, and apoptosis was found in both D-18 and C-2 cells (Fig. 6B, top panels). The apoptosis induction was also detected in two native GD2-expressant lines after the transfection of FAK siRNA (Fig. 6B, bottom panels).

Phosphorylation of p38 Was Induced by the Suppression of FAK—The phosphorylation level of p38 increased by the suppression of FAK via FAK siRNA (Fig. 6C). Further increase in p38 phosphorylation was found by the addition of anti-GD2 mAb in immunoblotting with anti-phospho-p38.

Apoptosis Induced by Anti-GD2 mAb Was Protected with Inhibition of p38—Apoptosis activity by UV light exposure (100 J/m²) was inhibited via pre-exposure to SB203580 at 100 μM (but not at concentrations of 50 μM or less, as determined by Western immunoblotting of phospho-p38) (Fig. 7A). After treatment with SB203580 (100 μM) or Me₃SO (vehicle), cells were treated with mAb 220–51 (60 μg/ml) for 1 h at 37 °C and used for apoptosis assay by FACS. Apoptosis in D-18 was almost completely protected by SB203580 treatment (Fig. 7B, right panels). In the vector control cells, no apoptosis was induced by anti-GD2 mAb regardless of SB203580 treatment (Fig. 7B, left panels). As for the native GD2-expressant lines, both of the two cell lines examined showed marked suppression of apoptosis by SB203580 treatment (Fig. 7C).

SB203580 Showed No Effect on the Phosphorylation Levels of FAK—The effects of p38 inhibition with SB203580 on the phosphorylation levels of FAK was examined by immunoblotting. It was shown that SB203580 had almost no effect on the phosphorylation levels of FAK, suggesting that p38 was positioned at the downstream of FAK in the apoptosis signaling pathway (Fig. 8).

Apoptosis Signaling Pathway Triggered by Anti-GD2 mAbs—An apoptosis signaling pathway triggered by anti-GD2 mAbs was proposed based on the results demonstrated in this study in Fig. 9.

DISCUSSION

It is unusual that only an antibody binding to a surface molecule can induce apoptotic cell death, except for anti-Fas antibodies, which bind the Fas antigen associated with sequential death signaling molecules, such as FADD (13, 14). On the other hand, gangliosides are embedded in the outer layer of the plasma membrane, and there appears to be difficulty in transmitting signals from outside the cell into the cell nucleus.
FIG. 2. Suppression of cell growth and apoptosis induction by anti-GD2 mAb. A, suppression of cell growth. D-18 and C-2 were treated with mAb 220–51 diluted at the concentrations indicated for 24 h and then counted with the trypan blue exclusion method. B, apoptosis induction in the GD3 synthase gene transfectant cells (left) and three native GD2-expressant cell lines (right) by anti-GD2 mAb. Shown is double staining of Annexin V and propidium iodide (PI) in the cells after anti-GD2 mAb treatment. D-18 and C-2 were treated with 60 μg/ml mAb 220–51 for 2 h and stained with FITC-conjugated Annexin V (x-axis) and PI (y-axis) and then analyzed by flow cytometry, as described under “Materials and Methods.” Three cell lines were also similarly treated with mAb 220–51 and analyzed by flow cytometry. C, cytoplasmic DNA prepared from D-18 after treatment with or without 60 μg/ml of mAb 220–51 for 42 h was analyzed by agarose gel electrophoresis. Similar results were obtained in at least three experiments. D, morphological changes of a transfectant and a control treated with or without 60 μg/ml of mAb 220–51 for 5 h were observed under a phase-contrast microscope at ×400 (left). Those of the native cell lines were also examined after treatment with/without mAb 220–51, and typical pictures are shown (right).
Therefore, we need to consider a quite novel mechanism for apoptosis induced via gangliosides. Although Cheresh et al. (15) already report the cell rounding and detachment of melanoma cells from fibronectin substrate with anti-GD2 mAbs, we demonstrated in this study that the apoptosis of SCLC cells induced with anti-GD2 mAbs was caused by dephosphorylation of FAK with various results, i.e., reduction of phosphorylated FAK and morphological changes of GD2+ cells after the treatment with anti-GD2 mAbs and apoptosis induction with knock down of FAK. The formation of spaces between cells under antibody treatment seemed to be caused by the detachment of apoptotic cells. However, the precise cause of the cell shrinkage that simultaneously occurred (probably due to detachment) remains to be clarified. Apoptosis induced with disruption of cell-matrix interaction has been called “anoikis” (16). The anti-apoptotic or anti-anoikis activity of FAK has been reported by a number of studies using overexpression of wild type FAK (17), introduction of the constitutively activated form of FAK (18), or enforced activation of β1 integrin (19). The mechanisms for these anti-apoptotic activities of FAK include its binding to a death domain of receptor-interacting protein, augmentation of phosphatidylinositol 3-kinase (p85), phosphorylation, and Akt activity. Interestingly, FAK phosphorylation is also critical in the protection of apoptosis induced with ultraviolet irradiation (20). Moreover, FAK induces resistance to apoptosis induced with hydrogen peroxide in glioma cells (21) and even in a leukemia cell line (HL-60) (22). In turn, apoptosis induction with disruption of FAK has also been reported using a peptide of the FAK binding site of the integrin β1 tail, microinjection of anti-FAK antibody (23), mutated FAK at a tyrosine-phosphorylation site (24), and the N-terminal domain of FAK (25). All of these results indicate that the activation levels of FAK are
considered to be a crucial and universal factor in determining the fates of those cells treated with preapoptotic stresses in both monolayer cells and suspension cells.

In addition to the dephosphorylation of FAK, increased phosphorylation of p38 was one more conspicuous change during the apoptosis induction. The signaling pathway between FAK dephosphorylation and p38 activation is not known at this moment. In the previous report, we have found that JNK activation was observed at 6 h after anti-GD2 antibody treatment (26). In this study, activation of another MAPK, p38, could be detected at a much earlier time, suggesting p38 is primarily involved in the apoptosis induction. It has been reported that inhibition of FAK resulted in the activation of p38 and anoikis in a colon cancer cell line (27) and in myotube (28). However, it is not clear whether dephosphorylation of FAK causes p38 activation in a single direction. In our results, knock down of FAK induced p38 activation, and p38 inhibition failed to affect the phosphorylation levels of FAK, suggesting that p38 is located at the downstream of FAK antibody against integrin β1 combined with HRP-conjugated anti-mouse IgG as a second antibody. B, co-precipitation of GD2 with FAK and integrin. Cells were solubilized in 100 μl of the lysis buffer and were immunoprecipitated separately with 5 μl (1 μg of IgG) of anti-GD2, anti-integrin β1, or normal rabbit IgG. Thereafter, mAb 220–51 was used in the immunoblotting combined with peroxidase-conjugated anti-rabbit IgG as a second antibody to detect co-precipitated GD2. C, three native GD2-expressant cell lines were also used for the immunoprecipitation and subsequent immunoblotting to detect the co-precipitated GD2, as described in B.

The most intriguing issue in understanding the apoptotic process with anti-GD2 antibodies is how the antibody binding to gangliosides can trigger the initial signal for cell death. The fact that GD2 was physically associated with integrins (Fig. 5) strongly suggested that the antibody binding to GD2 causes conformational changes in integrin molecules, leading to the dephosphorylation of FAK, because integrins and FAK had
been known to be tightly associated in both physical and functional senses (32). The formation of a tertiary complex consisting of GD2, integrin, and FAK seemed to be a critical substantial basis to generate and/or maintain the malignant properties of SCLC cells (7). Insults destroying the intact form of the molecular complex might, therefore, result in the reduction of FAK-mediated anti-anoikis signals with its dephosphorylation. Functional modulation of integrins by the expression of glycosphingolipids was previously suggested by Cheresh et al. (33) and our group (34) using human melanoma cells and murine lung cancer cells, respectively.

Although gangliosides have been considered to be tumor-associated molecules and have been used as tumor markers, no clear and specific biological function in the malignant properties of human cancer cells have been elucidated. Colocalization of GD2/GD3 gangliosides at the adhesion plaque of melanoma cells was reported by Cheresh et al. (35). At that time, they demonstrated that these disialo-gangliosides were involved in the adhesion of melanoma cells to the extracellular matrix (15, 36). The critical effects of a peptide containing the ECM binding motif of integrins in the ganglioside-associated adhesion was also shown (33), indicating that disialo-gangliosides are functionally involved in the cell adhesion mediated by the interaction between integrins and the extracellular matrix. Burns et al. (37) also reported synergism between gangliosides and Arg-Gly-Asp-directed glycoprotein receptors in attachment to the matrix protein by melanoma cells, whereas Kojima and Hakomori (38) interpreted the synergism as the result of a combination of two cell recognition systems, i.e., interaction of glycosphingolipid-glycosphingolipid and that of adhesive matrix protein-integrin.

The mechanisms and implication of the interaction of gangliosides with integrin molecules on the cell surface is not well understood. Wang et al. (39) report that GT1b induced apopto-
sis of SCC12 cells by binding to integrin α5β1 and the resultant inhibition of the integrin-linked kinase/protein kinase B/Akt pathway. They also report that GM3 blocks epidermal growth factor receptor activation induced by disrupting the association of integrin α5β1 with the epidermal growth factor receptor (40). They raised the possibility of the modification of caveolin-1 with GM3 as a mechanism, but no evidences were demonstrated. Kawakami et al. (41) also report the promotion of interaction between tetraspanin CD9 and α3 integrin in microdomain with GM3, leading to inhibition of laminin-5-dependent cell motility. This regulation seems to be based on the modified organizational status of the glycolipid-enriched microdomain with glycosylation status. Actually, alteration of glycosylation in glycolipids affects intracellular localization of integrin, Src, and caveolin into or out of the glycolipid-enriched microdomain (42). Many of these studies indicate that gangliosides suppress integrin functions in cell adhesion, and mainly monosialyl structures have been analyzed in those studies. However, many so-called “cancer-associated glycolipids” are polysialyl compounds, as described in the Introduction. Therefore, modes of interaction with integrin and resulting effects should be different depending whether the glycolipids are monosialyl or polysialyl structures. In previous studies, direct interaction between gangliosides and integrin has never been clearly demonstrated, although the possibility that GD2 associates with integrins was suggested by Cheresh et al. (33) on the basis of their co-purification with an affinity column containing either an Arg-Gly-Asp-containing peptide, concanavalin A, or lentil lectin. However, actual molecular interaction has never been demonstrated. Results in this study elucidated, at least partly, the molecular association of GD2 with a membrane-penetrating molecule and an intracellular kinase molecule, i.e. GD2/integrin/FAK, although it is not yet clear whether the interaction between GD2 and integrins is direct or indirect. Precise modes of interaction between polysialo gangliosides and integrins should be clarified with more effective approaches, such as cross-linking, to further understand the mechanisms for apoptosis with anti-ganglioside mAbs.

Whatever the mechanisms are, the fact that not only GD2-overexpressing cells but native GD2-expressant SCLC cell lines underwent equivalent degrees of apoptosis with anti-GD2 mAb strongly encourages us to apply the antibody therapy for the treatment of SCLC patients. The ganglioside nomenclature followed was that of Svennerholm (43).
Similar results were obtained in at least three experiments. The binding of anti-GD2 mAbs might trigger the conformational changes of integrin molecules resulting in the dephosphorylation of FAK and subsequent activation of p38. Inhibition of p38 showed no effect on the dephosphorylation of FAK before and after anti-GD2 mAb treatment, respectively. Intensities of phospho-FAK bands (top) were normalized with those of total FAK (bottom) and plotted (middle). SB203580 showed almost no effect on the phosphorylation and dephosphorylation of FAK before and after anti-GD2 mAb treatment, respectively. Similar results were obtained in at least three experiments.

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