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Attenuation of telomerase activity does not increase sensitivity of human melanoma cells to anticancer agents

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Abstract

In tumour cells, replicative immortality is attained through stabilisation of telomeres by telomerase. Recent evidence suggests that telomerase plays an anti-apoptotic role. Since apoptosis is the primary mode of cell death induced by several drugs, telomerase could be involved in determining the chemosensitivity profile of tumour cells. We investigated whether inhibition of telomerase activity through a hammerhead ribozyme targeting the RNA template of telomerase influences the susceptibility of human melanoma cells to a variety of anticancer agents (platinum compounds, taxanes, topoisomerase I inhibitors). The ribozyme sequence was inserted into an expression vector and the JR8 human melanoma cell line was transfected with it. The cell clones obtained showed a reduced telomerase activity. Growth inhibition curves generated after exposure of ribozyme-transfectant clones to individual drugs were superimposable to those obtained from parental cells. Moreover, telomerase inhibition did not promote apoptosis as a cellular response to drug treatment. Overall, our results indicate that downregulation of telomerase activity does not increase the sensitivity of melanoma cells to anticancer drugs.

Keywords: Telomerase; Telomere; Ribozyme; Apoptosis; Drug sensitivity; Melanoma cells

1. Introduction

Normal human cells lose telomeric repeats at each round of cell division [1]. When telomeres shorten to a critical length, cells undergo senescence and cease to proliferate [2]. In tumour cells, stabilisation of telomere length seems to be attained through the expression of telomerase, which maintains and elongates telomeres by the *de novo* synthesis of telomeric DNA [3]. Human telomerase is a ribonucleoprotein complex, composed of a catalytic reverse transcriptase subunit (hTERT), an RNA component (hTR) that serves as a template for the synthesis of telomeric repeats, and an associated protein subunit (TEP1) [3].

The observation that telomerase activity is present in 80–90% of human cancers [4] has led to the hypothesis that telomerase is involved in cellular immortality and carcinogenesis. The notion that telomeric maintenance is essential for the formation of human tumours has been recently substantiated by Hahn and colleagues [5], who found that ectopic expression of hTERT in cooperation with the oncogenes SV40 large-T and H-ras resulted in direct tumorigenic conversion of human epithelial and fibroblast cells.

On the basis of such findings, telomerase has been proposed as a novel target for anticancer therapies. By taking advantage of the evolving understanding of the composition and function of telomerase, a number of potential inhibitors have been developed including antisense oligonucleotides against the template region of hTR [6–8], traditional reverse transcriptase inhibitors [9,10], and agents able to promote or stabilise G-quadruplex formation in telomeric DNA [11,12]. However, the long lag time expected before the telomere would shorten sufficiently to produce detrimental effects on cell growth suggests that telomerase will be a challenging target for drug development.

Recent evidence indicates that telomerase plays a role in cellular resistance to apoptosis [13,14]. Since apoptosis is the primary mode of cell death induced by several drugs in different experimental tumour models [15], telomerase could be involved in determining the chemosensitivity profile of tumour cells. As a consequence, the possibility to sensitise tumour cells to the antiproliferative activity of anticancer agents through the downregulation of telomerase could be pursued.

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In the present study, we set out to inhibit telomerase activity in human melanoma cells with the final aim of increasing their susceptibility to anticancer drugs. Human melanoma was selected as the tumour model since telomerase reactivation has been detected in a large percentage of melanoma lesions [16,17]. Moreover, melanoma is highly refractory to all conventional anticancer treatments.

We generated a hammerhead ribozyme targeting the RNA template of telomerase. The ability of such a ribozyme to significantly inhibit telomerase's catalytic activity in melanoma cell lines and surgical specimens has previously been described [7]. Melanoma cell clones were obtained by transfection of the JR8 melanoma cell line with an expression vector containing the ribozyme sequence; we then comparatively analysed the sensitivity profiles of ribozyme transfectant clones and parental cells to anticancer agents with different mechanisms of action, including platinum compounds (cisplatin, oxaliplatin and the trinuclear platinum complex BBR 3464 [18]), taxanes (paclitaxel and docetaxel) and topoisomerase I inhibitors (topotecan and the active metabolite of irinotecan, SN-38). We also investigated whether telomerase inhibition may influence the induction of programmed cell death as a cellular response to drug treatment.

2. Materials and methods

2.1. Cell line

The JR8 human melanoma cell line [19] was maintained in the logarithmic growth phase at 37°C in a 5% CO_2 humidified atmosphere using Roswell Park Memorial Institute (RPMI) 1640 (BioWhittaker, Verviers, Belgium) supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine and 0.25% (v/v) gentamycin.

2.2. Construction of the ribozyme expression vector and transfection

The oligonucleotide sequences encoding the ribozyme were the following: Rztelo− (5′-GGCCGCGGCGGCCGCCATTTTTTGTTTCGTCCTCACGGACTCTTCAGTAACCCTAACGCGGCCGCA-3′) and Rztelo+ (5′-AGCTTGCGGCCGCGTTAGGGTTACTGAAGAGTCCGTGAGGACGAAACAAAAAATGGCGGCCGCCGC-3′) (M-Medical S.r.l., Florence, Italy). The annealed oligonucleotides produced a fragment with *Hind*III and *Not*I protruding ends. The fragment was inserted into the pRC/CMV expression vector (Invitrogen, San Diego, CA, USA) previously digested with *Hind*III and *Not*I restriction enzymes. The presence and the correct orientation of the insert was verified by DNA sequencing (AmpliCycle™, Perkin Elmer-Roche Molecular System, Inc., Branchburg, NJ, USA). The resulting plasmid was named pRcRzB. (N-(2,3dioleoxyloxy)propyl)-N,N,N trimethylammonium methylsulphate (DOTAP)-mediated transfection of JR8 cells was performed as follows: cells were seeded at a density yielding approximately 50% confluency at the time of transfection, and then transfected with 5 μg of pRcRzB vector (or pRc/CMV control vector) that had been complexed with 30 μg of DOTAP. Six hours after transfection the culture medium containing the DOTAP/DNA mixture was replaced by a selection medium containing G418 to a final concentration of 2 mg/ml. The transfected cells were exposed to G418 for 1 month.

2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of ribozyme and telomerase RNA expression

Total RNA isolated from the transfectants and parental JR8 cell line was reverse-transcribed using a GeneAmp RNA polymerase chain reaction (PCR) core kit (Perkin Elmer) according to the manufacturer's instructions. To analyse ribozyme expression, the resultant cDNA was amplified using T7 and Rztelo (5′-CCTCACGGACTCTTCAG-3′) primers (M-Medical) and by performing 35 cycles of PCR in the presence of 1 μCi/sample of [α^{32}P] deoxycytidine triphosphate (3000 Ci/mmol, Amersham).

For the analysis of *hTR* expression, RT mixtures were diluted 10 000 fold. One μl of each solution was amplified in the presence of teloS and teloA primers [7] and 1 μCi of [α^{32}P] deoxycytidine triphosphate, by performing 26 cycles of PCR as previously described [7].

β-actin, used as a standard of amplification, was coamplified with *hTR* RNA as previously described [7]. The PCR products were analysed by electrophoresis on a 10% non-denaturating polyacrylamide gel. The gel was dried and autoradiographed.

2.4. Telomerase activity detection assay

Telomerase activity was measured by the telomeric repeat amplification protocol (TRAP) [20]. Five to 10^6 cells were resuspended in ice-cold lysis buffer and kept on ice for 30 min. After centrifugation at 25 000*g* at 4°C for 30 min the supernatants were quick-frozen in liquid nitrogen and stored at −80°C. Protein concentrations of the lysates were determined using standard procedures. Proteins (2 μg) from each extract were assayed in 50 μl of TRAP reaction mixture composed of 20 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$, 63 mM KCl, 0.005% Tween-20, 1 mM EGTA, 50 mM each of deoxynucleoside triphosphates, 0.1 μg telomerase substrate (TS) oligonucleotide, 1 μg of T4g32 protein (Boehringher Mannheim, Mannheim, Germany), 0.1 mg/ml bovine serum albumin, 2 U Taq DNA polymerase (AmpliTaq, Perkin Elmer) and 0.2 μl of [α-^{32}P] deoxycytidine

triphosphate (10 μCi/μl, 3000 Ci/mmol, Amersham, Buckinghamshire, UK). After 20 min of incubation at 23°C the reaction mixture was heated to 94°C for 30 s and then subjected to 30 cycles of PCR [20]. The reaction mixtures were analysed by electrophoresis on a 10% non-denaturating polyacrylamide gel. To verify the specific telomerase activity, 2 μg of protein was pretreated with 20 μg/ml RNase for 20 min at 37°C.

For quantitative analysis, assays were repeated using the Oncor TRAPeze Telomerase Detection Kit (Oncor Appligene, Heidelberg, Germany). Each reaction product was amplified in the presence of a 36-bp internal TRAP assay standard (ITAS), and each sample extract was tested for RNase sensitivity. A TSR8 quantitation standard was included for each set of TRAP assays. The TSR8 control is provided in the kit and serves as a standard for estimating the amount of product extended by telomerase in a given extract. Quantitative analysis was performed with the Image-QuanT software (Molecular Dynamics, Sunnyvale, CA, USA), which allowed densitometric evaluation of the digitised image. Telomerase activity was quantified by measuring the signal of telomerase ladder bands, and the relative telomerase activity was calculated as the ratio to the internal standard using the following formula:

$$\text{relative telomerase activity} : ((X - X_0)/C) \times ((R - R_0)/Cr)^{-1}$$

where X is the untreated sample, X_0 is the RNase-treated sample, C is the internal control of untreated samples, Cr is the internal control of TSR8, R is the TSR8 quantitation control, and R_0 is the negative control.

2.5. Telomere length measurement

For each sample, 10 μg of total DNA was digested with 40 units of *Hin*fI and then electrophoresed on 0.8% (w/v) agarose gels. Following electrophoresis, the gels were denatured, neutralised, transferred to a nylon membrane (Hybond N; Amersham) and then cross-linked with ultraviolet (UV) light. The membrane was hybridised with a 5′-end [γ-^{32}P]deoxyadenosine triphosphate-labelled telomeric oligonucleotide probe $(TTAGGG)_4$. Hybridisation was performed at 42°C for 1 h in rapid hybridisation buffer (Amersham). The membrane was washed and then autoradiographed. The autoradiographs were scanned (ScanJet IIcx/T; Hewlett Packard, Milan, Italy) and the mean telomere length was calculated as previously reported [7].

2.6. Drugs and cytotoxicity assay

Cisplatin (Bristol-Myers, Evansville, IL, USA), BBR 3464 (Roche Boehringer, Milan, Italy) and oxaliplatin (Sanofi Winthrop, Gentilly, France) were dissolved in 0.9% NaCl solution. Paclitaxel (Sigma Chemical Co., St Louis, MO, USA), docetaxel (Rhône-Poulenc Rorer, Vitry-sur-Seine, France) and the active metabolite of irinotecan, SN-38 (Rhône-Poulenc Rorer), were dissolved in dimethylsulphoxide and then diluted in sterile water. Topotecan (SmithKline Beecham Parmaceuticals, King of Prussia, PA, USA) was dissolved in sterile water.

After harvesting in the logarithmic growth phase, cells were seeded in 6-well plates for 24 h and then exposed for 1 h to cisplatin, oxaliplatin or BBR 3464 and for 24 h to paclitaxel, docetaxel, SN-38 or topotecan. At the end of the exposure, adherent cells were washed with phosphate-buffered saline (PBS, BioWhittaker) and incubated at 37°C in a 5% CO_2 humidified atmosphere for 3 days. Cells were then trypsinised and counted in a particle counter (Coulter Counter, Coulter Electronics, Luton, UK). Each experimental sample was run in triplicate. The results were expressed as the total number of adherent cells in treated samples compared with control samples in which only drug solvent was added. *In vitro* drug activities were expressed in terms of concentrations able to inhibit cell proliferation by 50% IC_{50}.

2.7. Evaluation of apoptotic morphology by fluorescence microscopy

Cells were harvested, washed in PBS and stained with a solution containing 50 μg/ml propidium iodide, 50

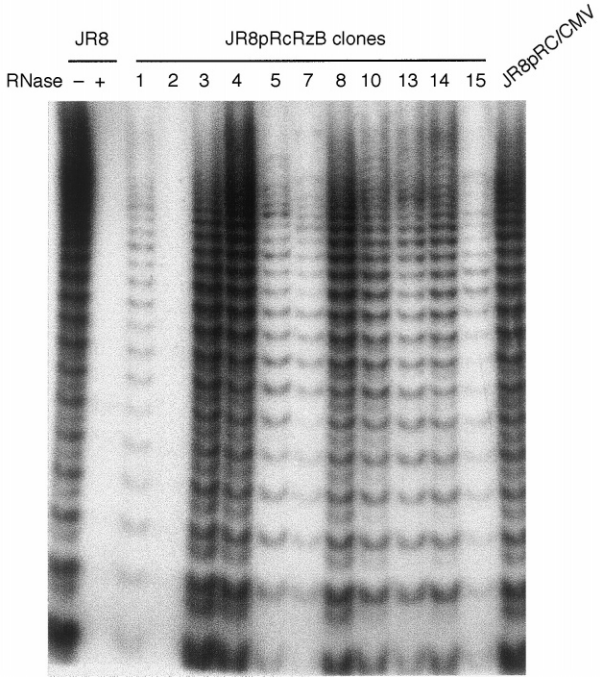

Fig. 1. Telomerase activity in transfectants and parental cells. Lane 1, JR8 parental cells; lane 2, JR8 cell extract pretreated with 20 μg/ml RNase; lanes 3–13, ribozyme-transfectant clones (numbered according to their clone numbers); lane 16, vector-transfectant clone.

mg/ml RNase, and 0.05% (v/v) Nonidet P40 (NP40). After staining, the slides were observed by means of fluorescence microscopy. The percentage of apoptotic cells was determined by scoring at least 500 cells in each sample.

3. Results

The catalytic potential of the ribozyme was previously verified as the ability to cleave an internally ^{32}P-labelled synthetic RNA substrate obtained by cloning a portion of the RNA component of human telomerase [7].

JR8 cells were then transfected with the pRcRzB vector containing the ribozyme sequence. The transfectants were treated with G418 for 1 month, and 11 G418-resistant clones were selected. Telomerase activity of the clones and of control cells transfected with the pRc/CMV (cytomegalovirus) vector lacking the ribozyme sequence was studied. As shown in Fig. 1, some clones were characterised by a reduced telomerase activity compared with that of the vector-transfectant control and the parental JR8 cells. On the basis of these results, we selected for further studies clones 2 and 15, in which the maximum decline of telomerase activity was observed. RT-PCR analysis of ribozyme expression was performed and the presence of the ribozyme was detected to a similar degree in both ribozyme-transfectant clones, but not in the vector-transfectant clone JR8 pRc/CMV (data not shown).

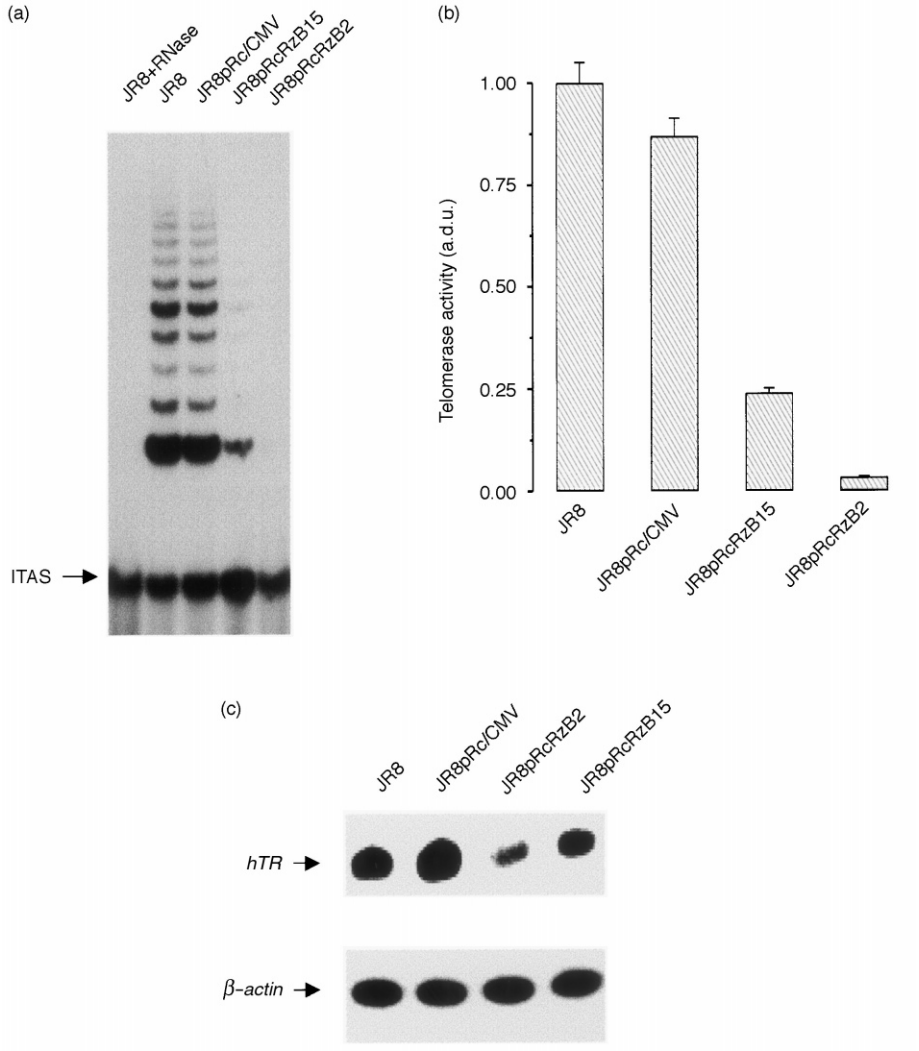

Fig. 2. (a) Telomerase activity in ribozyme-transfectants after 45 days of growth in culture. Lane 1, JR8 parental cells plus RNase; lane 2, JR8 cells; lane 3, vector-transfectant clone; lane 4, ribozyme-transfectant clone 15; lane 5, ribozyme-transfectant clone 2. The location of the internal amplification standard (ITAS) is indicated. (b) Quantitation of telomerase activity, in terms of arbitrary densitometric unit (a.d.u.), in JR8 parental cells, vector-transfectant clone and ribozyme-transfectant clones 2 and 15. Each value is the average±standard deviation (S.D.) of three independent experiments. (c) *hTR* expression as detected by reverse transcriptase-polymerase chain reaction (RT-PCR) in ribozyme-transfectants after 45 days of growth in culture. Lane 1, JR8 parental cells; lane 2, vector-transfectant clone; lane 3, ribozyme-transfectant clone 2; lane 4, ribozyme-transfectant clone 15. *β-actin* was used as a control for amplification.

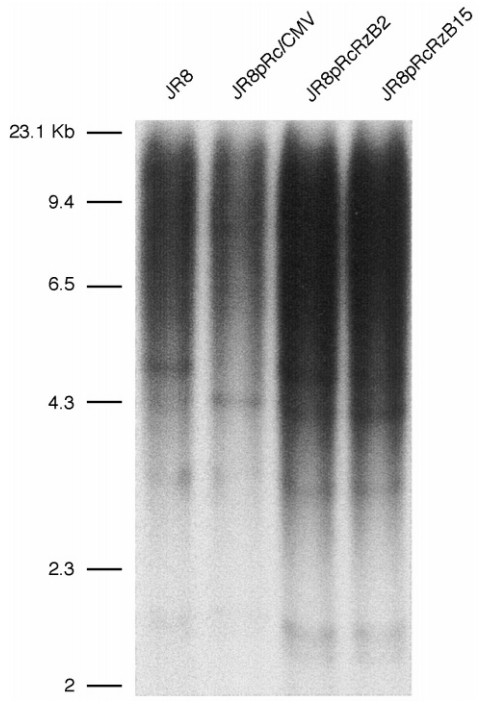

Fig. 3. Telomere length of ribozyme transfectants after 45 days of growth in culture, as detected by Southern blot hybridisation. Lane 1, JR8 parental cells; lane 2, vector-transfectant clone; lane 3, ribozyme-transfectant clone 2; lane 4, ribozyme-transfectant clone 15.

After 45 days in culture, telomerase activity was still inhibited in the two ribozyme-transfectant clones (Fig. 2a). Moreover, since similar internal standard bars were detected in all samples, the possibility that the reduction of TRAP signals in ribozyme-transfectant clones is the consequence of the presence of Taq polymerase inhibitors was excluded. The intensity of TRAP signals of JR8 control cells was assigned an arbitrary value of 1.00, whereas the relative signal intensity was 0.86±0.05 for the vector-transfectant clone, and 0.035±0.005 and 0.24±0.02 for the ribozyme-transfectant clones 2 and 15, respectively (Fig. 2b). RT-PCR analysis showed a reduced level of telomerase RNA in clone 2. Conversely, *hTR* was steadily expressed in clone 15 (Fig. 2c). Following this culture period, the ribozyme-transfectant clones 2 and 15 were still able to proliferate, even though they had significantly ($P < 0.01$) longer doubling times than JR8 parental cells (29.5±0.52 h and 30.1±1.02 h versus 24.0±1.08). As regards morphology, JR8 and vector-transfectant cells were spindle- or triangle-shaped, whereas ribozyme-transfectant cells showed a dendritic appearance (data not shown). The telomere length of the ribozyme-transfectant clones analysed by Southern blot hybridisation showed no telomere shortening compared with JR8 cells (Fig. 3). Fluorescence microscopy analysis of cells stained with propidium iodide (Fig. 4) indicated the presence of a small, but significant, percentage of cells with an apoptotic nuclear morphology in the ribozyme-transfectants (4.5±1.2% and 6.0±1.5% of the overall cell population in clones 2 and 15, respectively). Conversely, in the JR8 parental cell line and the vector-transfectant clone, the fraction of apoptotic cells was significantly ($P < 0.01$) lower and accounted for only 0.2±0.1% and 0.5±0.3% of the overall cell population.

To investigate whether or not attenuation of telomerase activity may influence the susceptibility of melanoma cells to anticancer agents, we comparatively assessed the activity of drugs with different mechanisms of action such as platinum compounds (cisplatin, oxaliplatin and BBR 3464), taxanes (paclitaxel and docetaxel) and topoisozmerase I inhibitors (SN-38 and topotecan) in ribozyme-transfectant clones and parental cells. As shown in Figs. 5–7, no appreciable difference was found in the growth inhibition curves obtained in ribozyme-transfectant and control cells after exposure to individual drugs, and similar IC_{50} values were obtained for the different agents (Table 1). In these cell lines, the major mode of drug activity for all classes of compounds proved to be inhibition of cell division. In fact,

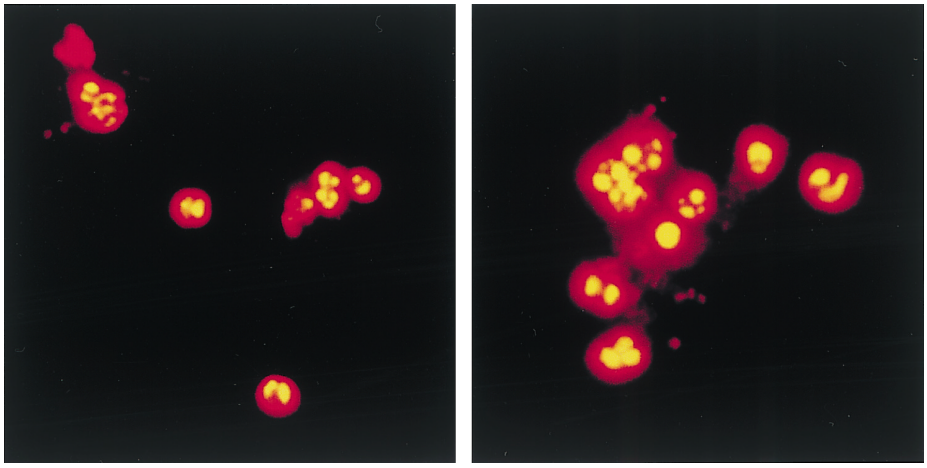

Fig. 4. Propidium iodide staining of ribozyme-transfectant clone 2. Cells were collected, stained with propidium iodide and viewed under a fluorescence microscope. Two representative fields are shown.

the presence of cells with an apoptotic morphology was only occasionally observed in the drug-treated JR8 cells. In ribozyme-transfectant clones, no significant difference was observed in the percentage of apoptotic cells in drug-treated samples (always $<10\%$ of the overall cell population) as compared with untreated (no drug) controls.

4. Discussion

Melanoma is considered to be a chemotherapy-refractory tumour type and the commonly used anti-cancer drugs do not modify the prognosis of metastatic disease. The molecular and cellular mechanisms that sustain the chemoresistance of melanoma cells have not yet been elucidated [21], even though a possible role of altered DNA repair pathways has been suggested [22].

The finding that many chemotherapeutic agents kill susceptible cancer cells through the induction of apoptosis would suggest resistance to undergo programmed cell death to be a possible mechanism of melanoma chemoresistance [21]. In fact, it has been reported that even a drug like paclitaxel, which is known to be a potent inducer of apoptosis in several experimental tumour models [23], failed to induce programmed cell death in the SK-MEL-28 human melanoma cell line. In these cells, paclitaxel exerted its cytotoxic effect only through the inhibition of cell division [24]. Moreover, the assessment of the expression of proteins involved in the control of apoptosis in a series of surgical specimens of cutaneous melanoma showed increased levels of the apoptotic inhibitors Mcl-1 and Bcl-X_L. Overexpression of Mcl-1 and Bcl-X_L was also observed in thin primary melanomas, suggesting that upregulation of these proteins represents a relatively early event associated with malignant transformation in human melanoma [25].

Telomerase, an RNA-dependent DNA polymerase which contributes to maintaining the stability of telomeres by compensating for their shortening [3], is reactivated in many cancer and immortal cells [4]. Specifically, telomerase reactivation has been detected in a high percentage of clinical melanoma lesions [16,17]. Because of its differential expression pattern in normal and tumour cells telomerase has been proposed as a promising target for innovative anticancer strategies. Many potential inhibitors of telomerase have been developed [26]; however, thus far it has not been clearly demonstrated how telomerase inhibition actually affects the proliferative potential of tumour cells.

Several lines of evidence suggest that telomerase might play a role in the cellular resistance to apoptosis.

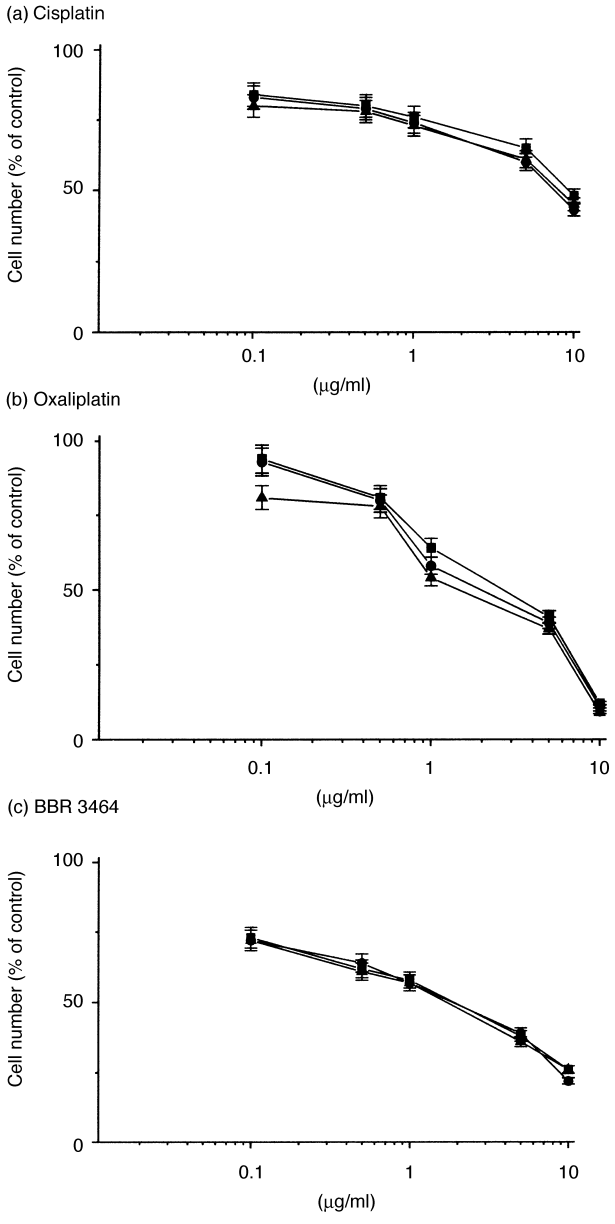

Fig. 5. Growth inhibition curves of JR8 parental cells (■); ribozyme-transfectant clone 2 (●); and ribozyme-transfectant clone 15 (▲) exposed to (a) cisplatin, (b) oxaliplatin and (c) BBR 3464 for 1 h. Each value is the average±standard deviation (S.D.) of three independent experiments.

Table 1
Drug sensitivity profiles of parental cells and ribozyme-transfectant clones

Drug	Exposure time (h)	IC_{50} (μg/ml)		
		JR8	Clone 2	Clone 15
Cisplatin	1	9.24±0.98	7.47±1.01	8.13±1.15
Oxaliplatin	1	2.70±0.25	1.95±0.18	1.45±0.20
BBR 3464	1	1.90±0.18	1.90±0.20	1.73±0.16
Paclitaxel	24	0.0065±0.0006	0.0064±0.0007	0.0064±0.0004
Docetaxel	24	0.0016±0.0003	0.0017±0.0003	0.0014±0.0002
SN-38	24	0.0026±0.0004	0.0022±0.0004	0.0024±0.0003
Topotecan	24	0.029±0.003	0.027±0.004	0.027±0.004

The IC_{50} value was determined graphically from the survival curves as the concentration inhibiting cell growth by 50%. Data represent average values±standard deviation (S.D.) of three independent experiments.

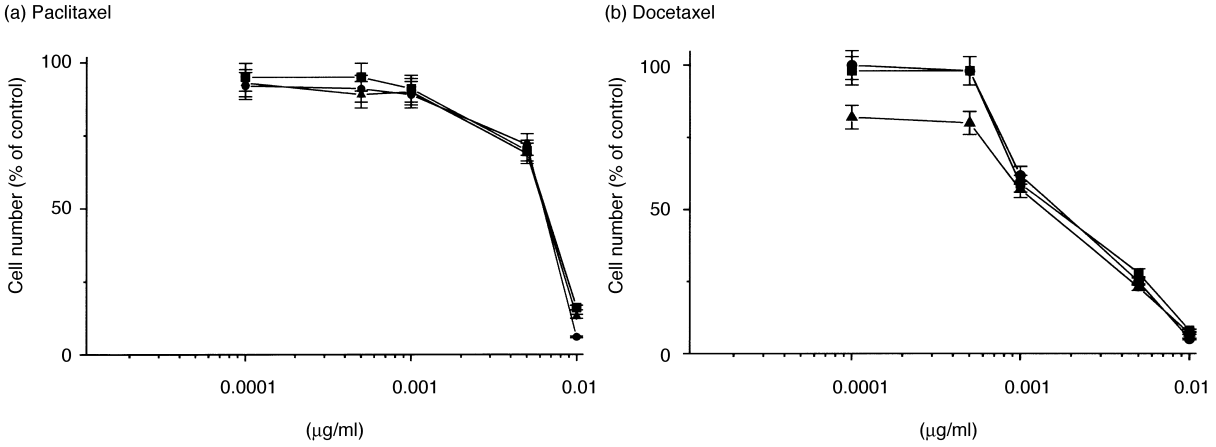

Fig. 6. Growth inhibition curves of JR8 parental cells (■); ribozyme-transfectant clone 2 (●); and ribozyme-transfectant clone 15 (△) exposed to (a) paclitaxel and (b) docetaxel for 24 h. Each value is the average±standard deviation (S.D.) of three independent experiments.

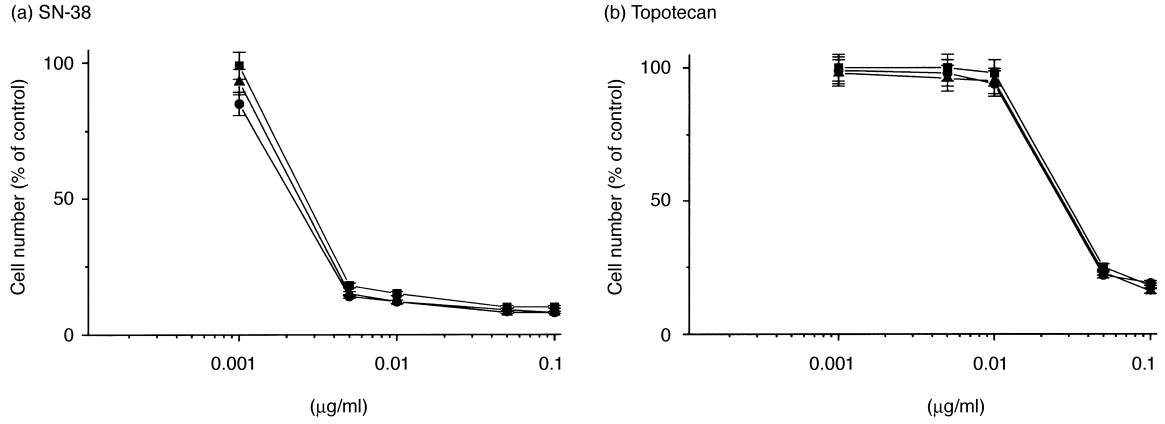

Fig. 7. Growth inhibition curves of JR8 parental cells (■); ribozyme-transfectant clone 2 (●); and ribozyme-transfectant clone 15 (▲) exposed to (a) SN-38 and (b) topotecan for 24 h. Each value is the average±standard deviation (S.D.) of three independent experiments.

In fact, it has been demonstrated that an antisense oligonucleotide against telomerase RNA linked to a 2′,5′-oligoadenylate (2-5A), which activates the endoribonuclease RNAse L, not only decreased telomerase activity, but also induced a rapid (within 4 days) loss of cell viability with concomitant apoptosis in U251-MG human glioblastoma cells [6]. Moreover, a 7-day exposure to a similar 2-5A antisense oligonucleotide induced apoptotic cell death in telomerase-expressing HEY-1B human ovarian cancer cells, but not in telomerase-negative normal ovarian epithelial cells [27]. Again, exposure for 3 days of OMA-BL1 Burkitt's lymphoma cells to a phosphorothioate oligonucleotide with a sequence identical to the repeat sequence of the mammalian telomere d(TTAGGG) resulted in inhibition of telomerase activity, increased doubling time and induction of apoptosis [28]. Results from the studies are not explainable with the classical model which predicts that long-term exposure of tumour cells to telomerase inhibitors should induce telomere shortening after a certain number of rounds of cell division (the number being dependent upon initial telomere length) and growth arrest. In fact, in the aforementioned studies, it is unlikely that cell death was related to telomere erosion since the cells would not have undergone enough divisions to significantly shorten their telomeres. As a consequence, there is the possibility that interfering with telomerase activity can affect aspects in the control of cell proliferation and apoptosis other than telomere length. Such a hypothesis has also been corroborated by results recently obtained in PC12 human pheochromocytoma cells [14]. In fact, in these cells the inhibition of telomerase activity observed after a 24-h exposure to the oligonucleotide TTAGGG or to 3,3′-diethyloxadicarbocyanine was associated with increased susceptibility to apoptosis induced by different stimuli, such as staurosporine, amyloid β-peptide and oxidative insult. Moreover, caspase inhibitors protected PC12 cells against the pro-apoptotic action of telomerase inhibitors, thus suggesting a site of action of telomerase prior to caspase activation [14]. It has also been reported that stable overexpression of Bcl-2 in HeLa human cervical

carcinoma cells resulted in increased telomerase activity and resistance to apoptosis [29].

Considering that apoptosis is the primary mode of cell death induced by several classes of anticancer agents [15], a role of telomerase in determining the chemosensitivity profile of tumour cells can be hypothesised. Although the mechanisms responsible for resistance to apoptosis of cells with high levels of telomerase activity have not yet been established, telomerase inhibition could be used as a means to enhance the susceptibility of tumour cells to chemotherapy. In the present study, we set out to inhibit telomerase activity in human melanoma cells with the final aim of sensitising the cells to the effect of anticancer agents. For this purpose, we generated a hammerhead ribozyme designed to specifically cleave a site located at the end of the telomerase template element. The specificity of the ribozyme's catalytic activity, as well as its ability to inhibit telomerase activity in human melanoma cell lines and surgical specimens have previously been documented [7]. When the ribozyme sequence was cloned into the pRc/CMV vector and the JR8 cells were transfected with the ribozyme expression vector, we were able to select ribozyme-transfectants successfully expressing the ribozyme RNA and characterised by a reduced telomerase activity. However, in accordance with data obtained in a previous study also dealing with the use of a ribozyme targeting the RNA component of telomerase [30], a reduction of *hTR* expression was not observed in one ribozyme transfectant clone. Moreover, ribozyme-transfectant cells grew more slowly than the parental cell line and expressed an altered morphology; in a small percentage of them apoptotic nuclear morphology was observed. The finding is in agreement with previous reports indicating the induction of apoptosis as a consequence of telomerase decline, although a marked variability in the extent of the phenomenon (with percentages of apoptotic cells ranging from 6% [28] to 41–92% [31]) was found in the different studies in which a quantitative analysis was carried out.

We did not find any shortening of telomere length in ribozyme-transfectants displaying reduced telomerase activity. Moreover, after 45 days in culture, they were still able to proliferate. Such a finding might be tentatively explained by the emergence of resistant cells through the activation of ALT (alternative lengthening of telomeres) mechanisms, which are responsible for the maintenance of telomeres and have been demonstrated to be present in a few tumours [32], even though our ribozyme transfectant clones did not show the very long and heterogeneous telomeres typical of ALT cells [32]. Several other attempts aimed at inhibiting telomerase through interference with its RNA component were not able to demonstrate telomere shortening [6,33] or cell growth arrest [30]. Conversely, recent reports dealing with the use of dominant-negative mutants of the telomerase catalytic subunit (hTERT) showed complete inhibition of telomerase activity, reduction of telomere length and death of tumour cells [31]. Such evidence should indicate hTERT as a more promising target than the telomerase RNA component for the development of novel antineoplastic therapies.

Results from chemosensitivity experiments indicated that inhibition of telomerase activity failed to sensitise ribozyme-transfectant clones to the cytotoxic effects of a panel of anticancer agents including the platinum compounds cisplatin, oxaliplatin and BBR 3464, the topoisomerase I inhibitors topotecan and SN-38, and the taxanes paclitaxel and docetaxel. All drugs produced their antitumour effects by inhibiting cell division in the parental JR8 melanoma cells. In fact, no evidence of apoptosis was observed in these cells after drug treatment. Similarly, in ribozyme transfectant clones exposure to the different drugs did not appreciably modify the percentage of apoptotic cells with respect to that observed before drug treatment.

Such results do not agree with the findings by Kondo and associates [33], who reported that inhibition of telomerase with an antisense telomerase expression vector not only decreased telomerase's catalytic activity, but also increased the susceptibility to cisplatin-induced apoptotic cell death in U251-MG human glioblastoma cells. However, the pathway by which attenuation of telomerase activity leads to an increase in the apoptotic response of glioblastoma cells after drug treatment has not been elucidated. Moreover, since telomere shortening after telomerase inhibition has not been established, it is not possible to ascertain whether the influence of telomerase on the sensitivity of glioblastoma cells is telomere-mediated. The controversial results obtained in these studies could be tentatively explained on the basis of the possible differences in the status and/or expression of genes involved in the control of programmed cell death in the two tumour cell models. It is known that the JR8 melanoma and U251-MG glioblastoma cell lines are both characterised by the presence of a mutated *TP53* gene [19,33]. However, it is unknown how the two cell lines compare with each other for the expression of anti-apoptotic proteins such as Bcl-2, Bcl-X_L and survivin [34].

In conclusion, the results from our study indicate that specific inhibition of telomerase activity by a hammerhead ribozyme targeting the RNA template of the ribonucleoprotein does not result in an increased susceptibility of human melanoma cells to drug-induced growth arrest and apoptosis.

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