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Phenotypic alterations of small cell lung carcinoma induced by different levels of wild-type p53 expression

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Abstract

p53 induces both growth arrest and apoptosis in cancer cells. To clarify whether the level of p53 expression determines the response of small cell lung carcinoma (SCLC) cells, we assessed the effect of various p53 levels on a p53-null SCLC cell line, N417, using a tetracycline (Tc)-regulated inducible p53 expression system. Apoptosis was induced in SCLC cells with high p53 expression. Although low levels of p53 induced G1 arrest accompanied by p21 expression, cells with G1 arrest seemed to undergo apoptosis after further cultivation. Expression of exogenous p21 induced G1 arrest but not apoptosis in SCLC cells, suggesting that p53-mediated G1 arrest was induced through p21 expression. Moreover, high level of p53 expression down-regulated Bcl-2 expression in SCLC cells, while Bax was consistently expressed irrespective to the level of p53 expression. These results suggest that p53-mediated apoptosis and G1 arrest depend on level of p53 expression in SCLC cells and that the relative dominancy of Bax to Bcl-2 is involved in the induction of apoptosis by high level of p53 expression.

Keywords: p53; apoptosis; G1 arrest; lung carcinoma

Abbreviations: SCLC, small cell lung carcinoma; RB, retinoblastoma; CDK, cyclin-dependent kinase; Tc, tetracycline; FACS, fluorescence-activated cell sorter; MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide

Introduction

The *p53* tumor suppressor gene is frequently mutated in a wide variety of human cancers (Hollstein *et al*, 1991; Levine *et al*, 1991), and restoration of p53 function can often suppress the growth of cancer cells (Baker *et al*, 1990; Mercer *et al*, 1990; Chen *et al*, 1990; Diller *et al*, 1990; Yonish-Rouach *et al*, 1991; Vogelstein and Kinzler, 1992; Lin *et al*, 1992; Shaw *et al*, 1992; Ko and Prives, 1996). This growth inhibition has been explained by at least two distinct mechanisms. One is to inhibit the growth of cancer cells by blocking cell cycle progression, and the other is to induce apoptosis in cancer cells. Cell cycle arrest caused by p53 expression is believed to be achieved partly through the transcriptional activation of $p21^{WAF1/CIP1}$, a broad-spectrum inhibitor of cyclin-dependent kinases (Harper *et al*, 1993; El-Deiry *et al*, 1993; Xiong *et al*, 1993). On the other hand, the molecular mechanism of p53 induced apoptosis is still poorly understood. Although p53 transcriptionally up-regulates the *Bax* gene which suppresses the activity of Bcl-2 to block apoptosis (Oltvai *et al*, 1993; Miyashita and Reed, 1995), there have been only a few reports that Bax expression directly induced apoptosis in cancer cells (Bargou *et al*, 1995; Xiang *et al*, 1996; McCurrach *et al*, 1997; Sabbatini *et al*, 1997). Furthermore, it is unclear what factors determine whether cells undergo cell cycle arrest or apoptosis following p53 expression.

Recently, using a Tc-regulated inducible p53 expression system, it was shown that the cellular response, arrest or apoptosis, depended on the level of p53 expression in cells. Chen *et al* (1996) demonstrated that high level of p53 expression induced apoptosis, while low level of p53 expression induced cell cycle arrest in Saos-2 osteosarcoma cells and H1299 non-small cell lung carcinoma cells. These data suggest that high level but not low level of p53 gives rise to p53-mediated apoptosis. However, in some cases, considerable amounts of induced p53 could result in only growth arrest of not only p53-positive but also p53-negative cells (Mercer *et al*, 1990; Kuerbitz *et al*, 1992; DiLeonardo *et al*, 1994; Kobayashi *et al*, 1995; Polyak *et al*, 1996). Therefore, the cellular response to p53 may be different among cells of different origin. Thus, in order to understand the biological function of p53, it would be of great importance to elucidate the phenotypic alterations induced by different levels of p53 in various types of cells.

Inactivation of the *p53* gene is a frequent event in lung carcinoma, especially in SCLC (Takahashi *et al*, 1989; Sameshima *et al*, 1992). To assess the biological significance of p53 inactivation in SCLC, we previously established Tc-regulated p53-inducible SCLC cells and obtained the evidence that the high level of p53 expression induced apoptosis but not G1 arrest in SCLC cells (Adachi *et al*, 1996). The results indicate that p53 inactivation assists the SCLC cells to proliferate by repressing apoptosis. However, it remains unclear whether other phenotypic alterations occur in SCLC cells in association with the level of p53 expression. Therefore, to further clarify p53 function in SCLC cells, we attempted to induce different levels of p53 expression in SCLC cells using a Tc-regulated inducible p53 expression system. Here we demonstrate that high level of p53 expression induces apoptosis in SCLC cells, while low level of p53 expression

does the appearance of G1-arrested cells, which ultimately undergo apoptosis.

Results

High levels of p53 expression induce apoptosis in SCLC

A human SCLC cell line, N417, fails to express wild-type p53 protein due to two mutational events in the *p53* gene (Bodner *et al*, 1992). We previously established wild-type p53-inducible p53w-2 and -3 cells, which were clonal cells derived from N417 (Adachi *et al*, 1996). They harbor the chimeric Tc-responsive transactivator, which allows the induction of wild-type p53 expression under the control of a Tc-repressible promoter (Gossen *et al*, 1992). These clones express high level of p53 protein after the removal of Tc from the growth medium. To ascertain that p53 expression can be quantitatively regulated in this system, we cultured p53w-2 and -3 cells in various Tc concentrations and evaluated the level of p53 expression by Western blot analysis. The level of p53 expression was regulated by Tc concentrations in the medium (Figure 1). At the concentrations of 0.1 μg/ml or 1.0 μg/ml Tc, expression of the *p53* gene was completely repressed and p53 protein was not detected by Western blot analysis (Figure 1, lanes 8 and 9). The level of p53 increased in proportion to the decrease of Tc concentrations in the medium, and the p53 level reached to the maximum when Tc concentration was 0.00001 μg/ml (Figure 1, lanes 4–7).

Then, the effect of p53 expression on the growth of SCLC cells was assessed at various Tc concentrations by flow cytometry (Figure 2, Table 1). Cell debris, which were fractionated in sub-G1 population, appeared at 12 h after high level p53 was induced by removal of Tc in p53w-2 and -3 cells. The percentage of sub-G1 population further increased after additional 12 and 36 h. By 10 days after induction, viable cells almost vanished. However, the cells in G1 or G2/M phases did not increase after high level p53 induction. Next, we analyzed the cell cycle distribution of both clones under various level of p53 expression. Cells fractionated in sub-G1 phase decreased in proportion to the level of induced p53. At the Tc concentration of 0.1 μg/ml, sub-G1 population mostly disappeared in both clones. At 12 h of induction by 0.01 and 0.001 μg/ml of Tc, the population of S phase cells was reduced to 62–86% of that of the respective uninduced cells and G1 phase cells slightly increased (Table 1). However, in any levels of induced p53, neither increase of G1 or G2/M phases cells nor decrease of S phase cells were distinctly observed at either 24 h or 48 h after induction. These results indicated that the level of p53 was critical to induce apoptosis and G1 arrest in SCLC cells. High level of p53 induces apoptosis, while low level of p53 might induce G1 arrest in N417 cells.

Low level of p53 expression transiently induces G1 arrest, but eventually resulted in the accumulation of apoptotic cells

N417 cells have short doubling time of 18.5 h (Ookawa *et al*, 1993). It is possible that cells arrested in G1 are indistinguishable from cells recycling back from the G2/M phase to the G1 phase under the experimental condition above. To clarify whether or not G1 arrest occurred after p53 induction in SCLC cells, we added nocodazol to p53-induced cells. Nocodazol is one of rapidly-reversible inhibitors of microtubule polymerization which can block the cell cycle at G2/M phase (Zieve *et al*, 1980; Cross *et al*, 1995; Ookawa *et al*, 1997), and thus prevents the cells recycling back from the G2/M phase to the G1 phase. Cells exposed to the induced state for 24 h were treated with nocodazol for additional 12, 24 and 48 h and were analyzed by flow cytometry (Figure 3).

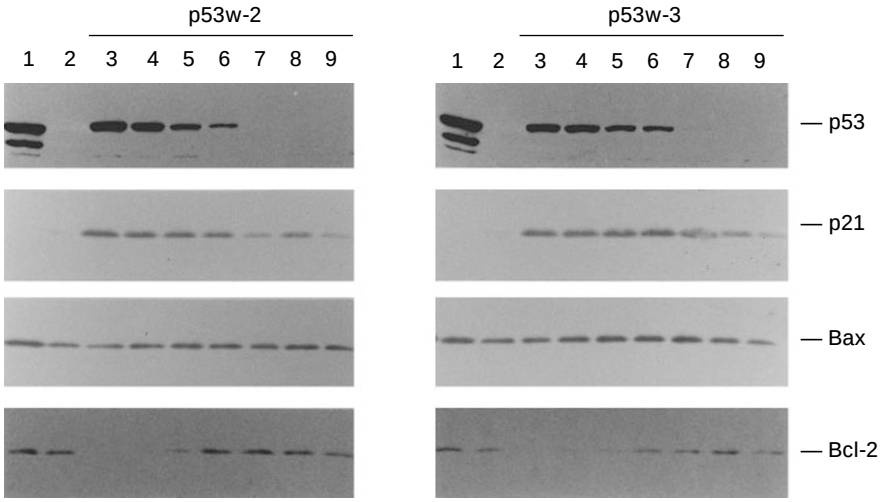

Figure 1 Regulation of p21, Bax and Bcl-2 expression by exogenous wild-type p53 in N417 SCLC cells. Cells were cultured in various Tc concentrations (lane 3, 0 μg/ml; lane 4, 0.00001 μg/ml; lane 5, 0.0001 μg/ml; lane 6, 0.001 μg/ml; lane 7, 0.01 μg/ml; lane 8, 0.1 μg/ml; lane 9, 1 μg/ml) at 37.0°C for 24 h, then cells were lysed, and total proteins were separated with 4–20% gradient SDS gels and transferred to nitrocellulose membranes. The membranes were incubated with the indicated antibodies. Lane 1, SCLC cell line Lu-135, which expresses abundant mutant p53 protein; lane 2, parental N417

At 12 h after nocodazol treatment, most of p53-uninduced cells progressed to the G2/M phase. In contrast, the G1 arrest was observed in cells with p53 expression. G1 arrest was detected more notably in cells with low p53 levels than in cells with high p53 levels. Induction of high p53 levels led to the rapid decrease of G1 and G2/M phase cells together with a more significant increase of sub-G1 population at 24 h than that without nocodazol treatment. On the other hand, low levels of p53 expression (that is 0.01, 0.001, 0.0001 μg/ml of Tc) still left a fraction of cells in the G1 phase at 24 h after nocodazol treatment. However, cells arrested in G1 phase disappeared after additional 24 h, while cells in G2/M phase was almost unchanged and cells in sub-G1 phase substitutively increased. Thus, although low level of p53 expression induced G1 arrest, cells were not persistently remained in G1 phase and apoptotic cells gradually increased.

Induction of the *p21* gene resulted in G1 arrest in SCLC cells

As shown in Figure 1, very little p53 was required to induce maximal upregulation of the *p21* gene. Low level of p21 protein was expressed in 1 μg/ml of Tc, in which p53 expression was completely repressed (Figure 1, lane 9), probably due to serum stimulation after the exchange of culture media. p21 expression apparently increased even in 0.1 μg/ml of Tc, and the level of p21 expression reached to the maximum in 0.001 μg/ml of Tc (Figure 1, lanes 6, 7 and 8).

To further investigate the biological function of p21 protein in apoptosis and G1 arrest of SCLC cells, we attempted to establish clones in which expression of the *p21* gene was regulated under Tc control. For this purpose, we transfected the p21 expression vector pT2p21/neo into N417-derived NTA4 cells (Adachi *et al*, 1996), in which high

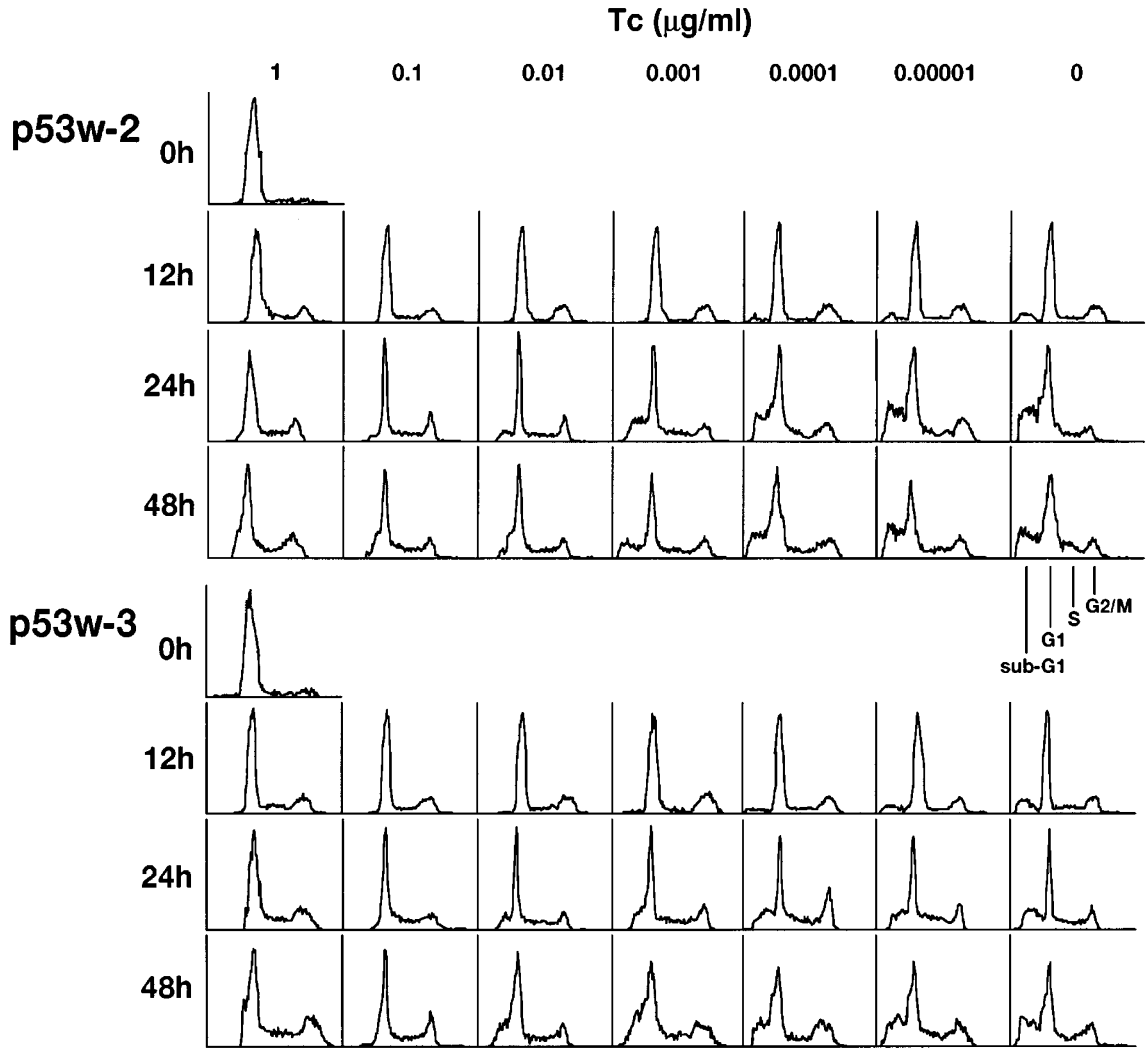

Figure 2 Apoptosis induced by various level of p53 expression in N417 SCLC cells. Cells were harvested at the indicated time points, stained with propidium iodide, and analyzed using the flow cytometer. *Horizontal lines* and *vertical lines*, DNA content and cell number, respectively. The peaks corresponding to the different phases of the cell cycle are indicated on one of the DNA histograms

Table 1 Effect of different level of p53 expression on the cell cycle distribution of N417 SCLC cells

Cells	Tc (μg/ml)	Time (h)[a]	Cell cycle distribution (%)[b] G0/G1	S	G2/M
p53 w-2	1	0	89.22	9.49	1.29
	1	12	66.78	14.29	18.93
		24	53.44	23.38	23.18
		48	59.79	19.35	20.86
	0.1	12	66.41	14.53	19.06
		24	52.34	24.94	22.72
		48	58.62	25.56	15.82
	0.01	12	70.05	9.96	19.99
		24	59.15	23.27	17.58
		48	58.94	25.72	15.34
	0.001	12	75.97	9.81	14.22
		24	52.70	29.21	18.09
		48	58.03	23.84	18.13
	0.0001	12	71.54	15.19	13.27
		24	55.23	27.40	17.37
		48	60.88	21.76	17.36
	0.00001	12	69.23	16.60	14.17
		24	57.89	28.72	13.39
		48	61.89	23.88	14.23
	0	12	64.31	17.77	17.92
		24	66.11	20.24	13.65
		48	67.76	17.23	15.01
p53 w-3	1	0	83.52	8.48	8.00
	1	12	64.72	15.26	20.02
		24	56.91	22.06	21.03
		48	46.34	27.69	25.97
	0.1	12	67.51	13.76	18.73
		24	56.11	23.80	20.09
		48	47.68	27.53	24.79
	0.01	12	69.06	13.10	17.84
		24	59.23	21.90	18.87
		48	63.20	19.52	17.28
	0.001	12	68.10	9.44	22.46
		24	56.17	20.34	23.49
		48	56.28	19.63	24.09
	0.0001	12	69.26	11.54	19.20
		24	53.81	22.15	24.04
		48	55.55	18.85	25.60
	0.00001	12	71.57	13.93	14.50
		24	57.63	20.51	21.86
		48	58.23	20.66	21.11
	0	12	68.44	16.07	15.49
		24	57.16	25.74	17.10
		48	59.61	19.85	20.54

[a]Time after induction. [b]Determined with flow cytometry for DNA content using the CellQuest and Modfit Lt softwares

level of Tc-responsive transactivation was expressed. We were able to obtain several p21-inducible clones that expressed nearly equal amounts of p21 protein to p53-induced cells in the absence of Tc. Using one (p21-1, Figure 4a) of these clones, we analyzed the effect of p21 expression on the growth of N417 cells.

The growth rate of p21-1 cells was moderately reduced after the removal of Tc (Figure 4b). Flow cytometric analysis revealed that S phase cells of the induced p21-1 cells was reduced to 48–51% of that of the uninduced cells and the induced cells were arrested in G1 phase (Figure 4c, d, Table 2). However, there was no evidence for the presence of sub-G1 peak or G2/M arrest. Therefore, it was suggested that p21 itself has a function to induce G1 arrest but not apoptosis or G2/M arrest to SCLC cells.

High level of p53 expression down-regulated Bcl-2 expression in SCLC cells

The *Bax* gene has p53-responsive elements in its promoter and Bax protein heterodimerizes with cell death repressor, Bcl-2 protein (Oltvai *et al*, 1993; Miyashita and Reed, 1995). We subsequently evaluated the levels of Bax, and Bcl-2 expression following the induction of various p53 levels (Figure 1). Western blot analysis revealed that uninduced N417 cells steadily expressed Bax and Bcl-2 proteins. Although the level of Bax protein was almost constant independently of induced p53 levels, that of Bcl-2 protein significantly decreased in cells cultured in 0.00001 and 0 μg/ml of Tc, suggesting that Bcl-2 was down-regulated by high level of p53 expression. Since another member of the Bcl-2 family, Bcl-x_L, was reported to protect against p53-induced apoptosis (Schott *et al*, 1995), we examined whether the level of Bcl-x_L was altered after induction of different p53 levels. However, different p53 levels did not influence the expression level of Bcl-x_L protein in N417 cells (data not shown).

Discussion

We demonstrated here that N417 SCLC cells underwent apoptosis with transient G1 arrest by exogenous p53 expression. Chen *et al* (1996) and Polyak *et al* (1996) also demonstrated that G1 arrest transiently occurred prior to massive apoptotic response. In our assay system, it is likely that high level of p53 expression causes cells to undergo apoptosis. Especially, G2/M arrested cells after nocodazol treatment were preferentially reduced to apoptosis by induction of high p53 expression. This may imply that G2/M arrested cells become more sensitive to p53-induced apoptosis. On the other hand, it is also possible that cells treated with nocodazol are more sensitive to apoptosis. A further study is necessary to resolve this question. G1 arrest occurred for a certain period of time after induction of low p53 expression. The percentage of G1-arrested cells afterwards decreased and that of sub-G1 population substitutively increased, suggesting that cells in G1 phase were reduced to apoptosis. Thus, although low level of p53 expression induced persistent G1 and G2/M arrests to osteosarcoma Saos-2 cells (Chen *et al*, 1996), it failed to induce persistent growth arrest to SCLC cells. At present, it is unknown why the effect of low level p53 is different in these two cell lines. It could be due to the difference in the status of genes downstream of p53. Further studies are now in progress to elucidate the molecular mechanism of p53-induced growth arrest and apoptosis in each cell type.

p53 plays a critical role as a tumor suppressor by transcriptionally regulating the target genes (Vogelstein and Kinzler, 1992; Ko and Prives, 1996). The negative cell cycle regulator p21, which was one of the molecules downstream of p53, was sufficiently upregulated in p53w-2, -3 cells even after low level of p53 induction. Using p21-inducible SCLC cells, we obtained the results that p21 had a function to induce G1 arrest. Therefore, it is likely that G1 arrest initially occurred after p53 induction in SCLC was due to p21 induction. Chen *et al* (1996) and Polyak *et al* (1996) also reported that the initial response after exogenous p53

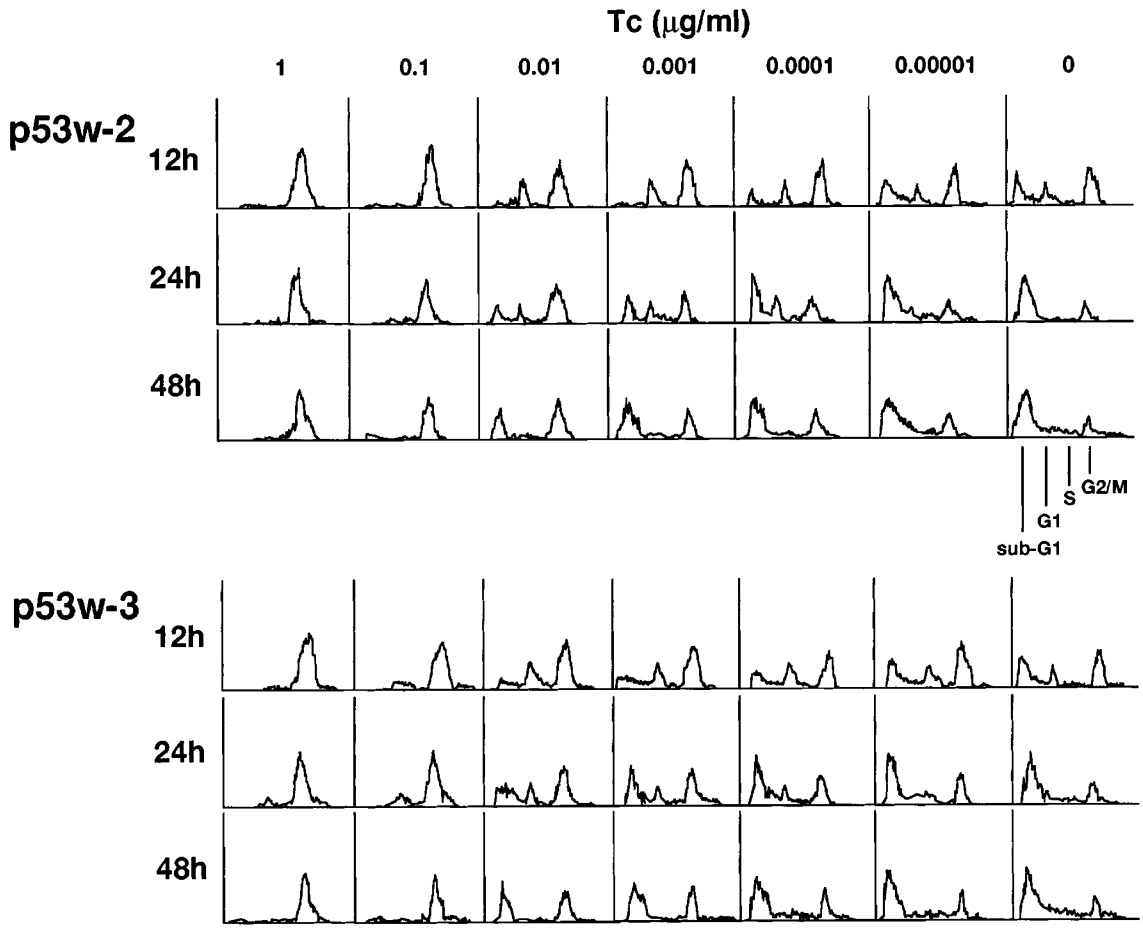

Figure 3 G1 arrest proceeds apoptosis after induction of p53 expression in N417 SCLC cells. Cells were cultured in the indicated Tc concentrations at 37.0°C for 24 h, then treated with 40 ng/ml nocodazol for additional indicated hours. The following procedure was performed as described in the legend to Figure 2. The peaks corresponding to the different phases of the cell cycle are indicated on one of the DNA histograms

expression was growth arrest mediated by p21. Our results also indicated that p21 was not implicated in p53-mediated apoptosis of SCLC.

The level of Bax mRNA (data not shown) and protein expression were almost consistent irrespective to induced p53 levels in SCLC cells. However, the levels of Bcl-2 markedly decreased when high levels of p53 were induced. It was previously shown that Bcl-2 expression was negatively regulated by p53 and that the decrease of Bcl-2 would control the susceptibility of cells to undergo apoptosis (Oltvai *et al*, 1993; Yin *et al*, 1994; Miyashita *et al*, 1994a, b; Miyashita and Reed, 1995). Although Bcl-x_L functionally resembles Bcl-2 as a potent inhibitor of apoptosis (Boise *et al*, 1993) and was found to protect cancer cells from p53-mediated apoptosis (Schott *et al*, 1995), different levels of p53 had no effect on the level of Bcl-x_L in SCLC cells. Therefore, the relative dominancy of Bax for Bcl-2 may play an important role in apoptosis by high p53 expression in SCLC. Recently Yin *et al* (1997) demonstrated that Bax could be a noble component of the p53 mediated apoptotic response in transgenic mouse brain tumors. Induction of low p53 expresion ultimately caused SCLC cells to undergo apoptosis. However, since Bcl-2 expression was not down-regulated in cells with low p53 expression, p53-mediated apoptosis would proceed in SCLC through the different molecular mechanism betwen high and low p53 expression.

Although some differences are present between SCLC and osteosarcoma in response to high and low levels of p53 expression, the results obtained here substantially support the conclusions to which other groups reached. Namely the cellular response by p53 induction depended upon the level of p53 expression in cells. Immediate apoptotic response would be brought about to cells when p53 is expressed in higher dose than a certain level, while low level of p53 expression would be sufficient to induce G1 arrest in cells.

Materials and Methods

Cells and culture conditions

Human small cell lung carcinoma cell lines, N417 and Lu135, were grown in RPMI 1640 (Nissui) supplemented with 10% fetal calf serum

(FCS) in 5% CO_2 at 37.0°C. N417-derived cells, p53w-2 and p53w-3, were maintained in media containing 0.8 mg/ml of G418 (Geneticin, Gibco BRL-Life Technologies), 0.3 mg/ml of hygromycin (Calbiochem) and 1 μg/ml of Tc (Sigma).

Plasmids and transfection

Plasmid pT2GN is a tTA responsive β-galactosidase reporter construct generated by subcloning the cDNA encoding β-galactosidase from pSV-gal (Promega) and the neomycin-resistant gene from pcDNAIneo (Invitrogen) into a tTA-responsive expression vector pT2 (Adachi *et al*, 1996; Ookawa *et al*, 1997) derived from pUHD10-3 (Gossen *et al*,

Table 2 Effect of p21 expression on the cell cycle distribution of N417 SCLC cells

			Cell cycle distribution (%)[b]		
Cells	**Tc (μg/ml)**	**Time (h)[a]**	**G0/G1**	**S**	**G2/M**
p21-1	1	0	84.41	7.02	8.57
	1	24	61.38	18.67	19.95
		48	56.23	22.31	21.46
	0	24	73.32	8.99	17.69
		48	69.41	11.48	19.11

[a]Time after induction. [b]Determined with flow cytometry for DNA content using the CellQuest and ModFit Lt softwares

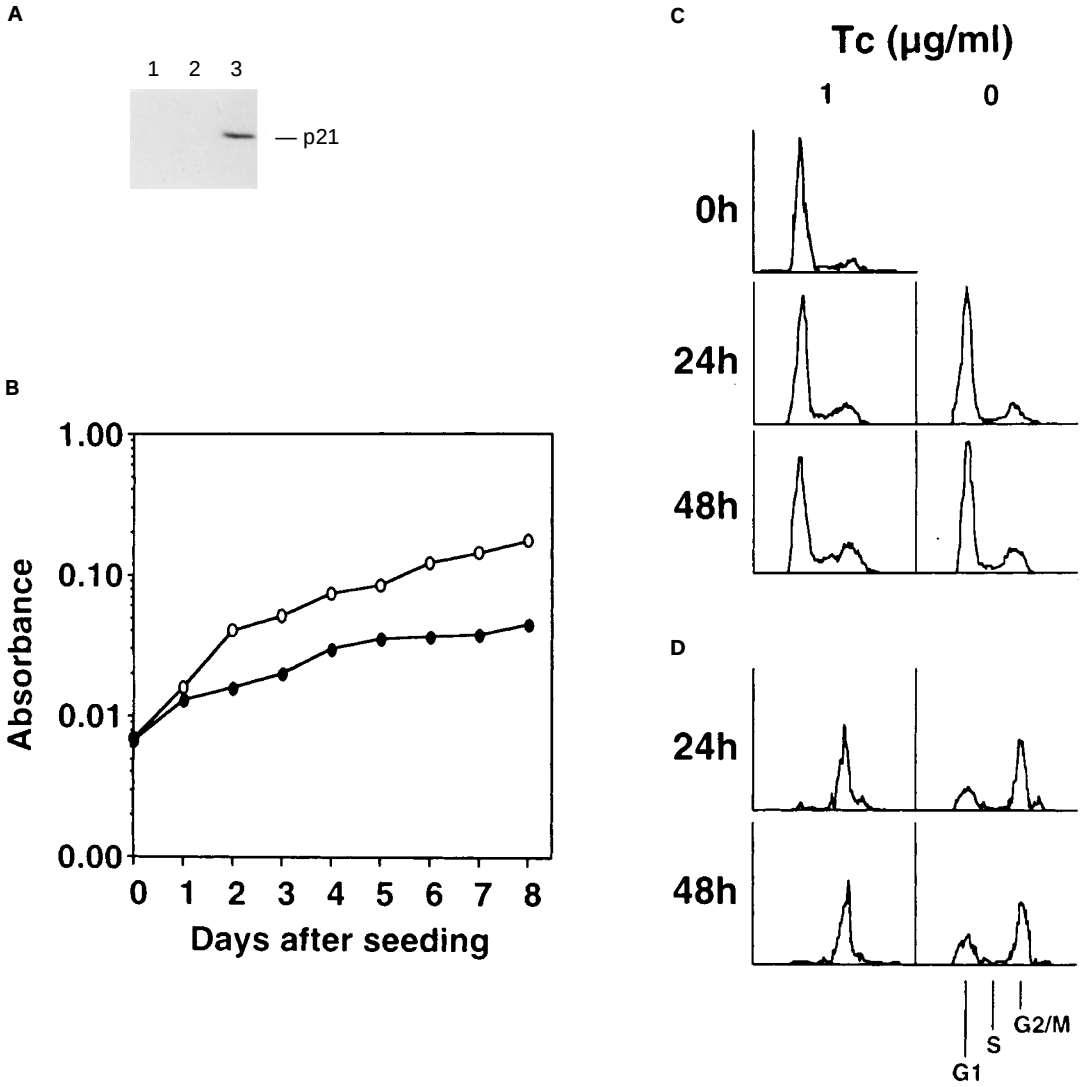

Figure 4 Effect of p21 induction on the growth and cell cycle of N417 SCLC cells. (**A**) Induction of p21 expression under Tc control. p21-1 cells were incubated in the presence or absence of 1 μg/ml Tc at 37°C for 24 h. The following procedure was performed as described in the legend to Figure 1, and then the membrane was incubated with the anti-p21 polyclonal antibody. Lane 1, parental N417; lane 2, p21-1 cells in the presence of 1 μg/ml Tc; lane 3, p21-1 cells in the absence of Tc. (**B**) Growth curve of p21-1 cells in the presence (open circles) and absence (closed circles) of Tc. 5×10^3 cells/ml were seeded. At each indicated time point (every 24 h), cell viability was determined and represented as the degree of absorbance using the MTT assay. The mean absorbance per triplicate wells were plotted against the number of days after seeding. (**C** and **D**) Cell cycle analysis of p21-1 cells after induction in the indicated Tc concentrations (**C**) and after nocodazol treatment (**D**). The experiments of **C** and **D** were performed as those in Figure 2 and Figure 3, respectively. The peaks corresponding to the different phases of the cell cycle are indicated on one of the DNA histograms

1992). Plasmid pT2p21/neo was constructed by subcloning the full-length p21 cDNA into the *Sal*I/*Bam*HI sites of pT2GN. N417-derived NTA4 cells (Adachi *et al*, 1996) were transfected with pT2p21/neo by DIMRIE-C reagent (Gibco BRL-Life Technologies) according to manufacturer's protocol. Cells were selected in medium containing 0.8 mg/ml of G418 and 0.3 mg/ml of hygromycin in the presence of 1 μg/ml of Tc. Hygromycin- and G418-resistant colonies were cloned and expanded for further analyses.

Western blot analysis

Cellular protein was extracted by lysing 1×10^6 cells with 40 μl; of lysis buffer (50 mM HEPES-NaOH, pH 7.0, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM dithiothreitol, 1 mM PMSF, and 50 μg/ml aprotinin). Fifty μg of total protein was separated in a 4–20% gradient sodium dodecyl sulfate (SDS)/polyacrylamide gel and electroblotted to Hybond-Enhanced Chemiluminescence (ECL) nitrocellulose membrane (Amersham). Equal loading of protein was confirmed by staining the membrane after detection. After being blocked with 5% nonfat dry milk and 0.1% Tween 20 in Tris-buffered saline, membranes were incubated at 37°C for 2 h with the human-p53 specific monoclonal antibody (Ab-2) (Oncogene Science), the human p21-specific polyclonal antibody (C-19) (Santa Cruz Biotechnology), the human Bax-specific polyclonal antibody (P-19) (Santa Cruz Biotechnology), the human Bcl-2-specific monoclonal antibody (SC-509) (Santa Cruz Biotechnology), or the human Bcl-$x_{S/L}$-specific polyclonal antibody (S-18) (Santa Cruz Biotechnology). The blot was subsequently probed by the ECL Western blotting detection system (Amersham).

Cell cycle analysis

The cells were serum starved for 48 h in the presence of 1 μg/ml Tc, and then released into growth media in different Tc concentrations. At the time of 0, 12, 24 and 48 h, cells were harvested and fixed with 50% ethanol. When the treatment with nocodazol was performed, nocodazol was added to a final concentration of 40 ng/ml at 24 h after induction and the cells were incubated for additional 12, 24 and 48 h followed by fixation. Cells were then treated with 5 mg/ml RNase A (Sigma), stained with 50 μg/ml propidium iodide (Sigma), and analyzed by flow cytometry for DNA synthesis and cell cycle status. Flow cytometric assay was performed with a FACSCalibur (Beckton Dickinson, San Jose, CA). FACS profiles of cells were calculated using the CellQuest and ModFit Lt softwares.

In vitro growth rate

Cells were assayed for viability using the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay as described by Mosmann (1983). 5×10^3 cells/ml of a p21 inducible clone, p21-1, were seeded in culture media containing 10% FCS and cultured in the presence or absence of 1 μg/ml tetracycline in 24-well plates (day 0). MTT was added to the cultures (500 mg/ml) and incubated at 37°C for 4 h. The intracellular formazan crystals formed were solubilized with dimethyl sulfoxide (DMSO) and the absorbance of the solution at 560 nm was measured using a spectrophotometer. The wells were harvested on each of day 0 to day 8. Doubling times were calculated from lines fitted to log cell numbers by linear regression analysis.

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