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Effects of Wild-Type p53 Expression on the Quantity and Activity of Topoisomerase IIα and β in Various Human Cancer Cell Lines

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Abstract The p53 null HL-60 cell line was transfected with plasmids coding for either the wild-type p53 or mutant p53 gene. The stable expression of wild-type p53 resulted in a significant increase in sensitivity to the topoisomerase II poisons etoposide and doxorubicin, but not to the topoisomerase II inhibitors razoxane and ADR-529. HL-60 cells expressing wild-type p53 demonstrated 8- to 10-fold more VP-16 induced DNA breaks by the alkaline elution assay. The effect of inducible expression of wild-type p53 was also studied in the p53 null erythroblastoid cell line K562 and in the human squamous carcinoma cell line SqCC. The inducible expression of wild-type p53 in the K562 cell line resulted in a 3-fold increase in sensitivity to VP-16. The quantity of topoisomerase IIα was not altered by the transfection as determined by immunoblotting, while the amount of the β isoform was increased 2.5-fold in HL-60 cells. The topo II catalytic activity present in nuclear extracts was measured as the decatenation of kinetoplast DNA, and found to be unaltered by p53 expression. Immunostaining for topoisomerase IIα was substantially diminished in both stable and inducible wild-type p53 expressing cells when three different antibodies were used (two polyclonal and one monoclonal). However, the addition of VP-16 resulted in a rapid appearance of nuclear fluorescence for topoisomerase IIα. No changes in topoisomerase IIβ immunostaining were observed. These results suggest that an epitope for topoisomerase IIα is concealed in cells expressing wild-type p53 and that a complex between topoisomerase IIα and p53 may be disrupted by the addition of antitumor drugs. J. Cell. Biochem. 75:245–257, 1999. © 1999 Wiley-Liss, Inc.

Key words: topoisomerase II; p53; drug sensitivity; immunofluorescence; VP-16

There is increasing evidence that alterations of cell cycle checkpoints in cell lines and tumors may result in resistance to chemotherapeutic agents. More than 60% of human tumors have alterations of p53 that are manifested by dysfunction of the p53-dependent transactivation and apoptotic pathways. Several mutant p53 expressing cell lines and tumors display an intrinsic resistance to most chemotherapeutic drugs, including alkylating agents, topo I and II poisons, and antimetabolites [Lowe et al., 1993, 1994; Fan et al., 1994; Sugrue et al., 1997; O'Connor et al., 1997; Preudhomme and Fenaux, 1997; Iwadate et al., 1998; Wilson et al., 1997], while fewer examples [Trepel et al., 1997] argue for the opposite correlation. These cells exhibit elevated levels of mutant p53 that is stably accumulated, while wild-type p53 has a short half-life. DNA damage activates the nuclear accumulation of p53, which may induce G_1 cell cycle arrest through the cyclin dependent kinase inhibitor p21waf1/cip1 [El-Deiry et al., 1993]. This arrest is considered vital for the

Abbreviations used: topo II, DNA topoisomerase II (EC 5.99.1.3); PBS, phosphate-buffered saline; BSA, bovine serum albumin; kDNA, kinetoplast DNA; DMSO, dimethylsulfoxide; VP-16 (etoposide), 9-(4,6,-*O*-ethylidene-β-D-glucopyranosyl)-4′-demethylepipodophyllotoxin; tet, tetracycline.

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repair of DNA damage, otherwise the cell will undergo apoptosis. Putative molecular mechanisms by which alterations in cell cycle genes, and specifically p53, affect the expression of cellular targets of chemotherapeutic agents have recently been reported [Samuelson and Lowe, 1997; Agarwal et al., 1998]. So far, the involvement of p53 in the cellular response to antitumor drugs has been limited to its modulating effect on the apoptotic pathway. The impact of the expression of wild-type p53 on well-established cytocidal enzymes like topoisomerases remains unclear.

The topo II inhibitors are agents that act by stabilizing a covalent "cleavable complex" between the enzyme and DNA, thus inhibiting DNA religation and causing an apparent increase in DNA double-strand breaks. The molecular event which leads to cellular death from topo II inhibitors appears to be the arrest of replication fork progression by the drug-induced DNA-topo II covalent complex [Qiu et al., 1996]. A correlation between levels of topo II and drug sensitivity has been described [Webb et al., 1991; Davies et al., 1988; Deffie et al., 1989]. We previously reported that transfection of wild-type p53 into null HL-60 cells increases their sensitivity to VP-16 and induces monocytoid differentiation [Banerjee et al., 1995]. Other studies confirm the possibility of stable expression of wild-type p53 in p53-null leukemic HL-60 and U-937 cells and its effect on differentiation [Ehinger et al., 1996; Ronen et al., 1996]. Thus, these data suggest that the level of expression of wild-type p53 may determine whether cells differentiate or undergo apoptosis [Ronen et al., 1996]. This may help explain the conflicting results concerning the effect of wild-type p53 expression in null leukemic cell lines, that is, whether it leads to a decrease or increase in drug sensitivity. To further explore the effect of wild-type p53 expression on sensitivity to topo II inhibitors, both the stable and inducible expression of p53 were studied with respect to topo II activity and drug sensitivity. The increased sensitivity of wild-type p53 transfected HL-60 and K562 cells to topo II inhibitors suggests that p53 and topo II are interrelated and may be associated in vivo. A similar association of p53 with topo I has already been described [Gobert et al., 1996].

MATERIALS AND METHODS

Cell Culture

The human HL-60 cell line was obtained from the American Type Culture Collection (ATCC; Rockville, MD) and maintained in RPMI 1640 media containing 10% fetal bovine serum (FBS). The transfection and isolation of stable cell lines expressing p53 was previously described [Bannerjee et al., 1995]. The parental HL-60 line is referred to as S_1, the wild-type p53 transfected line is SN_3, while the mutant p53 transfected cell line is 248. The human K562 cell line was stably transfected for inducible wild-type p53 expression by use of a two-plasmid co-transfection protocol, so that a tet responsive system for controlled expression of p53 was established [Fan and Bertino, 1997]. The human squamous carcinoma cell line SqCC/Y1.53.5 [Brenner et al., 1993] was kindly provided by Dr. Michael Reiss (Yale University School of Medicine). The inducible expression of wild-type p53 in these cells is accomplished by exposing the cells to 1 μM dexamethasone for 24 h.

Cytotoxicity Assays

Doxorubicin and etoposide were obtained from Sigma Chemical Co. (St. Louis, MO), while razoxane and ADR-529 were a kind gift of Pharmacia (Columbus, OH). Cytotoxicity assays were a modified version of the MTT assay, described previously as XTT [Scudiero et al., 1993]. Cells were treated with drug, seeded into 96-well plates and incubated for 96 h before assay. Clonogenic assays were performed by seeding 5,000 exponentially growing cells in 1 ml of 0.3% agar in medium supplemented with 15% FBS. The cells were grown for 15 days at 37°C in a humidified 5% CO_2 atmosphere. The number of colonies containing more than 40 cells was determined. The IC_{50} is defined as the concentration of drug inhibiting cell growth by 50%.

Western Blotting

Immunoblotting of whole cell extracts obtained from parental S_1, 248, and SN_3 HL-60 cells, as well as from K562 (±tet) was performed as previously described [Feldhoff et al., 1994]. Log-phase HL-60 cells were exposed to either 25 μM VP-16, 1 μM mitoxantrone, 50 μM cis-platinum or solvent only (control) for 1 h at

37°C. K562 cells were exposed only to VP-16. DNA topo IIα was detected with our rabbit polyclonal anti-topo IIα antibody 454 raised against the recombinant M_r 70,000 C-terminus of HeLa topo IIα [Sullivan et al., 1993], and topo IIβ was detected with a new rabbit polyclonal anti-topo IIβ antibody (JB-1) raised against recombinant human topo IIβ overexpressed in yeast. The expression system for this human topo II isoform [Austin et al., 1995] was generously provided by Dr. Caroline Austin (Medical School, University of Newcastle-upon-Tyne, United Kingdom) and 5 mg of the antigen were purified by FPLC and used for polyclonal antibody production and characterization. This new antiserum recognizes topo IIβ exclusively by immunoblotting and immunofluorescence, and quantitatively and selectively immunoprecipitates this isoform as well.

To quantify the expression of p53, whole cell lysates of one million S_1, SN_3, K562 (±tet), and Raji cells (positive control) were probed with several antibodies for p53. These included the monoclonal antibodies pAb 122 (Roche Molecular Biochemicals, Indianapolis, IN), DO-1 (Santa Cruz Biotechnology, Santa Cruz, CA), and DO-12 (Novocastra Laboratories, Newcastle-upon-Tyne, UK), as well as the polyclonal antibody CM1 (Novocastra Laboratories, Newcastle upon Tyne, UK).

Immunoprecipitation of Metabolically Labeled Cells

For metabolic labeling of p53, five million S_1, SN_3, and K562 (±tet) cells were grown in RPMI 1640 media lacking methionine and cysteine, (Fisher, Pittsburgh, PA) that was supplemented with 10% FBS, 4 mM glutamine, and 10 µM methionine (10% "cold" methionine). The cells were allowed to grow for 16 h in the presence of 0.05 µCi/ml of ^{35}S-methionine/cysteine EXPRES^{35}S^{35}S protein-labeling mix (DuPont NEN, Wilmington, DE). After washing the cells twice in 10 ml ice-cold phosphate-buffered saline (PBS), the pellet was suspended in 800 µl lysis buffer (50 mM Tris, 125 mM NaCl, 50 mM NaF, 5 mM EDTA, 1 mM Na_3VO_4, 0.1% Nonidet P-40 [NP-40], 2 mM PMSF, pH 7.5 and 1× protease inhibitors cocktail, consisting of 20 µg/ml each of antipain, aprotinin, chymostatin, leupeptin, soy bean trypsin inhibitor, benzamidine, and pepstatin A) for 20 min on ice with frequent vortexing. The lysate was centrifuged for 5 min at 13,000*g* and the supernatant was used for immunoprecipitation with protein A-Sepharose preincubated with monoclonal antibodies against p53 (DO-1 and pAb 122).

To complex protein A-Sepharose with antibodies, approximately 2.5 µg of monoclonal antibody were mixed with 50 µl of a protein A-Sepharose slurry and diluted to 200 µl with binding buffer (0.1% NP-40, 1% bovine serum albumin [BSA] in PBS). After a 2-h incubation with rocking at 4°C, the beads were collected at 13,000*g* for 1 min and washed 3 times with binding buffer and brought to a final volume of 200 µl. The lysate was incubated 2 h with the preformed complex by rocking at 4°C; the beads were then collected at 13,000*g* for 1 min and washed 3 times in 1 ml 1% Triton X-100 in PBS. The pellet was boiled in 4× sample buffer (8% SDS, 0.4 M DTT, 40% glycerol, 4% bromophenol blue) and the solubilized proteins run on a 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. The gel was dried and autoradiographed for 5–90 h.

Immunofluorescent Microscopy

Parental S_1 HL-60, mutant p53 transfected 248, and wild-type p53 transfected SN_3 cells were treated with various drugs, that were added from stock solutions to the growth media in the flasks. K562 cells were grown in the presence and absence of tetracycline for as long as 2 days before treatment with drugs. It is the absence of tetracycline that induces wild-type p53 expression. At different time points after drug addition (5, 15, 30, and 60 min), aliquots of the suspension were withdrawn and immediately processed for immunocytochemistry. Drug concentrations were as follows: VP-16, 25 µM; mitoxantrone, 25 nM; topotecan, 400 nM; cisplatin, 10 µM and vincristine, 0.1 µM. These concentrations are approximately 4-fold higher than the IC_{50} of many cell lines.

Fifty thousand S_1, 248, and SN_3 log-phase HL-60 cells, or K562 cells (±tet for 48 h) drug-treated or untreated, were cytospun on slides with a Shandon cytocentrifuge (Pittsburgh, PA) for 3 min at 500 rpm, dried, and immediately fixed at −20°C with methanol/acetone (3:1) for 20 min. The adherent squamous carcinoma cell line was grown in Petri dishes onto sterile charged Superfrost Plus slides. The induction of wild-type p53 in SqCC cells was accom-

plished by adding 1 μM dexamethasone for 24 h. Drug treatment was performed by adding stock solutions to the media, and after the incubation the slides were washed and fixed as described above.

All slides were air-dried and rehydrated for 2 h in several changes of PBS. Rabbit polyclonal antibody 454 against topo IIα [Sullivan et al., 1993] was diluted 1:100 with 0.1% NP-40, 1% BSA in PBS, and the cells were incubated with this antibody for 1 h at room temperature. Rabbit polyclonal antibody IIA was kindly provided by Dr. Leroy F. Liu (University of Medicine and Dentistry of New Jersey—Robert Wood Johnson Medical School) [Kaufmann et al., 1991] and used also at a dilution of 1:100 as described for the other polyclonal antibody. Monoclonal antibody Ki-S1 against the C-terminal domain of topo IIα (Roche Molecular Biochemicals, Indianapolis, IN) was diluted to 5 μg/ml in the same buffer as above, and the incubation was identical to the polyclonal antibodies. p53 expression was assessed with monoclonal antibody pAb 122 or DO-1. Combining a monoclonal anti-p53 antibody with the polyclonal anti-topo IIα antibody permitted simultaneous labeling of both proteins.

After primary antibody incubation and several washes with PBS for 2 h, the slides were incubated with a 1:80 diluted goat anti-rabbit IgG-TRITC-labeled antibody (Sigma) for the polyclonal primary antibody, and/or a 1:125 diluted goat anti-mouse IgG-FITC-labeled antibody (Sigma) for the monoclonal primary antibody in 0.1% NP-40, 1% BSA in PBS for 25 min at room temperature. After several washes in PBS, the slides were dried and covered with coverslips in Vectashield mounting media of antifade/DAPI (1:1) (Vector Laboratories, Burlingame, CA). Immunofluorescence was observed with a Leitz Orthoplan 2 microscope and images were captured by a CCD-camera with the Smart Capture program (Vysis, Downers Grove, IL).

Topoisomerase II Catalytic Activity

The DNA topo II catalytic activity present in 1.0 M NaCl nuclear extracts obtained from S_1 parental HL-60, 248, and SN_3 cells was measured by the decatenation of kinetoplast DNA (kDNA) into minicircles [Sullivan et al., 1989]. Nuclear extracts of 2–3 × 10^8 log-phase HL-60 cells were prepared as previously described for human lymphoblastic CCRF cells [Sullivan et al., 1987]. Before the nuclear extraction procedure, cells were treated with either 25 μM VP-16 dissolved in DMSO for 1 h at 37°C or 0.025% dimethylsulfoxide (DMSO) alone (control). The cells were washed twice with 20 ml cold PBS and a routine nuclear extraction procedure carried out with 1.0 M NaCl. The decatenation assays were incubated for 30 min at 30°C in the presence of 100 mM NaCl with 0.5 μg kDNA substrate (agarose gel assay), or 15 s to 5 min, using 15,000 cpm [^{3}H]kDNA (centrifugation assay; approximately 0.5 μg kDNA).

Alkaline Elution

Alkaline elution was performed as previously described [Kohn et al., 1981] with cells that had been labelled for 16 h with ^{14}C-thymidine (final concentration 0.0165 mCi/mM). Drug treatment was for 2 h and alkaline elution was performed for 15 h at pH 12.1 with the inclusion of proteinase K.

Determination of Hemoglobin in K562 Cells

Hemoglobin was detected in K562 cells as described previously [Ehinger et al., 1997]. Briefly, 5 × 10^5 cells were washed twice with PBS (without calcium, magnesium or bicarbonate) and resuspended in 100 μl of 0.9% NaCl. Then 50 μl of 0.2% tetramethylbenzidine (Sigma) solution in 0.5 M acetic acid and 0.6% H_2O_2 were added and cells were gently shaken for 30 min at room temperature. The number of cells containing blue crystals (benzidine-positive cells) was determined by counting 400 cells.

RESULTS

Levels of p53 Protein in Stably Transfected Cells and in an Inducible Expression System

The expression of p53 determined by Western blotting with four different antibodies against p53 (pAb 122, DO-1, DO-12, and CM 1) in the parental HL-60 cell line was null, and in the K562 cell line was 1% of that seen in the highly overexpressing mutant p53 Raji cell line. The stable expression of wild-type p53 in SN_3 HL-60 cells (relative to parental HL-60 cells) was increased from ≈0 to 3.7%, while the inducible expression of wild-type p53 in K562 cells was found to increase from 1% to 4% (both calculated using Raji cells as a standard). There is a technical difficulty in determining such a small change in protein amount by Western blotting, as the protein A used in the immuno-

precipitation experiments gives a strong chemiluminescent signal very near to p53. Therefore, the increase in p53 was confirmed by immunoprecipitating whole cell lysates obtained from cells metabolically labeled with ^{35}S-methionine (Fig. 1). We have previously shown that the wild-type p53 expressed in SN_3 HL-60 cells is functional [Ju et al., 1998].

The differentiation that occurs in HL-60 cells stably transfected with wild-type p53 has been previously shown to lead to a monocytoid phenotype [Banerjee et al., 1995]. The erythroblastoid K562 cell line has the potential to differentiate and initiate hemoglobin synthesis. The number of benzidine-positive cells is a convenient and useful marker of the differentiation that occurs during a transient inducible expression of wild-type p53. After a 48 h induction of wild-type p53 (−tet) the number of benzidine-positive K562 cells increases from 0.25% to 2% as determined by counting 400 cells. A time course estimating the level of p53 expression relative to the differentiation status demonstrated after the initial increase of benzidine-positive cells (days 2–4), that at 5 days under inducible conditions, the level of benzidine-positive cells decreased to the baseline level. This phenotypic change suggests that wild-type p53 may change the drug sensitivity of the more differentiated state of the parental cell line.

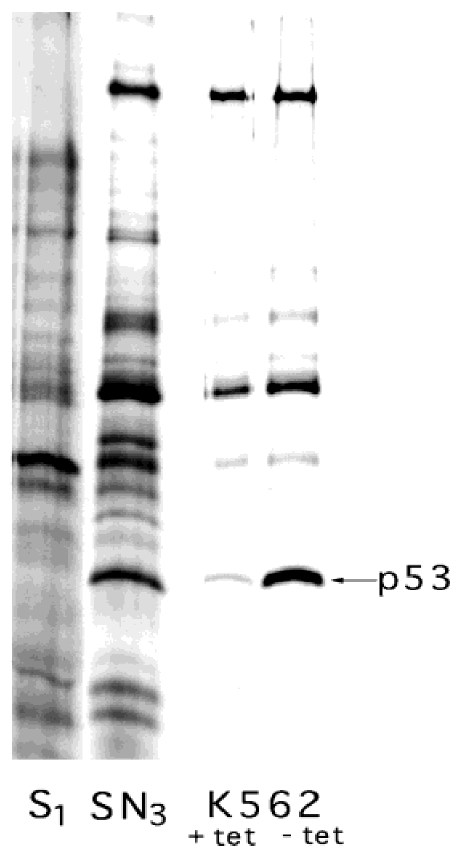

Fig. 1. Autoradiography of immunoprecipitates of whole cell lysates obtained from HL-60 and K562 cells. S_1, SN_3, and K562 (±tet) cells were labeled with ^{35}S-methionine and p53 was immunoprecipitated with monoclonal antibody DO-1 and separated on a 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel as described under Methods. The signal in all lanes represents 5×10^6 cells.

Sensitivity of Parental and p53 Transfected HL-60 Cells to Topo II Poisons

The three HL-60 cell lines were exposed to cleavable complex stabilizing topo II inhibitors (VP-16 and doxorubicin), as well as to the topo II catalytic inhibitors [Roca et al., 1994] ADR-529 and razoxane. The IC_{50} for each cell line with these drugs is shown in Table I. Cells expressing wild-type p53 were 7-fold more sensitive to VP-16 and 3-fold more sensitive to doxorubicin, as compared to the parental line and mutant p53 transfected line. There was no significant difference in sensitivity between the three lines when exposed to the catalytic inhibitors ADR-529 and razoxane.

To confirm our findings with the HL-60 cell lines, we examined the drug sensitivity of another leukemic cell line (K562) that has an inducible expression of wild-type p53 (in the absence of tet). Drug sensitivity to VP-16 was found to increase 3- to 4-fold after a 48-h induction of wild-type p53, as measured by both MTT and clonogenicity assays (data not shown). After 5 days of inducible expression of wild-type p53, however, the drug sensitivity to VP-16 returned to the initial level.

Measurement of DNA Strand Breaks by Alkaline Elution

Alkaline elution was used in the HL-60 cell lines to study the effect of p53 status on the

TABLE I. Cytotoxicity of Topo II Inhibitors in HL-60 Cell Lines*

	Doxorubicin	VP-16	Razoxane	ADR529
HL-60 S_1	25 ± 2[a]	102 ± 14	0.0018	0.0020
HL-60 SN_3	8 ± 5	15 ± 9	0.0015	0.0045
HL-60 248	30 ± 15	95 ± 35	0.0012	0.0015

*Log phase cells were exposed to the drugs for 96 h before the XTT assay.
[a]Numbers (± SEM) are IC_{50} values in nM concentrations.

formation of DNA strand breaks by a 2-h exposure to different concentrations of VP-16 (Fig. 2). Increased formation of protein-associated DNA strand breaks in cells expressing wild-type p53 was observed. For a 2-h exposure to VP-16, the wild-type p53 transfected SN_3 cells displayed an 8- to 10-fold increase in the formation of single-strand DNA breaks as compared with the parental p53 null HL-60 cell line. It is unlikely that the DNA damage seen in the wild-type p53 expressing SN_3 cells is attributable to apoptotic endonucleolytic strand breaks, as these DNA strand breaks were revealed by proteinase treatment. These data are consistent with the presence of wild-type p53 mediating an increased number of strand breaks after treatment with chemotherapeutic agents.

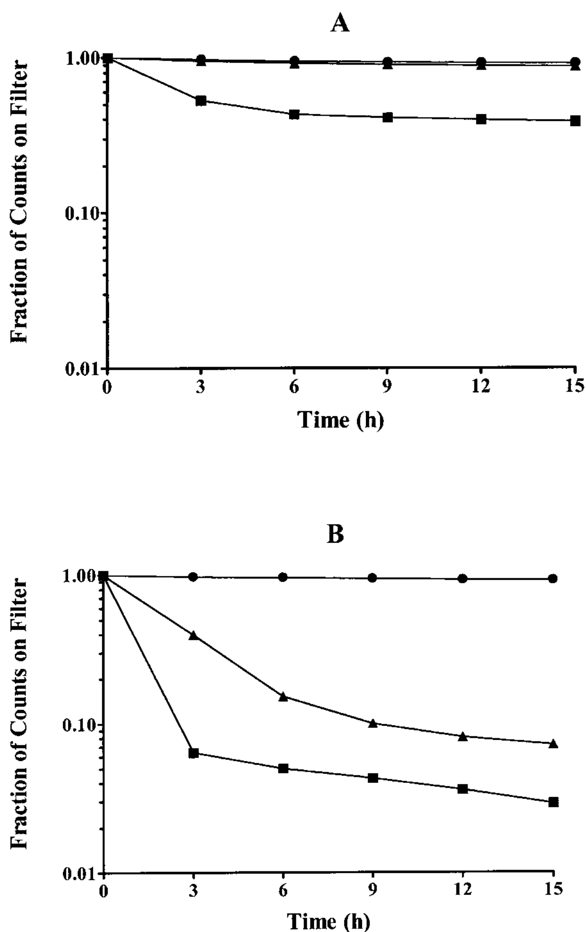

Fig. 2. Alkaline elution profile of S_1 and SN_3 cells after treatment with VP-16 for 2 h. **A:** Parental S_1 cells. **B:** Wild-type p53 transfected SN_3 cells. Untreated control cells (●); cells treated with 1 μM VP-16 for 2 h (▲); and cells treated with 20 μM VP-16 for 2 h (■). These results were reproduced in three separate experiments.

Quantity of Topo IIα and β by Immunoblotting

Immunoblots of whole cell extracts from S_1 and SN_3 HL-60 cell lines were performed and were probed with specific antibodies for topo IIα and topo IIβ (Fig. 3). Similar levels of topo IIα were detected in parental S_1 HL-60 log-phase cells (lane 1) as compared with wild-type p53 transfected SN_3 cells (lane 3). The levels of topo IIα mRNA were also found to be equivalent in parental HL-60 cells and the transfectants (data not shown). A reproducible finding in SN_3 cells is the presence of lower-molecular-weight peptides immunoreactive for topo IIα. Treatment with 25 μM VP-16 for 1 h did not result in significant quantitative changes in 170 kDa topo IIα in either cell line (lanes 2 and 4). However, the appearance of higher-molecular-weight bands, that could be due to a mobility shift from either a covalent association between topo II and DNA fragments or ubiquitination of topo II, was observed. Treatment of S_1 and SN_3 cells with mitoxantrone and cis-platinum resulted in similar findings with topo IIα (data not shown). Distinct from the findings with topo IIα, the levels of topo IIβ were consistently found to be 2.5-fold greater in SN_3 cells by immunoblotting (Fig. 3, lanes 5 and 6).

The inducible expression of wild-type p53 in the K562 cell line did not result in quantitative changes in topo IIα content (±tet), as assayed by immunoblotting of whole cell lysates (Fig. 3, lanes 7 and 9). Exposure to VP-16 did not change the amount of topo IIα, in either the absence or presence of wild-type p53. The only difference noted was the appearance of a lower-molecular-weight band of immunoreactive topo IIα in K562 cells expressing wild-type p53. However, similar to the HL-60 cell lines, the presence of wild-type p53 for two days resulted in a 1.5-fold increase in the level of topo IIβ (Fig. 3, lanes 11 and 12).

Immunocytochemistry of Topo IIα

The distribution of topo IIα was studied in the three HL-60 cell lines by immunolabeling with two specific polyclonal antibodies, as well as with one monoclonal antibody that recognizes an epitope in the C-terminal domain of topo IIα, spanning amino acid residues 1512–1530. All three antibodies produced an identical immunofluorescent staining pattern. Parental and mutant p53 transfected cell lines exhibited bright nuclear staining with detect-

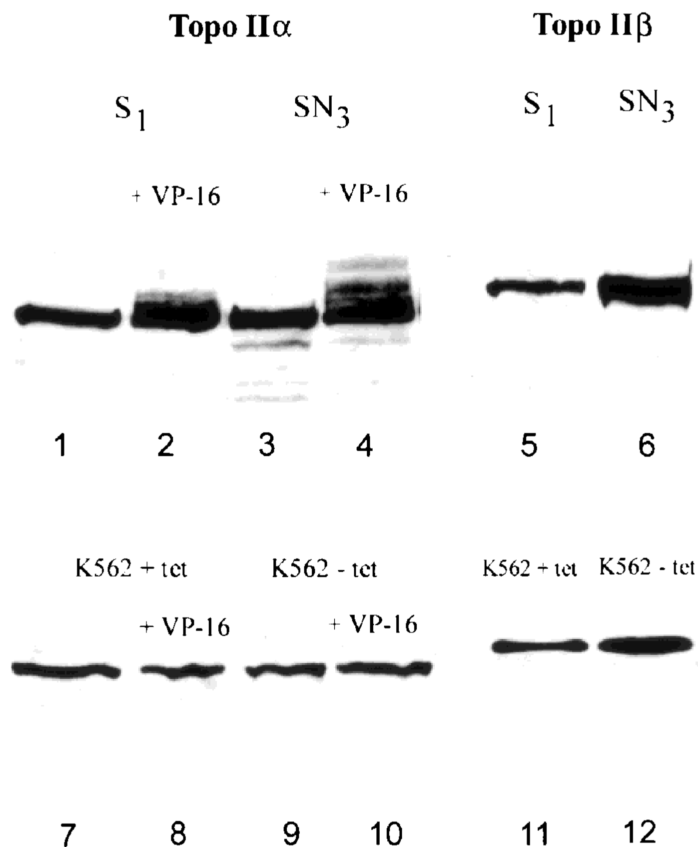

Fig. 3. Western blot analysis of cell lysates obtained from log-phase parental HL-60 cells (S_1), wild-type p53 transfected cells (SN_3), and K562 cells probed with either polyclonal antibody 454 raised against topo IIα **(lanes 1–4,7–10)**, or polyclonal antibody JB-1 raised against topo IIβ **(lanes 5,6,11,12)**. **Lanes 1,5**, untreated S_1 cells; **lane 2**, S_1 cells treated with 25 μM VP-16 for 1 h; **lanes 3,6**, untreated SN_3 cells; **lane 4**, SN_3 cells treated with 25 μM VP-16 for 1 h. K562 cells not expressing p53 (+tet, **lanes 7,8,11**), and K562 cells expressing wild-type p53 (−tet, **lanes 9,10,12**). **Lanes 8,10**, cells treated with 25 μM VP-16 for 1 h. All lanes are loaded with lysate obtained from 5×10^5 cells. The ECL signals for topo IIα and β were quantified densitometrically.

able nucleolar labeling (Fig. 4a,c). By contrast, cells transfected with wild-type p53 were faintly labeled, with near absence of a nuclear signal, and a prominent perinuclear rim of staining (Fig. 4b). The exposure of cells to VP-16 evoked a dramatic and rapid change in the immunofluorescent labeling of topo IIα (Fig. 4d–f). The parental cell line and mutant p53 transfected cells displayed negative nucleolar staining (arrowheads) after only 5 min of drug exposure, while SN_3 cells, after 5-min exposure to 25 μM VP-16, displayed a nuclear staining pattern in nearly 90% of the cell nuclei that had previously been devoid of topo IIα labeling. K562 cells when induced to express wild-type p53 demonstrated the same phenomenon, a marked reduction of the topo IIα and p53 immunofluorescence in the presence of wild-type p53 (Fig. 4i,j) that is significantly increased after VP-16 exposure (Fig. 4m,n). No difference was seen in the noninduced K562 cells (Fig. 4g,h,k,l). Identical results were obtained with the inducible expression of wild-type p53 in the squamous carcinoma cell line (Fig. 4o–v). Quantitative measurements of the signal from the immunofluorescence studies are presented in Figure 5.

The same phenomenon was observed when the SN_3 HL-60 cells were exposed to a wide variety of antineoplastic agents, including cis-platinum, topotecan, mitoxantrone, and vincristine. Similar to VP-16, the first three drugs elicited a rapid appearance of topo IIα after 5 min of drug exposure, while vincristine required a 30 min incubation period. The rapid reappearance of topo IIα in these immunocytochemistry experiments may reflect a more general mechanism involving drugs that interact with DNA (cis-platinum and topo I and II inhibitors).

Topo II Catalytic Activity in Nuclear Extracts

Nuclear extracts (1.0 M NaCl) were obtained from log-phase parental, 248, and SN_3 HL-60 cells and topo II activity was determined by the decatenation assay. The results for nuclear extracts obtained from wild-type p53 transfected SN_3 cells preincubated in the absence and presence of 25 μM VP-16 are displayed in Figure 6. Figure 6A shows that the topo II catalytic activity present in the extracts from SN_3 cells treated with VP-16 is equivalent to that of extracts obtained in the absence of VP-16 (cf. lanes 4

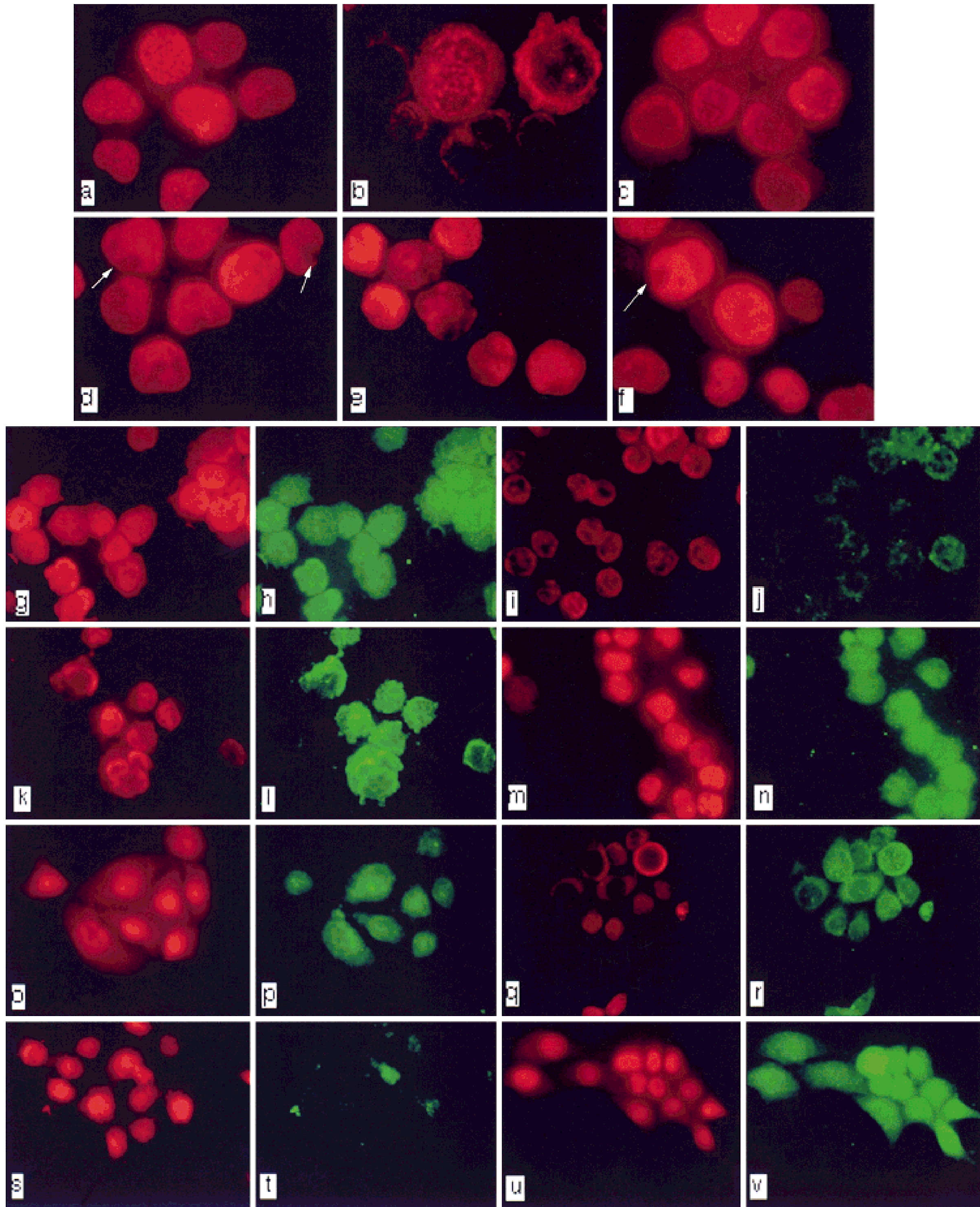

Fig. 4. Immunofluorescent labeling of parental HL-60 S_1 cells **(a)**, wild-type p53 transfected SN_3 cells **(b)**, and mutant p53 transfected 248 cells **(c)** with specific anti-topo IIα antibody 454. Untreated parental S_1 and mutant p53 transfected cells display a bright nuclear and detectable nucleolar staining **(a,c)**, while SN_3 cells, containing wild-type p53, are practically devoid of nuclear staining **(b)**. Incubation of cells with 25 μM VP-16 for 5 min results in rapid depletion of nucleolar staining (arrowheads) in S_1 and 248 cells **(d,f)** but leads to a dramatic emergence of nuclear staining in SN_3 cells **(e)**. Immunofluorescent staining of parental K562 (+tet) for topo IIα **(g)**, and p53 **(h)** and K562 cells transiently expressing wild-type p53 (−tet) for topo IIα **(i)** and p53 with the monoclonal antibody DO-1 **(j)**. Treatment of the cells with 25 μM VP-16 for 5 min resulted in the rapid reappearance of topo IIα **(m)** and p53 **(n)** staining in wild-type p53 expressing K562 cells, but no change in K562 cells grown in the presence of tet **(k,l)**. Immunostaining for topo IIα **(o)** and p53 **(p)** in squamous cell carcinoma cells not expressing p53 (−dexamethasone) and in the presence of dexamethasone **(q,r)**. Note the minimal immunofluorescence for topo IIα in p53 expressing SqCC cells **(q)**. The addition of 25 μM VP-16 leads to an increased signal for both topo IIα **(u)** and p53 **(v)** in wild-type p53 expressing SqCC cells, but minimal changes in uninduced cells **(s,t)**. **g–v:** ×400; **a–f:** ×600.

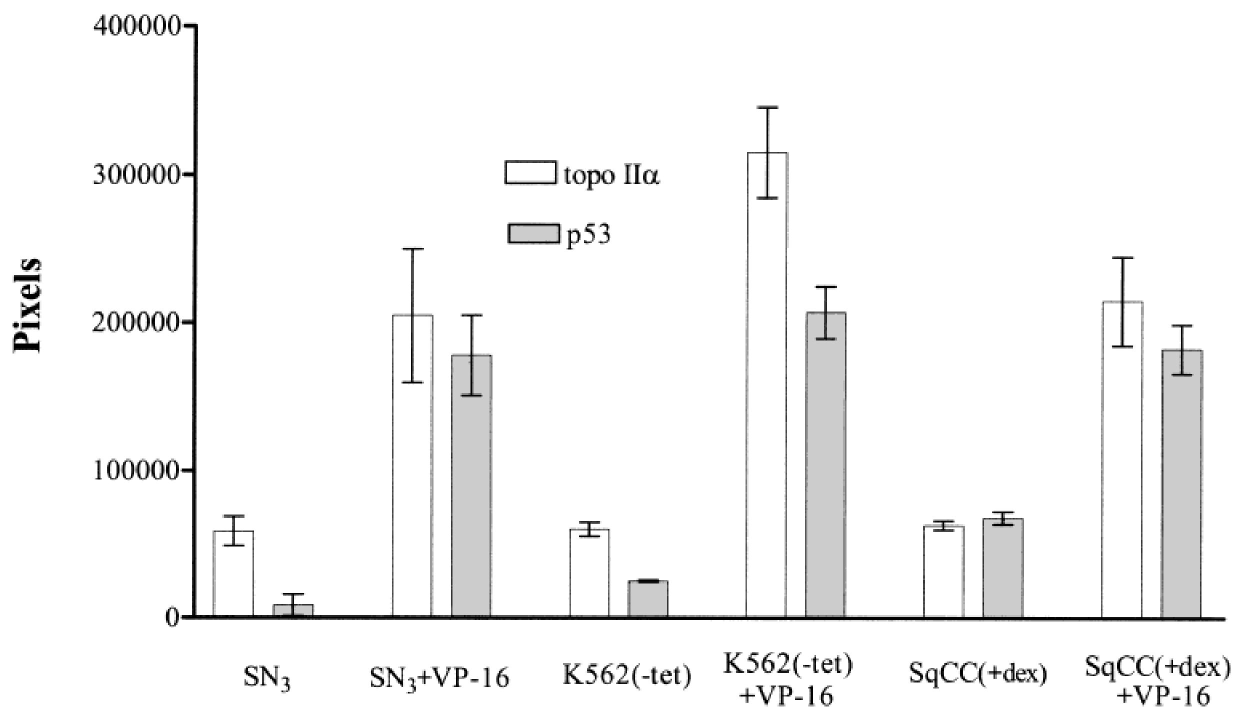

Fig. 5. Statistical evaluation of topo IIα and p53 immunofluorescence in cells expressing wild-type p53 (±25 μM VP-16 for 1 h) as measured in pixels from 100 treated and untreated cells from each cell line. Data are derived from six separate experiments with HL-60 S_1 and SN_3 cells, from 3 separate experiments with the inducible K562 (±tet) cell line and one experiment with the squamous cell carcinoma cell line (±dexamethasone).

and 9; each required 500 ng nuclear extract protein to totally decatenate 0.5 μg kDNA). The decatenation activity of nuclear extracts obtained from 248 and parental HL-60 cells was equivalent to that seen with SN_3 extracts (data not shown). Figure 6B shows the initial decatenation velocities of SN_3 extracts (obtained ±VP-16) over a time frame of 15 s to 5 min with a fixed amount of nuclear extract protein (775 ng). Again, the initial decatenation activities in this [^{3}H]kDNA assay were equivalent for SN_3 extracts previously exposed or unexposed to 25 μM VP-16. Thus, although topo IIα is virtually absent in wild-type p53 transfected SN_3 cells by immunocytochemistry, and reappears after exposure of cells to VP-16, the amount of decatenation activity present in SN_3 nuclear extracts does not depend on prior exposure to VP-16. In addition, the modest increase in topo IIβ seen in SN_3 cells by immunoblotting did not significantly augment the topo II catalytic activity of nuclear extracts from this cell line.

DISCUSSION

HL-60 and K562 cells are reported to have negligible expression of p53 protein due to a loss of one p53 allele and point mutations in the other allele [Prokocimer et al., 1986], although some studies do not exclude the presence of mutant p53 peptides [O'Connor et al., 1997]. The status of these leukemic cell lines provides a model system to assess the impact of transfection with plasmids coding for wild-type p53. Wild-type p53 expression has been shown to induce various differentiation pathways in leukemic cell lines and squamous carcinoma cell lines [Banerjee et al., 1995; Ehinger et al., 1995, 1996, 1997; Ronen et al., 1996; Brenner et al., 1993; Soddu et al., 1994]. We studied both the stable and inducible expression of wild-type p53, and observed detectable protein levels that resulted in significant differentiation events in the two leukemic cell lines. This was accompanied by an increase in sensitivity of wild-type p53-expressing leukemic cells to topo II poisons.

It is known that the formation of DNA strand breaks is a potent stimulus for the expression and nuclear accumulation of wild-type p53, which results in increased apoptosis [Nelson and Kastan, 1994]. The null p53 HL-60 cell line is an appropriate model to determine the effect

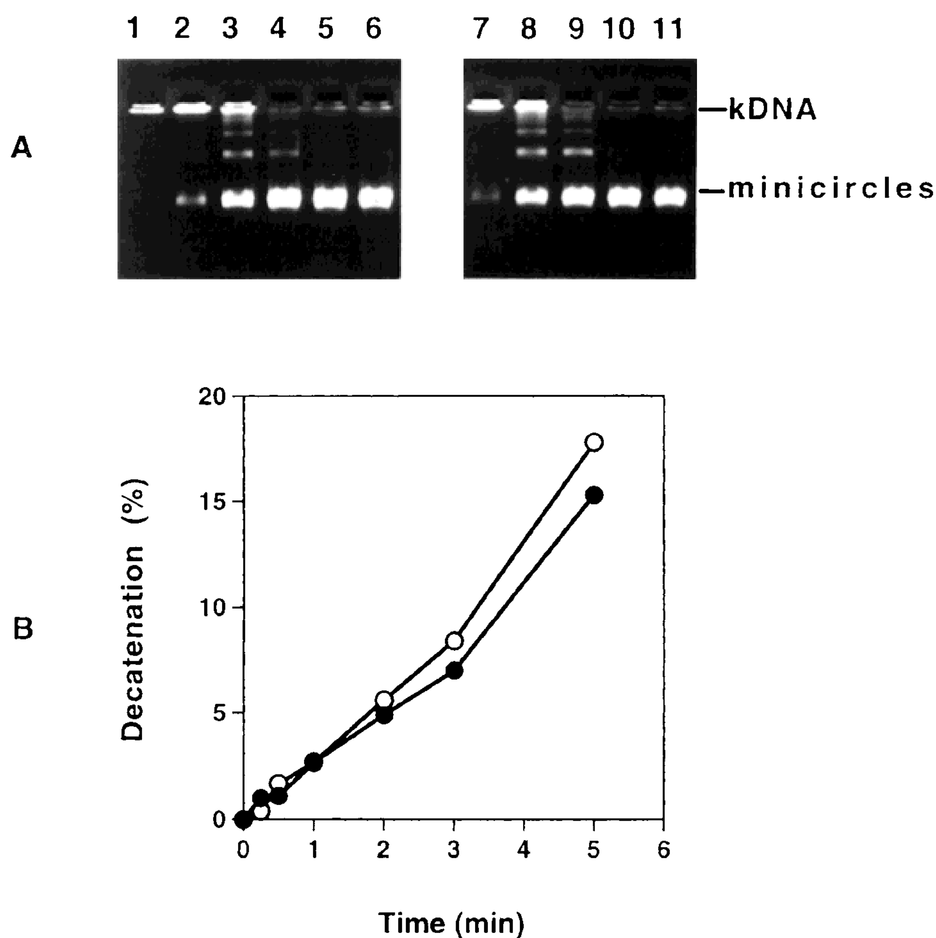

Fig. 6. Topo II activity present in 1.0 M NaCl nuclear extracts of SN_3 cells measured by decatenation. **A:** Nuclear extracts were incubated with 0.5 µg kinetoplast DNA (kDNA) for 30 min at 30°C in decatenation buffer at a final NaCl concentration of 100 mM. The reaction products were electrophoresed in a 1% agarose gel for 2 h at 100 V, which was subsequently stained with ethidium bromide. Nuclear extracts were obtained from cells preincubated with 25 µM VP-16 for 1 h **(lanes 7–11)** or solvent alone **(lanes 2–6)**. Nuclear extract protein amount: **Lane 1**, kDNA alone; **lanes 2,7**, 0.1 µg; **lanes 3,8**, 0.25 µg; **lanes 4,9**, 0.5 µg; **lanes 5,10**, 1.5 µg; **lanes 6,11**, 2.5 µg. **B:** Nuclear extracts (0.775 µg protein each time point) obtained from cells preincubated with 25 µM VP-16 (○) or solvent alone (●) for 1 h were used to decatenate 15,000 cpm [^{3}H]kDNA (approximately 0.5 µg) for the times indicated. The kinetoplast networks were separated from the released minicircles by centrifugation as described under Materials and Methods.

of wild-type p53 presence on the formation of DNA strand breaks and associated cytotoxicity induced by two types of topo II inhibitors. The non-intercalative topo II inhibitors, VP-16 and VM-26, are postulated to exert their cytotoxic effect by producing protein-associated DNA strand breaks. Our results suggest that the presence of wild-type p53 leads to increased sensitivity to cleavable complex forming topo II inhibitors. Mutated p53 does not appear to potentiate cytotoxicity, a phenomenon that may partially explain the clinical response to anticancer agents in many tumors with p53 alterations, i.e., the emergence of drug resistance. However, different mutants of p53 are reported to have a divergent effect on viability and differentiation [Kremenetskaya et al., 1997]. The presence of various p53 mutants in human tumors has recently been reported to confer VP-16 resistance and is considered a “gain of function” in establishing drug resistance [Blandino et al., 1999].

In many cell lines, a correlation between topo II levels and sensitivity to topo II inhibitors has been described [Webb et al., 1991; Davies et al., 1988; Deffie et al., 1989; Kaufmann et al., 1991; Sullivan et al., 1987], where elevated levels of topo II are associated with increased sensitivity to VP-16 and doxorubicin. We examined the possibility that the presence of functional p53

may increase the quantity or activity of topo II. Wild-type p53 transfected HL-60 cells have an 8-fold increase in both VP-16 sensitivity and VP-16 induced DNA damage. However, the decatenation assays suggest that the in vitro catalytic activity of topo II from the wild-type and null p53 cells are the same. The observation that there is no difference in topo II catalytic activity in nuclear extracts from wild-type and p53 null cells reflects the nature of the in vitro assay of strand-passing activity, which measures the catalytic activity of salt-extractable topo II, and not the in situ drug-induced cleavage measured by alkaline elution. The in situ assay may reflect the cleavage activity of a complex of DNA, topo IIα, and p53. The difference in alkaline elution profiles after treatment with drugs suggests that p53 may modulate downstream effects of DNA damage as well as respond to cellular DNA damage. The significance of this downstream effect remains to be clarified.

It is unlikely that the increase in SN_3 topo IIβ accounts for the VP-16 hypersensitivity, as there were no differences in topo II catalytic activity in the HL-60 cell lines, nor did the amount of this isoform change significantly in immunocytochemistry experiments in response to VP-16 exposure. To confirm the wild-type p53-dependent collateral sensitivity of the HL-60 leukemic cells, we also studied the effect of short-term inducible expression of p53 in K562 cells and observed a 3- to 4-fold increase in VP-16 sensitivity during days 2–4 of p53 induction. Within 5 days, baseline levels of VP-16 sensitivity were restored in K562 cells, perhaps due to a counteracting system activated in the presence of wild-type p53, i.e., MDM-2, which has been shown to be overexpressed in K562 cells [Bueso-Ramos et al., 1993]. Again, the increased sensitivity to topo II inhibitors was not due to an increased amount of topo IIβ, as immunoblotting demonstrated no change in enzyme quantity. Our data do not support the theoretical [Wang et al., 1997] possibility of p53 suppression of the topo IIα promoter that evidently requires high (perhaps physiologically intolerable) levels of p53.

The most significant observation related to the presence of wild-type p53 in the two leukemic and one squamous cell carcinoma cell lines, was the concealing of wild-type p53 and topo IIα epitopes such that these two proteins could not be detected by immunofluorescence. This masking of topo IIα and p53 epitopes was observed with three monoclonal antibodies against p53 (pAb 122, DO-1, and DO-12), and with two polyclonal and one monoclonal antibody to topo IIα (data not shown). These data suggest a possible interaction between the two molecules, either of which has the capacity for multimerization. The possibility of forming poly-heteromers is under investigation in our laboratory. The addition of drugs for very short time periods restored the fluorescent signal. In addition to the Western blot results, we further excluded the possibility that the "return" of topo IIα in the presence of VP-16 in HL-60 SN_3 cells was due to de novo synthesis. The drug-induced increased topo IIα signal was not inhibited by 100 µM cycloheximide, a concentration which was found to inhibit >90% protein synthesis in the same cells (data not shown). Identical immunofluorescence results were obtained with both polyclonal antibodies and the one monoclonal antibody, suggesting that an epitope (presumably in the C-terminal domain, as mapped by the monoclonal antibody) [Boege et al., 1995] of topo IIα is concealed in cells expressing wild-type p53, and that this protein-protein and/or protein-DNA interaction is quickly disrupted by the addition of chemotherapeutic agents. We have excluded the possibility of a nuclear-cytoplasmic translocation, which has been described for p53 [Martinez et al., 1997], as neither p53 nor topo IIα were detected in the cytoplasmic fraction of the cells. Immunofluorescent studies with a topo IIβ specific rabbit polyclonal antibody did not show any qualitative differences in the labeling pattern between the three HL-60 cell lines, nor did the addition of antitumor drugs lead to changes in the immunofluorescence of topo IIβ (data not shown).

A similar phenomenon to what we have observed in HL-60 cells has been described in other cells [Wolverton et al., 1992], solely as an increase of topo II fluorescence in the presence of VP-16 or m-AMSA. In most studies dealing with topo II localization, the content of p53 is undetermined and its possible impact on topo II immunofluorescence is unknown. The existence of a dynamic complex in cells containing wild-type p53, that could conceal topo IIα from immunofluorescent labeling, may provide an explanation for the technical difficulties in immunolabeling topo IIα, especially in quiescent cells and differentiated tissues.

Stable protein-protein interactions are already described between p53 and heat-shock proteins [Fourie et al., 1997; Hansen et al., 1996]. The rapid restoration of immunofluorescence after drug treatment (8-fold increase in the fluorescence as measured in pixels) suggests that a protein-protein and/or protein-DNA interaction underlies this epitope masking. The cell lines described here may be useful in defining these molecular interactions involving p53 and enzymes that participate in the maintenance of DNA integrity and topology. A complex between p53 and topo IIα may augment the sensitivity of cells to antineoplastic drugs due to elevated DNA damage by this complex. Such dynamic interactions of p53 with different proteins could explain its otherwise elusive role in so many cellular events [Wiman, 1997; Ko and Prives, 1996]. It is tempting to speculate that the covalent binding of topo IIα to 5′-DNA ends enables the inherent 3′-exonuclease activity of p53 [Mummenbrauer et al., 1996] to convert the lesion into an unrepairable event that leads to apoptosis. A similar interaction was recently proposed for the occurrence of translocations in leukemia [Megonigal et al., 1998]. A determination of the protein components of such a putative supramolecular complex may help define the basis for the sensitivity of cells towards antitumor drugs.

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