Biochemical and Biophysical Research Communications **276,** 278–285 (2000)
doi:10.1006/bbrc.2000.3341, available online at http://www.idealibrary.com on IDEAL®

Norcantharidin-Induced Post-G_2/M Apoptosis Is Dependent on Wild-Type p53 Gene

Chi-Yuan Hong,*[,1] Shen C. Huang,*[,1] Sze-Kwan Lin,* Jihjong J. Lee,† Ling-Ling Chueh,† Chien-Hsin K. Lee,‡ Jen-Hsou Lin,§ and Michael Hsiao†[,¶,2]

**Department of Dentistry, †Department of Veterinary Medicine, ‡Department of Biochemistry, and §Department of Animal Science, National Taiwan University, Taipei, Taiwan, Republic of China; and ¶Department of Medical Education and Research, Kaohsiung Veterans General Hospital, Kaohsiung, Taiwan, Republic of China*

Received July 10, 2000

Norcantharidin (NCTD), a synthetic analogue of phosphatase type 2A inhibitors, cantharidin, was shown to have limited effects in treating human and animal tumors. The tumor cell killing mechanisms by norcantharidin, however, remain unclear. In this report, we wished to investigate the mechanisms of norcantharidin-mediated cytotoxicity. Effort was made to investigate whether norcantharidin exerted its cytotoxicity through a p53-dependent or -independent mechanism. RT-2 (wtp53) and U251 (mutant p53) glioblastoma cell lines were exposed to norcantharidin at different dosages. Time-course fluorescent-activated cell sorting (FACS) analysis showed that high doses of norcantharidin arrested the cells at the G_2/M phase and subsequent post-G_2/M apoptosis in RT-2 cell line. In comparison, the U251 cell line was found resistant to norcantharidin-induced cytotoxicity. Restoring wild-type p53 gene function in the U251 cell line after adenoviral infections induced tumor cell cytotoxicity after exposure to norcantharidin. These results showed that norcantharidin kills tumor cells efficiently corresponding to their endogenous p53 gene status. The results also showed the feasibility of using adenoviral p53 gene therapy to enhance chemosensitivity of tumor cells to norcantharidin. © 2000 Academic Press

Key Words: **norcantharidin; glioblastoma; p53; G_2/M arrest; apoptosis.**

Norcantharidin (NCTD), a demethylated analogue of cantharidin which is the toxic constituent of blister beetles and the active ingredient of the purported aphrodisiac Spanish fly, appeared to inhibit protein phosphatase type 2A (PP2A) (1, 2). Besides the toxic effect due to the binding, NCTD inhibits several tumor cell types including HeLa cells, murine ascites hepatoma and reticulocell sarcoma. There were, however, some cell types such as murine erythroid leukemia cells *in vitro,* S180 *in vivo* and Walker tumor in rats where the drug showed little effect (3). Clinical trials indicated that cantharidin, the parent formed of NCTD, had effects on patients with primary hepatoma. The application, however, was limited by its severe nephrotoxic and inflammatory side effects and mainly been used in the gastrointestinal tract, ureter, and kidney (3). The NCTD appeared to improve the down side of cantharidin that made the drug safer in application. It had shown the effect in inhibiting the proliferation of several tumor cell lines including HeLa, CHO, CaEs-17, BEL-7402, SMMC7721, HEP-2, and human epidermoid laryngocarcinoma (3).

Although NCTD was shown effective in inhibiting tumor cell proliferation and was used to treat cancers in China since 1984 (3). There were, however, very few reports investigating the cytotoxic effects of NCTD on tumor cells. The molecular and cellular effects of NCTD have not yet been elucidated. Recent studies indicated that this new antitumor agent might induce G_1/S transition at lower dosage and caused murine myeloblast mitotic arrest at higher dosage (4). In this study, we investigated the antitumor molecular mechanism of NCTD and sought to control the growth of tumor cells with the new antitumor agent. We showed the effects of NCTD in the RT-2 and U251 cells. At high dose, norcantharidin killed tumor cells through apoptosis correspond to their p53 status. Evidences indicated that p53 plays a role in the induction of G_2/M arrest as well as apoptosis after expose to norcantharidin.

[1] The first two authors contributed equally to this paper.

[2] To whom correspondence and reprint requests should be addressed at Department of Medical Education and Research, Kaohsiung Veterans General Hospital, 386 Ta-Chung 1st Road, Kaoshiung, Taiwan, Republic of China. Fax: (886)7-3468056. E-mail: mhsiao@isca.vghks.gov.tw.

0006-291X/00 $35.00

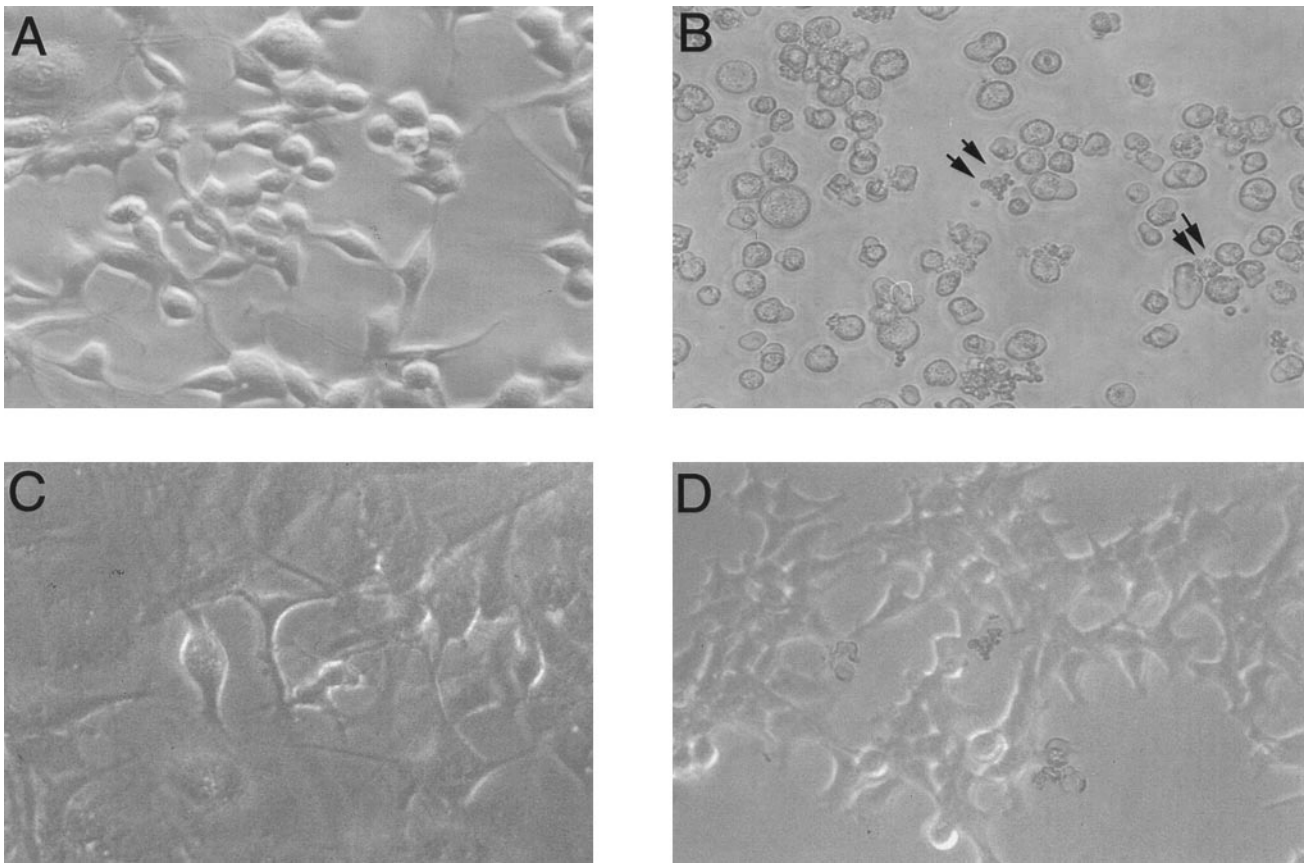

FIG. 1. Morphology of RT-2 and U251 cells exposed to NCTD. (A) RT-2 cells without NCTD exposure. (B) RT-2 cells exposed to NCTD for 24 h. Note significant cell rounding-up and occasional membrane blebbing (double arrow). (C) U251 cells without NCTD exposure. (D) U251 cells exposed to NCTD for 24 h. Note most cells still attached to the plate.

MATERIALS AND METHODS

Cell line. RT-2 (wild-type p53) and U251 (mutant p53) glioblastoma cell lines were grown in Dulbecco's modified Eagle medium (DMEM, high glucose) with 10% bovine calf serum (Gibco/BRL, MD) (5). The cell line has a 95% plating efficiency with doubling time about 12–18 h. Both cell lines produced p53 protein which can be detected with p53-specific antibody (DO1 for U251 and Pab 421 for RT2, Santa Cruz Biotechnology, CA).

Flow cytometry analysis. Tumor cells were harvested and stained with propidium iodine (PI) (Sigma, MO) to determine the DNA indexes after flow cytometry analysis. Briefly, 18 h after plating, tumor cells were treated with different doses of NCTD (ranging from 10 μg/ml to 100 μg/ml). Cells were collected at the indicated time interval and fixed in ice-cold methanol/PBS (2:1 ratio) for a minimum of 30 min. Tumor DNA was then stained in 500 μl of 50 μg/ml PI and 5 μl of 100 mg/ml RNase (Sigma, MO) for 30 min in the dark. DNA contents of the tumor cells were then determined by Fluorescent Activated Cell Sorter (FACS, Becton–Dickinson, CA).

TdT-mediated dUTP nick end-labeling (TUNEL) analysis. TUNEL staining (Boehringer-Mannheim, IN) was used to determine the percentage of the cells undergoing apoptosis. This can be done by enzymatic *in situ* labeling of apoptosis induced DNA strand breaks. DNA polymerase as well as terminal deoxynucleotidyl transferase (TdT) has been used to incorporate labeled nucleotides to DNA strand breaks in situ. Cells were spun onto slides coated with adhesion agent (VectaBond, Vector, CA) using Cytospin Centrifuge (Shandon, England) at 500 rpm for 5 min. Cells were dried and fixed in 4% freshly prepared paraformaldehyde solution in PBS at pH 7.4 for 30 min at room temperature. Cells were then permeabilized in 0.1% Triton X-100 and 0.1% sodium citrate solution on ice for 15 min. After washing twice with PBS, TUNEL reaction mixtures (add enzyme solution 5 μl to the 45 μl label solution to obtain 50 μl TUNEL reaction mixture for each sample) were added. The samples with staining mixtures were kept in the dark and incubated for 60 min at 37°C. PI solution (2.5 μg/ml) was used to counterstain the nucleus for 2 min and subjected to fluorescent microscope (Nikon, Japan) observation.

Construction, propagation, and infection of high titered recombinant wtp53 adenovirus. Full-length human wild-type p53 gene (a 2.1 kb *Eco*RI fragment) (6) was cloned into pCA4 adenoviral shuttle vector (Microbix, Ontario, Canada) to generate adenoviral vector encoding wtp53 gene. pCA4wtp53 plasmid and pJM17 adenoviral parental plasmid were cotransfected into 293 cells by $CaCl_2$ method recommended by the manufacturer. The recombinant viruses were purified from isolated plaques and amplified. The adenoviruses containing the transgene were identified by restriction fragment analysis, and tested for the transgene activity (data not shown). High titered adenoviruses were obtained after purified by two CsCl gradient centrifugations, dialyzed against buffer containing 10 mm Tris–Cl, pH 7.5, 1 mm $MgCl_2$ and 135 mm NaCl and stored in 10% glycerol at −80°C. The viral titers were determined using plaque forming assay in 293 cells. The high-titered wtp53 adenovirus (5×10^{11} pfu/ml) was used to insert wtp53 gene function in glioblastoma cells. The purified high titered wtp53 adenovirus was used to infect glioblastoma cell line *in vitro.* Briefly, 1×10^6 tumor cells were plated

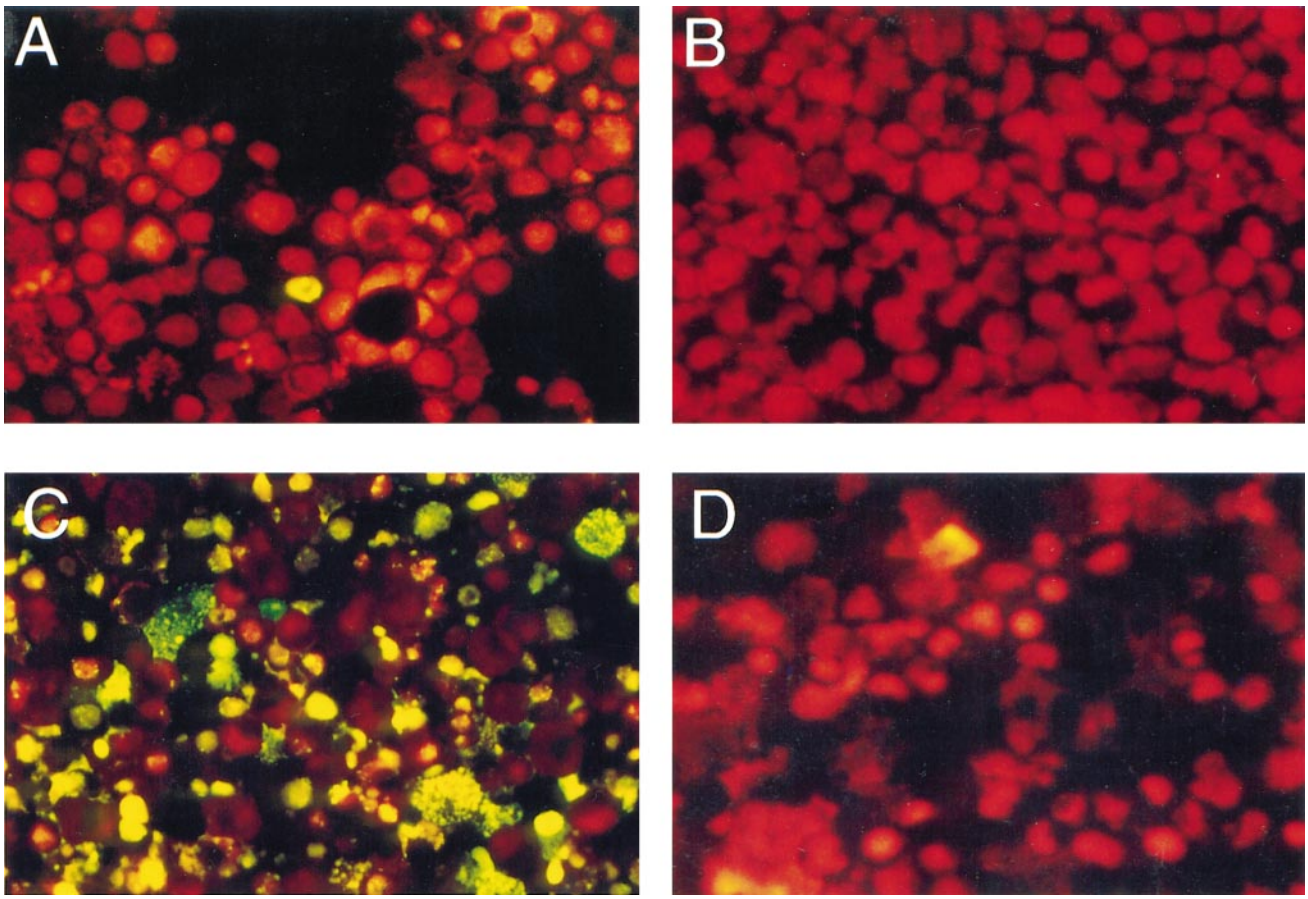

FIG. 2. Induction of apoptosis by NCTD in RT-2 and U251 tumor cells by TUNEL analysis. (A) U251 cells exposed to NCTD for 24 h. (B) U251 cells without NCTD exposure. (C) RT-2 cells exposed to NCTD for 24 h. Note that more than 70% cells were TUNEL positive. (D) RT-2 cells without NCTD (control).

for 18 h in 6-well plates. Adenoviruses at various multiplicities of infection (MOI = 1–1000) were used to infect the cells for 2 h in 1 ml of infection medium composed of DMEM with 2% L-glutamine (Gibco/BRL, MD), 2% fetal bovine serum (FBS, Hyclone, UT), and 1% antibiotic–antimycotic at 37°C in 5% CO_2. The infected tumor cells were fed by the addition of 1 ml of 20% FBS DMEM and returned to culture at 37°C in 5% CO_2. Fifteen hours after infection, cells were exposed to various doses of norcantharidin. The tumor cell cytotoxicity was determined by MTT assay and FACS analysis.

RESULTS AND DISCUSSION

This report aimed at investigating whether wild-type p53 gene played a role in norcantharidin induced tumor cytotoxicity. Morphological observations, flow cytometry, and TUNEL analyses were employed to determine whether norcantharidin induced growth arrest and/or apoptosis through p53 activation.

Norcantharidin-induced significant changes in tumor morphology. RT-2 (wild-type p53) and U251 (mutant p53) glioblastoma cell lines were treated with various doses of NCTD (10 μg/ml to 100 μg/ml) for 24 h to determine whether NCTD-induced apoptosis in these cell lines. At 50 μg/ml dose, NCTD induced significant proportion of the cells rounding up and membrane blebbing morphology (Fig. 1B, double arrow, 200×) in RT-2 cells compared to control parental spindle cell morphology (Fig. 1A, 200×) by light microscopic observation. In comparisons, mutant p53 cell line U251, retained parental morphology after exposed to 50 μg/ml NCTD for 24 h (Fig. 1D, 200×, controls in Fig. 1C). ID 50 of RT-2 tumor cells was found to be at 32 μg/ml compared to U251 at 125 μg/ml (Hong *et al.*, manuscript in preparation). Norcantharidin, the demethylated analogue of cantharidin, appeared to cause cell death effectively. It had been shown that NCTD inhibited the proliferation of several tumor cell lines. When long-term incubations with NCTD, tumor cell lines including HL-60 and K562 had shown to be inhibited the mass cell production implicated the efficacy of its antitumor ability (4). There was, however, little mention about the morphologic changes in these articles. In this experiment, we had shown an apoptotic change dependent to the status of p53 in the cells where NCTD acted on.

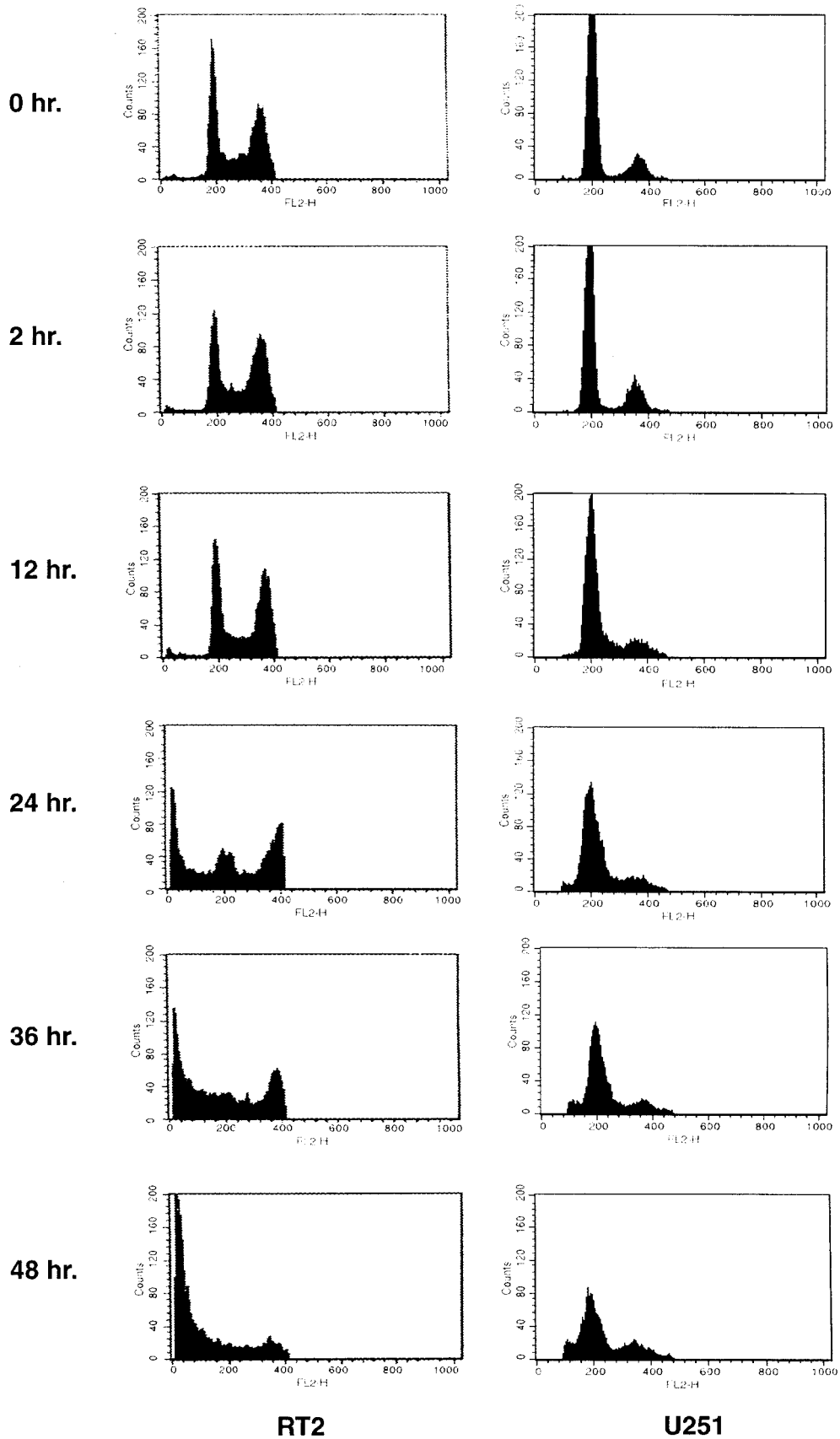

FIG. 3. Time-course histograms showing cell cycle analysis in RT-2 and U251 cell lines after exposed to NCTD. Horizontal and vertical axes indicated DNA content and cell number, respectively. The peaks on channel 200 and 400 representing G_1 and G_2/M were shown. Note RT-2 cells accumulated at G_2/M phase 24 h after exposed to NCTD. The subsequent accumulation of cells at sub-G_1 phase and increasing with time were also noticed.

Induction of apoptosis in tumor cells by norcantharidin. TUNEL staining analysis was employed to confirm whether RT-2 cells with rounding-up and membrane blebbing morphology underwent apoptosis. RT-2 after exposed to NCTD for 24 h were cytospun onto glass slides and stained by TUNEL staining solutions. The results showed more than 70% RT-2 cells underwent apoptosis (Fig. 2C, yellow fluorescence cells, 200×) compared to controls (Fig. 2D, 200×). In comparisons, less than 5% of U251 cells underwent apoptosis by TUNEL analysis (Fig. 2A, yellow fluorescence; controls Fig. 2B, 200×). Propidium iodide (PI) was used to stain the nucleus of the normal tumor cells (large, round morphology and red fluorescence, Figs. 2A, 2B, and 2D) and apoptotic tumor cells (small, dense morphology overlapping red and green fluorescence, Fig. 2C). Some RT-2 cells after exposed to NCTD showed typical apoptotic bodies (small, dense and fragmented morphology with yellow fluorescence, Fig. 2C). Claims of neuronal apoptosis induced by various agents and condition had regularly been stated, however, little data indicated their molecular mechanisms. Our results showed that glioblastoma cell line with wild-type p53 genotype (RT-2) was more sensitive to NCTD induced cytotoxicity through apoptosis. Mutant p53 encoding glioblastoma cell line (U251) was resistant to NCTD induced cytotoxicity. These observations implied a possible involvement of p53 gene product in regulating NCTD induced apoptosis.

Norcantharidin induced G_2/M arrest and subsequent apoptosis in tumor cells. To determine whether NCTD affected the cell cycle distributions, wild-type and mutant p53 tumor cells were exposed to NCTD and analyzed by fluorescent activated cell sorter (FACS) at different time points (0, 2, 12, 24, 36, and 48 h). RT-2 cells arrested at G_2/M at 12 h and peak at 24 h (61 ± 6%) after exposed to NCTD at 50 μg/ml (Figs. 3 and 4A). In comparison, U251 cells did not showed either G_1 or G_2/M growth arrest after exposed to NCTD at 50 μg/ml for 48 h (Figs. 3 and 4B). In addition, high percentage of RT-2 cells (55 ± 4%) accumulated at sub-G_1 phase (apoptosis) 48 h after exposed to NCTD (Fig. 4C). U251 cell line, however, only showed very few cells at sub-G_1 phase (3.1 ± 1.3%) after expose to NCTD for 48 h (Fig. 4C). This interesting feature of NCTD indicated that it could induce G_2/M arrest and post-G_2/M apoptosis in tumor cells with endogenous wild-type p53 genotype and not in tumor cells with mutant p53 genotype. The G_2/M growth arrest was speculated to be resulting from the activation of a protein kinase, Chk1, which phosphorylated and inhibited the function of the protein phosphatase Cdc25C (7). The inhibition of Cdc25C would further prevent the removal of inhibitory phosphates from Cdc2, a protein kinase that complexes with mitotic cyclins and was required for mitotic entry, which might result in G_2/M

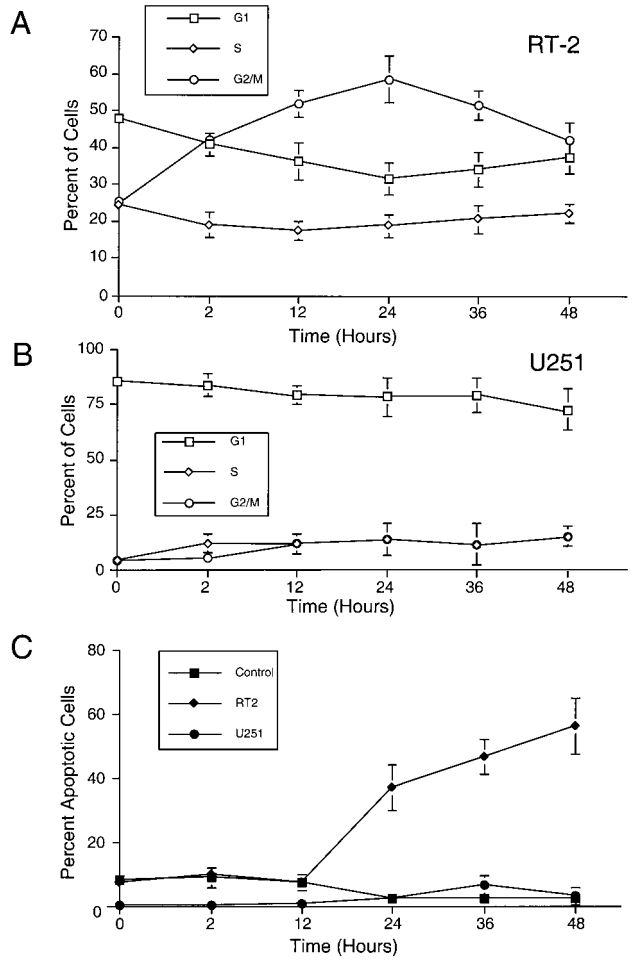

FIG. 4. Percent of RT-2 and U251 cells in cell cycle distribution and apoptosis after exposed to NCTD by flow cytometry analysis. (A) RT-2 cell cycle distribution. Note significant G_2/M arrest 24 h after exposed to NCTD. (B) U251 cell cycle distribution. (C) Percentage of apoptosis cells in RT-2 and U251 cells after exposed to NCTD. Note the induction of apoptosis in RT-2 cells and not in U251 cells or control.

arrest (7–9). Research, however, had also shown that G_2/M arrest after DNA damage in both human and murine tumor cells in the absence of functional p53 or p21 gene (7, 10). In this report, we showed that NCTD induced G_2/M arrest and post-G_2/M apoptosis through wild-type p53-dependent pathway.

Wild-type p53 gene insertions/expressions enhanced norcantharidin cytotoxicity in tumor cells with mutant p53. To investigate if wild-type p53 insertions enhanced mutant p53 tumor cell cytotoxicity to NCTD, high titered p53 adenovirus (5×10^{11} pfu/ml) was used to infect U251 glioblastoma cells resistant to NCTD treatment. More than 90% of the tumor cells were found expressing wild-type p53 (detected by staining positive for wild-type p53 specific antibody pAb1620) after infected with 100 m.o.i. (multiplicity of infection) of p53 adenovirus (data not shown). This m.o.i. was

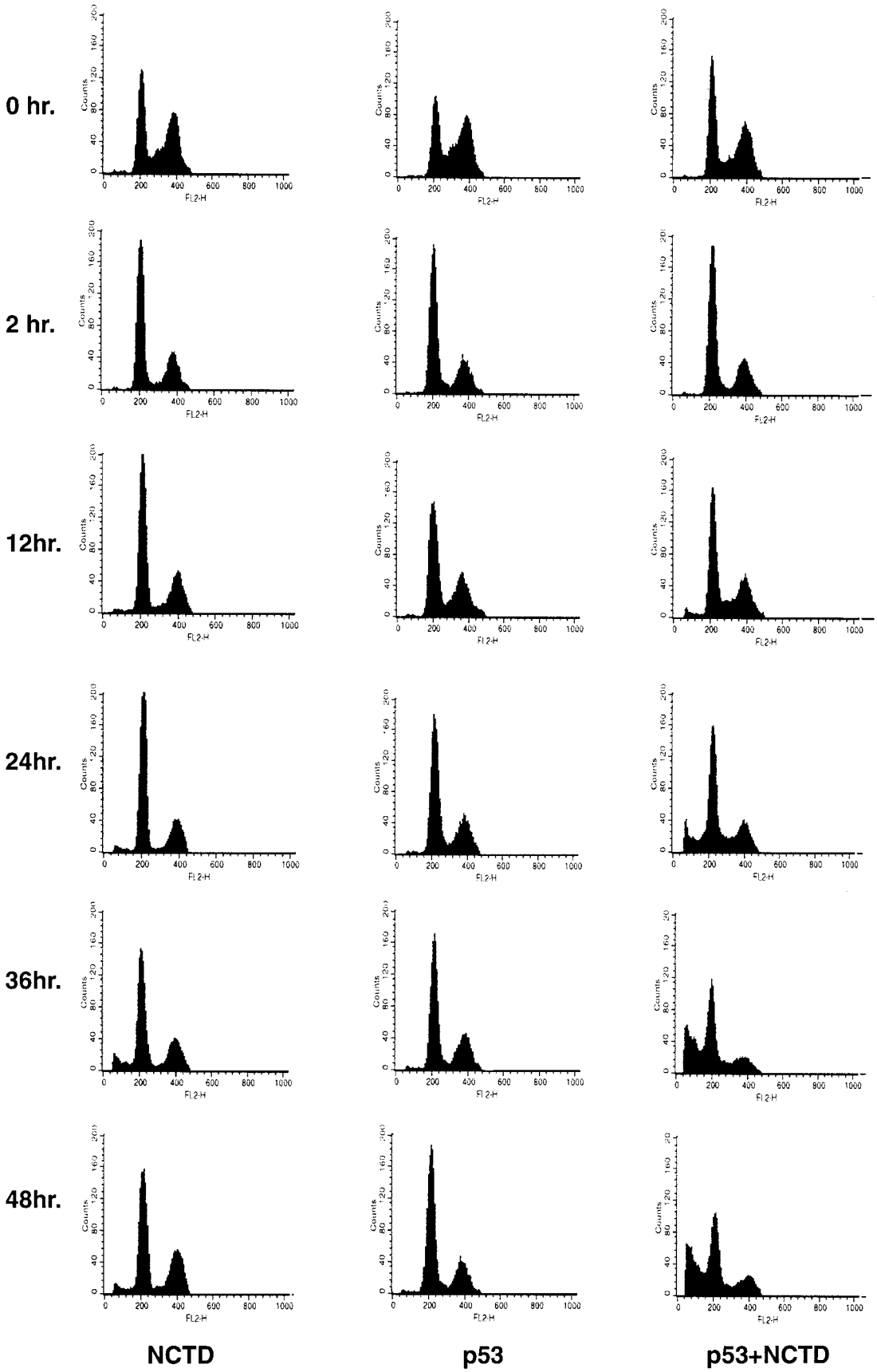

FIG. 5. Time-course FACS histograms showing cell cycle analysis in U251 cell line infected with wtp53 adenovirus and subsequent exposure to NCTD. Horizontal and vertical axes indicated DNA content and cell number, respectively. The peaks on channel 200 and 400 representing G_1 and G_2/M were shown. NCTD, U251 cells exposed to norcantharidin; p53, U251 cells infected with wtp53 adenovirus; p53 + NCTD, U251 cells infected with wtp53 adenovirus and exposed to norcantharidin.

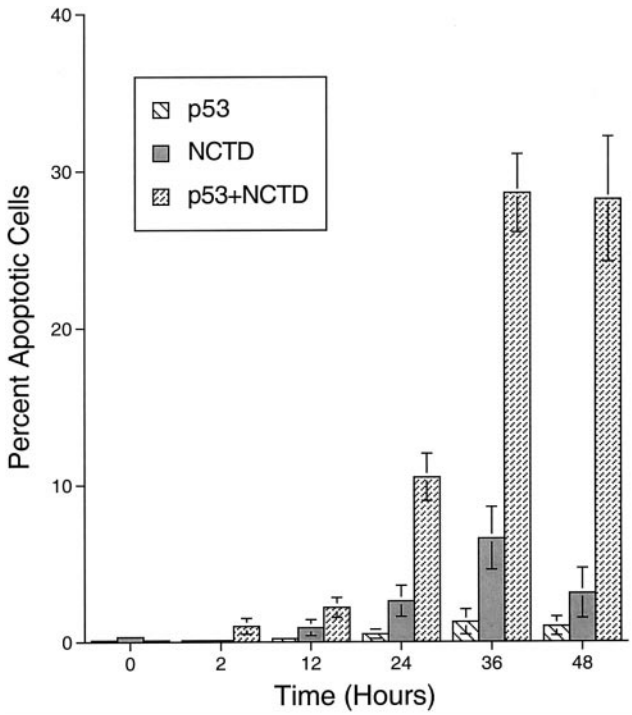

FIG. 6. Percentage of apoptosis cells induced by NCTD in U251 cell line after wtp53 adenovirus infection. The results were from the average of triplicate experiments.

used to perform the all chemosensitivity experiments. Fifteen hours after infecting the U251 tumor cells with the wild-type p53 adenovirus, NCTD (50 μg/ml) was added to the culture medium. Percent of tumor cells at sub-G_1 phase (apoptosis) was analyzed by FACS at different time points (0, 2, 12, 24, 36, 48 h) afterwards (Fig. 5). U251 showed very few cells at sub-G_1 phase 48 h after exposed to NCTD (4.2 $\pm$ 1.6%, Figs. 5 and 6). The result was comparable to the findings in Fig. 3. p53 gene insertions/expressions in U251 showed little or no effect in inducing apoptosis in U251 cells (1.5 $\pm$ 0.8%, Figs. 5 and 6). Interestingly, 24 h after p53 gene insertion/expression and norcantharidin exposure, More than 10% of U251 cells were found in sub-G_1 phase. Apoptosis of U251 cells peaked at 36 h after NCTD exposure and maintained stable until 48 h. Close to 30% (29.0 $\pm$ 1.8%) of wtp53 inserted/expressed U251 tumor cells underwent apoptosis after expose to NCTD for 36 h and maintained until 48 h (28.1 $\pm$ 3.2%, Figs. 5 and 6). These results showed wtp53 gene insertions/expressions significantly enhanced U251 tumor cells chemosensitivity to NCTD compared to NCTD control (28.1% vs 4.2%, 6.7-fold increase in NCTD chemosensitivity; $P < 0.001$) and wtp53 gene inserted/expressed control (28.1% vs 1.5%, 18.8-fold increase in NCTD chemosensitivity; $P < 0.001$).

Many DNA-damaging agents such as cisplatin, UV, ionizing radiation exposure (11–17), under most circumstances, signals which activate the p53 response led to rapid elevation of the p53 protein, principally through stabilization of the protein, and activation of the DNA binding function of p53. Posttranslational modification of p53 by phosphorylation was proposed to be an important mechanism by which p53 stabilization and function are regulated (18). In recent studies using phospho-specific antibodies, serines 15 and 37 were identified as sites of phosphorylation in cells following DNA damage (19–21). Phosphorylation of serine 33 in response to UV or ionizing radiation was shown recently with evidence that N-terminal phosphorylation of p53 directed C-terminal acetylation of the protein. NCTD is one of the protein phosphotase 2A (PP2A) inhibitor (4). No previous reports demonstrated the correlation between PP2A inhibitors and p53 gene. Our results showed NCTD induced apoptosis in glioblastoma cells with functional wild-type p53 and not in tumor cells with mutant p53 gene. Functional expressions of wtp53 through adenoviral infections in mutant p53 tumor cells restored NCTD cytotoxicity in these tumor cells. These results further indicate the feasibility of using wild-type p53 gene therapy to enhance the chemosensitivity of mutant p53 tumor cells to norcantharidin.

ACKNOWLEDGMENTS

We thank Dr. Guang-Sheng Wang for providing the Norcantharidin. This work was supported in part by Grants DOH89-TD-1134 and NSC89-2314-B-002-191 (C.H.) and NSC89-2318-B-075B-001-M51,and NSC89-NU-7-075B-001 (M.H.) from National Science Council, Grants VGHKS89-19 and VGHKS89-107 from Kaohsiung Veterans General Hospital (M.H.), and Grant 89-B-FA08-1-4 from the Academic Excellent Program of the Ministry of Education (M.H.), Taiwan, Republic of China.

REFERENCES

1. Graziano, M. J., Pessah, I. N., Matsuzawa, M., and Casida, J. E. (1988) *Mol. Pharmacol.* **33,** 706–712.
2. Graziano, M. J., Waterhouse, A. L., and Casida, J. E. (1987) *Biochem. Biophys. Res. Commun.* **149,** 79–85.
3. Wang, G. S. (1989) *J. Ethnopharmacol.* **26,** 147–162.
4. Liu, X. H., Blazsek, I., Comisso, M., Legras, S., Marion, S., Quittet, P., Anjo, A., Wang, G. S., and Misset, J. L. (1995) *Eur. J. Cancer* **31A,** 953–963.
5. Hung, K. S., Hong, C. Y., Lee, J., Lin, S. K., Huang, S. C., Wang, T. M., Tse, V., Sliverberg, G. D., Weng, S. C., and Hsiao, M. (2000) *Biochem. Biophys. Res. Commun.* **269,** 718–725.
6. Hsiao, M., Tse, V., Carmel, J., Tsai, Y., Felgner, P. L., Haas, M., and Silverberg, G. D. (1997) *Biochem. Biophys. Res. Commun.* **233,** 359–364.
7. O'Connor, P. M. (1997) *Cancer Surv.* **29,** 151–182.
8. Innocente, S. A., Abrahamson, J. L., Cogswell, J. P., and Lee, J. M. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 2147–2152.
9. Winters, Z. E., Ongkeko, W. M., Harris, A. L., and Norbury, C. J. (1998) *Oncogene* **17,** 673–684.
10. Sedivy, R., and Mader, R. M. (1997) *Cancer Invest.* **15,** 601–607.
11. Roth, J. A., Swisher, S. G., and Meyn, R. E. (1999) *Oncology (Huntingt)* **13,** 148–154.

12. Dabholkar, M., and Reed, E. (1996) *Cancer Chemother. Biol. Response Modif.* **16,** 88–110.
13. Lakin, N. D., and Jackson, S. P. (1999) *Oncogene* **18,** 7644–7655.
14. Herrlich, P., Sachsenmaier, C., Radler-Pohl, A., Gebel, S., Blattner, C., and Rahmsdorf, H. J. (1994) *Adv. Enzyme Regul.* **34,** 381–395.
15. McGregor, W. G. (1999) *J. Invest. Dermatol. Symp. Proc.* **4,** 1–5.
16. Bold, R. J., Termuhlen, P. M., and McConkey, D. J. (1997) *Surg. Oncol.* **6,** 133–142.
17. Zamble, D. B., Jacks, T., and Lippard, S. J. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 6163–6168.
18. Meek, D. W. (1994) *Semin. Cancer Biol.* **5,** 203–210.
19. Shieh, S. Y., Ikeda, M., Taya, Y., and Prives, C. (1997) *Cell* **91,** 325–334.
20. Kharbanda, S., Saleem, A., Yuan, Z. M., Kraeft, S., Weichselbaum, R., Chen, L. B., and Kufe, D. (1996) *Cancer Res.* **56,** 3617–3621.
21. Kharbanda, S., Yuan, Z. M., Rubin, E., Weichselbaum, R., and Kufe, D. (1994) *J. Biol. Chem.* **269,** 20739–20743.