Jpn. J. Cancer Res. **93**, 816–824, July 2002

An Anti-GD2 Monoclonal Antibody Enhances Apoptotic Effects of Anti-cancer Drugs against Small Cell Lung Cancer Cells via JNK (c-Jun Terminal Kinase) Activation

Shoko Yoshida,[1,2] Haruhiko Kawaguchi,[2] Shigeki Sato,[2] Ryuzo Ueda[2] and Koichi Furukawa[1,3]

[1]Department of Biochemistry II, Nagoya University School of Medicine, 65 Tsurumai, Showa-ku, Nagoya 466-0065 and [2]Department of Internal Medicine II, Nagoya City University School of Medicine, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601

Small cell lung cancer (SCLC) cell lines specifically express ganglioside GD2, and anti-GD2 monoclonal antibodies (mAbs) caused suppression of cell growth and induced apoptosis of SCLC cells with single use. Here, enhancement of the cytotoxic effects of various anti-cancer drugs with an anti-GD2 mAb was demonstrated. The cytotoxicity of all six drugs examined was markedly enhanced, i.e. 2.4–7.8-fold increase of cell sensitivity in terms of IC_{50}. In particular, the combination of cisplatin (CDDP) with an anti-GD2 mAb resulted in prominent enhancement of cytotoxicity even in low–moderate GD2-expressing lines. The anti-GD2 mAb induced weak activation of c-Jun terminal kinase (JNK) in SCLC cells, and all anti-cancer drugs also induced its activation to various degrees. When CDDP and an anti-GD2 mAb were used together, significantly stronger JNK activation was observed corresponding to the cytotoxic effects, suggesting that synergistic phosphorylation of JNK with two reagents induced prominent apoptosis. The essential role of JNK in the induction of SCLC apoptosis with CDDP and anti-GD2 mAb was confirmed by experiments with a JNK inhibitor, curcumin. These results suggest that anti-GD2 mAbs would be very efficient in combination with anti-cancer drugs, both to achieve SCLC-specific cytotoxicity and to enhance its magnitude.

Key words: Apoptosis — Small cell lung cancer — Chemotherapy — JNK — GD2

Gangliosides are enriched in nervous tissues of vertebrates[1] and their expression is spatio-temporally regulated.[2] In human malignant tumor cells, neuroectoderm-derived tumor cells such as melanomas, neuroblastomas, and gliomas frequently express characteristic gangliosides for individual tumor types.[3–6] Sarcomas,[7] gastric cancers,[8,9] T cell leukemias[10–12] and small cell lung cancer (SCLC) cells[13] also express specific gangliosides. Specific monoclonal antibodies (mAbs) to these tumor-associated ganglioside antigens have been utilized for diagnosis,[14,15] prediction of the prognosis,[16,17] and detection of residual tumors.[18] In particular, anti-ganglioside mAbs have been tried for immunotherapy of melanomas[19,20] and neuroblastomas.[21]

During the early stage of mAb application, anti-GD3 mAb R24 was used for the first time in the treatment of recurrent melanomas, and showed the ability to reduce the tumor size or halt the progression.[19] Anti-GD2 mAb was also applied in the treatment of neuroblastomas to extend remission.[22] Moreover, a human mAb reactive with GD2 was tried for cutaneous melanomas with some effects.[20] However, further development of antibody therapy has not been successful mainly due to xenogeneic immunogenicity of mouse antibodies and poor penetration of antibodies into tumor tissues.

Recently, application of mAbs for the treatment of various cancers has been re-evaluated based on the progress in recombinant molecular technology and protein engineering with site-directed mutagenesis. Anti-c-erbB2/HER2/neu mAb was used in the therapy of advanced stages of breast cancers[23] and anti-CD20 mAb for B cell lymphomas,[24] and many more applications of mAbs in the therapy of intractable malignant tumors are now expected.[25]

Several studies have suggested that gangliosides are involved in tumor cell adhesion,[26,27] proliferation,[28,29] motility,[29] and metastasis.[30] We also demonstrated that ganglioside GD2 is characteristically expressed on SCLC cells and is involved in proliferation and invasion.[31] Furthermore, we elucidated that anti-GD2 mAb could induce apoptosis of SCLC cells via activation of caspases. These results suggest that gangliosides play important roles in tumor phenotypes, and provide a rationale for a strategy of targeting gangliosides in cancer immunotherapy.

In the present study, we examined the effects of anti-GD2 mAb on the chemotherapy of SCLC, and showed that the mAb exhibited supra-additive effects on the action of various anti-cancer drugs. Furthermore, we show here that the combination of anti-GD2 mAb and anti-cancer

[3] To whom correspondence and reprint requests should be addressed.
E-mail: koichi@med.nagoya-u.ac.jp

drugs induced marked activation of c-Jun terminal kinase (JNK), leading to apoptosis much more strongly than when either agent was used alone. These results suggested that combination chemo-immunotherapy with anti-GD2 mAb is a promising approach to overcome intractable SCLC.

In this paper, ganglioside nomenclature is based on that of Svennerholm.[32)]

MATERIALS AND METHODS

Cell lines All human lung cancer cell lines were provided by Dr. T. Takahashi (Aichi Cancer Center Research Institute, Aichi) and were maintained in RPMI 1640 containing 10% fetal bovine serum in a humidified 5% CO_2 atmosphere at 37°C. To prepare the GD2 expressants (D-18 cells), human α 2,8-sialyltransferase expression vector[33)] was transfected into SCLC SK-LC-17 cells using "Lipofectamine" (Life Technologies, Inc., Rockville, MD). Stable transfectants were selected with 250 μg/ml G418 (Life Technologies, Inc.).

Drugs and chemicals Cisplatin (CDDP), doxorubicin, etoposide and paclitaxel were purchased from Sigma Chemical Co. (St. Louis, MO), SN-38 was provided by Daiichi Co. (Tokyo), and vinorelbine was obtained from Kyowa Hakko Kogyo Co., Ltd. (Tokyo). The anti-GD2 mAb (mAb 220-51, mouse IgG3) was generated in our laboratory.[34)] This antibody was purified using a Protein G affinity column, and the concentration of the protein was determined by Lowry's method.[35)]

Experimental design for drug testing We used the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as described previously.[31)] In brief, cells (8×10^4 cells/well) were prepared in 48-well plates in serum-containing medium and treated with or without 20 μg/ml of mAb 220-51, at 2 h before various anticancer drugs, appropriately diluted, were added. Following exposure to drugs for 48 h, MTT (Sigma Chemical Co.) was added to each well (0.5 mg/ml). After incubation for 2 h at 37°C, the supernatants were aspirated, and 100 μl of *n*-propyl alcohol containing 0.1% NP-40 and 4 m*M* HCl were added. The color reaction was quantitated using an automatic plate reader Immuno-Mini NJ-2300 (Nihon InterMed, Tokyo), at 590 nm with a reference filter of 620 nm. MTT assays were carried out in triplicate. For non-adherent SCLC lines, cells (5×10^4 cells/well) were prepared in 96-well plates in serum-containing medium and treated as described above, and then used for the MTT assay. In this case, lysis buffer (*n*-propyl alcohol with 0.1% NP-40 and 4 m*M* HCl) was added to each well without aspiration of the medium. Actual IC_{50} values, which were the concentrations of the drugs needed to produce 50% growth inhibition, were obtained from growth curves after exposure of cells to various concentrations of the drugs. IC_{50}s were determined as the means of three independently performed assays.

Flow cytometry The cell surface expression of ganglioside GD2 was analyzed by FACScan (Becton Dickinson, Mountain View, CA) using anti-GD2 mAb (mAb 220-51). The cells were incubated with anti-GD2 mAb for 45 min on ice and then stained with FITC-conjugated goat anti-mouse IgG (Cappel, Durham, NC). Control cells for flow cytometry were prepared using the second antibody alone. For quantification of positive cells, the CELLQuest program was used.

Western immunoblotting Cells (9×10^5 cells) were plated in 60-mm tissue culture plates in serum-containing medium and then stimulated with various agents. After treatment, cells were harvested with 0.02% EDTA, washed twice with ice-cold PBS and solubilized in lysis buffer (20 m*M* Tris-HCl pH 7.4, 1% NP-40, 10% glycerol, 50 m*M* NaF, 1 m*M* Na_3VO_4, 1 m*M* phenylmethylsulfonyl fluoride, and 20 units/ml aprotinin). Soluble proteins (50 μg/lane) were subjected to 10% or 15% SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was incubated with 5% dry milk in PBS overnight at 4°C, washed with PBS containing 0.05% Tween 20 and then incubated with antibodies reactive with either JNK, phospho-JNK (Cell Signaling Technology, Beverly, MA) or active caspase-3 (Pharmingen, San Diego, CA). Bands were detected with peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology) combined with an ECL kit (Amersham Pharmacia Biotech, Uppsala, Sweden).

DNA fragmentation assay Cells (3×10^6 cells) were treated with or without 20 μg/ml of mAb 220-51 in the presence of 50 μ*M* CDDP. After 48 h, cells were harvested and lysed in 100 μl of lysis buffer (10 m*M* Tris-HCl pH 7.4, 10 m*M* EDTA, and 0.5% Triton X-100) for 10 min at 4°C. After centrifugation, the supernatants were collected, and 2 μl of RNAase (10 mg/ml) and 2 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 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 m*M* Tris-acetate, 1 m*M* EDTA). The gel was observed under UV light.

TUNEL assay Cells (6×10^5 cells) were plated in 60-mm tissue culture plates in serum-containing medium and then treated with or without 80 μ*M* CDDP, 20 μg/ml of mAb 220-51, or 5 μ*M* curcumin (Sigma Chemical Co.) for 30 h at 37°C. After treatment, cells were harvested, fixed in 4% paraformaldehyde for 30 min at 4°C and permeabilized in 70% EtOH overnight at −20°C. Cells were then stained with FITC-conjugated terminal deoxynucleotidyl TUNEL reaction mixture (MEBSTAIN Apotosis Kit Direct; MBL, Nagoya) for 1 h at 37°C. The numbers of apoptotic cells were monitored by flow cytometry (FACScan; Becton

Dickinson) and determined using the CELLQuest program. Control cells for samples with or without curcumin were prepared by treatment with curcumin alone or DMSO (solvent of curcumin), respectively.

Statistical analysis Significance was determined by using Student's *t* test.

RESULTS

Enhancement of chemosensitivity of GD2-expressing lung cancer cells by anti-GD2 mAb To investigate the effects of anti-GD2 mAb on chemosensitivity, the cytotoxic effects of six currently used anticancer agents, CDDP, doxorubicin, etoposide, SN-38, paclitaxel and vinorelbine, against a GD2-expressing transfectant D18[31] (95.3% positive) cells in the presence of anti-GD2 mAb were analyzed by MTT assay (Fig. 1, A–F). Since anti-GD2 mAb alone gave 50% survival at approximately 20 μg/ml (Fig. 2A), we used the antibody at this concentration hereafter. In the case of CDDP, there was a great dif-

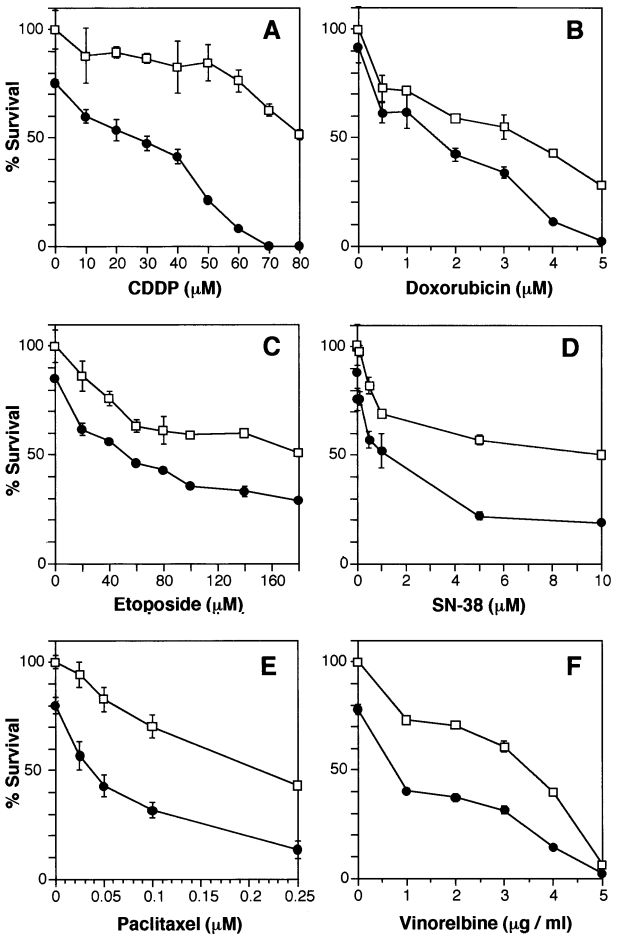

Fig. 1. Enhancement of the cytotoxic effects of various anticancer agents with anti-GD2 mAb. A GD2 expressant, D-18, was treated with or without mAb 220-51 (20 μg/ml) in the presence of anticancer agents, CDDP (A), doxorubicin (B), etoposide (C), SN-38 (D), paclitaxel (E) and vinorelbine (F), at the concentration indicated. After 48 h treatment, cells were subjected to MTT assay and the percentages of surviving cells were presented. Values are means of triplicate samples in a representative experiment; bars, SD. Similar results were obtained in three independent experiments. □, none; ●, mAb 220-51.

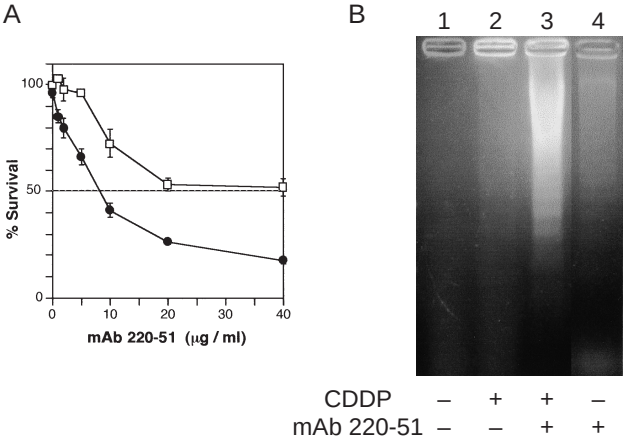

Fig. 2. Anti-GD2 mAb prominently enhanced the cytotoxicity of CDDP. A, dose dependency of the anti-GD2 mAb effect in the enhancement of the cytotoxic effects of CDDP. D-18 was treated with or without 50 μM CDDP in the presence of mAb 220-51 diluted to the concentration indicated for 48 h and subjected to MTT assay. Values are means of three independent experiments; bars, SD. □, none; ●, CDDP 50 μM. B, CDDP combined with anti-GD2 mAb showed prominent apoptosis induction. Cytoplasmic DNA prepared from D-18 after incubation with reagents as indicated in the figure (mAb 220-51, 20 μg/ml; CDDP, 50 μM) for 48 h was analyzed by agarose gel electrophoresis.

Table I. Enhancement of Drug Sensitivity by Anti-GD2 mAb

Drug	IC_{50}[a] (μM)		Enhancement of sensitivity (fold)
	Drug alone	Drug+mAb 220-51	
CDDP	86.66±4.16	18.66±8.08*	4.6
Doxorubicin	3.73±0.30	1.53±0.50*	2.4
Etoposide	171.6±36.8	43.66±10.06*	3.9
SN-38	10.66±3.05	1.36±0.37*	7.8
Paclitaxel	0.256±0.045	0.039±0.002*	6.6
Vinorelbine	3.76±0.37[b]	0.726±0.155[b],*	5.2

a) The IC_{50}s were determined as the concentrations of drugs that showed 50% growth inhibition in MTT assay. Values are means±SD of three independent experiments. Statistical significance when compared with the drug alone samples is presented. *, $P<0.01$.

b) Unit for vinorelbine is μg/ml.

ference in the dose-response curves between CDDP alone and CDDP plus anti-GD2 mAb combination treatment (Fig. 1A). Taking into account the IC_{50} values obtained from growth-inhibition curves, anti-GD2 mAb enhanced the chemosensitivity of the GD2 expressants for SN-38, paclitaxel and vinorelbine by 5- to 8-fold (Table I). On the other hand, when enhancement of the cytotoxic effects of anticancer drugs by anti-GD2 mAb was calculated from growth-inhibition curves, CDDP showed the highest value among these six drugs. Enhancement of cytotoxicity of CDDP and the other drugs amounted to 68% and 26–39%, respectively (Table II).

Table II. Enhancement of Cytotoxicity with Drugs by Anti-GD2 mAb

Drug	Survival rate (%)		Enhancement of cytotoxicity[a)] (%)
	Drug alone	Drug+mAb 220-51	
CDDP	76.29±5.02	8.08±0.48	68.2
Doxorubicin	42.69±0.79	11.32±0.30	31.4
Etoposide	59.88±1.04	33.19±2.07	26.7
SN-38	56.92±2.35	21.88±1.80	35.0
Paclitaxel	82.9±5.50	43.00±5.00	39.9
Vinorelbine	70.72±0.92	37.28±1.60	33.4

a) Enhancement of cytotoxic effects of anticancer drugs by anti-GD2 mAb was calculated from the growth inhibition curves presented in Fig. 1. Enhancement of cytotoxicity=% survival by drug alone−% survival by drug plus anti-GD2 mAb at the points where the subtracted values were maximum for individual drugs.

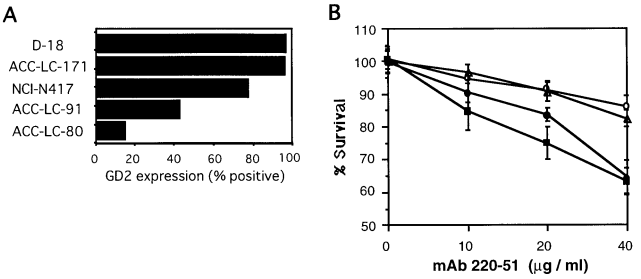

Fig. 3. Dose dependency of cytotoxic effect of anti-GD2 mAb alone against lung cancer cell lines with various levels of GD2 expression. A, GD2 expression levels of lung cancer cell lines determined by flow cytometry. ACC-LC-171, NCI-N417 and ACC-LC-80, small cell lung carcinoma; ACC-LC-91, large cell lung carcinoma. B, Cells (5×10^4 cells/well for ACC-LC-171 (●), NCI-N417 (■) and ACC-LC-80 (○), 2.5×10^4 cells/well for ACC-LC-91 (△)) were seeded in 96-well plates and treated with mAb220-51 at the concentration indicated for 48 h, then subjected to MTT assay. Values are means of triplicate samples in a representative experiment; bars, SD. Similar results were obtained in three independent experiments.

Anti-GD2 mAb enhanced apoptosis of lung cancer cells induced by CDDP in a dose-dependent manner This enhancement effect of CDDP cytotoxicity by anti-GD2 mAb depended on the antibody concentration (Fig. 2A). When the degree of apoptosis induced by CDDP alone and CDDP plus anti-GD2 mAb treatment was compared by DNA fragmentation assay, cells treated with the two agents showed apparently more DNA ladder formation than cells treated with CDDP alone (Fig. 2B). Cells treated with only anti-GD2 mAb also showed a low level of DNA ladder formation. The dose-dependent cytotoxic effect of anti-GD2 mAb against four lung cancer cell lines, in addition to D18, with various levels of GD2 expression was analyzed. GD2 expression was presented as % positive as determined with flow cytometry (Fig. 3A). Two SCLC cell lines with high GD2 levels showed lower % survival, and the other two lines showed higher % survival (Fig. 3B). The cytotoxicity was dependent on the antibody concentration. Moreover, enhancement of the chemosensitivity of these cells by anti-GD2 mAb was tested by MTT assay (Fig. 4, A–D). Not only high GD2 expressants such

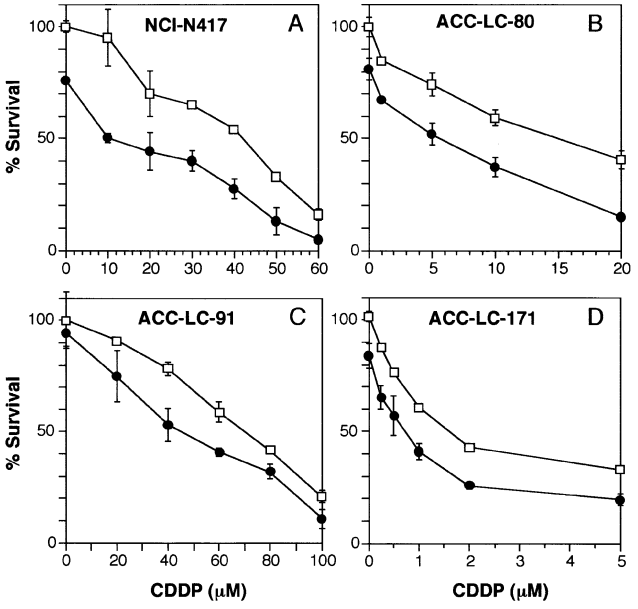

Fig. 4. Enhancement of chemosensitivity of lung cancer cell lines with various expression levels of GD2 by an anti-GD2 mAb. Cells (5×10^4 cells/well for NCI-N417 (A), ACC-LC-80 (B) and ACC-LC-171 (D), 2.5×10^4 cells/well for ACC-LC-91 (C)) were seeded in 96-well plates, treated with or without 20 μg/ml of mAb 220-51 in the presence of CDDP at the concentration indicated for 48 h, and subjected to MTT assay. Results are presented as in Fig. 1. GD2 expression levels are presented in Table III. NCI-N417, ACC-LC-80 and ACC-LC-171, small cell lung carcinoma; ACC-LC-91, large cell lung carcinoma. □, none; ●, mAb 220-51.

as NCI-N417 and ACC-LC-171, but also those with low GD2 levels, such as ACC-LC-80, showed reduced IC_{50} values of CDDP in the presence of anti-GD2 mAb (Table III). These findings suggested that the CDDP plus anti-GD2 mAb combination would be useful in chemo-immunotherapy for GD2-expressing lung cancers. Whether large cell lung cancers are also susceptible to this approach remain to be clarified.

JNK activation is a key event in apoptotic cell death induced by CDDP plus anti-GD2 mAb combination treatment To investigate the mechanisms of enhancement of CDDP cytotoxic effects by anti-GD2 mAb, phosphorylation levels of JNK, which is often activated during apoptosis induced by stress stimulation, were analyzed after exposure of cells to the six anticancer drugs tested in this study. At the concentration of drugs which showed 20% growth inhibition in 24 h, the highest phosphorylation of JNK was observed with CDDP (Fig. 5). To examine whether JNK activation is an important event for the combination effects of CDDP plus anti-GD2 mAb, the time course of the phosphorylation levels of JNK after drug exposure was analyzed. In the case of CDDP alone, JNK activation was observed at 12 h after the addition of CDDP, whereas it was already seen at 6 h after the addition of both CDDP and anti-GD2 mAb (Fig. 6, A and C). On the other hand, using anti-GD2 mAb alone, at high concentrations, but not apparently at lower concentrations, weak activation of JNK, 54 kDa isoform dominant, was observed after 1 h incubation (Fig. 6, B and D). These results suggest that JNK activation is a key event in the combination of CDDP plus anti-GD2 mAb.

To confirm this hypothesis, the effect of the JNK pathway inhibitor, curcumin, on the cytotoxicity of the combination treatment was studied. Phosphorylation of JNK was more prominent with the combination than with CDDP alone and activation of caspase-3 was also stronger in this case (Fig. 7A, lane 3). However, curcumin inhibited not

Table III. Enhancement of Sensitivities to CDDP by Anti-GD2 mAb in Lung Cancer Cell Lines with Various GD2 Levels

Cell line	GD2 expression (% positive)	IC_{50}[a)] (μM)		Enhancement of sensitivity (fold)
		CDDP alone	CDDP+mAb 220-51	
NCI-N417	77.2	42.66±3.05	9.33±1.15**	4.6
ACC-LC-80	14.2	17.6±2.62	6.16±1.60*	2.9
ACC-LC-91	42.0	76.66±7.63	37.00±8.54**	2.1
ACC-LC-171	95.6	1.96±0.47	0.80±0.17**	2.5

a) The IC_{50}s were determined as the concentration of CDDP that showed 50% growth inhibition in MTT assay. Values are means±SD of three independent experiments. *, $P<0.05$; **, $P<0.01$ compared with the drug alone samples.
GD2 expression levels (%) were determined by flow cytometry.

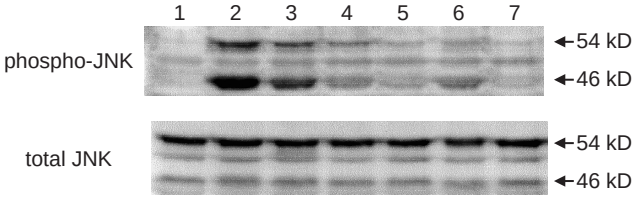

Fig. 5. Phosphorylation levels of JNK induced by anticancer agents. D-18 was incubated with anticancer drugs; none (lane 1), 80 μM CDDP (lane 2), 2.5 μg/ml of vinorelbine (lane 3), 140 μM etoposide (lane 4), 10 μM SN-38 (lane 5), 0.5 μM paclitaxel (lane 6), 4 μM doxorubicin (lane 7) for 24 h and the lysates were used for the 10% SDS-PAGE and immunoblotting with an antiphosphorylated JNK antibody (upper) or an anti-JNK antibody (lower) as described in "Materials and Methods."

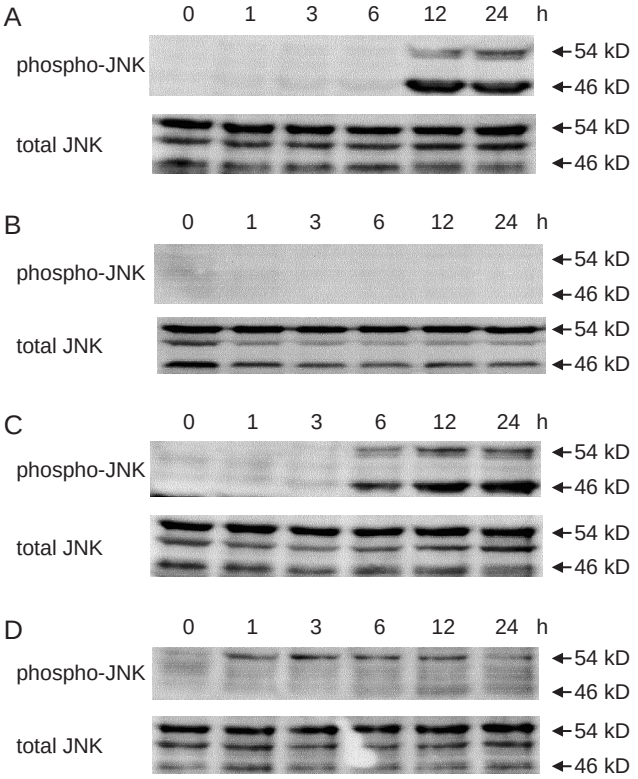

Fig. 6. Enhancement of JNK activation by the combination treatment with CDDP plus anti-GD2 mAb. D-18 was treated with or without mAb 220-51 (20 μg/ml) and 80 μM CDDP for the time indicated and the lysates were subjected to 10% SDS-PAGE and immunoblotting with an antiphosphorylated JNK antibody (upper) or an anti-JNK antibody (lower) as described in "Materials and Methods." A, CDDP 80 μM. B, mAb 220-51 20 μg/ml. C, mAb 220-51 20 μg/ml+CDDP 80 μM. D, mAb 220-51 40 μg/ml.

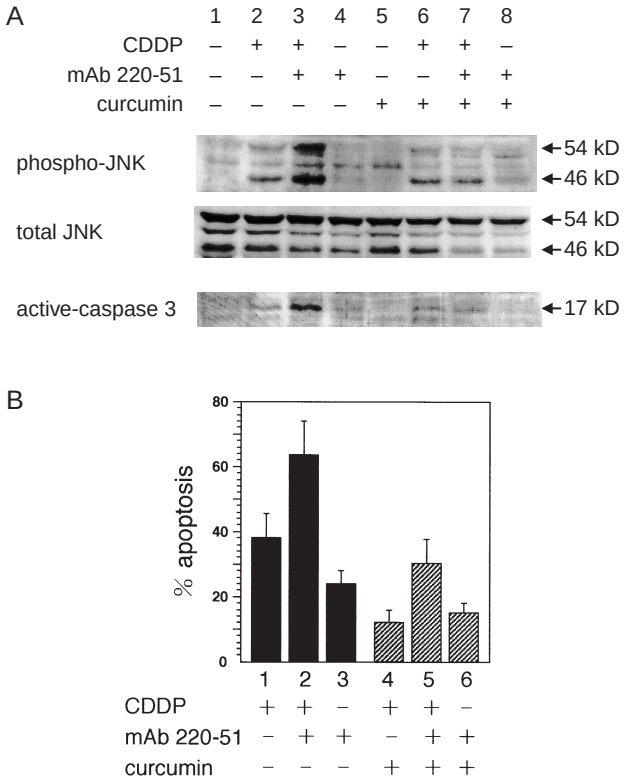

Fig. 7. JNK activation is a key process in the enhanced apoptotic cell death induced by the combination of CDDP plus anti-GD2 mAb. A, D-18 was treated with various combinations of 80 μM CDDP, 20 μg/ml of mAb 220-51, or 5 μM JNK pathway inhibitor, curcumin. Top, combination of the drugs used. After treatment (JNK for 12 h, caspase-3 for 20 h), samples were subjected to SDS-PAGE and immunoblotting (JNK for 10%, caspase-3 for 15%) as described in "Materials and Methods." Upper, phosphorylated JNK; middle, JNK; lower, active caspase-3. B, Suppression of apoptosis by a JNK pathway inhibitor. D-18 was treated with or without 80 μM CDDP, 20 μg/ml of mAb 220-51, 5 μM JNK pathway inhibitor, curcumin, by the combination as indicated at the bottom. After 30 h treatment, apoptotic cells were determined by TUNEL assay as described in "Materials and Methods."

only JNK phosphorylation, but also caspase-3 activation that was enhanced by anti-GD2 mAb (Fig. 7A, lane 7). Moreover, in the TUNEL assay, an increase in apoptotic cells induced by combination treatment was also suppressed by curcumin by 63.6% to 30.4% (Fig. 7B), suggesting that the combination effect was mainly mediated via the JNK pathway.

DISCUSSION

In attempts to apply mAbs reactive with cancer-associated antigens for the treatment of cancer patients, a number of devices have been employed to enhance the tumor-killing effects, to increase the accessibility of antibodies to tumor tissues,[36] and to avoid the xenogeneic immune reaction against mouse immunoglobulins. For example, mAbs have been conjugated with anti-cancer drugs such as neocarcinostatin, doxorubicin or toxin molecules such as ricin A, leading cytotoxic agents to the target sites matching the binding specificity of the mAbs. Conjugates with a radioisotope or immunoliposomal drug have also been tried. Therefore, chemotherapy has been widely combined with mAbs which suppress advantageous factors for tumor cell growth or survival, e.g. anti-glycoprotein P,[37] anti-interleukin 6,[38] anti-EGF receptor,[39, 40] or anti-VEGF.[41] Many of them enhanced the cytotoxicity of chemotherapeutic agents *in vitro* and *in vivo*. The combination of chemotherapy with anti-c-erbB2/HER2 or anti-CD20 antibodies has also been attempted[42–45] with outstanding results. These approaches are especially significant to increase the tumor-specific destructive effects of drugs and to enhance the magnitude of the toxic process.

Even when mAbs are combined with chemotherapy, it is likely to be better if the antigens recognized by mAbs are critical for the survival or viability of cells. A representative example is the Fas/Fas ligand system. A single anti-Fas mAb can, of course, induce apoptosis of cancer cells, and many anti-cancer drugs can sensitize tumor cells to Fas-mediated apoptosis.[46, 47] The reason why this approach is very efficient appears to be because the apoptosis induced with Fas/Fas ligand and that with anti-cancer drugs share the same pathway in their later phase.

Thus, the combination of anti-GD2 mAbs with chemotherapy can be expected to be markedly effective in the therapy of SCLC for many reasons. As reported previously, GD2 is scarcely expressed in the normal human body, while the expression in the tumor cells is clear. Furthermore, GD2 has been demonstrated to play important roles in cell proliferation and invasion of cultured SCLC cells, although the mechanisms remain to be clarified. The most attractive point of anti-GD2 mAbs is that a single use of the antibody can induce apoptosis of SCLC cells via the caspase cascade activation.[31] In view of the antibody specificity to SCLC cells, more efficient and exclusive destruction of tumor cells can be expected when the antibody is used together with anti-cancer agents. In fact, results obtained in the present study clearly showed the enhancing effects of anti-GD2 mAb on tumor suppression with various anti-cancer drugs, based on the unexpected amplification of JNK activation. Among anti-cancer drugs examined, CDDP showed the highest activation of JNK, though all of them induced its activation to some degree. This result corresponds well to the results on the enhancement of cytotoxicity, as shown in Fig. 1 and Table II. Fortunately, the combination of anti-GD2 mAb with CDDP showed a definite enhancement of sensitivity even for

low-GD2 expressants and a large cell lung carcinoma (Fig. 3 and Table III).

In the analysis of the mechanisms of cytotoxicity of anti-cancer drugs, many of them have turned out to be associated with apoptosis.[48, 49] Activation of the *p53* gene is frequently involved in the apoptotic process with anti-cancer drugs.[50–52] However, the *p53* gene is inactivated in the majority of SCLC cells, and another pathway of apoptosis induction in which *p53* is not involved might be present. Indeed, the SK-LC-17 cells used in the present study underwent apoptosis with CDDP via the activation of JNK and caspases. This was confirmed in the experiments with inhibitors of JNK.

JNK/SAPK, a member of mitogen-activated protein kinases (MAPK), is activated by various forms of environmental stresses.[53] JNK activation is one of the responses to genotoxic stresses, including some anti-cancer drugs,[54, 55] and Seimiya *et al.* demonstrated that JNK positively regulated drug-induced protease activation and apoptosis of U937 cells.[56] In the present study, anti-cancer drugs caused JNK activation to various extents, and anti-GD2 mAb weakly activated JNK. Inhibition analysis showed that this mAb effect was only partly mediated by JNK. Nevertheless, when anti-GD2 mAb and CDDP were combined, markedly enhanced activation of JNK was observed. This effect might be generated by different triggering pathways leading to JNK activation, resulting in synergistic phosphorylation of JNK and subsequent apoptotic events.

The roles of the Fc portions of antibodies in enhancing the antibody function via complement binding, effector recruitment for antibody-dependent cell-mediated cytotoxicity (ADCC) or effector inhibition need to be considered when we think of the *in vivo* effects of anti-ganglioside antibodies.[57] If these antibody-mediated mechanisms work well, more efficient anti-tumor effects than those observed *in vitro* may be obtained in *in vivo* application. Experiments to clarify these points are currently on-going in our laboratory.

Gangliosides, such as GD2 are amphipathic molecules enbedded in the outer layer of the plasma membrane.[1] Therefore, it appears difficult for them to directly mediate apoptosis signals to cytoplasmic signaling molecules. Whether GD2 clustering is important for the apoptosis induction, whether the apoptotic process is triggered in lipid rafts, and what molecule associates with the GD2/anti-GD2 complex are totally unknown, and remain fascinating themes in research to understand the apoptotic mechanisms of anti-glycolipid mAb.

ACKNOWLEDGMENTS

This work was supported by Grants-in-Aid for Scientific Research on Priority Areas (12217127, 10178104) and by a COE Research Grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan. This work was also supported by Grants-in-Aid from the Ministry of Health, Labour and Welfare of Japan. We thank Dr. K. Shitara at Kyowa Hakko Kogyo Co., Ltd. for helpful discussions, and Dr. Tougo at Daiichi Seiyaku Co. for kindly providing SN-38. We also thank Ms. T. Ando for technical assistance.

(Received February 4, 2002/Revised May 1, 2002/2nd Revised July 11, 2002/Accepted July 12, 2002)

REFERENCES

1) Wiegandt, H. Gangliosides. *In* "Glycolipids," ed. H. Wiegandt, pp. 199–260 (1985). Elsevier Science Publishers, B. V., Amsterdam.
2) Suzuki, K. The pattern of mammalian brain gangliosides. II. Evaluation of the extraction procedures, postmortem changes and the effect of formalin preservation. *J. Neurochem.*, **12**, 629–638 (1965).
3) Portoukalian, J., Zwingelstein, G. and Dore, J. F. Lipid composition of human malignant melanoma tumors at various levels of malignant growth. *Eur. J. Biochem.*, **94**, 19–23 (1979).
4) Carubia, J. M., Yu, R. K., Macala, L. J., Kirkwood, J. M. and Varga, J. M. Gangliosides of normal and neoplastic human melanocytes. *Biochem. Biophys. Res. Commun.*, **120**, 500–504 (1984).
5) Cheung, N. K., Sarrinen, U. M., Neely, J. E., Landmeier, B., Donovan, D. and Coccia, P. F. Monoclonal antibodies to a glycolipid antigen on human neuroblastoma cells. *Cancer Res.*, **45**, 2642–2649 (1985).
6) Fredman, P., von Holst, H., Collins, V. P., Ammar, A., Dellheden, B., Wahren, B., Granholm, L. and Svennerholm, L. Potential ganglioside antigens associated with human gliomas. *Neurol. Res.*, **8**, 123–126 (1986).
7) Chang, H. R., Cordon-Cardo, C., Houghton, A. N., Cheung, N. K. and Brennan, M. F. Expression of disialogangliosides GD2 and GD3 on human soft tissue sarcomas. *Cancer (Phila.)*, **70**, 633–638 (1992).
8) Yuyama, Y., Dohi, T., Morita, H., Furukawa, K. and Oshima, M. Enhanced expression of GM2/GD2 synthase mRNA in human gastrointestinal cancer. *Cancer*, **75**, 1273–1280 (1995).
9) Dohi, T., Hanai, N., Yamaguchi, K. and Oshima, M. Localization of UDP-GalNAc:NeuAc α 2,3Gal-R β1,4(GalNAc to Gal)N-acetylgalactosaminyltransferase in human stomach. Enzymatic synthesis of a fundic gland-specific ganglioside and GM2. *J. Biol. Chem.*, **266**, 24038–24043 (1991).
10) Merritt, W. D., Sztein, M. B. and Reaman, G. H. Detection of GD3 ganglioside in childhood acute lymphoblastic leukemia with monoclonal antibody to GD3: restriction to immunophenotypically defined T-cell disease. *J. Cell. Biochem.*,

37, 11–19 (1988).
11) Furukawa, K., Akagi, T., Nagata, Y., Yamada, Y., Shimotohno, K., Cheung, N. K., Shiku, H. and Furukawa, K. GD2 ganglioside on human T-lymphotropic virus type I-infected T cells: possible activation of β-1,4-*N*-acetylgalactosaminyltransferase gene by $p40^{tax}$. *Proc. Natl. Acad. Sci. USA*, **90**, 1972–1976 (1993).
12) Okada, M., Furukawa, K., Yamashiro, S., Yamada, Y., Haraguchi, M., Horibe, K., Kato, K., Tsuji, Y. and Furukawa, K. High expression of ganglioside α-2,8-sialyltransferase (GD3 synthase) gene in adult T-cell leukemia cells unrelated to the gene expression of human T-lymphotropic virus type I. *Cancer Res.*, **56**, 2844–2848 (1996).
13) Cheresh, D. A., Rosenberg, J., Mujoo, K., Hirschowitz, L. and Reisfeld, R. A. Biosynthesis and expression of the disialoganglioside GD2, a relevant target antigen on small cell lung carcinoma for monoclonal antibody-mediated cytolysis. *Cancer Res.*, **46**, 5112–5118 (1986).
14) Dnistrian, A. M. and Schwartz, M. K. Lipid-bound sialic acid as a tumor marker. *Ann. Clin. Lab. Sci.*, **13**, 137–142 (1983).
15) Sung, C. C., Pearl, D. K., Coons, S. W., Scheithauer, B. W., Johnson, P. C. and Yates, A. J. Gangliosides as diagnostic markers of human astrocytomas and primitive neuroectodermal tumors. *Cancer*, **74**, 3010–3022 (1994).
16) Nakamori, S., Furukawa, H., Hiratsuka, M., Iwanaga, T., Imaoka, S., Ishikawa, O., Kabuto, T., Sasaki, Y., Kameyama, M., Ishiguro, S. and Irimura, T. Expression of carbohydrate antigen sialyl Le(a): a new functional prognostic factor in gastric cancer. *J. Clin. Oncol.*, **15**, 816–825 (1997).
17) Sung, C. C., Pearl, D. K., Coons, S. W., Scheithauer, B. W., Johnson, P. C., Zheng, M. and Yates, A. J. Correlation of ganglioside patterns of primary brain tumors with survival. *Cancer*, **75**, 851–859 (1995).
18) Lode, H. N., Handgretinger, R., Schuermann, U., Seitz, G., Klingebiel, T., Niethammer, D. and Beck, J. Detection of neuroblastoma cells in CD34+ selected peripheral stem cells using a combination of tyrosine hydroxylase nested RT-PCR and anti-ganglioside GD2 immunocytochemistry. *Eur. J. Cancer*, **33**, 2024–2030 (1997).
19) Houghton, A. N., Mintzer, D., Cordon-Cardo, C., Welt, S., Fliegel, B., Vadhan, S., Carswell, E., Melamed, M. R., Oettgen, H. F. and Old, L. J. Mouse monoclonal IgG3 antibody detecting GD3 ganglioside: a phase I trial in patients with malignant melanoma. *Proc. Natl. Acad. Sci. USA*, **82**, 1242–1246 (1985).
20) Irie, R. F. and Morton, D. L. Regression of cutaneous metastatic melanoma by intralesional injection with human monoclonal antibody to ganglioside GD2. *Proc. Natl. Acad. Sci. USA*, **83**, 8694–8698 (1986).
21) Cheung, N. K., Cheung, I. Y., Canete, A., Yeh, S. J., Kushner, B., Bonilla, M. A., Heller, G. and Larson, S. M. Antibody response to murine anti-GD2 monoclonal antibodies: correlation with patient survival. *Cancer Res.*, **54**, 2228–2233 (1994).
22) Cheung, N. K., Kushner, B. H., Yeh, S. D. and Larson, S. M. 3F8 monoclonal antibody treatment of patients with stage 4 neuroblastoma: a phase II study. *Int. J. Oncol.*, **12**, 1299–1306 (1998).
23) Pegram, M. and Slamon, D. Biological rationale for HER2/neu (c-erbB2) as a target for monoclonal antibody therapy. *Semin. Oncol.*, **27**, 13–19 (2000).
24) Treon, S. P. and Anderson, K. C. The use of rituximab in the treatment of malignant and nonmalignant plasma cell disorders. *Semin. Oncol.*, **27**, 79–85 (2000).
25) Dickman, S. Antibodies stage a comeback in cancer treatment. *Science*, **280**, 1196–1197 (1998).
26) Cheresh, D. A., Pierschbacher, M. D., Herzig, M. A. and Mujoo, K. Disialogangliosides GD2 and GD3 are involved in the attachment of human melanoma and neuroblastoma cells to extracellular matrix proteins. *J. Cell Biol.*, **102**, 688–696 (1986).
27) Nakano, J., Yasui, H., Lloyd, K. O. and Muto, M. Biologic roles of gangliosides G (M3) and G (D3) in the attachment of human melanoma cells to extracellular matrix proteins. *J. Investig. Dermatol. Symp. Proc.*, **4**, 173–176 (1999).
28) Dippold, W. G., Knuth, A. and Meyer Zum, B. K. H. Inhibition of human melanoma cell growth *in vitro* by monoclonal anti-GD3-ganglioside antibody. *Cancer Res.*, **44**, 806–810 (1984).
29) Nakano, J., Raj, B. K., Asagami, C. and Lloyd, K. O. Human melanoma cell lines deficient in GD3 ganglioside expression exhibit altered growth and tumorigenic characteristics. *J. Invest. Dermatol.*, **107**, 543–548 (1996).
30) Thurin, J., Thurin, M., Herlyn, M., Elder, D. E., Steplewski, Z., Clark, W. H., Jr. and Koprowski, H. GD2 ganglioside biosynthesis is a distinct biochemical event in human melanoma tumor progression. *FEBS Lett.*, **208**, 17–22 (1986).
31) Yoshida, S., Fukumoto, S., Kawaguchi, H., Sato, S., Ueda, R. and Furukawa, K. Ganglioside G (D2) in small cell lung cancer cell lines: enhancement of cell proliferation and mediation of apoptosis. *Cancer Res.*, **61**, 4244–4252 (2001).
32) Svennerholm, L. Chromatographic separation of human brain gangliosides. *J. Neurochem.*, **10**, 613–623 (1963).
33) Haraguchi, M., Yamashiro, S., Yamamoto, A., Furukawa, K., Takamiya, K., Lloyd, K. O., Shiku, H. and Furukawa, K. Isolation of GD3 synthase gene by expression cloning of GM3 α-2,8-sialyltransferase cDNA using anti-GD2 monoclonal antibody. *Proc. Natl. Acad. Sci. USA*, **91**, 10455–10459 (1994).
34) Miyazaki, H., Fukumoto, S., Okada, M., Hasegawa, T. and Furukawa, K. Expression cloning of rat cDNA encoding UDP-galactose:GD2 β1,3-galactosyltransferase that determines the expression of GD1b/GM1/GA1. *J. Biol. Chem.*, **272**, 24794–24799 (1997).
35) Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275 (1951).
36) Hudson, P. J. Recombinant antibody constructs in cancer

therapy. *Curr. Opin. Immunol.*, **11**, 548–557 (1999).
37) Watanabe, T., Naito, M., Kokubu, N. and Tsuruo, T. Regression of established tumors expressing P-glycoprotein by combinations of adriamycin, cyclosporin derivatives, and MRK-16 antibodies. *J. Natl. Cancer Inst.*, **89**, 512–518 (1997).
38) Mizutani, Y., Bonavida, B., Koishihara, Y., Akamatsu, K., Ohsugi, Y. and Yoshida, O. Sensitization of human renal cell carcinoma cells to *cis*-diamminedichloroplatinum (II) by anti-interleukin 6 monoclonal antibody or anti-interleukin 6 receptor monoclonal antibody. *Cancer Res.*, **55**, 590–596 (1995).
39) Mendelsohn, J. Epidermal growth factor receptor inhibition by a monoclonal antibody as anticancer therapy. *Clin. Cancer Res.*, **3**, 2703–2707 (1997).
40) Ciardiello, F., Bianco, R., Damiano, V., De Lorenzo, S., Pepe, S., De Placido, S., Fan, Z., Mendelsohn, J., Bianco, A. R. and Tortora, G. Antitumor activity of sequential treatment with topotecan and anti-epidermal growth factor receptor monoclonal antibody C225. *Clin. Cancer Res.*, **5**, 909–916 (1999).
41) Margolin, K., Gordon, M. S., Holmgren, E., Gaudreault, J., Novotny, W., Fyfe, G., Adelman, D., Stalter, S. and Breed, J. Phase Ib trial of intravenous recombinant humanized monoclonal antibody to vascular endothelial growth factor in combination with chemotherapy in patients with advanced cancer: pharmacologic and long-term safety data. *J. Clin. Oncol.*, **19**, 851–856 (2001).
42) Pegram, M. D. and Slamon, D. J. Combination therapy with trastuzumab (Herceptin) and cisplatin for chemoresistant metastatic breast cancer: evidence for receptor-enhanced chemosensitivity. *Semin. Oncol.*, **26**, 89–95 (1999).
43) Burris, H. A., 3rd. Docetaxel (Taxotere) plus trastuzumab (Herceptin) in breast cancer. *Semin. Oncol.*, **28**, 38–44 (2001).
44) Crown, J. P. The platinum agents: a role in breast cancer treatment? *Semin. Oncol.*, **28**, 28–37 (2001).
45) Agus, D. B., Bunn, P. A., Jr., Franklin, W., Garcia, M. and Ozols, R. F. HER-2/neu as a therapeutic target in non-small cell lung cancer, prostate cancer, and ovarian cancer. *Semin. Oncol.*, **27**, 53–63 (2000).
46) Mizutani, Y., Okada, Y., Yoshida, O., Fukumoto, M. and Bonavida, B. Doxorubicin sensitizes human bladder carcinoma cells to Fas-mediated cytotoxicity. *Cancer*, **79**, 1180–1189 (1997).
47) Pace, E., Melis, M., Siena, L., Bucchieri, F., Vignola, A. M., Profita, M., Gjomarkaj, M. and Bonsignore, G. Effects of gemcitabine on cell proliferation and apoptosis in non-small-cell lung cancer (NSCLC) cell lines. *Cancer Chemother. Pharmacol.*, **46**, 467–476 (2000).
48) Makin, G. and Hickman, J. A. Apotosis and cancer chemotherapy. *Cell Tissue Res.*, **301**, 143–152 (2000).
49) Debatin, K. Activation of apoptosis pathways by anticancer treatment. *Toxicol. Lett.*, **112-113**, 41–48 (2000).
50) Lowe, S. W. Cancer therapy and p53. *Curr. Opin. Oncol.*, **7**, 547–553 (1995).
51) Fan, S., el-Deiry, W. S., Bae, I., Freeman, J., Jondle, D., Bhatia, K., Fornace, A. J., Jr., Magrath, I., Kohn, K. W. and O'Connor, P. M. p53 gene mutations are associated with decreased sensitivity of human lymphoma cells to DNA damaging agents. *Cancer Res.*, **54**, 5824–5830 (1994).
52) Kanamori, Y., Kigawa, J., Minagawa, Y., Irie, T., Oishi, T., Shimada, M., Takahashi, M., Nakamura, T., Sato, K. and Terakawa, N. A newly developed adenovirus-mediated transfer of a wild-type p53 gene increases sensitivity to *cis*-diamminedichloroplatinum (II) in p53-deleted ovarian cancer cells. *Eur. J. Cancer*, **34**, 1802–1806 (1998).
53) Kyriakis, J. M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E. A., Ahmad, M. F., Avruch, J. and Woodgett, J. R. The stress-activated protein kinase subfamily of c-Jun kinases. *Nature*, **369**, 156–160 (1994).
54) Liu, Y., Gorospe, M., Yang, C. and Holbrook, N. J. Role of mitogen-activated protein kinase phosphatase during the cellular response to genotoxic stress. Inhibition of c-Jun N-terminal kinase activity and AP-1-dependent gene activation. *J. Biol. Chem.*, **270**, 8377–8380 (1995).
55) van Dam, H., Wilhelm, D., Herr, I., Steffen, A., Herrlich, P. and Angel, P. ATF-2 is preferentially activated by stress-activated protein kinases to mediate c-jun induction in response to genotoxic agents. *EMBO J.*, **14**, 1798–1811 (1995).
56) Seimiya, H., Mashima, T., Toho, M. and Tsuruo, T. c-Jun NH2-terminal kinase-mediated activation of interleukin-1beta converting enzyme/CED-3-like protease during anticancer drug-induced apoptosis. *J. Biol. Chem.*, **272**, 4631–4636 (1997).
57) Houghton, A. N. and Scheinberg, D. A. Monoclonal antibody therapies—a 'constant' threat to cancer. *Nat. Med.*, **6**, 373–374 (2000).