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Telomere Dysfunction Increases Cisplatin and Ecteinascidin-743 Sensitivity of Melanoma Cells

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ABSTRACT

The aim of this study was to investigate the role of telomerase function on the chemosensitivity of melanoma cells. To this end, ecteinascidin-743 (ET-743) and cisplatin [*cis*-diamminedichloroplatinum(II) (CDDP)], two DNA-interacting drugs that invariably cause an arrest in the G_2/M phase, and 1-(2,4-dichlorobenzyl)-1H-indazole-3-carboxylic acid (LND), a mitochondria-targeting drug inducing a G_1 block, were used. As experimental model, human melanoma clones showing reduced human telomerase reverse transcriptase (hTERT) expression and telomerase activity and characterized by telomere dysfunction were used. Reconstitution of telomerase activity by exogenous hTERT expression improved telomere function and reduced the sensitivity to CDDP and ET-743 without affecting LND susceptibility. The decreased sensitivity to CDDP and ET-743 was mainly caused by the ability of cells to recover from drug-induced damage, evaluated in terms of both chromosomal lesions and cell survival. The ability of hTERT-reconstituted cells to recover from drug-induced damage was attributable to the restoration of cell cycle progression. In fact, the cells without hTERT restoration remained for a prolonged time in the G_2/M phase, and this cell cycle alteration made irreversible the drug-induced S-G_2/M block and led to the activation of apoptotic program. On the contrary, the hTERT-reconstituted cells progressed quickly through the cell cycle, thus acquiring the capacity to recover from drug-induced block and to protect themselves from the G_2/M phase-specific drug-triggered apoptosis.

Major obstacles for anticancer chemotherapy are the cytotoxicity of anticancer agents to normal cells and the occurrence of resistant tumor cells to chemotherapeutic agents. Therefore, new chemotherapeutic strategies, which could reduce the cytotoxicity of normal cells and reverse chemoresistance of tumor cells, represent an important goal for the development of selective cancer therapies. Telomerase activity has been found in almost all tumors but not in adjacent normal cells (Shay and Bacchetti, 1997). The most prominent hypothesis is that the maintenance of telomere stability is required for long-term proliferation of tumors. Therefore, telomerase has become a target for the development of new anticancer therapeutic agents.

The relationship between telomerase and the vulnerability to drug-induced apoptosis is poorly understood. Antisense inhibition of telomerase increases the susceptibility of glioblastoma cells to CDDP-induced apoptosis (Kondo et al., 1998) and enhances apoptosis in pheochromocytoma cells induced by a variety of stimuli (Fu et al., 1999). Similarly, telomerase inhibition by ribozyme cleavage of telomerase mRNA sensitizes breast epithelial cells to topoisomerase inhibitors through the activation of the apoptotic program (Ludwig et al., 2001). These findings suggest a protective role of telomerase against drug-induced apoptotic cell death. Recently, a fast-increasing number of articles seem to indicate that the simple inhibition of telomerase may not result in the anticancer effect (Bearss et al., 2000). Therefore, although telomerase may not be a universal target for cancer therapy, targeting the telomere maintenance mechanisms could be important for future successful therapeutic anticancer strategies. In this context, by using telomerase-deficient mouse, other studies have demonstrated that telomere dysfunction, rather than telomerase per se, is the principal determinant

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ABBREVIATIONS: CDDP, cisplatin [*cis*-diamminedichloroplatinum(II)]; ET-743, ecteinascidin-743; LND, lonidamine [1-(2,4-dichlorobenzyl)-1H-indazole-3-carboxylic acid]; hTERT, human telomerase reverse transcriptase; PD, population doubling; BrdU, bromodeoxyuridine; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; +hTERT, c-Myc low-expressing clones infected with retroviruses encoding hTERT; −hTERT, c-Myc low-expressing clones infected with the gene for the puromycin resistance only.

governing chemosensitivity against agents that induce double-strand DNA breaks (Wong et al., 2000; Lee et al., 2001).

The present study examines the impact of telomerase function on the sensitivity of melanoma cells to ET-743, a novel marine natural compound that shows a good activity against a variety of tumors (Izbicka et al., 1998; Valoti et al., 1998; Villalona-Calero et al., 2002); CDDP, one of the most effective and broadly used anticancer drugs; and LND, a dichlorinated derivative of indazole-3-carboxylic acid, which plays a significant role in reversing or overcoming multidrug resistance (Citro et al., 1991; Silvestrini et al., 1992). CDDP and ET-743 are DNA-interacting drugs that cause an arrest in the G_2/M phase (Sorenson and Eastman, 1988; Takebayashi et al., 2001a; Gajate et al., 2002), whereas LND is a mitochondria-targeting drug inducing a G_1 block (Del Bufalo et al., 1996). Unlike CDDP and LND, the mechanism of action of ET-743 is not yet fully elucidated. It binds to guanines at the N2 position in the minor groove (Pommier et al., 1996), and its cytotoxic effect and cell cycle perturbation (Erba et al., 2001) seem related to an alteration of the transcription regulation that occurs in a promoter-dependent fashion (Jin et al., 2000; Minuzzo et al., 2000). Moreover, cells with mismatch repair defects, which are tolerant to some alkylating agents and to CDDP, are sensitive to ET-743, whereas cells deficient in transcription-coupled nucleotide excision repair, which are very sensitive to CDDP, are resistant to ET-743 (Damia et al., 1996; Takebayashi et al., 2001b).

We demonstrate here that the reconstitution of telomerase activity, by exogenous hTERT expression, improved telomere function and decreased sensitivity to CDDP and ET-743 without affecting LND susceptibility. The reduced drug sensitivity was caused by the ability of hTERT to modify cell-cycle progression, enabling the cells to recover from drug-induced G_2/M block and consequently protecting them from apoptosis.

Materials and Methods

Cells and Culture Conditions. MAS51 and MAS53 c-Myc low-expressing clones were previously obtained by transfecting the M14 human melanoma line with an expression vector carrying the exon 2 + exon 3 of c-myc cDNA cloned in antisense orientation (Biroccio et al., 2001). The c-Myc low-expressing clones were then infected with retroviruses encoding hTERT (+hTERT cells) or the gene for the puromycin resistance only (−hTERT cells) and used after 14 and 35 population doublings (PD), corresponding to the second and fifth culture passages, respectively, after infection (Biroccio et al., 2002).

The −hTERT and +hTERT cells were grown at 37°C (5% CO_2/95% air atmosphere) in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum, 2 mM L-glutamin, and antibiotics and containing neomycin (0.8 mg/ml; Invitrogen) and puromycin (0.5 μg/ml; Sigma, Milan, Italy).

Treatments and Clonogenic Assay. Clinical grade CDDP, ET-743, and LND were obtained from Pharmacia (Milan, Italy), PharmaMar (Tres Cantos, Madrid, Spain), and Angelini (Rome, Italy), respectively. Drug dilutions were freshly prepared before each experiment. Cells were seeded in 60-mm Petri dishes (Nunc-Mascia Brunelli, Milan, Italy) at a density of 2×10^5 cells/dish. Cells were exposed to different doses of CDDP (ranging from 0.3 to 16 μM) for 2 h, ET-743 (ranging from 1 to 50 nM) for 1 h, and LND (ranging from 0.07 to 0.6 mM) for 24 h, and the analysis was performed at the end of treatments. The doses of CDDP and ET-743 that inhibit cell survival by approximately 50% (IC_{50}) in all the cellular lines were used as equitoxic doses. In particular, we used the doses of 7 μM CDDP and 20 nM of ET-743 for the treatment of M14 and +hTERT cells and 1.7 μM CDDP and 1 nM ET-743 for −hTERT cells. To evaluate cell colony-forming ability, aliquots of cell suspension from each sample were seeded into 60-mm Petri dishes with complete medium and incubated for 10 to 12 days. Colonies were stained with 2% methylene blue in 95% ethanol and counted (1 colony ≥ 50 cells). Surviving fractions were calculated as the ratio of absolute survival of the treated sample to the absolute survival of the control sample. All of the experiments were repeated four times in triplicate.

Western Blot. Western blot and detection were performed as reported previously (Biroccio et al., 2001). Briefly, 40 μg of total proteins were loaded on denaturing SDS-polyacrylamide gel electrophoresis. Immunodetection of the c-Myc protein was performed using a 1:1000 dilution of the anti–c-myc monoclonal antibody clone 9E10 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). To determine the amount of protein transferred onto nitrocellulose membrane, β-actin was used as control. The relative amounts of the transferred proteins were quantified by scanning the autoradiographic films with a gel densitometer scanner (Bio-Rad, Milan, Italy) and normalized to the related β-actin amounts.

Cytogenetic Analysis. To obtain chromosome preparation, cells, in the log phase of growth, were incubated with 0.1 μg/ml of colcemid for 1 h and trypsinized, and then they were incubated with hypotonic 0.075 M KCl for 20 min, fixed with methanol to acetic acid (3:1, v/v), dropped onto frosted microscope slides, and air-dried overnight. Chromosome aberration frequency was evaluated in at least 50 Giemsa-stained metaphases from two simultaneously grown cultures for each line and each treatment (7 μM of CDDP for 2 h and 20 nM ET-743 for 1 h). For all of the experiments, metaphase preparations of the different cells were performed simultaneously under the same conditions. The χ^2 test was used for statistical analysis.

Bromodeoxyuridine Labeling. Progression of cells through the cell-cycle phase was analyzed by flow cytometry (BD Biosciences, San Jose, CA) using bromodeoxyuridine (BrdU; BD Biosciences) incorporation, as described previously (Biroccio et al., 2001). Briefly, cells were pulsed with BrdU (10 μM for 15 min) 24 h after the end of CDDP and ET-743 treatments. At the end of BrdU pulse and at 4 h intervals after the pulse, the cells were fixed and the DNA was denatured. Cells were then incubated with 2 μg/ml of mouse anti-BrdU (clone BMC 9318, Roche Diagnostics, Indianapolis, IN) for 30 min at room temperature, and the BrdU-positive cells were revealed with fluorescein isothiocyanate-conjugated anti-mouse monoclonal antibody (1:20; DAKO, Glostrup, Denmark). To evaluate the percentage of cells in each phase of the cell cycle after propidium iodide staining (1 μg/ml), the fraction of BrdU-positive cells was divided into three regions by their DNA content (G_1, S, and G_2/M). The three compartments were chosen for each cell line at the end of the pulse and were left unchanged during the following intervals of the analysis.

Detection of Apoptosis. Apoptotic cells were detected by using the FlowTACS in situ terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)-based Apoptosis Detection Kit (R & D Systems, Minneapolis, MN) according to the manufacturer's instruction. Briefly, 2×10^6 cells were collected 48 h after the treatments with equitoxic doses of CDDP and ET-743, fixed with 3.7% formaldehyde/phosphate-buffered saline, washed with phosphate-buffered saline, and permeabilized with 100 μl of Cytonin (Trevigen, Gaithersburg, MD) for 30 min at room temperature. The fragmented DNA was revealed by incubating the samples with the labeling reaction mix for 1 h at 37°C, and the fluorescein isothiocyanate-labeled cells were then stained with 50 μg/ml propidium iodide solution containing 750 μg/ml RNAase for 30 min at room temperature and in the dark. The flow-cytometric analysis was performed using FACSCalibur (BD Biosciences).

Results

Reconstitution of hTERT Decreases CDDP and ET-743 Sensitivity. In this study, an experimental model obtained previously by our group was used (Biroccio et al., 2002). Briefly, we first generated M14-derived c-Myc low-expressing clones (MAS51 and MAS53), characterized by the reduction of hTERT expression, telomerase activity, and telomere shortening compared with the parental line (Biroccio et al., 2001). Second, telomerase activity was restored in the c-Myc low-expressing clones (Biroccio et al., 2002) by infection with amphotropic viruses, encoding either hTERT (+hTERT cells) or the puromycin resistance only (−hTERT cells).

In this article, +hTERT cells after 14 (MAS51/T_{14}, MAS53/T_{14}) and 35 PD (MAS51/T_{35}, MAS53/T_{35}) and the −hTERT cells after 35 PD (MAS51/V_{35}, MAS53/V_{35}) after infection were used to study the role of telomerase function on drug sensitivity. Figure 1 shows the survival curves of the different clones exposed to increasing doses of CDDP and ET-743, two DNA-interacting drugs that invariably cause an arrest in G_2/M phase, and LND, a mitochondria-targeting drug, inducing a G_1 block (Del Bufalo et al., 1996). No difference in cell survival was observed between −hTERT and +hTERT cells treated with LND. On the contrary, the survival curves of the −hTERT cells treated with CDDP and ET-743 showed an exponential decrease in cell survival with the increasing of doses and, at the highest drug concentrations, the curves decreased in the third decade, reaching values of approximately 0.1%. Reconstitution of hTERT decreased sensitivity to CDDP and ET-743 at all the doses of drugs used. The behavior of the survival curves was biphasic, characterized by a shoulder region followed by an exponential phase at the highest doses, even though the surviving fraction remained between the first and the second decade. The degree of sensitivity to both drugs was strictly associated with the PD of the +hTERT cells. In fact, at 7 μM CDDP, the surviving fraction of the +hTERT cells was approximately 20% after 14 PD and 60% after 35 PD. Similarly, at 20 nM ET-743, the surviving fraction of the +hTERT cells was approximately 15% after 14 PD and 50% after 35 PD. Moreover, after 35 PD, the sensitivity to both drugs was superimposable to that of the M14 parental line. To exclude the possibility that the decreased CDDP and ET-743 sensitivity observed in +hTERT cells with the increasing PD was caused by a reactivation of c-Myc expression, Western blot analysis was performed. As shown in Fig. 2, no difference in c-Myc protein expression was evident between −hTERT and +hTERT cells, as well as between +hTERT cells at different PD.

Because hTERT expression levels and telomerase activity did not change in the +hTERT cells with the PD increasing (Biroccio et al., 2002), to explain the difference in drug sensitivity, telomere status was analyzed. Data reported in Table 1 show that −hTERT cells had a high frequency of telomeric fusions with a mean value, between MAS51/V_{35} and MAS53/V_{35}, of 1.24. A significant decrease in telomeric fusion frequency appeared in +hTERT cells already after 14 PD and became more evident after 35 PD ($p < 0.001$) compared with −hTERT cells. The mean values of telomeric fusion frequency of the two +hTERT 14 and 35 PD were 0.79 and 0.23, respectively. A representative metaphase of −hTERT (MAS51/V_{35}) and +hTERT after 14 (MAS51/T_{14}) and 35 (MAS51/T_{35}) PD, showing the maximum number of telomeric fusions per metaphase observed (four, three, and two, respectively), is shown in Fig. 3.

Reconstitution of hTERT Enables the Cells to Recover from CDDP and ET-743-Induced Damage. We next analyzed the ability of cells to recover from CDDP and

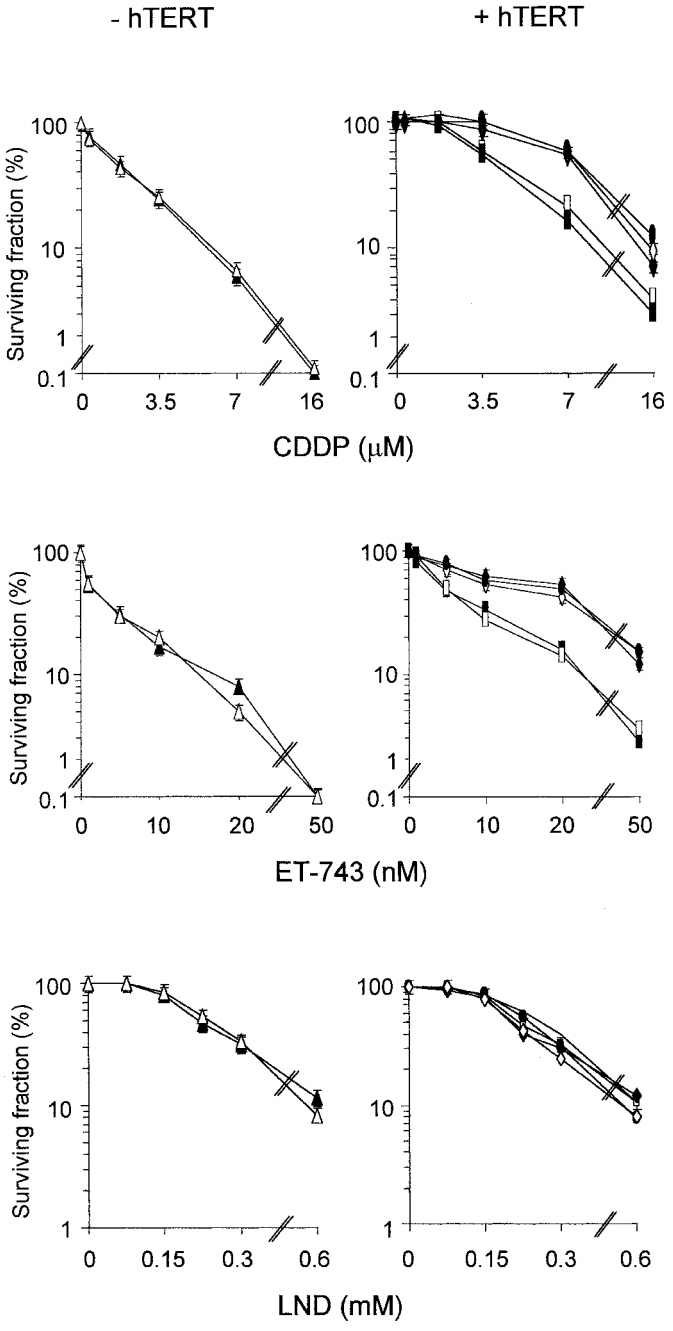

Fig. 1. Reconstitution of hTERT decreases sensitivity to CDDP and ET-743 but not to LND. Survival curves of the −hTERT cells (left; ▲, MAS51/V_{35}; △, MAS53/V_{35}), +hTERT (right) cells after 14 (■, MAS51/T_{14}; □, MAS53/T_{14}) and 35 (◆, MAS51/T_{35}; ◇, MAS53/T_{35}) PD, and M14 parental line (●; right) exposed for 2 h to different doses of CDDP ranging from 0.3 to 16 μM (top), for 1 h to different doses of ET-743 ranging from 1 to 50 nM (center), and for 24 h to increasing doses of LND ranging from 0.07 to 0.6 mM (bottom). Surviving fractions were calculated as the ratio of absolute survival of the treated sample to the absolute survival of the control sample. The data represent the mean and S.D. of four independent experiments.

ET-743–induced damage. The experiments were performed by using −hTERT (MAS51/V_{35}) and +hTERT (MAS51/T_{35}) cells after 35 PD after infection treated with equitoxic doses of both drugs, chosen from the dose-response curves (see Fig. 1). The doses of 7 μM CDDP for +hTERT and M14 cells and 1.7 μM for −hTERT cells that reduced cell survival by approximately 50% (IC_{50}) were used. Figure 4 shows that immediately after the end of CDDP treatment, a similar behavior between the survival curves of −hTERT and +hTERT was observed. However, a different ability to recover from CDDP damage was evident. In fact, at 96 h, the surviving fraction of −hTERT cells was approximately 50%. On the contrary, +hTERT cells showed a surviving fraction of approximately 100%, indicating that these cells were able to completely recover from CDDP-induced damage. The doses of ET-743, corresponding to IC_{50}, were 20 nM for the +hTERT and M14 cells and 1 nM for the −hTERT cells. After a decrease of cell survival by approximately 70%, a plateau phase in the −hTERT cells was observed, indicating that they were unable to recover from the ET-743-induced damage. On the contrary, the survival fraction of +hTERT cells reached approximately 80% of cell survival at 96 h. The survival curves of the +hTERT cells treated with both drugs were superimposable to those of M14 cells.

The ability of different cells to recover the damage elicited by CDDP and ET-743 was also evaluated in terms of chromosome damage. Figure 5 shows the distribution of chromosome damage observed from 24 to 96 h after treatment with both drugs. The data showed that the treatment with CDDP or ET-743 significantly ($p < 0.001$) enhanced telomeric fusion frequency both in −hTERT and in +hTERT cells. In addition, both −hTERT and +hTERT cells treated with CDDP or ET-743 exhibited various chromosome aberrations such as aspecific chromatid and chromosome breaks and exchange figures involving two or more chromosomes. During the time after the treatment, the rate of induced chromosome damage significantly ($p < 0.01$) decreased in +hTERT cells, whereas no significant difference was observed in −hTERT cells. The results obtained when using the MAS51 clone, with and without hTERT reconstitution, were similar to those obtained with the MAS53 clone (data not shown).

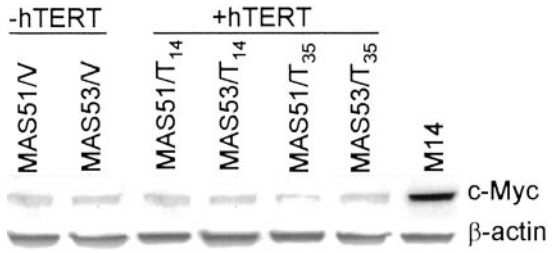

Fig. 2. Decreased sensitivity to CDDP and ET-743 is not attributable to a reactivation of c-Myc expression. Immunoblot analysis of c-Myc protein expression evaluated in −hTERT cells (MAS51/V_{35}, MAS53/V_{35}), +hTERT cells after 14 (MAS51/T_{14}, MAS53/T_{14}) and 35 (MAS51/T_{35}, MAS53/T_{35}) PD, and M14 parental line.

Reconstitution of hTERT Enables the Cells to Recover from CDDP and ET-743-Induced G_2/M Block. To evaluate whether the inability to recover the drug damage elicited by the −hTERT cells was related to alterations in cell-cycle progression, BrdU incorporation assay was performed. Figure 6 shows the bivariate DNA/BrdU distribution, performed at different times after 15 min of BrdU labeling, in −hTERT (MAS51/V_{35}), +hTERT (MAS51/T_{35}), and M14 cells untreated or treated with CDDP and ET-743 at equitoxic doses. It is evident that both drugs caused a similar block in the S-G_2/M phase of cell cycle 24 h after the end of treatments (0 h), regardless of hTERT expression. However, a significant difference among the lines was observed during the progression through the cell-cycle phases. In fact, although the +hTERT cells were able to recover from the CDDP and ET-743–induced block to the same extent as M14 cells, a strong perturbation of the cell cycle was still evident in the −hTERT cells 16 h after labeling. This effect was mainly caused by a different progression through the cell cycle between −hTERT and +hTERT cells. In fact, the analysis of the percentage of BrdU-positive cells in the different phases of cell cycle (Fig. 7) demonstrated that −hTERT cells remained in the G_2/M phase for a prolonged time with respect to +hTERT or M14 cells, thus causing a delay in the repopulation of the G_1 and S phases. As a consequence, the CDDP and ET-743–induced G_2/M block was irreversible in the −hTERT cells, in which approximately 80% of BrdU-positive cells were still arrested in G_2/M. On the contrary, the restored progression into cell cycle in the +hTERT cells permitted them to overcome the drug-induced G_2/M block, as observed in the M14 parental line.

To determine whether the permanence in the G_2/M phase of CDDP- and ET-743–treated −hTERT cells led them to activate the apoptotic program, a TUNEL assay was carried out by flow cytometry, in which the fragmented DNA is simultaneously analyzed with DNA content. Two-parameter flow-cytometry analysis (Fig. 8) demonstrated that the −hTERT cells treated with both drugs gave a TUNEL-positive signal in the G_2/M-phase region (approximately 20%),

TABLE 1

Telomeric fusions in −hTERT (MAS51/V_{35} and MAS53/V_{35}) and +hTERT after 14 (MAS51/T_{14} and MAS53/T_{14}) and 35 (MAS51/T_{35} and MAS53/T_{35}) population doublings after hTERT infection

95% of telomeric fusions observed involve two chromosomes, resulting in a dicentric chromosome, whereas 5% involve both end of the same chromosome. Frequency of telomeric fusions was calculated as total number of telomeric fusions/total number of metaphases analyzed.

Lines	Cells Analyzed	Cells with Telomeric Fusions					Total Telomeric Fusions	Frequency of Telomeric Fusions
		0	1	2	3	4		
−hTERT								
MAS51/V_{35}	100	26	41	25	5	3	118	1.18
MAS53/V_{35}	88	18	40	20	6	4	114	1.30
+hTERT								
MAS53/T_{14}	99	48	33	12	6		75	0.76
MAS51/T_{14}	100	44	36	14	6		82	0.82
MAS51/T_{35}	92	75	15	2			19	0.21
MAS53/T_{35}	91	71	17	3			23	0.25

whereas no apoptotic cells were detected in either the +hTERT or M14 cells. Therefore, it follows that CDDP and ET-743 treatments induced DNA fragmentation in the G_2/M phase in the −hTERT cells only, suggesting that the prolonged permanence in G_2/M of the −hTERT cells was responsible for the CDDP and ET-743 sensitivity.

The progression through the cell cycle and the induction of apoptosis obtained by using the MAS51 clone, with and without hTERT reconstitution, were similar to those obtained with the MAS53 clone (data not shown).

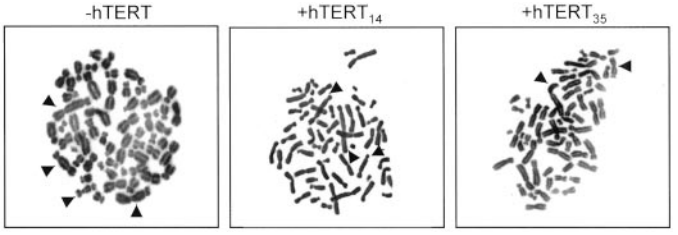

Fig. 3. Reconstitution of hTERT improves telomere function. Metaphase spreads of −hTERT (MAS51/V_{35}) and +hTERT after 14 (MAS51/T_{14}) and 35 (MAS51/T_{35}) PD after infection. The arrows indicate telomeric fusions. A representative of four independent experiments with similar results is shown.

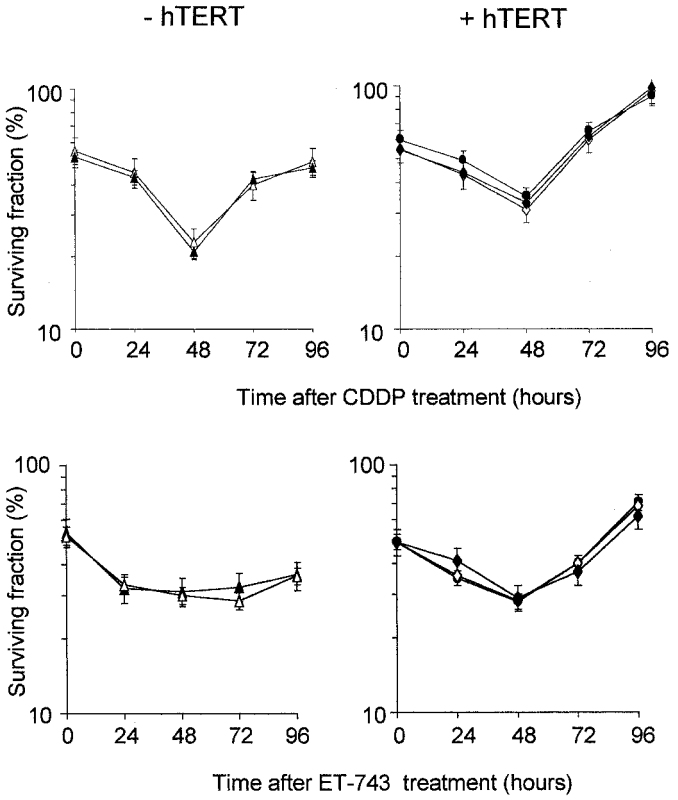

Fig. 4. Reconstitution of hTERT enables the cells to recover from CDDP and ET-743–induced damage. Survival curves of the −hTERT cells (left; ▲, MAS51/V_{35}; △, MAS53/V_{35}), +hTERT after 35 PD (right; ◆, MAS51/T_{35}; ◇, MAS53/T_{35}), and M14 cells (●; right) exposed to CDDP (top) and ET-743 (bottom) at equitoxic doses (see *Materials and Methods*). The analysis was performed from 0 to 96 h after the end of treatment. Surviving fractions were calculated as the ratio of absolute survival of the treated sample to the absolute survival of the control sample. The data represent the mean and S.D. of four independent experiments.

Discussion

We report that the reconstitution of telomerase activity was able to modify the functional status of telomeres. In fact, the +hTERT cells showed a number of total end-to-end fusions significantly lower than those of the −hTERT cells. The improved telomere function gradually occurred with the increase of population doublings. The restored telomere function rendered the cells less sensitive to CDDP and ET-743 without affecting LND susceptibility. In particular, −hTERT cells containing dysfunctional telomeres were more sensitive to CDDP and ET-743 than were +hTERT cells possessing functional telomeres. Moreover, we demonstrated that telomere dysfunction, rather than telomerase activity, determined the chemosensitivity. In fact, by using +hTERT cells at two different population doublings showing the same level of telomerase activity (Biroccio et al., 2002) but different telomere status, we demonstrated that the degree of chemosensitivity strictly depended on the severity of telomere dysfunction. Our results are in agreement with the data reported by De-Pinho et al., demonstrating that telomere dysfunction alters the radio- and chemotherapeutic profile of cells derived from telomerase RNA-null mice (Wong et al., 2000;

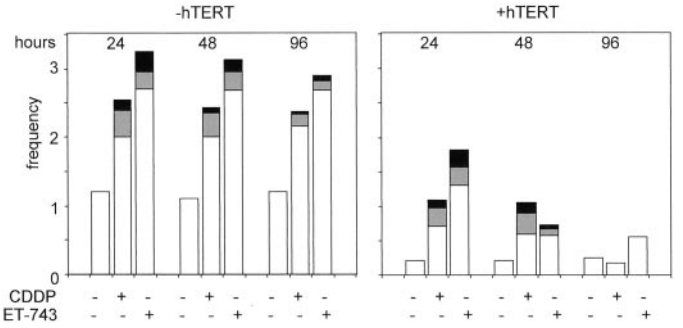

Fig. 5. Reconstitution of hTERT enables the cells to recover from CDDP and ET-743–induced chromosomal damage. Distribution of chromosome damage in the −hTERT (MAS51/V_{35}) and +hTERT cells after 35 PD (MAS51/T_{35}) untreated or treated with CDDP and ET-743. The analysis was performed from 24 to 96 h after the end of both treatments. Frequency of telomeric fusions (□), exchange figures (▨), and breaks (■) are shown. The data represent the mean of three independent experiments with standard deviations of less than 10%.

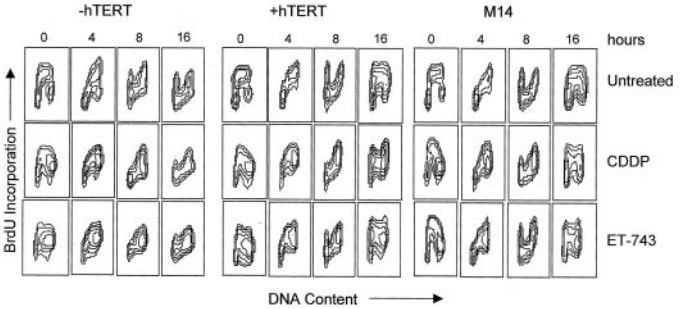

Fig. 6. Reconstitution of hTERT enables the cells to recover from CDDP and ET-743–induced G_2/M block. Kinetics of progression through the stages of the cell cycle after BrdU-labeling in the −hTERT (MAS51/V_{35}), +hTERT after 35 PD (MAS51/T_{35}), and M14 cells untreated and treated with CDDP or ET-743 at equitoxic doses (see *Materials and Methods*). BrdU was added 24 h after the end of CDDP and ET-743 treatment, and cytofluorometric analysis was performed at the end of the 15-min pulse with BrdU (0 h) and from 4 to 16 h after the end of the pulse. A representative of three independent experiments with similar results is shown.

Lee et al., 2001). On the other hand, attenuation of telomerase activity without telomere shortening does not increase sensitivity to anticancer agents (Folini et al., 2000).

The decreased sensitivity to CDDP and ET-743 after reactivation of telomerase function was caused by the ability of cells to recover from drug-induced damage in terms of cell survival. Similarly, the persistence of structural chromosomal lesions was evident only in −hTERT cells treated with both drugs, whereas these aberrations were largely resolved in +hTERT cells. The ability of the +hTERT cells to recover from drug-induced damage was attributable to the restored cell cycle progression. In fact, although the −hTERT cells remained for a prolonged time in the G_2/M phase with a delay in the repopulation of the G_1 and S phases, hTERT reconstitution restored normal cell cycle. The delayed progression of the G_2/M phase, observed in −hTERT cells, rendered irreversible the S-G_2/M block induced by both drugs. In fact, at equitoxic doses, both drugs caused a similar arrest of the S-G_2/M phases, which is recovered only in +hTERT cells, although a strong perturbation of the cell cycle was evident in the cells without hTERT reconstitution. Our data are strengthened by the use of the G_1-blocking drug LND, because the susceptibility to this compound was similar in all the cell lines regardless of hTERT expression and telomere dysfunction. The decreased drug sensitivity was mainly caused by telomere dysfunction rather than by telomerase activity per se, because, as we demonstrated previously, the mutant biologically inactive hTERT, which is catalytically active but unable to maintain telomeres, was unable to restore cell cycle (Biroccio et al., 2002).

The cell cycle deregulation after alteration of telomere function has been reported by other authors. In particular, the expression of mutant telomerase in immortal telomerase-negative human cells results in chromosome fusion and abnormal cell cycle, with the ratio between the percentage of cells in G_1 and G_2/M being decreased (Guiducci

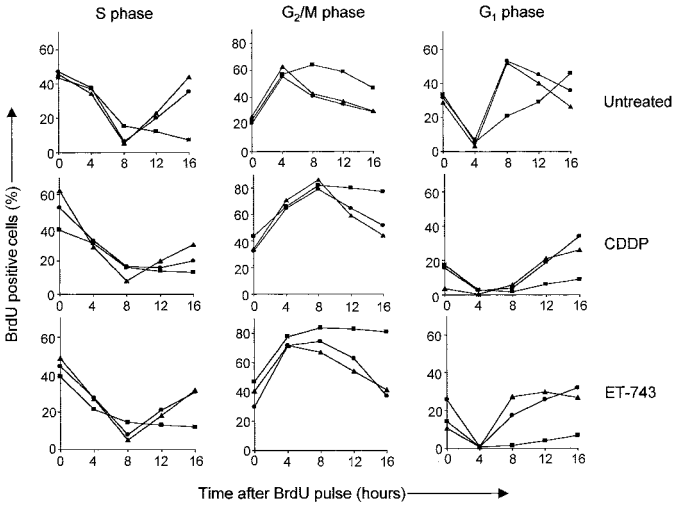

Fig. 7. Reconstitution of hTERT restores the progression through the cell cycle. BrdU-positive S, G_2/M, and G_1 −hTERT (■, MAS51/V_{35}), +hTERT after 35 PD (●, MAS51/T_{35}), and M14 cells (▲) untreated and treated with CDDP or ET-743 at equitoxic doses (see *Materials and Methods*). BrdU was added 24 h after the end of CDDP and ET-743 treatment, and cytofluorometric analysis was performed at the end of the 15-min pulse with BrdU (0 h) and from 4 to 16 h after the end of the pulse. The data represent the mean of three independent experiments with standard deviations of less than 10%.

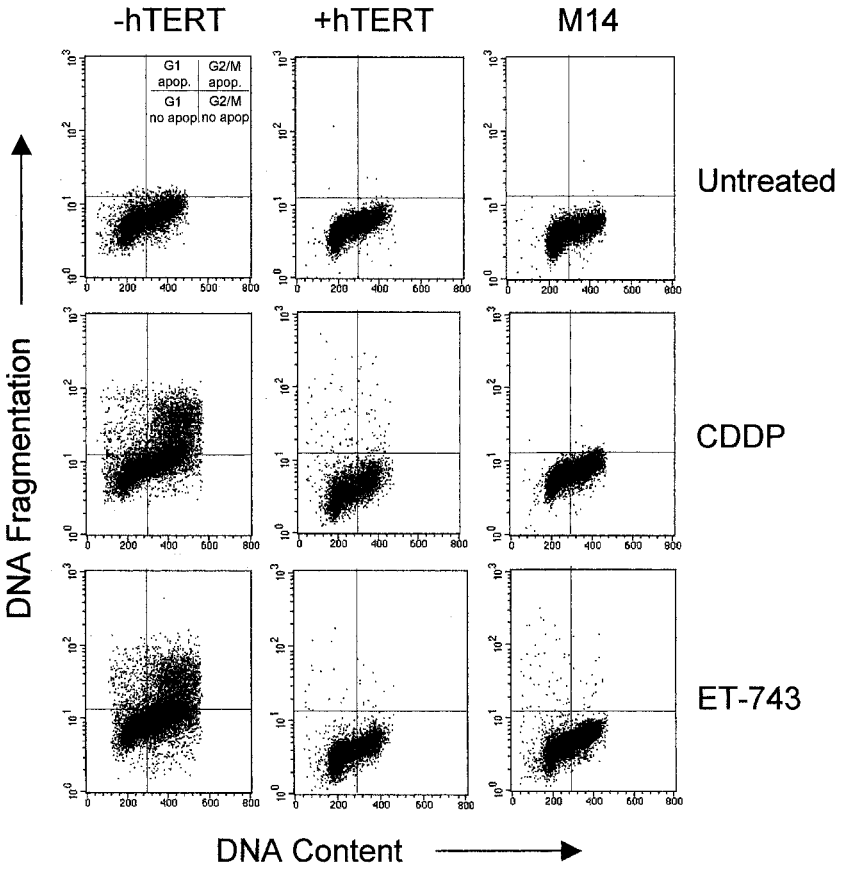

Fig. 8. Reconstitution of hTERT protects cells from CDDP and ET-743–induced apoptosis. DNA fragmentation determined by TUNEL assay in the −hTERT (MAS51/V_{35}), +hTERT after 35 PD (MAS51/T_{35}), and M14 cells untreated and treated with CDDP or ET-743 at equitoxic doses (see *Materials and Methods*). TUNEL analysis was performed 48 h after the end of the treatments. A representative of three independent experiments with similar results is shown.

et al., 2001). Similar cell cycle deregulation has been reported by altering the expression of telomere function-regulating proteins, such as Pin2/TRF1 (Shen et al., 1997), or the new Pin2/TRF1-interacting protein PinX1 (Zhou and Lu, 2001).

However, even if an alteration in the cell cycle after telomere dysfunction has already been described, it has not been correlated with drug sensitivity. We demonstrated that the permanence in G_2/M phase of CDDP- and ET-743–treated cells without hTERT reconstitution led the cells to activate the apoptotic program. Apoptosis was observed only in −hTERT cells treated with both drugs and, by using a two-parameter flow-cytometric analysis, we demonstrated that apoptosis occurred at the G_2/M phase of cell cycle, strengthening the hypothesis that irreversible G_2/M block induced by treatment was responsible for the CDDP and ET-743 sensitivity.

To the best of our knowledge, this is the first evidence demonstrating that telomere dysfunction is involved in the susceptibility to CDDP and ET-743 in melanoma cells. Therefore, on the basis of previous results demonstrating the efficacy of telomerase inhibitors in cells with short telomeres (Kelland, 2001) and recent evidence showing the antitumoral activity of CDDP and ET-743 combination in several histotypes, including melanoma (D'Incalci et al., 2002), a new approach consisting of the use of telomerase inhibitors able to induce telomere dysfunction, followed by CDDP/ET-743 treatment, could improve the chemotherapeutic response of melanoma.

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