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p16^{INK4} Expression Is Associated with the Increased Sensitivity of Human Non-small Cell Lung Cancer Cells to DNA Topoisomerase I Inhibitors

Kazuya Fukuoka,[1,3] Jun-ichi Adachi,[2] Kazuto Nishio,[1] Hitoshi Arioka,[1] Hirokazu Kurokawa,[1] Hisao Fukumoto,[1] Tomoyuki Ishida,[1] Taisuke Nomoto,[1] Hideyuki Yokote,[1] Akira Tomonari,[1] Nobuhiro Narita,[3] Jun Yokota[2] and Nagahiro Saijo[1,4]

[1]*Pharmacology Division and* [2]*Biology Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104 and* [3]*Second Department of Internal Medicine, Nara Medical University, 840 Shijo-cho, Kashihara-city 634*

Inactivation of p16^{INK4}, an inhibitor of cyclin-dependent kinases 4 (CDK4) and 6 (CDK6), may be essential for oncogenesis in non-small cell lung cancer (NSCLC). We examined the sensitivity of two clones of *p16^{INK4}*-transfected NSCLC cell line with homozygous deletion of *p16^{INK4}*, A549/p16-1 and 2, to DNA topoisomerase I (topo I) inhibitors. A549/p16-1 and -2 showed 7.7- and 9.1-fold increases in sensitivity to CPT-11 (11,7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin), respectively, compared with A549 cells. Ectopic p16^{INK4}-expressing cells also showed ~4.0-fold increase in sensitivity to SN-38 (7-ethyl-10-hydroxycamptothecin), the active metabolite of CPT-11, compared to the parent cells. The topo I-mediated DNA relaxation activities of ectopic p16^{INK4}-expressing cells were approximately 5 times higher than those of the parent cells. Northern and western blot analyses indicate that these increased topo I activities of ectopic p16^{INK4}-expressing cells were due to an elevated topo I mRNA level and an increase in topo I protein. The chemosensitivity to topo I inhibitors, topo I mRNA level, protein content and activity of a *p16^{INK4}* revertant, lacking functional p16^{INK4}, tended to be restored toward those of the parental phenotype to some extent. These results suggest that p16^{INK4} expression is closely associated with the increased sensitivity of ectopic p16^{INK4}-expressing NSCLC cells to topo I inhibitors. The up-regulation of topo I mRNA level, protein content and activity may be responsible for this hypersensitivity.

Key words: p16^{INK4} — DNA topoisomerase I — DNA topoisomerase I inhibitor — Non-small cell lung cancer cells — Drug sensitivity

Recent studies provide evidence that the expression of some oncogenes or tumor suppressor genes modulates the sensitivity of tumor cells to anticancer agents.[1] A tumor suppressor gene, *p16^{INK4}* encoding p16^{INK4}, an inhibitor of cyclin-dependent kinases (CDK) 4 and 6, is located on chromosome 9p21.[2,3] The *p16^{INK4}* itself can be deleted, mutated, or silenced by promoter methylation in a wide variety of tumor-derived cell lines[2–7] and in some primary tumors, including familial melanoma,[8] pancreatic adenocarcinoma,[9] esophageal cancer[10] and non-small cell lung cancer (NSCLC).[6,7,11] p16^{INK4}, specifically binds to CDK4 and CDK6/cyclin D and inhibits their catalytic activities, which mediate phosphorylation of pRb and results in cell cycle progression through a G_1-S restriction point.[12] If *p16^{INK4}* is deleted or functionally inactivated, deregulation of CDK4 (CDK6)/cyclin D activity causes persistent phosphorylation of pRb and this may lead to uncontrolled cell growth and tumorigenesis.[13] The p16^{INK4}, CDK4 (CDK6), cyclin D and pRb constitute a common growth-suppressor pathway regulating cell cycle progression through G_1 to S-phase.[4,11] It is of interest that in lung cancer cell lines and in primary lung cancer, a reciprocal relationship has been observed between loss of p16^{INK4} expression and inactivation of pRb.[4,11,14] p16^{INK4} expression is usually detectable in small cell lung cancers (SCLCs), in which pRb is almost always deleted and mutated, whereas p16^{INK4} is frequently altered in NSCLCs, which commonly express an intact pRb. SCLC is much more responsive to the cytotoxic effects of chemotherapy than NSCLC in the treatment of primary lung cancer. Since a pleiotropic tumor suppressor gene, *p53*, is frequently inactivated in both tumor cell-types,[15] we hypothesize that the status of p16^{INK4} or pRb expression may, in part, account for the differences in biological behavior and sensitivity to anticancer agents between SCLC and NSCLC. However, it remains unclear whether the status of p16^{INK4} expression plays a role as a determinant of chemosensitivity in lung cancer cells. To test this hypothesis, we investigated the sensitivity of ectopic p16^{INK4}-expressing NSCLC cells to anticancer agents and then demonstrated that the presence of p16^{INK4} expression is closely related to the cytotoxicity of DNA topoisomerase (topo) I inhibitors.

The DNA topos are nuclear enzymes that catalyze the systematic breaking and rejoining of DNA strands,

[4] To whom correspondence and reprint requests should be addressed.

thereby controlling the topological state of DNA. Topo I catalyzes the relaxation of DNA strands through transient single-strand breaks, whereas topo II catalyzes that of DNA double strands through transient double-strand breaks. These topos are known to be involved in many DNA metabolic processes, including replication, recombination, transcription and chromosome segregation at mitosis.[16] Because of these critical functions, the DNA topos have been considered as important target molecules for anticancer agents.[17] Camptothecin and its synthetic derivatives, such as 11,7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin (CPT-11) and 7-ethyl-10-hydroxycamptothecin (SN-38), which inhibit topo I, induce topo I-mediated DNA cleavage and exert cytotoxic effects on eukaryotic cells.[18] Recent clinical trials of camptothecin derivatives have shown that topo I inhibitors are promising anticancer agents for a wide variety of malignant tumors including lung cancer.[19]

In the present study, we have demonstrated that $p16^{INK4}$ expression is associated with the increased sensitivity of ectopic $p16^{INK4}$-expressing NSCLC cells to topo I inhibitors and analyzed the underlying mechanisms responsible for this hypersensitivity.

MATERIALS AND METHODS

Materials CPT-11 and SN-38 were obtained from Yakult Co., Ltd. (Tokyo). Plasmid pBR322 DNA was purchased from Toyobo Co., Ltd (Osaka).

Cell line and culture The human NSCLC (adenocarcinoma) cell line, A549, was obtained from the American Type Culture Collection (ATCC, Rockville, MD). In A549, *p16*^{INK4} is homozygously deleted, while *Rb* and *p53* are wild-type.[6] The cells were grown as attached cultures in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical Co., Ltd., Tokyo) supplemented with 0.1% sodium bicarbonate, 2 m*M* L-glutamine, 10% heat-inactivated fetal bovine serum (FBS) (Sigma Chemical Co., St. Louis, MO), penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO_2 at 37°C.

Transfection and characterization of *p16*^{INK4}-transfected cells Plasmid construction and transfection were described elsewhere.[20] Briefly, A549 was stably transfected with a wild-type *p16*^{INK4} expression vector, pCDKN2WT (a gift of Dr. H.-J. Su Huang) or pcDNA3 (no insert) (Invitrogen, San Diego, CA) and transfectants were selected by the use of G418 (Geneticin, Sigma). The selected cell lines, transfected with pCDKN2WT or pcDNA3, were designated A549/p16 or A549/Neo (mock transfectant), respectively. A549/p16-1 expressed a high level of $p16^{INK4}$, while A549/p16-2 expressed a relatively low level. A *p16*^{INK4} revertant, A549/p16-1R, was cloned from A549/p16-1 cells following culture after the removal of G418 and the absence of *p16*^{INK4} expression was verified by western and northern blot analyses. With regard to cell growth rate, there were no marked differences between A549, A549/Neo and A549/p16-1R, though the cell growth rate of *p16*^{INK4}-transfectants was reduced compared to that of the parent cells in culture.

Growth inhibition assay Cytotoxic effects of anticancer agents were measured by using the tetrazolium dye assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)] of Mosmann.[21] Briefly, 180 μl/well of the cell suspension (5 to 7.5×10^3/ml) was plated in 96-well microculture plates (Falcon 3072). For the growth inhibition assay, the culture plates were treated with 20 μl/well of drugs at various concentrations and incubated at 37°C in a humidified atmosphere for 4 days. After the incubation period, 20 μl/well of MTT solution [5 mg/ml in phosphate-buffered saline (PBS)] was added. The produced formazan was dissolved in 200 μl/well dimethyl sulfoxide (DMSO) and the optical density was measured at 562 and 630 nm using Delta-soft enzyme-linked immunosorbent assay analysis for a Macintosh computer interfaced to a Bio-Tek Microplate Reader (EL-340, Bio Metallics, Princeton, NJ). Wells containing only DMEM-FBS and MTT were used as controls. Each experiment was performed using 6 replicate wells for each drug concentration and three independent experiments were carried out. The IC_{50} value was defined as the drug concentration required to inhibit cell proliferation by 50% compared with control cultures. In order to abrogate an influence of difference in the cell growth rate among the cells on this growth inhibition assay, the optical density in the drug-free control wells was adjusted to approximately 1.0–1.5 by varying the plating cell numbers.

Preparation of nuclear extracts and topo I activity assay Crude nuclear extracts were prepared as described by Deffie *et al.*[22] The cells were collected by centrifugation, washed twice with ice-cold nuclear buffer (NB), comprising 2 m*M* KH_2PO_4, 5 m*M* $MgCl_2$, 150 m*M* NaCl, 1 m*M* ethyleneglycol-bis(β-aminoethyl ether)-*N*, *N*, *N'*, *N'*-tetraacetic acid and 0.1 m*M* dithiothreitol (adjusted to pH 6.5), and resuspended in 1 ml ice-cold NB. Then 9 ml of ice-cold NB containing 0.35% (v/v) Triton X-100 and 1 m*M* phenylmethylsulfonyl fluoride was added. The cell suspension was kept on ice for 10 min, then washed with Triton X-100-free ice-cold NB, and the nuclear protein was eluted for 1 h at 4°C with ice-cold NB containing 0.35 *M* NaCl. A nuclear protein solution was obtained by centrifugation at 18,000*g* for 10 min and the protein concentration of the supernatant was determined by using the method of Bradford[23] with bovine serum albumin (BSA, Sigma) as a standard.

The topo I activity of the cells was determined by measuring the relaxation of supercoiled plasmid DNA

(pBR322), essentially as described by Liu and Miller.[24] The reaction mixtures for measuring the total topo I activity of the cells comprised 100 m*M* KCl, 10 m*M* $MgCl_2$, 1 m*M* dithiothreitol, 0.1 m*M* EDTA, 10% (v/v) glycerol, 50 m*M* Tris-HCl (pH 7.4), 1.7 μg of pBR322 DNA and crude nuclear extracts (0.025–1.0 μg protein). These reaction solutions were diluted with distilled water to 20 μl, and incubated at 37°C for 15 min. The reaction was terminated by adding 5 μl of dye solution comprising 2.5% (w/v) sodium dodecyl sulfate (SDS), 0.01% (w/v) bromophenol blue, and 50% glycerol. These mixtures were electrophoresed in 1.0% (w/v) agarose gel, stained with ethidium bromide (2 μM), transilluminated with 300-nm UV light and photographed.

RNA extraction and northern blot analysis Total RNA was extracted from the cells by the acid guanidium thiocyanate-phenol-chloroform extraction method.[25] Approximately 25 μg of total RNA was electrophoresed on a 1% agarose-6% formaldehyde gel at 30V for 20 h. Electrophoresed agarose gel was stained with SYBR Green II (Molecular Probes, Inc., OR), transilluminated with 300-nm UV light and photographed. The RNA was transferred to a positively charged nylon membrane (Hybond-N+, Amersham), which was then hybridized for 20 h at 42°C with a ^{32}P-labeled topo I cDNA probe, a gift of Dr. L. F. Liu, in a solution comprising 50% deionized formamide, 10× Denhardt's buffer, 5× SSC [1× SSC; 0.15 *M* NaCl and 0.015 *M* sodium citrate (pH 7.0)], and 0.1% SDS. After hybridization, the membrane was washed twice with 1× SSC and 0.1% SDS for 20 min at 53°C, followed by a wash with 0.1× SSC and 0.1% SDS under the same conditions and then autoradiographed on X-ray film (Amersham Hyperfilm-MP). The mRNA size of topo I was reported to be 4.1 kb.[26]

Western blot analysis of topo I protein Nuclear protein was extracted from each cell line as described above. The nuclear extracts, which had been stored at −80°C, were analyzed using polyacrylamide slab gels containing SDS. Two μg of nuclear protein from each type of cell was applied to the gel, electrophoresed, then transferred to polyvinylidene difluoride membranes (Immobilon, 0.45-μm pore diameter, Nihon Millipore Kogyo, Tokyo). The membranes were blocked by incubating them in 5% (w/v) BSA in PBS for 2 h at room temperature, then they were incubated at 4°C for 2 h with human anti-topo I antibody (TopoGEN Inc., Columbus, OH), which was diluted with PBS to the required concentration, and then rinsed with PBS containing 0.1% (v/v) Triton X-100. Next, the membranes were incubated with anti-human immunoglobulin G (Fc)-horseradish peroxidase conjugate diluted with PBS containing 1% BSA for 1 h at room temperature, and finally washed with PBS containing 0.1% Triton X-100. The membranes were developed with western blotting enhanced chemiluminescence detection reagents (Amersham, Buckinghamshire).

Statistical analysis Statistical comparisons were made by using the unpaired Student's *t* test. A significant difference was considered to exist when the *P* value was <0.01.

RESULTS

Sensitivity of p16INK4-expressing cells to topo I inhibitors In order to elucidate whether p16INK4 expression is associated with the sensitivity to topo I inhibitors, we examined the sensitivity of *p16INK4* transfectants to CPT-11 and SN-38 using the MTT assay. *p16INK4* transfectants showed 7.7- to 9.1-fold increased sensitivity to CPT-11 as compared with the parent cells (Fig. 1A). The IC_{50} values of CPT-11 against A549, A549/p16-1 and A549/p16-2 were 22.4, 2.89 and 2.49 μM, respectively, with the former being significantly different from the others ($P=0.0002$). *p16INK4* transfectants also showed a 4.0- to 4.2-fold increase in sensitivity to SN-38, compared to the parent cells (Fig. 1B). The IC_{50} values of SN-38 against A549, A549/p16-1 and A549/p16-2 were 0.032, 0.0081 and 0.0078 μM, respectively, and again the former is significantly different from the other two ($P=0.006$). A 549/p16-1R showed a 3.1- to 3.6-fold resistance to CPT-11 compared to *p16INK4* transfectants, indicating that the sensitivity of A549/p16-1R to CPT-11 tended to be restored to that of the parent A549 cells (Fig. 1A). This reversional tendency of a *p16INK4* revertant was also observed in the sensitivity to SN-38 (Fig. 1B). There was no significant difference in the sensitivity to each topo I inhibitor between A549 and A549/Neo. These results indicate that ectopic p16INK4-expression is associated with increased sensitivity of human NSCLC cells to topo I inhibitors, such as CPT-11 and SN-38.

Increased activity of topo I in p16INK4-expressing cells In order to clarify whether the enhanced cytotoxicity of topo I inhibitors in ectopic p16INK4-expressing cells was due to increased activity of topo I, we compared the topo I activities between A549, A549/Neo, *p16INK4* transfectants and A549/p16-1R using a DNA relaxation assay. The total cellular activities of topo I in crude nuclear extracts eluted with 0.35 *M* NaCl were measured. The relaxation of pBR322 DNA incubated with different amounts of cell nuclear protein extracts is shown in Fig. 2. Some supercoiled forms of DNA remained in the presence of 0.1 μg of A549 nuclear extract, whereas the same amount of nuclear extract from *p16INK4* transfectants completely converted the supercoiled DNA to the relaxed form. The amount of topo I protein required to relax almost all of the supercoiled DNA was 0.25 μg for A549 and A549/Neo, 0.1 μg for A549/p16-1R, and 0.05 μg for A549/p16-1 and A549/p16-2. These results suggest that the topo I activities of *p16INK4* transfectants are approximately 5 times higher than those of the parent

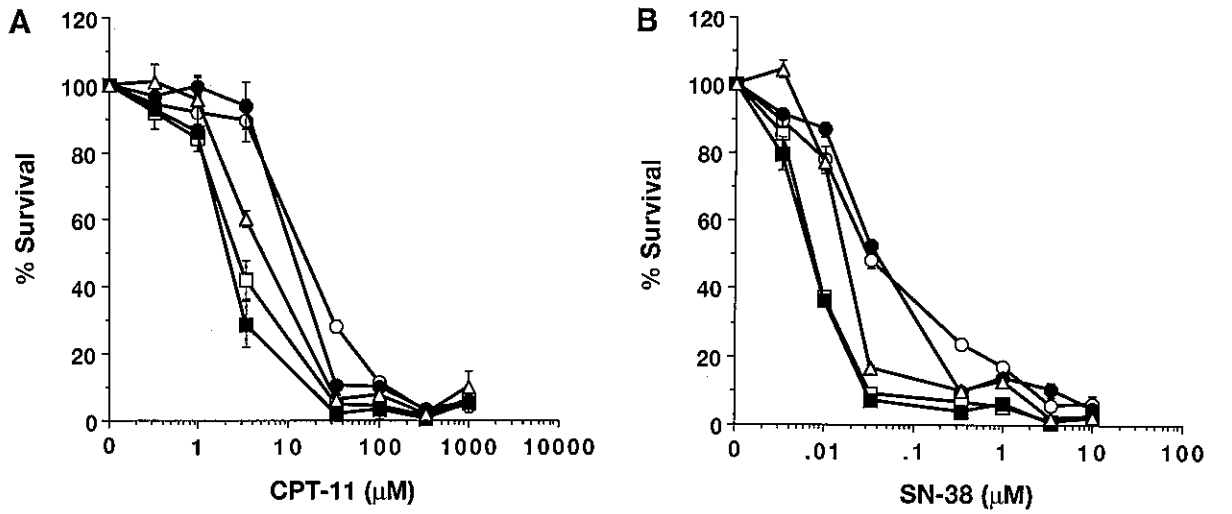

Fig. 1. Concentration-response relationship for the action of topo I inhibitors on the growth of A549, A549/Neo, A549/p16-1, -2 and A549/p16-1R measured using the MTT assay. Exponentially growing cells including A549 (○), A549/Neo (●), A549/p16-1 (□), -2 (■) and A549/p16-1R (△) were seeded in 96-well microtiter plates and exposed to CPT-11 (A) or SN-38 (B). After a 4-day culture period, MTT was added to each well, then the plates were incubated for a further 4 h, and centrifuged; the medium was removed, DMSO was added to each well to dissolve the pigment (formazan) crystals, and the absorbances of the solutions were measured at 562 and 630 nm using a Bio-Tek Microplate Reader. The IC_{50} value was defined as the drug concentration required for 50% reduction of the optical density in each test.

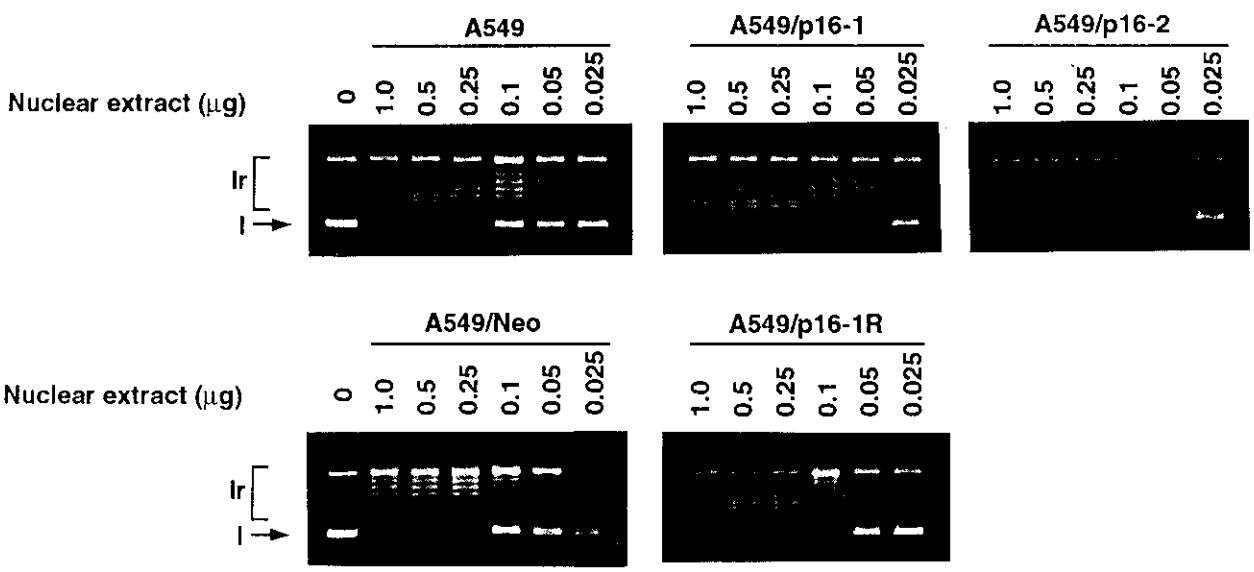

Fig. 2. Topo I activities of A549, A549/Neo, A549/p16-1, -2 and A549/p16-1R. The enzyme activity was determined by measuring the relaxation of supercoiled DNA (pBR322) during incubation with nuclear extract at 37°C for 15 min, using electrophoresis on a 1.0% agarose gel for 4 h. *I* and *Ir*, supercoiled and relaxed forms of DNA, respectively. Amounts of 1.0, 0.5, 0.25, 0.1, 0.05 and 0.025 μg of nuclear protein from A549, A549/p16-1, A549/p16-2, A549/Neo and A549/p16-1R were added. No nuclear extract was added in the control lane.

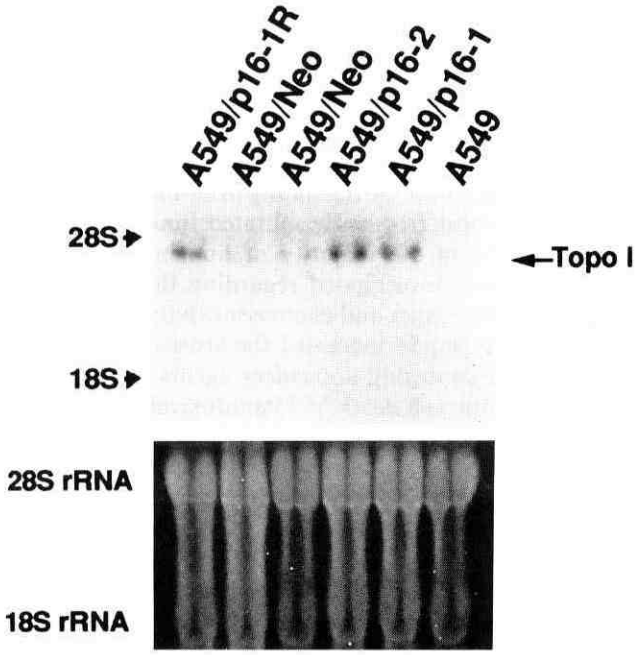

Fig. 3. Northern blot analysis of topo I mRNA extracted from A549, A549/Neo, A549/p16-1, -2 and A549/p16-1R. Samples of total RNA (25 μg) from A549, A549/Neo, A549/p16-1, -2 and A549/p16-1R were subjected to electrophoresis in a 1% agarose-6% formaldehyde gel, stained with SYBR Green II, transferred to a nylon membrane and hybridized with ^{32}P-labeled topo I cDNA probe. The arrow represents an approximately 4.1 kb mRNA transcript identical to topo I mRNA. The arrowheads represent 28S and 18S rRNA. Equivalent loadings of total RNA were verified by examination of the 28S and 18S rRNA bands.

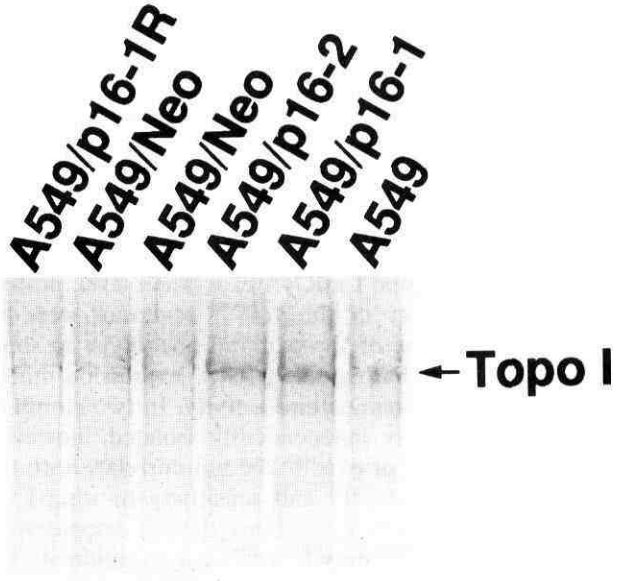

Fig. 4. Western blot analysis of topo I protein extracted from A549, A549/Neo, A549/p16-1, -2 and A549/p16-1R. Aliquots of 2 μg of nuclear extracts from A549, A549/Neo, A549/p16-1, -2 and A549/p16-1R were subjected to 10% (w/v) SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, incubated with anti-topo I antibody and visualized using a chemiluminescence-based system. The arrow represents an approximately 100 kD molecular weight protein identical to topo I protein.

cells. This increased activity of topo I observed in ectopic p16^{INK4}-expressing cells might account for the enhanced cytotoxicity of topo I inhibitors.

Increased expression of topo I mRNA in p16^{INK4}-expressing cells To determine whether the increased topo I activity was due to an elevation of topo I gene expression, topo I mRNA was measured using northern blot analysis (Fig. 3). The topo I mRNA levels of *p16^{INK4}* transfectants were higher than those of the parent A549 cells, the mock transfectant and a *p16^{INK4}* revertant. These data suggest that an elevated level of topo I mRNA expression resulted in the increased catalytic activity of topo I, leading to the enhanced sensitivity to topo I inhibitors of human NSCLC cells expressing ectopic p16^{INK4}.

Increased topo I content in p16^{INK4}-expressing cells In order to confirm that the increased expression of topo I mRNA leads to the increased protein content of topo I in ectopic p16^{INK4}-expressing cells, topo I contents of the nuclear extracts were measured using western blot analysis (Fig. 4). *p16^{INK4}* transfectants contained an increased amount of topo I protein as compared with the parent cells, mock transfectant or a *p16^{INK4}* revertant. There was no significant difference in the topo I contents between A549, A549/Neo and A549/p16-1R.

DISCUSSION

Since the loss of p16^{INK4} expression, caused by a variety of genetic or epigenetic events, is frequently observed in *Rb*-positive NSCLCs, inactivation of *p16^{INK4}* may be essential for the oncogenesis in NSCLC, biologically characterized as an inherently chemoresistant tumor.[4-7, 11, 14] In this study, we present evidence that *p16^{INK4}* is closely associated with the increased sensitivity of ectopic p16^{INK4}-expressing NSCLC cells to topo I inhibitors, such as CPT-11 and its active metabolite, SN-38. Furthermore, increased topo I activity, caused by an elevated level of topo I mRNA expression and an increased content of topo I protein, has been observed in ectopic p16^{INK4}-expressing cells. Previous reports provide evidence that increased topo I expression or activity in mammalian cells results in cellular hypersensitivity to

topo I inhibitors.[27] On the other hand, decreased cellular content of topo I or mutation of topo I gene correlates with the development of resistance to topo I inhibitors.[28-30] Therefore, our data indicate that the increased topo I expression and activity may be responsible for the enhanced cytotoxicity of topo I inhibitors to ectopic $p16^{INK4}$-expressing cells. In order to confirm the effect of *p16*^{INK4} on topo I expression and activity, we analyzed a *p16*^{INK4} revertant, lacking functional $p16^{INK4}$. Since the chemosensitivity, topo I mRNA expression level, protein content and activity of the *p16*^{INK4} revertant were restored toward those of the parental phenotype to some extent, it is clear that ectopic $p16^{INK4}$ expression modulates the topo I expression and activity. In two clones of *p16*^{INK4} transfectants independently isolated, however, the expression level of $p16^{INK4}$ did not correlate with the degree of topo I activity and sensitivity to topo I inhibitors. Thus, there is a possibility that an ectopic overexpression of $p16^{INK4}$ may be sufficient to modulate the topo I expression and activity.

The molecular mechanism by which ectopic $p16^{INK4}$ expression increases topo I mRNA expression level and protein content is not clear at present. $p16^{INK4}$ overexpression inhibits cyclin D/CDK4 (CDK6)-mediated phosphorylation of pRb and then dephosphorylated pRb can not release the E2F transcription factor, resulting in the inactivation of transcription implicated in DNA synthesis. If these molecular events inhibit the transcription of the topo I gene itself, $p16^{INK4}$ overexpression should cause down-regulation of topo I mRNA expression level and protein content, and consequently decrease topo I activity, leading to drug resistance to topo I inhibitors. However, it was surprising that $p16^{INK4}$ overexpression induced up-regulation of the topo I mRNA expression level and protein content, increasing the activity of topo I, and resulting in the enhanced cytotoxicity of topo I inhibitors. One possible explanation for this discrepancy is that the molecular mechanism concerning the regulation of topo I gene transcription has not been fully elucidated. It is still uncertain whether the transcription of the topo I gene is also inhibited when $p16^{INK4}$ overexpression induces the inactivation of transcription involved in DNA synthesis. Another possibility is that up-regulation of the topo I mRNA expression level and protein content induced by $p16^{INK4}$ overexpression may occur via an *Rb*-independent mechanism. We have demonstrated that the H-*ras* oncogene product modulated the topo I-mediated DNA relaxation activity and caused a qualitative change of topo I protein in H-*ras*-transformed murine fibroblast cell lines.[31] These observations indicate that the topo I activity is modulated by a H-*ras* gene-mediated signal transduction pathway. Moreover, the topo I expression levels in malignant tumors differed from those in normal tissues.[32] Taken together, a wide variety of molecular mechanisms modulating topo I expression and activity may be involved in oncogenesis. These and our results suggest that there may be a biological and/or biochemical interaction between 16^{INK4} expression and topo I activity.

Some tumor suppressor genes modulate the chemosensitivity of tumor cells by regulating drug-induced apoptosis. *p53*, the most frequently mutated tumor suppressor gene identified in the majority of human cancers, has been extensively investigated regarding the relationship between its expression and chemosensitivity. The expression of wild-type *p53* increased the sensitivity of tumor cells to DNA-damaging anticancer agents by the induction of apoptotic cell death.[1,33] Paradoxically, the loss of normal p53 function in normal fibroblasts conferred sensitization to a variety of chemotherapeutic agents, including an antimicrotubule agent, by increasing G_2/M arrest and apoptosis.[34,35] It is not yet clear whether $p16^{INK4}$ expression modulates the sensitivity of tumor cells to anticancer agents by influencing cellular susceptibility to apoptosis. We have previously reported that CPT-11 induced apoptosis in a human SCLC cell line, SBC-3, while a *bcl-2*-transfected SBC-3 cell line showed higher resistance to CPT-11 compared with the parent cell line owing to inhibition of drug-induced apoptosis.[36] Since the resistance of topo I inhibitors has been found to correlate with decreased topo I activity,[30] the topo I activity would be expected to be reduced in *bcl-2*-expressing SCLC cells. These findings and the present results suggest that, different from *bcl-2*, *p16*^{INK4} may activate the signalling pathway of CPT-11-induced apoptosis by mediating increased topo I expression and activity. However, further investigation will be required to elucidate the relationship between $p16^{INK4}$ expression and the topo I-mediated apoptotic pathway.

In some cancer cell lines with wild-type *Rb*, introduction of *p16*^{INK4} blocks entry into the S-phase of the cell cycle and inhibits cell proliferation.[4,5,13] In agreement with this finding, a DNA histogram of ectopic $p16^{INK4}$-expressing cells showed cell cycle arrest at the G_1-phase, as previously described.[20] The expression of topo IIα is increased in S or G_2-phase and decreased in quiescent cells, in a cell cycle-dependent manner.[37] In contrast, topo I expression has been considered to be unaffected by cell growth conditions in culture. Therefore, the increased topo I expression observed in ectopic $p16^{INK4}$-expressing cells is unlikely to be due to alterations of cell cycle progression.

In conclusion, *p16*^{INK4} is considered to play an important role as a determinant of chemosensitivity of topo I inhibitors in NSCLC cells, modulating topo I mRNA expression level, protein content and activity. Restoration of $p16^{INK4}$ expression in NSCLC may render this inherently resistant tumor more sensitive to topo I in-

hibitors. The elucidation of the molecular mechanism of the interaction between tumor suppressor genes and target molecules for anticancer agents should make it possible to overcome cellular resistance to chemotherapy.

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