Pergamon

European Journal of Cancer, Vol. 35, No. 2, pp. 309–315, 1999

Printed in Great Britain
0959-8049/99/$ - see front matter

PII: S0959-8049(98)00365-7

Original Paper

Cytostatic Concentrations of Anticancer Agents do not Affect Telomerase Activity of Leukaemic Cells *In Vitro*

M. Akiyama,[1,2] J. Horiguchi-Yamada,[3] S. Saito,[2] Y. Hoshi,[1] O. Yamada,[4] H. Mizoguchi[4] and H. Yamada[2]

[1]Department of Paediatrics; [2]Department of Molecular Genetics; [3]Department of Oncology, Institute of DNA Medicine, Jikei University School of Medicine, 3-25-8 Nishi-shinbashi, Minato-ku, Tokyo 105-8461; and [4]Department of Haematology, Tokyo Women's Medical College, Tokyo 162, Japan

Telomerase, the enzyme that maintains the ends of linear eukaryotic chromosomes, is more active in the majority of malignant tumours than in normal somatic cells. Telomerase plays a key role in the maintenance of chromosomal stability in tumours, but it still remains unknown whether anticancer agents can inhibit telomerase activity. In this study, we evaluated the effect of various anticancer agents (etoposide, cisplatin, irinotecan, mitomycin C and daunorubicin) on the telomerase activity of three human haematopoietic cancer cell lines (Daudi, K562 and U937). A decrease of telomerase activity was not observed in cells treated with IC_{50} doses of the drugs, except for irinotecan-treated Daudi cells and daunorubicin- and irinotecan-treated U937 cells. Propidium iodide staining disclosed that the cells with decreased telomerase activity were severely damaged. U937 cells exposed to 5 μM (IC_{90}) etoposide showed three different stages of cell viability during treatment. Apoptotic cells with an intact plasma membrane still maintained high telomerase activity, while cells with plasma membrane damage lost telomerase activity. The mRNA of the telomerase catalytic subunit (hTERT) showed a decrease in expression along with the decline of telomerase activity. These results indicate that the concentrations of drugs resulting in cytostatic effects on cells do not affect telomerase activity.

Key words: telomerase, anticancer agent, apoptosis, hTERT
Eur J Cancer, Vol. 35, No. 2, pp. 309–315, 1999

INTRODUCTION

TELOMERASE IS a ribonucleoprotein DNA polymerase that elongates the telomeres of chromosomes to compensate for losses that occur with each round of DNA replication [1–4]. Telomerase consists of a template RNA component (hTERC) containing the complementary sequence of TTAGGG and two proteins, a human homologue of *Tetrahymena* telomerase p80 (TEP-1) and a catalytic subunit (hTERT) [5–7]. Somatic cells express low or undetectable levels of telomerase, except for germ cells and stem cells such as those in the bone marrow, skin and intestine [8–12], and cessation of cell division occurs after the telomeres become shortened to a critical length [13–17]. In contrast, most human malignancies possess high levels of telomerase activity, because unlimited cell proliferation requires this enzyme to prevent the lethal loss of telomeres [18]. Therefore, it is of interest to investigate whether anticancer agents inhibit telomerase activity along with their antiproliferative effect.

Cells treated with anticancer agents respond in two different manners: one is growth arrest while maintaining cell viability and the other is apoptosis. Apoptotic cells can be further categorised into two groups by the presence or absence of plasma membrane damage in addition to nuclear fragmentation or chromatin condensation, and it is possible to investigate these differences by two-colour fluorescent staining [19, 20].

Correspondence to M. Akiyama.
Received 15 May 1998; revised 10 Aug. 1998; accepted 24 Aug. 1998.

In the present study, we investigated the effects of various anticancer agents on telomerase activity in relation to cell viability and telomerase-related gene expression.

MATERIALS AND METHODS

Cell lines and culture

Daudi cells (derived from Burkitt's lymphoma) were a kind gift from Sumitomo Pharmaceuticals (Osaka, Japan). K562 cells (derived from a patient with chronic myelogenous leukaemia in blastic crisis) and U937 cells (derived from the leukaemic cells of a patient with histiocytic lymphoma) were obtained from the Riken cell bank (Tsukuba, Japan). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah, U.S.A.) containing 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO BRL, Gaithersburg, Maryland, U.S.A.) in a 5% CO_2 incubator at 100% humidity.

Determination of drug concentrations

Exponentially growing cells (2×10^5/ml) were suspended in 10 ml of culture medium with the following anticancer agents, which were representative of various drug classes: irinotecan (CPT-11: Sigma, St. Louis, Missouri, U.S.A.), etoposide (VP-16: Sigma), cisplatin (CDDP: Sigma), mitomycin C (MMC: Kyowa, Tokyo) and daunorubicin (DNR: Sigma). The drug concentrations used in this study were examined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Briefly, cells were cultured in 96-well plates for 48 h, and the concentrations which showed the 50% reduction of absorbance by the MTT assay were determined as the IC_{50} of each drug [20, 21]. Daudi cells were exposed to 5 μM VP-16, 5 μM CPT-11, 3 μM CDDP, 1 μM MMC and 0.1 μM DNR; K562 cells were exposed to 5 μM VP-16, 0.5 μM CPT-11, 30 μM CDDP, 5 μM MMC and 1 μM DNR; and U937 cells were exposed to 0.5 μM

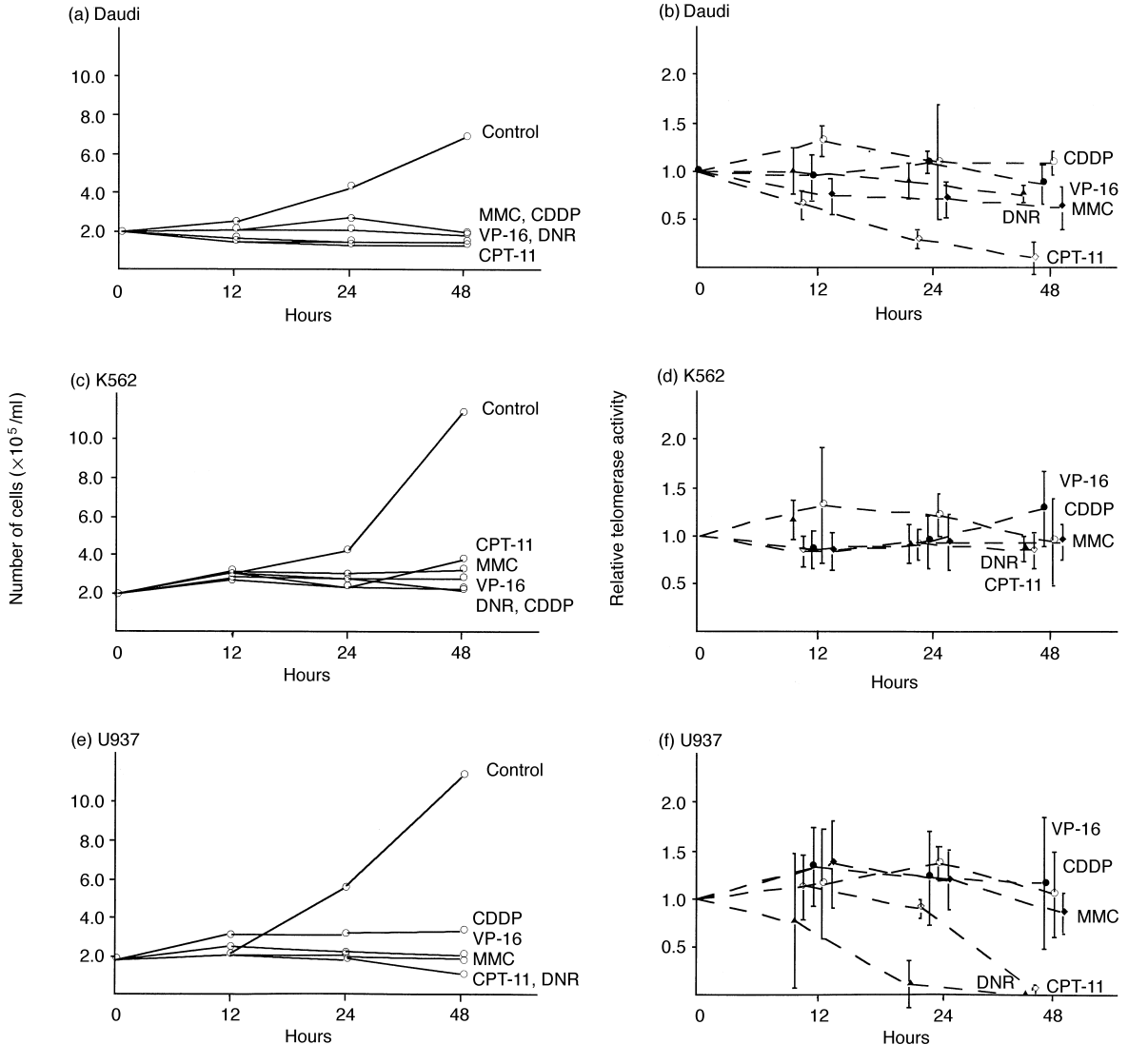

Figure 1. Growth curves and telomerase activity. (a,c,e) Exponentially growing cells were treated with each drug at the IC_{50} concentration as described in Materials and Methods. The number of cells was counted under a microscope using a haemocytometer. All treated cells showed growth inhibition. (b,d,f) Telomerase activity was measured as explained in the text. Relative telomerase activity was calculated by comparison with the initial level. Experiments were performed three times, and the mean and standard deviation (S.D.) are shown.

VP-16, 0.05 μM CPT-11, 3 μM CDDP, 1 μM MMC and 0.2 μM DNR. Some drugs were also applied at the IC_{90} concentration to study the cytocidal effect on telomerase activity.

Analysis of cell growth and viability

Exponentially proliferating cells were treated continuously with each agent and the number of cells was counted using a haematocytometer and a phase-contrast microscope at 0, 12, 24 and 48 h without staining. To evaluate viability, harvested cells were double stained for 10 min with 10 μM Hoechst 33342 (Sigma) and 10 μM propidium iodide (PI: Sigma). Stained cells were excited by exposure to 360 nm UV, and at least 500 cells were counted under a fluorescence microscope (Zeiss, Oberkochen, Germany) [19]. With this method, the morphological changes of cell nuclei related to apoptosis could be studied by Hoechst 33342 staining, while damage to the plasma membrane indicating cell death was observed by PI staining. Additionally, flow cytometric analysis (FACS Calibur: Becton Dickinson, San Jose, California, U.S.A.) was performed to distinguish apoptotic cells from necrotic cells using Annexin V (Boehringer Mannheim, Mannheim, Germany) and PI [22]. By combining the results of these double stainings, the cells were classified into three categories: intact, apoptosis without plasma membrane damage, and plasma membrane damage with or without apoptotic nuclear fragmentation.

Telomerase assay

The telomerase assay was performed using a $TRAP_{EZE}$® Telomerase Detection Kit (Oncor, Gaithersburg, Maryland, U.S.A.) [3, 23, 24]. The samples were treated with 1×CHAPS (3-[3-cholamidopropyl dimethylammonio]-1-propane-sulphonate) lysis buffer 10^6 cells/100 μl; 10 mM Tris–HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM ethylene glycol tetraacetic acid (EGTA),

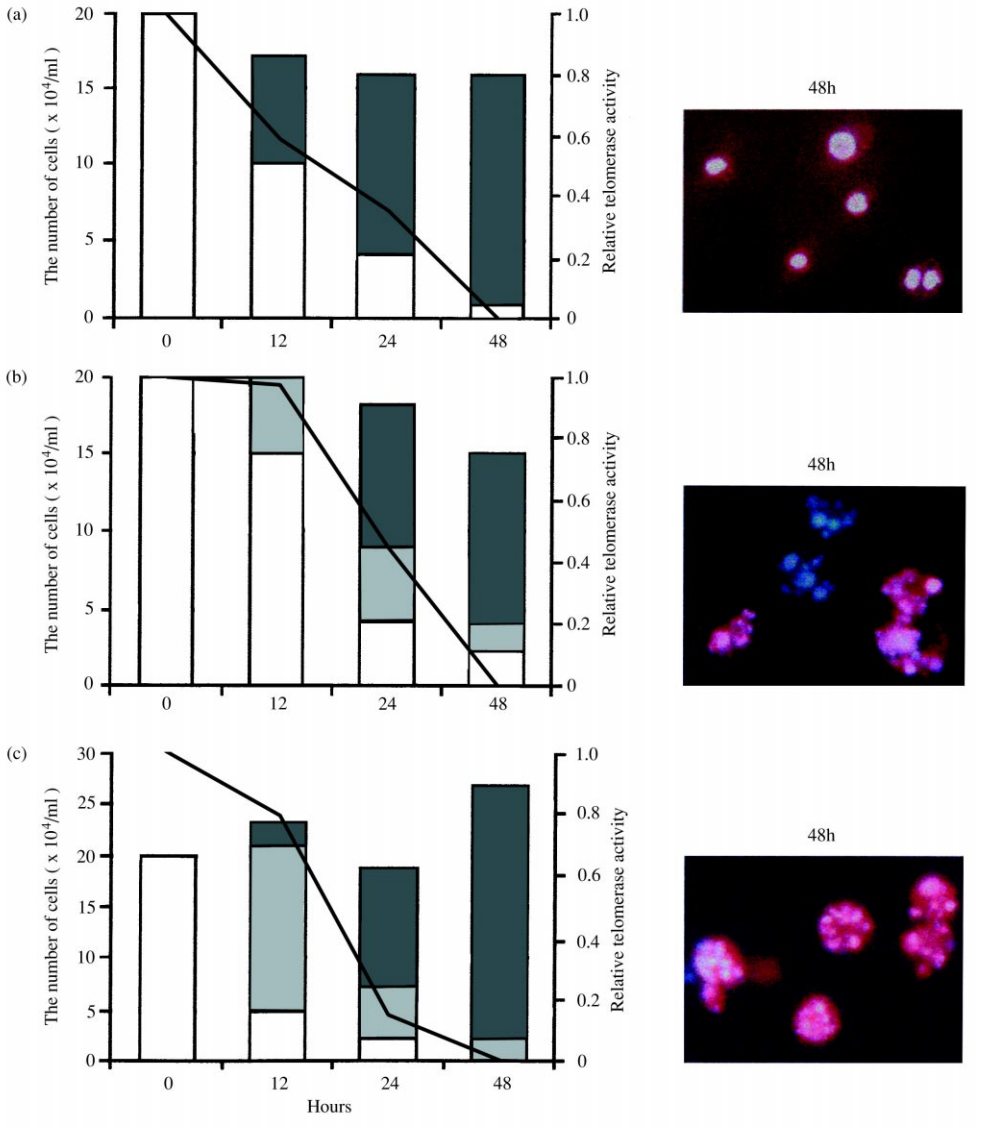

Figure 2. Analysis of telomerase activity and cell viability by Hoechst 33342 and propidium iodide (PI) double staining. The cells were sequentially harvested at the indicated times, stained with Hoechst 33342 and PI, and photographed under a fluorescent microscope. Cell viability was classified into the three categories explained in the text (□, intact cells; ▩, apoptotic cells without PI staining; ■, PI-stained cells). The bold line shows relative telomerase activity. (a) 5 μM irinotecan (CPT-11)-treated Daudi cells; (b) 0.2 μM daunorubicin (DNR)-treated U937 cells; (c) 0.05 μM CPT-11-treated U937 cells.

0.1 mM benzamidine, 5 mM β-mercaptoethanol, 0.5% CHAPS, 10% glycerol), incubated on ice for 30 min and centrifuged at 12 000 ***g*** for 20 min at 4°C. Then the supernatant was transferred into a fresh tube. Cell extracts were assayed in a 50 µl reaction mixture containing 10×TRAP (telomeric repeat amplification protocol) buffer (0.2 mM Tris–HCl (pH 7.3), 15 mM $MgCl_2$, 630 mM KCl, 0.5% Tween 20, 10 mM EGTA, 0.1% bovine serum albumin (BSA)), 2.5 mM of each deoxynucleoside triphosphate, 0.1 µg of TS (telomerase substrate) primer, 0.1 µg of Primer Mix, 2 units of *Taq* DNA polymerase and 0.5 µl of CHAPS cell extract. For a more sensitive and semiquantitative telomerase assay, Daudi and K562 cell extracts were diluted 1:20, so that 0.5 µl contained 250 cells, while the U937 cell extract was not diluted, and 0.5 µl was equivalent to 5000 cells. After 10 min of incubation at 30°C, polymerase chain reaction (PCR) amplification was performed with 30 cycles of 94°C for 30 sec and 60°C for 30 sec. The PCR products were analysed by electrophoresis on 12% polyacrylamide nondenaturating gels and stained with SYBR® Green I (Molecular Probes, Eugene, Oregon, U.S.A.). The gels were photographed using a CCD camera (Fuji Film, Tokyo, Japan) and a UV transilluminator (Ultra-Lum, California, U.S.A.). Telomerase ladders were analysed using NIH image software. The telomerase activity of each cell line was measured by taking the ratio of the entire telomerase ladder to that of the internal control [24]. The relative telomerase activity was obtained by comparison with that of the control (0 h). The direct effect of the anticancer agents on telomerase activity was analysed by adding the drugs directly to the telomerase reaction mixture, and the samples were incubated for 10 min at 30°C and then processed for the telomerase assay.

RNA isolation and reverse transcription–PCR (RT–PCR)

Total RNA was isolated using an ISOGEN RNA extraction kit (Wako, Tokyo, Japan). The first strand cDNA was synthesised using M-MLV reverse transcriptase (GIBCO BRL), as described previously [25]. Briefly, 6 µg of total RNA was transcribed using a random hexamer primer (Pharmacia Biotech, Uppsala, Sweden) in a reaction mixture with a total volume of 30 µl. PCR was performed using 1.5 µl of first strand cDNA and a thermal cycler (Gene Amp PCR system 2400-R: Perkin Elmer, Norwalk, Connecticut, U.S.A.). The reaction sequence was 94°C for 30 sec, 60°C for 30 sec and 72°C for 60 sec (25 cycles for hTERT, hTERC and TEP-1 and 21 cycles for β-actin). The amplified products were separated on 2% agarose gel. The gels were stained with ethidium bromide and photographed using a UV transilluminator and a CCD camera (Toyobo, Tokyo, Japan). The primer sets were as follows: for hTERT [26, 27], 5′-TGAACTTGCGGAAGACAGTGG-3′ (sense) and 5′-ATGCGTGAAACCTGTACGCCT-3′ (antisense); for hTERC [5], 5′-TTTGTCTAACCCTAACTGAGAAG-3′ (sense) and 5′-TTGCTCTAGAATGAACGGTGGA-3′ (antisense); for TEP-1 [27], 5′-TCAAGCCAAACCTGAATCTGAG-3′ (sense) and 5′-CCCCGAGTGAATCTTTCTACGC-3′ (antisense); for β-actin [25], 5′-GTGGGGCGCCCCAGGCACCA-3′ (sense) and 5′-CTCCTTAATGTCACGCACGATTTC-3′ (antisense).

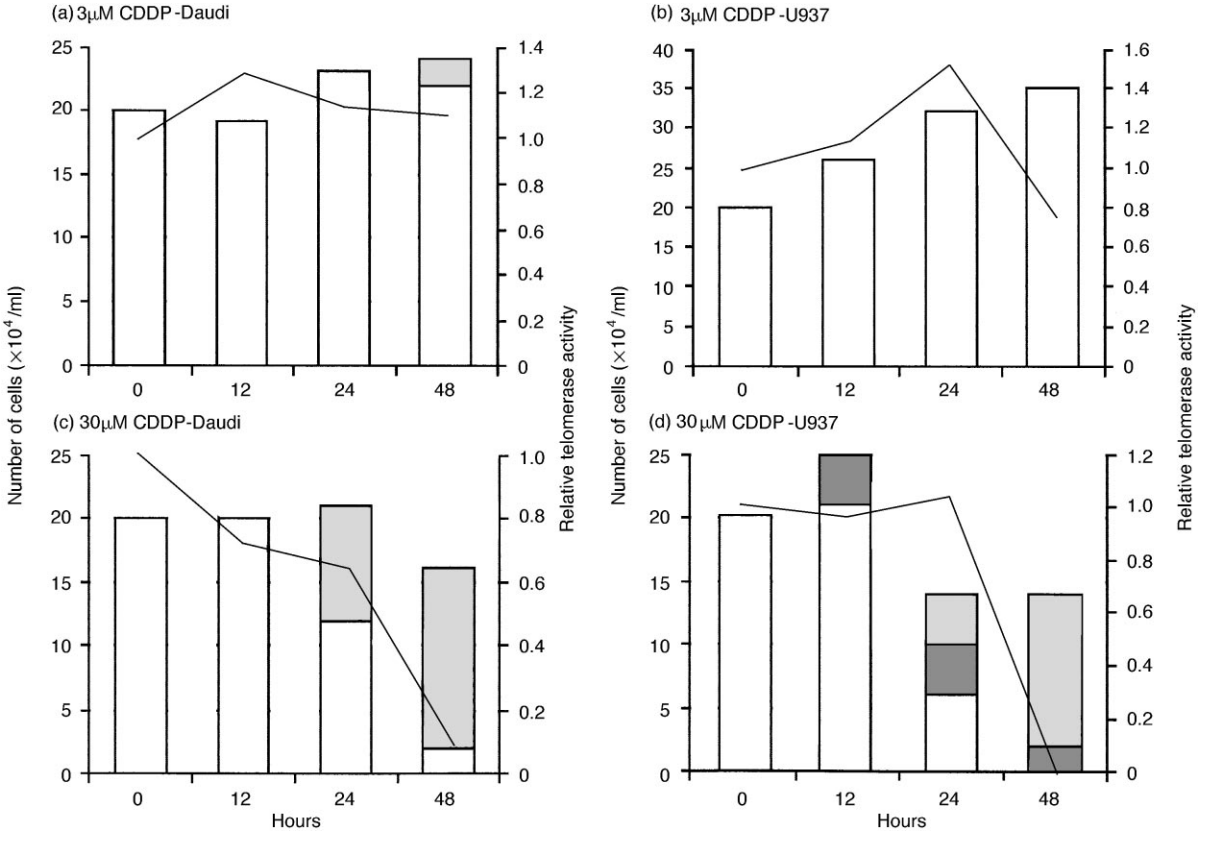

Figure 3. Concentration-dependent effects on telomorase activity and cell viability. Daudi and U937 cells were treated with 3 µM and 30 µM cisplatin (CDDP). The cell viability categories are the same as in Figure 2. (a) 3 µM CDDP-treated Daudi cells; (b) 3 µM CDDP-treated U937 cells; (c) 30 µM CDDP-treated Daudi cells; (d) 30 µM CDDP-treated U937 cells. Light grey shading: PI stained cells; dark grey shading: apoptotic cells without PI staining.

The RT–PCR products were confirmed at least once by DNA sequencing.

RESULTS

Effect of anticancer agents on telomerase activity

Each cell line was treated with the drug concentrations (IC_{50}) calculated by the MTT assay (data not shown) as mentioned in Materials and Methods, and the number of cells was counted under a phase-contrast microscope without any staining. Treated cells did not proliferate and remained at the initial level, except for a slight decrease of U937 cells when treated with CPT-11 and DNR (Figure 1e). Telomerase activity was studied using whole cell extracts, corresponding to 250 Daudi cells (Figure 1b), 250 K562 cells (Figure 1d) and 5000 U937 cells (Figure 1f). Most treated cells still possessed equivalent telomerase activity to that before treatment, demonstrating that cytostatic growth inhibition was not associated with a reduction of telomerase activity. Only CPT-11-treated Daudi cells, CPT-11-treated U937 cells and DNR-treated U937 cells showed a decrease of telomerase activity from 24 to 48 h (Figure 1b,f). Because cell numbers were counted under a phase-contrast microscope without staining, we studied the relationship between cell viability and telomerase activity. Cells which showed a decrease of telomerase activity were stained with both Hoechst 33342 and PI. The reduction of telomerase activity with treatment showed an inverse correlation with the increase of PI-stained cells. At 48 h, most cells were stained by PI, demonstrating that disruption of the plasma membrane had occurred (Figure 2), although the nuclei of CPT-11-treated Daudi cells were still oval (Figure 2a), while those of both DNR- and CPT-11-treated U937 cells were fragmented or condensed (Figures 2b,c). To study further the relationship between drug concentration and telomerase activity, Daudi cells and U937 cells were exposed to 3 μM or 30 μM of CDDP, corresponding to the IC_{50} and IC_{90}, respectively. We found a significant reduction of telomerase activity in both Daudi and U937 cells treated with 30 μM CDDP in accordance with the loss of cell viability (Figure 3). Moreover, most of the 30 μM CDDP-treated cells were stained with PI. When each cell extract prepared from untreated cells was co-incubated with 30 μM CDDP, no reduction of telomerase activity was observed (data not shown). These results demonstrate that the decline of telomerase activity induced by anticancer agents was clearly related to a decrease of cell viability, characterised by disrupted plasma membrane.

Relationship between cell death, telomerase activity and hTERT, hTERC and TEP-1 expression

As mentioned above, the decline of telomerase activity was closely related to cell death, so we studied the changes of telomerase activity and hTERC, hTERT and TEP-1 expression during the course of drug-induced cell death. When U937 cells were treated with 5 μM VP-16 (IC_{90}), there were three distinct stages of cell viability (Figure 4). Cells were initially morphologically normal, but apoptotic cells (cells with either chromatin condensation or fragmented nuclei that were only stained by Hoechst 33342) appeared from 2 h

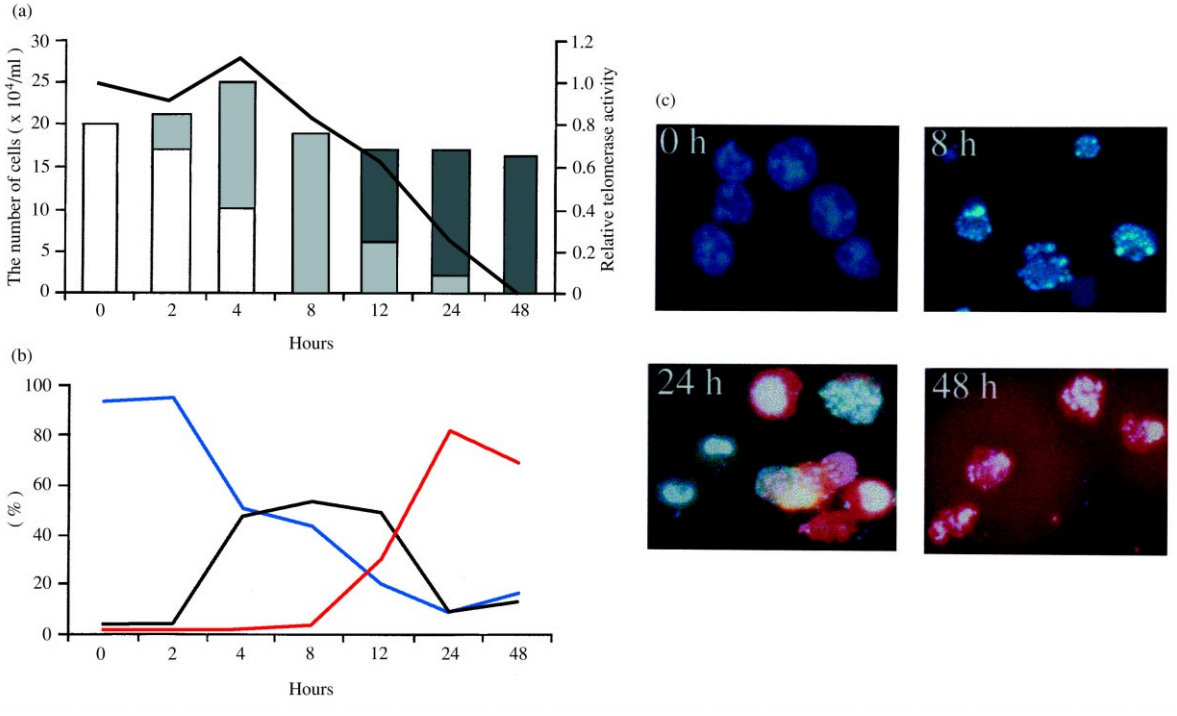

Figure 4. Correlation between cell death and telomerase activity. U937 cells were treated with 5 μM etoposide (VP-16) (IC_{50}), and harvested sequentially at the indicated times. (a) Relationship between cell viability and telomerase activity. The cell viability categories are the same as in Figure 2. (b) Flow cytometric analysis of cell viability by staining with Annexin V and propidium iodide (PI). Cell viability was classified into three categories (blue line, intact cells; black line, apoptotic cells without PI staining; red line, PI-stained cells). Cells at 48 h were damaged too much, and were not suitable for this analysis. All cells were morphologically confirmed to be stained with PI. (c) Morphological changes of the treated cells. Cells were stained with Hoechst 33342 and PI, and were photographed under a fluorescent microscope. Cells harvested at 8 h demonstrated the condensation of chromatin, but the cytoplasm was not stained by PI. At 24 h, more than half of the cells were stained by PI, and all cells were PI positive at 48 h.

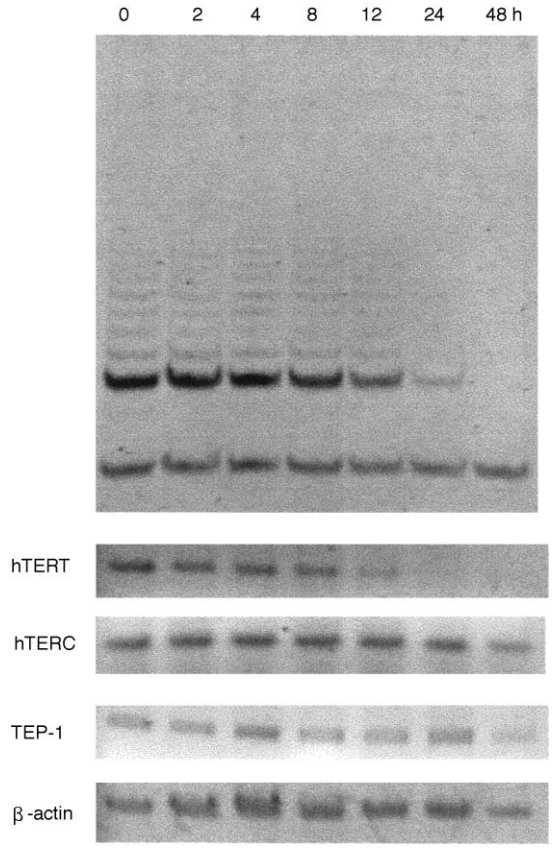

Figure 5. Sequential changes of telomerase activity and hTERT, hTERC and TEP-1 mRNA expression shown by reverse transcription–polymerase chain reaction (RT–PCR). Telomerase activity decreased markedly from 12 to 24 h, and no activity was observed at 48 h. The expression of hTERT mRNA decreased from 8 h and was not detected later than 24 h, while expression of hTERC and TEP-1 mRNA was not affected.

onwards. This apoptotic stage was maintained until 12 h, when PI-stained cells first appeared, and the number of PI-stained cells increased steeply from 12 to 24 h (Figure 4). These morphological changes were also confirmed by the flow cytometric analysis using Annexin V and PI (Figure 4b). At 8 h, nearly 60% of cells were Annexin V positive, while no PI-stained cells were observed. The increase of PI-stained cells analysed by both morphology and flow cytometry was well co-related until 24 h. Telomerase activity and hTERC, hTERT and TEP-1 mRNA were studied in these three different stages. No decline of telomerase activity and hTERT mRNA was observed until 8 h, when most of the treated cells showed signs of apoptosis. Interestingly, telomerase activity and the level of hTERT expression were inversely correlated with the increase of PI-stained cells, whereas no change of hTERC and TEP-1 mRNAs was observed (Figure 5). These results demonstrate that telomerase activity did not decrease in the initial stage of apoptosis.

DISCUSSION

More than 85% of all human cancers express telomerase activity, whereas most normal somatic cells have little or none [28]. Mice lacking telomerase activity develop apoptosis in the testis after several generations [29]. If there was a drug that interfered specifically with telomerase, it would provide a promising treatment strategy. Because rapid replication results in critical shortening of telomeres, tumour cells should undergo apoptosis faster than normal cells after the inhibition of telomerase. In the present study, we investigated whether common anticancer drugs (at the IC_{50} concentration) could directly modulate telomerase activity in human tumour cell lines. The IC_{50} was used because it is easily achieved clinically when cancer patients are treated by conventional therapy. Our data demonstrated that most drugs did not inhibit telomerase activity directly, even if treated cells showed cytostatic growth inhibition. Faraoni and associates described a decrease of telomerase activity in parallel with cell growth impairment [30]. This discrepancy with our results may have been related to the difference of experimental strategies. In our study, the telomerase activity of treated cells was measured using a constant number of treated cells, while Faraoni and associates used various numbers of treated cells. Therefore, the telomerase activity reflected the number of cells reduced by treatment.

We also focused on the relationship between the stage of cell death and telomerase activity. As demonstrated in Figures 2 and 3, cell viability was clearly related to telomerase activity. In U937 cells, the initial stage of apoptosis was not associated with a reduction of telomerase activity, but the later stage was accompanied by reduced activity. Because telomerase is a stable protein complex with a half-life of more than 24 h in the presence of cycloheximide [9, 31], it still remains active in the early stage of apoptosis. Reduced telomerase activity was also associated with the downregulation of hTERT, whereas hTERC and TEP-1 expression remained constant. These results are consistent with those of Nakamura and colleagues [27], who demonstrated the importance of hTERT expression in telomerase regulation.

In conclusion, the anticancer agents examined in this study did not target telomerase directly when tested at cytostatic concentrations, but the detection of impaired telomerase activity after drug treatment always accompanied severe cell damage. This supports that the telomerase assay could be a new drug sensitivity testing method as proposed by Faraoni and associates [30]. In addition, investigating the regulation of hTERT expression should help to increase our knowledge about cancer.

1. Blackburn EH. Structure and function of telomeres. *Nature* 1991, **266**, 569–573.
2. Harley CB, Kim NW, Prowse KR, *et al.* Telomerase, cell immortality, and cancer. *Cold Spring Harbor Symp Quant Biol* 1994, **59**, 307–315.
3. Kim NW, ilatyszek MA, Prowse KR, *et al.* Specific association of human telomerase activity with immortal cells and cancer. *Science* 1994, **266**, 2011–2015.
4. Counter CM, Avilion AA, LeFeuvre CE, *et al.* Telomere shortening associated with chromosome instability in immortal cells which express telomerase activity. *EMBO J* 1992, **11**, 1921–1929.
5. Feng J, Funk WD, Wang S, *et al.* The RNA component of human telomerase. *Science* 1995, **269**, 1236–1241.
6. Nakayama J, Saito M, Nakamura H, Matsuura A, Ishikawa F. TLP1: a gene encoding a protein component of mammalian telomerase is a novel member of WD repeats family. *Cell* 1997, **88**, 875–884.
7. Harrington L, Mcphail T, Mar V, *et al.* A mammalian telomerase-associated protein. *Science* 1997, **275**, 973–977.
8. Broccoli D, Young JW, De Lange T. Telomerase activity in normal and malignant hematopoietic cells. *Proc Natl Acad Sci USA* 1995, **92**, 9082–9086.

9. Holt SE, Wright WE, Shay JW. Regulation of telomerase activity in immortal cell lines. *Mol Cell Biol* 1996, **16**, 2932–2939.
10. Hiyama E, Hiyama K, Tatsumoto N, Shay JW, Yokoyama T. Telomerase activity in human intestine. *Int J Oncol* 1996, **9**, 453–458.
11. Taylor RS, Ramirez RD, Ogoshi M, Chaffins M, Piatyszek MA, Shay JW. Telomerase activity in malignant and nonmalignant skin conditions. *J Invest Dermatol* 1996, **106**, 759–765.
12. Hiyama K, Hirai Y, Kyoizumi S, *et al*. Activation of telomerase in human lymphocytes and hematopoietic progenitor cells. *J Immunol* 1995, **155**, 3711–3715.
13. Rhyu MS. Telomeres, telomerase, and immortality. *J Natl Cancer Inst* 1995, **87**, 884–894.
14. Autexier C, Greider CW. Telomerase and cancer: revisiting the telomere hypothesis. *TIBS* 1996, **21**, 387–391.
15. Holt SE, Shay JW, Wright WE. Refining the telomere–telomerase hypothesis of aging and cancer. *Nature Biotec* 1996, **14**, 836–839.
16. Norton JC, Piatyszek MA, Wright WE, Shay JW, Corey DR. Inhibition of human telomerase activity by peptide nucleic acids. *Nature Biotec* 1996, **14**, 615–618.
17. Shay JW, Werbin H, Wright WE. Telomeres and telomerase in human leukemias. *Leukemia* 1996, **10**, 1255–1261.
18. Counter CM, Gupta J, Harley CB, Leber B, Bacchetti S. Telomerase activity in normal leukocytes and in hematologic malignancies. *Blood* 1995, **85**, 2315–2320.
19. Shimizu S, Eguchi Y, Kamiike W, *et al*. Retardation of chemical hypoxia-induced necrotic cell death by Bcl-2 and ICE inhibitors: possible involvement of common mediators in apoptotic and necrotic signal transductions. *Oncogene* 1996, **12**, 2045–2050.
20. James C, William GD, Adi FG, John DM, James BM. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res* 1987, **47**, 936–942.
21. James C, William GD, Adi FG, John DM, James BM. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of radiosensitivity. *Cancer Res* 1987, **47**, 943–946.
22. Koopman G, Reutelingsperger CPM, Kuijten GAM, Keehnen RMJ, Pals ST, van Oers MHJ. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood* 1994, **84**, 1415–1420.
23. Piatyszek MA, Kim NW, Weinrich SL, *et al*. Detection of telomerase activity in human cells and tumors by a telomeric repeat amplification protocol (TRAP). *Meth Cell Sci* 1995, **17**, 1–15.
24. Holt SE, Norton JC, Wright WE, Shay JW. Comparison of the telomeric repeat amplification protocol (TRAP) to the new TRAP-eze telomerase detection kit. *Meth Cell Sci* 1996, **18**, 237–248.
25. Yamada H, Ochi K, Nakada S, *et al*. Interferon modulates the messenger RNA of G1-controlling genes to suppress the G1-to-S transition in Daudi cells. *Mol Cell Biochem* 1995, **152**, 149–158.
26. Nakamura TM, Morin GB, Chapman KB, *et al*. Telomerase catalytic subunit homologs from fission yeast and human. *Science* 1997, **227**, 955–959.
27. Nakamura J, Tahara H, Tahara E, *et al*. Telomerase activation by hTRT in human normal fibroblast and hepatocellular carcinomas. *Nature Genet* 1998, **18**, 65–68.
28. Ohyashiki JH, Ohyashiki K, Iwama H, Hayashi S, Toyama K, Shay JW. Clinical implications of telomerase activity levels in acute leukemia. *Clin Cancer Res* 1997, **3**, 619–625.
29. Lee H, Blasco MA, Gottlieb GJ, Horner II JW, Greider CW, DePinho RA. Essential role of mouse telomerase in highly proliferative organs. *Nature* 1998, **392**, 569–574.
30. Faraoni I, Turriziani M, Masci G, *et al*. Decline in telomerase activity as a measure of tumor cell killing by antineoplastic agents in vitro. *Clin Cancer Res* 1997, **3**, 579–585.
31. Holt SE, Aisner DL, Shay JW, Wright WE. Lack of cell cycle regulation of telomerase activity in human cells. *Proc Natl Acad Sci USA* 1997, **94**, 10687–10692.

Acknowledgements—We thank Professor Kihei Maekawa (Jikei University School of Medicine) for helpful advice and critical reading of the manuscript. This work was supported in part by the special Coordination Fund for Research for the Future program from the Science and Technology Agency and by the grant in aid of High-Tech Research Center (Institute of DNA Medicine) from the Ministry of Education of Japan.