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Modulation of Cisplatinum Cytotoxicity by p53: Effect of p53-Mediated Apoptosis and DNA Repair

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ABSTRACT

A stable transfectant (S2SN7) of p53-null SaOS-2 (human osteosarcoma) cells expressing wild-type p53 under the tight control of a tetracycline-responsive promoter was used to study the functional roles of p53 in cellular response to cisplatinum (CP). When cells were grown in media containing normal concentrations (10%) of serum, induction of p53 by tetracycline withdrawal resulted in an 8-fold decrease in sensitivity to CP. In contrast, when cells were grown in lower serum (1%) media, induction of p53 led to a 10-fold increase in sensitivity to CP. The p53-mediated sensitivity to CP under lower serum conditions was attributed, at least in part, to increased susceptibility of p53-mediated apoptosis. Under lower serum (0.1–1%) but not normal serum conditions, p53 induction correlated with selective down-regulation of bcl-2, an inhibitor of apoptosis. In addition, a host-cell reactivation assay showed that induction of p53 caused a significant increase in repair of CP-induced DNA damage under normal serum but not low serum conditions. These data suggest that growth conditions may modulate and possibly reverse p53-mediated CP sensitivity by altering p53-mediated gene regulation and, as a result, susceptibility to apoptosis. They also suggest that a combined effect of p53-mediated apoptosis and DNA repair may be the ultimate determinant in p53-mediated cellular resistance or sensitivity to chemotherapeutic drugs.

The tumor suppressor protein p53, one of the most commonly altered gene products in human cancer (Levine et al., 1991) (by genomic mutation or deletion or by interaction of the gene product with oncoproteins such as E6), plays a pivotal role in cell cycle arrest induced by stress such as drug or irradiation-induced DNA damage (Lee et al., 1994). This p53-mediated cell cycle arrest allows the host cell to repair its damaged DNA before cell division while cells with excessive DNA damage undergo apoptosis. A major mechanism by which p53 mediates cell cycle arrest is through induction of $p21^{waf1}$, a potent inhibitor of cyclin-dependent kinases (Roberts et al., 1994). The normal p53 tumor suppressor protein, widely regarded as "the guardian of the genome" (Lane, 1992), also plays important roles in regulation of apoptosis (Liebermann et al., 1995), differentiation (Rotter et al., 1994), genetic instability (Almasan et al., 1995), and DNA repair (Sanchez and Elledge, 1995). p53 may modulate susceptibility of cells to apoptosis by causing up-regulation of bax and down-regulation of bcl-2 (Selvakumaran et al., 1994), two of the best known regulators of apoptosis (Reed, 1995).

Loss of functional p53 has been associated with poor clinical prognosis (i.e., high recurrence and death rate) in cancer treatment (el Rouby et al., 1993). To find the best possible treatment for different tumors, it is important to understand how p53 status is related to the therapeutic response of tumors to drugs. Several studies (vide infra), both in vitro and in vivo, have provided evidence for a link between the functional status of p53 and drug or radiation sensitivity. Earlier work with transgenic mice expressing no p53 or mutant p53 showed that, in tumors that these animals developed, mutations or absence of p53 correlated with increased resistance of tumor cells to irradiation or treatment with drugs such as etoposide, 5-fluorouracil, and doxorubicin (Clarke et al., 1993). This result (i.e., increased irradiation or drug resistance due to loss of or mutations in p53) was subsequently confirmed in studies involving various cell systems (Pardo et al., 1994) or animal tumor models (Fujiwara et al., 1994) and in treatment of patients with certain cancers (Rusch et al., 1995). Loss of p53 function by expression of either the viral protein E6 or mdm-2 [both of which inactivate p53 (Momand et al., 1992)] also correlated with resistance to irradiation and drug treatment (Kondo et al., 1995). Introduction of wild-type p53 in cells lacking p53 or containing mutant p53 was shown to confer sensitivity of these cells to radiation or drug treatment (Gjerset et al., 1995). All of the above-mentioned findings suggest that loss of functional p53

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ABBREVIATIONS: Tet, tetracycline; CAT, chloramphenicol transferase; FBS, fetal bovine serum; PBS, phosphate buffered saline; PCR, polymerase chain reaction; IC_{50}, concentration of drug that is responsible for 50% of cell kill; CP, cisplatinum.

can render cells more resistant to irradiation or to drug treatment.

In contrast, other studies showed that functional loss of p53 resulted in increased sensitivity to irradiation and to certain chemotherapeutic drugs. It was reported recently that disruption of p53 by E6 expression in a breast cancer cell line sensitized these cells to cisplatinum (CP) (Fan et al., 1995) and that inactivation of p53 enhances sensitivity to multiple chemotherapeutic drugs, including CP (Hawkins et al., 1996). It also was reported that p53 mutations were correlated with sensitivity to X-rays (Biard et al., 1994). Another study (Wahl et al., 1996) showed that loss of functional p53, as a result of E6 or SV40 T antigen expression in normal human fibroblasts, sensitized cells to taxol, a drug that stabilizes tubulin polymerization, resulting in mitotic arrest and apoptotic cell death (Donaldson et al., 1994)). Furthermore, other studies suggested that there is no apparent correlation between functional status and levels of p53 and drug or radiation resistance (Jung et al., 1992).

These seemly controversial results may occur because different cell systems were used in these studies, which may display varying degrees of susceptibility to drug-induced apoptosis. But this explanation may be overly simplified because susceptibility to apoptosis of different cell lines was not measured or compared in any quantitative manner in these studies. The mechanism(s) by which p53 confers either resistance or sensitivity to irradiation and anticancer drugs is yet to be determined. In the current study, we used a tightly regulated tetracycline (Tet)-inducible expression system (Gossen and Bujard, 1992) for wild-type p53 stably integrated into the p53-null human osteosarcoma cell line (SaOS-2) to study the relationship between p53 expression and sensitivity to CP. Our studies revealed that induction of p53 conferred resistance to CP when cells were cultured in media containing normal serum concentrations, whereas p53 induction led to CP sensitivity when cells were grown in lower serum media. p53-mediated CP sensitivity under lower serum conditions correlated with selective down-regulation of bcl-2 upon induction of p53. This growth condition-dependent modulation of drug sensitivity suggests that certain serum factors may determine and even reverse p53-mediated CP resistance or sensitivity by intervening with p53-mediated gene regulation. In addition, induction of p53 resulted in a significant increase in repair of CP-induced DNA damage. Thus, p53-mediated drug resistance and p53-mediated drug sensitivity were observed in the same cells under different growth conditions. A combination of p53-mediated apoptosis and DNA repair may determine p53-mediated cellular resistance or sensitivity to chemotherapeutic drugs.

Materials and Methods

Chemicals and Reagents. Tet, sulforhodamine B, Nonidet P-40 (Nacalai Tesque, Kyoto, Japan), and CP were obtained from Sigma Chemical Co. (St. Louis, MO). Geneticin (G418) was obtained from Life Technologies, Inc. (Gaithersburg, MD). Media and sera for cell culture were purchased from Grand Island Biological Co. (Grand Island, NY). Liposomes (DOTAP/DOPE) used for transient transfections (see below) were either purchased from Boehringer Mannheim Corp. (Indianapolis, IN) or made available by the Liposome Facility, Cornell University Medical College, NY (courtesy of Dr. A. Scotto). Antibodies to p53 (DO-1), bcl-2, bax, and bcl-x were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). All other chemicals were reagent grade and from standard commercial sources.

Cell Line. The SaOS-2 cell line was obtained from American Type Culture Collection (Rockville, MD) and is maintained as monolayer cultures at 37°C in a 5% CO_2/95% air incubator in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS).

Plasmids. The Tet-inducible system plasmids pUHD10–3 and pUHD15–1 (Gossen and Bujard, 1992) were generous gifts from Dr. Herman Bujard (Heidelberg, Germany). The plasmid pC53-SN3 containing the wild-type p53 cDNA was kindly provided by Dr. Arnold Levine (Princeton, NJ). All plasmid DNAs were prepared from *Escherichia coli* strain DH5α and purified by ion exchange chromatography with the plasmid midi kit from Qiagen, Inc. (Chatsworth, CA).

Construction of Inducible Vector System for Wild-Type p53. The Tet-responsive system (Gossen and Bujard, 1992), which is composed of two plasmids (pUHD10–3 and pUHD15–1), was used to construct the inducible vectors for controlled expression of wild-type p53. First, pUHD15–1 was modified to contain the selectable marker neo. This was accomplished by cloning the 1.1-kilobase tTA fragment from pUHD15–1 into the multiple cloning site of the plasmid pcDNA3 (Invitrogene, San Diego, CA); the resulting plasmid is designated as pcdtTA. Then pUHD10–3 was modified to contain the cDNA sequences for wild-type p53, which was derived from the 1.8-kilobase *Bam*HI fragment from the plasmid pC53-SN3 (Kern et al., 1992). The resulting plasmid is designated as pUHDp53.

Stable Transfection of p53 Expression System into Cell Lines. The transfection of SaOS-2 (a human osteosarcoma cell line) cells with the Tet-responsive system was accomplished with a two-plasmid cotransfection protocol and a lipofection procedure (Stamatatos et al., 1988). Cells were allowed to grow to ~50% confluence in a 100-mm Petri dish in a medium containing 1 μg/ml Tet. A total of 20 μg of the two plasmids (pUHDp53 and pcdtTA) at a molar ratio of 10:5 was mixed with 70 μg of the lipofectant DOTAP (Boehringer Mannheim Corp.) in a total of 0.5 ml HEPES buffered saline (20 mM HEPES, pH 7.4, and 150 mM NaCl) and incubated at room temperature for 10 min. Fourteen milliliters of fresh growth medium containing Tet was added and, after removal of old medium from cells, the mixture was then added to cells and incubation continued for 24 h. Cells were split 1:5 and grown in a medium containing 1 μg/ml Tet. G418 selection began 24 h later at a concentration just high enough to kill 100% of the parental (untransfected) cells. Approximately 2 to 5 weeks later, colonies (for monolayer cells only) were picked by cloning cylinder techniques and expanded into cell lines (SaOS-2-p53 cells).

Identification of p53-Positive Clones by Polymerase Chain Reaction (PCR). The protocol for specific PCR amplification of the inserted gene sequence from whole cells was adapted from Perkin Elmer Cetus *Amplifications* (May 1989, Issue 2). Approximately 1×10^4 to 1×10^5 cells, which may be grown in a 24-well plate right after colony isolation with cloning cylinders, were detached with trypsin digestion and washed two times with phosphate-buffered saline (PBS). The cell pellet was resuspended in 50 μl of the following PCR-detergent buffer: 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.45% Nonidet P-40, 0.45% Tween-20, and 0.1 mg/ml proteinase K. After incubation at 37°C for 1 h followed by inactivation of proteinase K at 95°C for 10 min, 25 ml of this reaction mixture was subjected to 37 cycles of PCR amplification with a SV40 promoter-specific primer and a p53-specific primer. The clones that show the expected 600-bp PCR product on the agarose gel have incorporated the inserted p53 cDNA.

Western Blot Analysis. Cells from a clone (S2SN7) of SaOS-p53 cells were grown to mid-log phase in the presence of 2 μg/ml Tet. After being washed extensively to remove extraneous Tet, the cells were collected by trypsinization and split equally into two culture flasks containing or lacking 2 μg/ml Tet, respectively. After a 24-h incubation, the medium in each flask was changed with fresh medium containing or lacking 2 μg/ml Tet. After an additional 48-h incubation, cells were harvested by trypsinization, washed with

PBS, and solubilized with a buffer (50 mM NaCl, 20 mM Tris-HCl, pH 7.4) containing 2% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, and 0.2% (w/v) SDS, plus a mixture of protease inhibitors (20 μg/ml leupeptin, 30 μg/ml aprotinin, 10 μg/ml pepstatin A, 20 μg/ml soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate). The extract was centrifuged at 60,000*g* for 30 min to remove any insoluble cellular debris and the protein concentration was determined by the bicinchoninic acid assay according to the manufacturer's instructions (Pierce Chemical Co., Rockford, IL). The cell extract (containing 150 μg of total protein) was mixed with equal volume of 2× SDS sample buffer (20% (v/v) glycerol, 125 mM Tris-HCl, pH 6.8, 1% (v/v) β-mercaptomethanol, and 0.05% (w/v) bromophenol blue) and loaded onto a 12.5% polyacrylamide gel containing SDS. After electrophoresis, the proteins were electrotransferred to a nitrocellulose membrane and the latter was probed with an anti-p53 antibody followed by a second antibody conjugated with peroxidase. The p53 protein was then visualized by treatment of the membrane with the enhanced chemiluminescence reagents (Amersham Corp., Arlington Heights, IL) followed by exposure to X-ray film.

Cytotoxicity Assays. Cytotoxicity of drugs was measured with a microculture technique and sulforhodamine B (SRB) binding assay. Mid-log phase SaOS-2-p53 cells (clone S2SN7) were harvested by trypsinization and plated at a density of 3×10^3 cells/well in a 96-well plate. Each well contained a total of 180 μl of fresh RPMI 1640 medium supplemented with 10, 1, or 0.1% (v/v) dialyzed FBS, with or without 1 μg/ml Tet. After incubation at 37°C overnight to allow cell attachment, CP in 20 μl of saline was added to each well to give final concentrations ranging from 1×10^{-4} to 3×10^{-10} M (3× serial dilutions, prepared in a separate 96-well plate) and the plate was incubated at 37°C for another 24 h. The drug was removed by washing the plate with serum-free medium and cells were allowed to grow for 72 h under the same conditions as those before the washing step. Cells were fixed to the bottom of the plate by addition of 50 μl of 50% (w/v) trichloroacetic acid to each well and incubation at 4°C for 1 h. The plate was washed five times with water and the fixed cells were stained with 0.4% sulforhodamine B in 1% acetic acid at room temperature for 30 min. After washing four times with 1% acetic acid followed by solubilization of SRB with 150 μl/well of 10 mM Tris base, the plate was read at 562 or 610 nm. The absorbance (i.e., relative cell survival) was plotted against the drug concentration. IC_{50} values were determined and the averages of triplicate or quadruplicate measurements were used. $IC_{50}(-Tet)$ represents the IC_{50} value obtained in the absence of Tet; $IC_{50}(+Tet)$ represents the IC_{50} value in the presence of Tet.

Host-Cell Reactivation Assay. The reporter plasmid pGL3luc (Promega Biotec, Madison, WI) containing the luciferase gene driven by the SV40 promoter was treated for 24 h at 37°C with increasing concentrations (0–4 μM) of CP in a diluted (1:50) PBS buffer at a DNA concentration of 100 μg/ml. The damaged DNA was recovered by precipitation with ethanol, and after being dissolved in H_2O, the concentration of the plasmid was determined. Before transfection, S2SN7 cells were grown to mid-log phase in the presence of 1 μg/ml Tet. After being washed extensively to remove extraneous Tet, the cells were collected by trypsinization and seeded at 1×10^6 cells/plate into two separate sets of 100-cm culture dishes, both of which contain 10 ml of RPMI 1640 medium supplemented with 10% serum; one group had 1 μg/ml Tet added and the other had no Tet. After incubation for 24 h at 37°C and 5 h before transfection, the medium in each set of dishes was replaced with media containing or lacking Tet, with 10 or 1% serum. DNA samples consisting of 8 μg of CP-treated reporter plasmid and 2 μg of control plasmid (PSV2CAT) were mixed with 0.5-ml serum-free RPMI 1640 medium and the resulting DNA mixture was incubated for 1 h at room temperature. In a separate tube, 50 μg of the lipofectant DOTAP/DOPE (1:1) was mixed with 0.5 ml of serum-free RPMI 1640 medium and the resulting liposome mixture was incubated for 1 h at room temperature. The DNA mixture and the liposome mixture were combined and incubated for another 30 min at room temperature. The DNA/liposome mixture, after being diluted by addition of 5 ml of RPMI 1640 medium supplemented with 10 or 1% serum, with or without Tet added, was added to cells in the culture dishes (see above) whose medium had been removed. After incubation at 37°C for 16 h, the medium from each dish was replaced with the same medium (with 10 or 1% serum, containing or lacking Tet), and incubation was continued for 48 h. After being washed twice with PBS, the transfected cells from each dish were recovered by use of 0.75 ml of Reporter Lysis Buffer (Promega Biotec) and by scraping with a rubber policeman. The cell lysate was transferred to a microfuge tube and centrifuged to pellet large cell debris. The supernatant was stored at −70°C or used immediately for luciferase and chloramphenicol acetyltransferase (CAT) assays (see below).

Luciferase and CAT Activity Assays. The luciferase activity of the lysate prepared as described above was measured by use of the luciferase assay reagent (Promega Biotec) according to the manufacturer's instructions. Briefly, 40 μl of cell lysate and 100 μl of the luciferase assay reagent, both of which were prewarmed to room temperature, were mixed and the relative light units produced for a 20-s period were measured immediately on a luminometer (Monolight 2010; Analytical Luminescence Laboratory, San Diego, CA). The CAT activity of the cell lysate prepared from the transfected cells (see above) was measured by use of [^{14}C] labeled acetyl CoA and chloramphenicol as substrates and a radio diffusion procedure according to manufacturer's instructions. Briefly, 50 μl of cell lysate was mixed with 0.2 ml of 1.25 mM chloramphenicol in 100 mM Tris-HCl buffer (pH 7.8) in a 7-ml glass scintillation vial. The reaction was initiated by adding 0.1 μCi [^{14}C]acetyl CoA and 22.5 μl 1 mM cold acetyl CoA. Five milliliters of Econofluor-2 (NEN Research Products, Boston, MA) was gently overlaid and the vials were incubated at room temperature. At timed intervals, the vials were counted with a Beckman liquid scintillation counter. Relative CAT activity was calculated by comparison of the increase in counts over time.

Results

Inducible Expression of Wild-Type p53 in Saos-2 Cells. Because expression of exogenous p53 may be detrimental to cells (Noble et al., 1992) and there is the likelihood of artifacts caused by clonal variation (a common problem associated with stable transfection with noninducible expression vectors), SaOS-2 cells lacking functional p53 due to a large deletion in the p53 gene were used to obtain stable transfectants expressing inducible wild-type p53, under the control of a Tet-responsive promoter. The G418-resistant p53-positive transfectants (designated S2SN7) were subsequently tested for p53 expression under the presence or absence of Tet. Western blotting analysis with a p53 specific antibody revealed that p53 was strongly induced by Tet withdrawal (lane 1, Fig. 1), whereas p53 expression was nearly completely suppressed when cells were grown in the presence of Tet (lane 2). The level of p53 induced by Tet withdrawal (lane 1) was comparable to that in HT1080 cells (lane 4), which are known to express wild-type p53 (Sharma et al., 1993). p53 was absent, as expected, in the control cell line (i.e., nontransfected wild-type SaOS-2 cells) (lane 3). These data indicate that p53 expression is tightly regulated by Tet. The p53 protein induced by Tet withdrawal proved to be functional (i.e., transcriptionally active) because induction of p53 was accompanied by increased expression of both $p21^{WAF1}$ and Gadd45, two p53 effector molecules (Cox and Lane, 1995) that are known to be transcriptionally regulated by p53 (data not shown).

Effect of Growth Conditions and p53 Expression on CP Cytotoxicity. There are several contradictory reports (see *Introduction*) as to how p53 modulates CP cytotoxicity. By taking advantage of a tightly regulated Tet-inducible system for p53 as described above, we were able to study the effect of p53 on CP sensitivity or resistance under different growth conditions. Cytotoxicity of CP was measured in S2SN7 cells grown in media containing high (10%) or low (1%) concentrations of serum under both induced (i.e., in the absence of Tet) and noninduced conditions (i.e., in the presence of Tet). As shown in Fig. 2, under normal growth conditions (i.e., 10% serum) (Fig. 2A), S2SN7 cells were 8-fold more resistant to CP when p53 was induced by Tet withdrawal compared with cells under noninduced conditions, with IC_{50} values (i.e., the concentration of drug that is responsible for 50% of cell kill) of 7.5×10^{-5} M and 9.0×10^{-6} M, respectively. In contrast, when cells were grown in media containing a lower percentage (1%) of serum (Fig. 2B), S2SN7 cells were found to be 10-fold more sensitive to CP when p53 was induced by Tet withdrawal compared with cells under noninduced conditions, with IC_{50} values of 8.5×10^{-5} M and 9.0×10^{-4} M, respectively. Similar data were obtained when cells were grown in media containing even a lower concentration (0.1%) of serum compared with cells grown with media containing 1% serum (data not shown). These data indicate that induction of p53 may render cells either more resistant or more sensitive to CP, depending on the growth conditions (i.e., serum concentrations) under which the cells were grown.

High Serum Concentrations Suppress p53-Mediated Cell Death. To understand how growth conditions modulate p53-mediated CP sensitivity or resistance, the effect of p53 expression in the absence of drugs on cell growth was studied in S2SN7 cells. Under normal growth conditions (i.e., in media containing 10% serum), induction of p53 in S2SN7 cells by Tet withdrawal led to a slightly decreased growth rate although it had no effect on morphology (data not shown). When cells were grown in media containing a lower percentage (0.5%) of serum, however, induction of p53 by Tet withdrawal for extended periods (>10 days) led to extensive cell death typical of apoptosis (Fig. 3C), whereas in the presence of Tet cells were completely viable under these conditions (Fig. 3D). Cell death was not evident when S2SN7 cells were cultured in low serum media lacking Tet for shorter times (<5 days) (data not shown). These data indicate that the presence of a higher concentration (10%) of serum in the growth media suppresses p53-mediated apoptotic cell death. This finding is consistent with previous studies showing that growth factors such as interleukin 3 (IL-3) and IL-6 modulate p53-mediated apoptosis in certain leukemia cell lines (Zhu et al., 1994). These data suggest that p53-mediated increase in sensitivity of S2SN7 cells to CP under lower serum conditions (but not under high serum conditions) is most likely due to p53-enhanced susceptibility to apoptosis under these conditions.

Selective Down-Regulation of bcl-2 by p53 under Lower Serum Conditions. It has been shown previously that overexpression of bcl-2 or bcl-x_L confers resistance to CP (Miyashita and Reed, 1993) and that p53 modulates the susceptibility of cells to drug-induced apoptosis by coordinately regulating the expression of members of the bcl-2 family, including bcl-2, bax, and bcl-x, major regulators of apoptosis. To further understand the mechanism(s) by which p53 mediates resistance or sensitivity to CP, we investigated the effect of p53 induction on expression of the bcl-2 family members under different growth conditions. As shown in Fig. 4A, when cells were cultured in media containing low concentrations of serum, induction of p53 with Tet withdrawal resulted in down-regulation of the bcl-2 protein (compare lanes 3 and 4, Fig, 4A). However, no change in the levels of the bcl-2 protein was evident when cells were grown in high serum media (lanes 1 and 2). In addition, Fig. 4, B and C showed that induction of p53 did not change the protein levels of the other two bcl-2 family members bax (Fig. 4B)

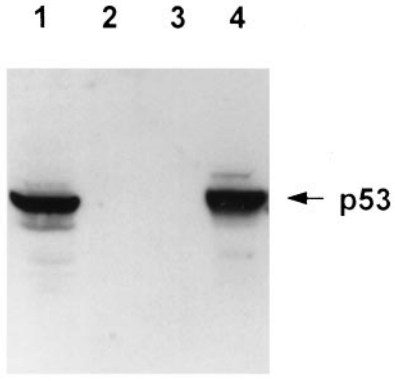

Fig. 1. Inducible expression of p53 in SaOS-2 cells. Mid-log cells from a clone (S2SN7) of SaOS-2 cells stably transfected with the Tet-inducible system for wild-type p53, which were maintained in the presence of Tet, were grown to mid-log phase (48-h incubation) in media containing or lacking Tet (1 μg/ml). Cells were collected by trypsinization and proteins from the total cellular extract were subjected to SDS-polyacrylamide gel electrophoresis followed by Western blotting with a monoclonal anti-p53 antibody (DO-1) (see *Materials and Methods* for details). Lane 1, S2SN7 cells grown in the absence of Tet; lane 2, S2SN7 cells grown in the presence of Tet; lane 3, nontransfected SaOS-2 cells; and lane 4, HT1080 (human fibrosarcoma) cells. The position of p53 is indicated by the arrow. The figure is representative of three different experiments.

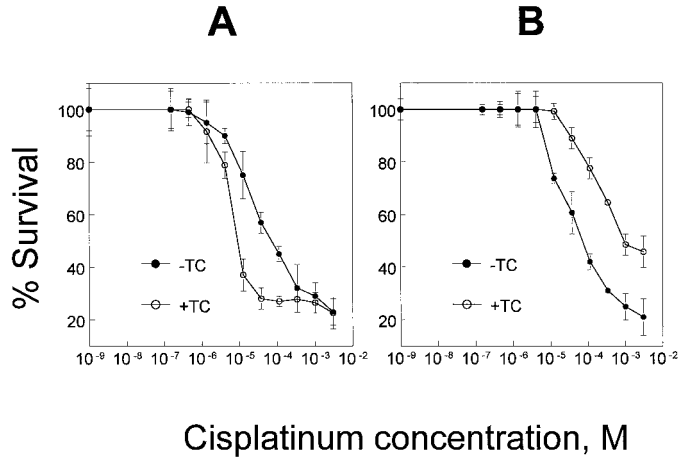

Fig. 2. Effect of p53 expression and serum concentrations on cytotoxicity of CP. Mid-log S2SN7 cells, maintained in the presence of Tet, were plated in 96-well plates in media containing 10% (A) and 1% (B) of FBS, in the presence or absence of Tet (1 μg/ml). After incubation for 24 h to allow cell attachment, CP was added to the indicated concentrations. Cell survival (after 72-h incubation) was measured by use of the SRB assay as described in *Materials and Methods*. Relative cell survival (i.e., precentage of that for cells grown in the absence of drug under each growth condition) was plotted against drug concentrations. Values (at each concentration point) represent means ± S.D. from four experiments. TC, tetracycline.

and bcl-x (Fig. 4C) under both low and high serum conditions. These data indicate that serum factors may suppress p53-mediated down-regulation of bcl-2, thereby reducing the susceptibility of cells to p53-mediated apoptosis.

p53 Enhances Repair of CP-Induced DNA Damage. Although p53-mediated apoptosis may well explain p53-induced sensitivity to CP under lower serum conditions, the mechanism by which p53 mediates resistance to CP under normal serum conditions remains to be determined. Sturzbecher et al. (1996) have shown that p53 is involved, either directly or indirectly, in repair of DNA damage induced either by drug treatment or UV irradiation. To test whether p53-mediated effect on DNA repair plays any role in CP resistance, a host-cell reactivation assay was conducted in which CP-damaged reporter plasmid (i.e., pGL3luc containing the luciferase gene driven by the SV40 promoter) was transiently transfected into S2SN7 cells grown in media containing or lacking Tet. As shown in Fig. 5A, induction of p53 by Tet withdrawal under normal (10%) serum conditions led to a significant (2- to 3-fold) increase in reactivation of the reporter activity from the plasmid damaged by various doses of CP. In contrast, when transfected cells were grown in media containing a lower concentration (i.e., 1%) of serum, p53 expression had no significant effect on reporter reactivation (Fig. 5B). These results indicate that induction of p53 indeed enhances repair of CP-induced DNA damage under normal serum conditions, thereby providing a possible explanation for p53-mediated CP resistance under these conditions.

Discussion

By taking advantage of a tightly controlled inducible expression system for p53 stably integrated into SaOS-2 cells, we studied the effect of p53 expression on sensitivity of these cells to CP, a commonly used chemotherapeutic drug that is suggested to kill cells by induction of strand breaks and apoptosis. Cells were more sensitive to CP upon p53 induction (by Tet withdrawal) when they were grown in media containing low concentrations of serum, whereas cells became actually more resistant to CP upon p53 induction under normal serum conditions. The p53-mediated sensitivity to CP under lower serum conditions is consistent with the observation that cells were more sensitive to p53-mediated cell death (mostly likely via apoptosis) when cultured for a long period (10 days) in low serum media (Fig. 3). Because no cell death was evident when S2SN7 cells were cultured in low serum media lacking Tet for short periods (e.g., 5 days) (data not shown), approximately the same duration as the cytotoxicity assay (72-h drug exposure), the observed cytotoxic effect of CP to S2SN7 cells grown in lower serum media lacking Tet was not attributed to the detrimental effect of p53 and rather reflected an increased sensitivity of these cells to the drug. It is likely that under low serum conditions expression of p53 increases sensitivity of S2SN7 cells to CP by enhancing CP-

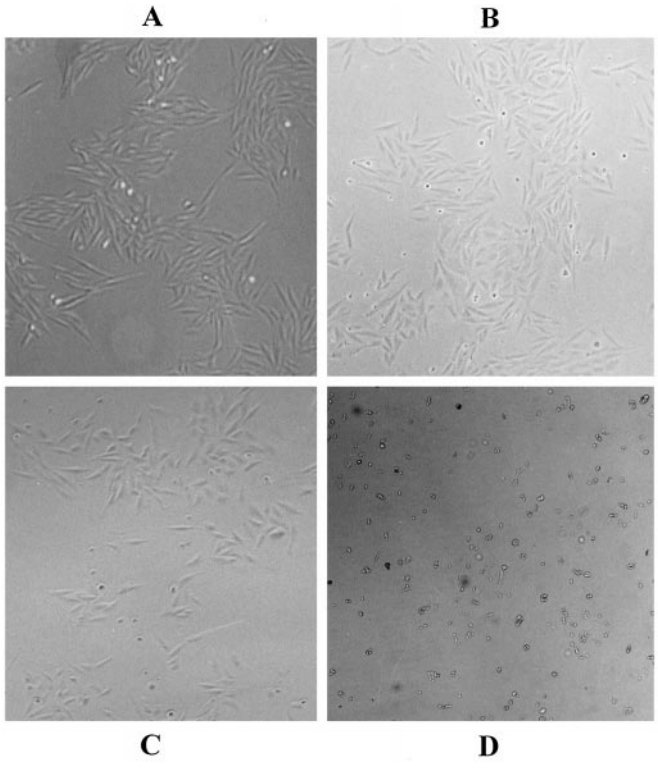

Fig. 3. Effect of p53 and serum concentrations on cell survival. Mid-log S2SN7 cells, maintained in the presence of Tet, were plated in 6-well plates in media containing 10 or 0.5% FBS with or without addition of Tet (1 μg/ml). After incubation at 37°C for 10 days with one change of the respective media, the plates were photographed under a phase-contrast microscope. Cells were grown in media with 10% FBS containing (A) or lacking Tet (B). Cells were grown in media with 0.5% FBS containing (C) or lacking Tet (D). This experiment was repeated four times and similar results were obtained.

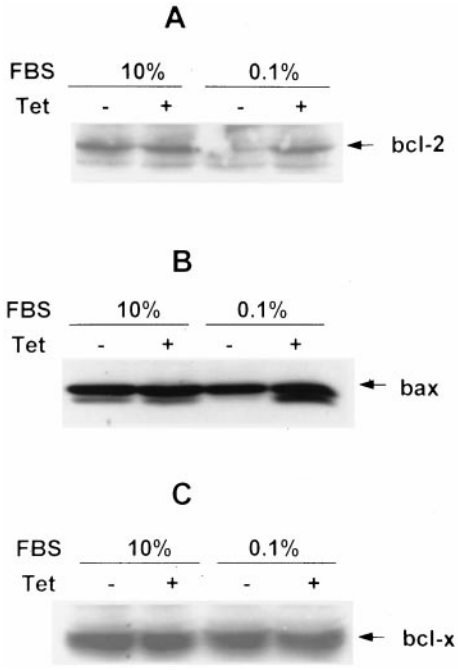

Fig. 4. Effect of serum concentrations on p53-mediated regulation of bcl-2. S2SN7 cells (mid-log), maintained in the presence of Tet, were grown to mid-log phase (48-h incubation) in media containing or lacking Tet (1 μg/ml), with multiple tissue culture flasks. Cells from one half of these flasks (growing in the presence or absence of Tet) were subjected to an additional 24-h serum deprivation by incubating cells with media containing 0.1% FBS, with or without Tet added, respectively. Cells were collected by trypsinization and proteins from the total cellular extract were subjected to SDS-polyacrylamide gel electrophoresis followed by Western blotting with polyclonal antibodies against bcl-2 (A), bax (B), or bcl-x (C). Positions of bcl-2, bax and bcl-x were indicated by arrows. The figure is representative of three independent experiments.

induced apoptosis. The latter correlated with the selective down-regulation of the apoptosis inhibitor bcl-2 upon p53 induction under low serum conditions (Fig. 4).

p53-Mediated resistance to CP when S2SN7 cells were grown in normal media (i.e., containing 10% serum) may be a result of the combined effects of p53-mediated increase in DNA repair (Fig. 5A) and suppression of p53-mediated apoptosis by serum factors (Fig. 3). It is demonstrated that the stimulative effect on DNA repair by p53 is minimized or abolished when cells are subjected to low serum conditions (Fig. 5B), suggesting that p53-mediated enhancement of DNA repair may be serum-dependent. Our result is consistent with that of a previous report showing that p53 is capable of enhancing DNA repair in other cell types (Smith et al., 1995). Serum factors such as EGF, insulin, or IL-3 are known to block p53-mediated apoptosis (Merlo et al., 1995). An in vitro study showed that endogenous IL-6 is a resistance factor for CP and etoposide-mediated cytotoxicity to two prostate carcinoma cell lines (Borsellino et al., 1995). These observations along with ours support the notion that p53-mediated resistance to CP under high serum conditions is a result of both inhibition of p53-mediated apoptosis by serum factors and p53-mediated enhancement in DNA repair. In addition, p53 was shown previously to enhance DNA repair probably by up-regulating Gadd45, a protein suggested to be involved in both cell cycle arrest and DNA repair (Hall et al., 1995). Interestingly, it was observed in the current study that induction of p53 resulted in up-regulation of Gadd45 even under high serum conditions (data not shown), suggesting that serum factors selectively suppress p53-mediated down-regulation of bcl-2 (thereby inhibiting p53-mediated apoptosis) but allow p53-mediated up-regulation of Gadd45 (thereby enhancing DNA repair).

At lower (1 or 0.1%) serum concentrations, the doubling times for either the parental or transfected S2SN7 cells (with or without Tet) are significantly increased over that of cells at normal (10%) serum concentrations. It also was observed that at lower serum concentrations, there are more cells at the G_1 phase of the cell cycle. These differences (i.e., G1 arrest and increase in doubling time) may explain, at lease partially, why cells at low serum concentrations are less sensitive to CP than cells at higher serum concentrations.

One possible reason for the controversial results obtained from previous studies concerning the relationship between the functional status of p53 and drug/irradiation resistance is that very different cell lines were used in each study, which may display variable levels of susceptibility to apoptosis and also may have other known or unknown genetic changes that contribute to affect p53-mediated response to drugs or irradiation. Data from the current study may provide an explanation for these seemingly controversial results. We have demonstrated, in one single cell line, that p53 may either increase or decrease cellular sensitivity to CP depending on the growth environment to which the cells are exposed. p53 may increase sensitivity of cells to CP when p53-induced apoptosis is not compromised and it may render cells more resistant to CP (via mechanisms such as enhanced DNA repair) when p53-mediated apoptosis is suppressed by conditions such as high serum concentrations. It is possible that this result may be extended to other DNA-damaging agents that kill cells by apoptosis and cause DNA damage whose repair can be enhanced by p53. Interestingly, as indicated in our previous study (Li et al., 1995), induced expression of functional pRb in the same (SaOS-2) cell line (lacking pRb), which renders cells more sensitive to antimetabolites such as methotrexate, does not change the sensitivity of cells to CP. Our data suggest that inhibition of the protective effect of certain serum factors (referred to as "survival factors" in some articles) on p53-mediated apoptosis (e.g., with IL-6 antagonists or other growth factor antagonists) in combination with conventional drug treatment may be an effective strategy in overcoming certain types of p53-mediated drug resistance.

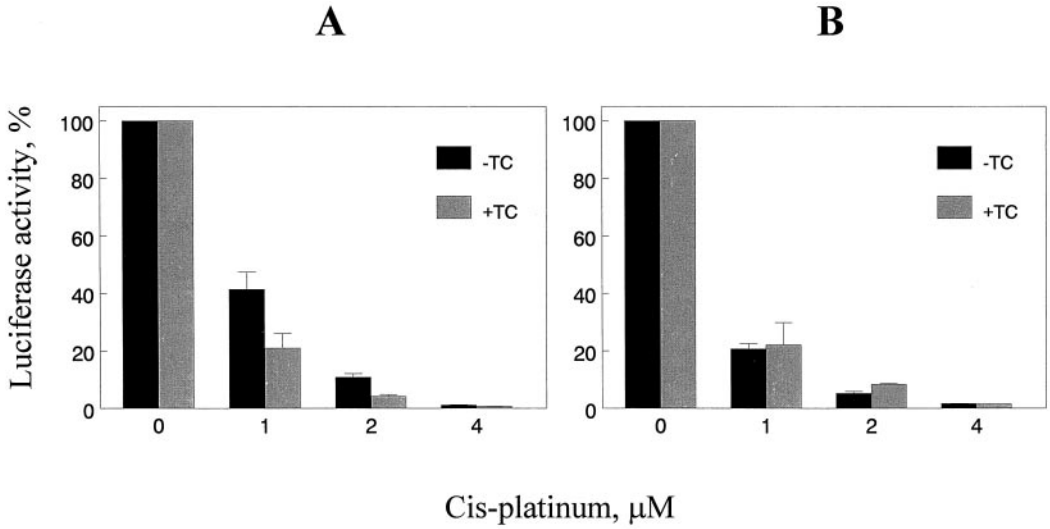

Fig. 5. Effect of p53 on the DNA repair capacity as measured by a host-cell reactivation assay. S2SN7 cells were plated in 100-mm culture dishes in media containing or lacking tetracycline (Tet) (1 μg/ml). After 24 h of growth, cells were cotransfected with the plasmid pGL3luc that had been damaged by increasing doses of CP and the plasmid pSV2CAT (as a control plasmid), in the presence or absence of Tet, respectively. After transfection and an additional 48 h of growth in their respective media, cells were collected and assayed for both luciferase and CAT activities as described in *Materials and Methods*. The luciferase activities were normalized to the CAT control. Values shown are percentages of luciferase activity relative to those obtained with undamaged plasmid. Bars represent S.D. from three experiments. A, transfected cells were grown in media with 10% serum. B, transfected cells were grown in media with 1% serum.

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