Decline in Telomerase Activity as a Measure of Tumor Cell Killing by Antineoplastic Agents in Vitro

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ABSTRACT

Telomerase activity is frequently associated with the malignant phenotype, and it can be considered an almost ubiquitous tumor marker. In this study, we evaluated telomerase activity in telomerase-positive human tumor cell lines exposed in vitro to antineoplastic agents. The results show that drug-induced cell killing of tumor cells is associated with a decline in detectable telomerase activity. The decrease of telomerase activity levels paralleled cell growth impairment evaluated by cell count or by measurement of cell ability to convert tetrazolium salt to colored formazan [3-(4,5-dimethylthiazol-2-yl)]2,5-diphenyl-tetrazolium bromide assay]. The observed telomerase activity remaining after treatment with antineoplastic agents is most likely to reflect activity from the remaining viable cells. When tumor cell lines resistant to the chemotherapeutic agents temozolomide or doxorubicin were treated with these compounds, no decline of telomerase activity or cell growth was observed. The results of the present study indicate that resistance of neoplastic cells to chemotherapeutic agents can be monitored by following telomerase activity. Moreover, the test can be performed with a limited number of neoplastic cells, such as those frequently obtained from tumor biopsies. These findings provide a rationale for developing new in vitro chemosensitivity assays, and detection of telomerase activity may be a novel marker of chemotherapy failure.

INTRODUCTION

One of the most challenging problems in cancer chemotherapy is to assess the clinical efficacy of antineoplastic drugs, especially in the case of poorly responsive tumors. Various in vitro chemosensitivity tests have been proposed for predicting tumor cell response to anticancer agents (1). However, technical difficulties and the limited number of prospective randomized studies do not always permit the establishment of a clear correlation between the results of in vitro assays and clinical responses to cancer chemotherapy.

In contrast to methods that rely on tumor cell culture (e.g., clonogenic assays), a chemosensitivity test, based on the analysis of the killing of total but not selected neoplastic cells, would be a more appropriate tool for predicting the individual therapeutic value of a drug. Such a test would require sensitivity and the use of an almost ubiquitous tumor marker to distinguish between normal and neoplastic cells.

The results of a number of recent studies indicate that expression of telomerase activity is strongly correlated with cell immortalization and the malignant phenotype (2). Telomerase is a ribonucleoprotein DNA polymerase that adds hexameric repetitive sequences (TTAGGG) to the ends of the chromosomes in continuously dividing cells (3). This enzyme helps ensure chromosomal stability and prevents cells from aging (4). In the absence of telomerase activity, telomeric sequences progressively shorten during cell divisions, until cells stop proliferating (5). Telomerase activity is minimal or absent in terminally differentiated cells of most organs, with the exception of stem cells of regenerating tissues such as blood, skin, and intestine (6–10). However, telomerase activity is expressed in more than 85% of tumor tissues, including primary and metastatic tumors of more than 12 different types (reviewed in Ref. 2).

A highly sensitive method based on PCR, the TRAP assay, has been developed for measuring telomerase activity in a limited number of cells (11, 12). Recent modifications of the assay have provided significant improvement in linearity and sensitivity, thus allowing detection of telomerase activity in as few as 1–10 neoplastic cells in heterogeneous differentiated cell populations. Thus far, this method has been largely used for the screening of tumor biopsies to evaluate telomerase activity in the diagnosis of neoplasia (reviewed in Ref. 2) and in some instances as a prognostic indicator of outcome (13–15).

In the present study, we have investigated the possibility of detecting tumor cell resistance to antitumor agents, using telomerase activity as a specific marker of neoplastic cell survival.

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1 This work was supported in part by a grant from the Italian Association for Cancer Research (AIRC) and in part by Applicazioni Cliniche delle Ricerche Oncologiche contract of the National Council of Research (U.O. Applicazioni Cliniche; Rome, Italy). The abbreviations used are: TRAP, telomerase repeat amplification protocol; CM, complete medium; DOXO, doxorubicin; CDDP, cis-diaminedichloroplatinum(II); TEMO, temozolomide; MTX, 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide; OGAT, O6-alkylguanine DNA alkyltransferase.
after drug treatment in vitro. Our preliminary results indicate that telomerase may have clinical utility in this respect.

**MATERIALS AND METHODS**

**Cells and in Vitro Cultures.** The cell lines used were the human T-cell leukemia Jurkat (ATCC CRL 8136), the histiocytic U937 (ATCC CRL 1593), the breast adenocarcinoma MCF-7 (ATCC HTB 22), and its subtype resistant to doxorubicin MCF-7/DOXO (16). These cell lines were maintained in RPMI 1640 (Life Technologies, Inc., Paisley, United Kingdom) supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine, and 100 IU penicillin and streptomycin (Flow Laboratories, McLean, VA; hereafter called CM). Normal endometrial cells were obtained from healthy donors in the course of routine examination.

**Drug Treatment in Vitro.** Jurkat and U937 cells (5 × 10^5 cells/ml) were incubated in vitro in 6-well plates in CM with the antitumor drugs at 37°C in a 5% CO₂ atmosphere for 5 h. In selected experiments, before drug treatment, Jurkat cells were mixed with normal endometrial cells at the ratio of neoplastic to normal cells of 1:9. The cells were then washed, resuspended in CM, and seeded at the concentration of 2 × 10⁵ cells/ml in three different plates to study cell viability (by cell count), cell proliferation (by MTT assay), and telomerase activity. In the case of MCF-7 and MCF-7/DOXO, the drug was not removed from the culture medium. Cell count, MTT assay, and telomerase activity assays (see below) were performed on days 1, 2, and 3 of culture. During the entire period of culture, untreated cells were in the exponential phase of growth. The antitumor agents used were: CDDP (Platinex; Bristol, Sermoneta, Latina, Italy), DOXO (Adriamycin; Farmitalia, Milan, Italy), and TEMO (Ref. 17; kindly supplied by Schering-Plough Research Institute, Kenilworth, NJ).

**Determination of Cell Proliferation and Cell Viability.** Cell proliferation was evaluated by the tetrazolium salt method (MTT assay) as described previously (18). This method allows an indirect measurement of cell growth/cell kill and is based on the ability of viable cells to convert tetrazolium salt to colored formazan. Briefly, Jurkat cell suspensions (2 × 10⁵ cells/ml), untreated or exposed to the drugs, were seeded in 96-well plates (eight wells for each group, 0.1 ml/well). MTT (Sigma Chemical Co., St. Louis, MO; 5 mg/ml, 20 μl/well) was added to each well, and plates were then incubated at 37°C for 5 h. The cells were then lysed using a buffer containing 20% w/v of SDS (Sigma) and 50% N,N-dimethyl formamide (Sigma), pH 4.7 (0.1 ml/well). After overnight incubation at room temperature, absorbance was read at 540 nm using a 96-well ELISA multiscaner (Labsystems Oy, Multiscan Bichromatic, Helsinki, Finland).

Viability counts (i.e., cells excluding trypan blue) were performed using a hemocytometer at 24, 48, and 72 h after drug treatment. All determinations were performed in quadruplicate.

**Telomerase Activity Assay.** Two hundred μl of CM containing 4 × 10⁴ Jurkat cells, untreated or exposed to the drugs, were added to each well of a 96-well plate. After 24, 48, or 72 h of incubation, cells were harvested and processed for TRAP assay as described previously (19). This method is based on PCR amplification of telomerase extension products. Briefly, cells were lysed in 400 μl of ice-cold extraction buffer containing 0.5% 3-[3-cholamidopropyl]-dimethyl-ammonio]-1-propanesulfonate, 10 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 1 mM EGTA, 5 mM β-mercaptoethanol, 0.1 mM [4-(2-aminoethyl)benzene-sulfonyl fluoride hydrochlorine], and 10% glycerol. Four μl of the cell extract were used for TRAP assay in 50 μl of reaction mixture [20 mM Tris-HCl (pH 8.3), 68 mM KCl, 1.5 mM MgCl₂, 1 mM EGTA, 0.05% Tween 20, 0.1 μg of TS [5’-AATCGGTCAAGCTTATG] primer, 0.5 μM T4 gene 32 protein, 50 μM of each deoxynucleotide triphosphate, 2 units of Taq DNA polymerase, and 2 μCi of [α-32P]dCTP (3000 Ci/mmol; Dupont NEN Research Products, Boston, MA)]. Each reaction was carried out in a single PCR tube containing 100 ng of CX oligonucleotide 5’-[CCCTTA]₅CCCTAA sealed at the bottom of the tube by a wax barrier. Samples were incubated at room temperature for 30 min to allow telomerase to extend TS primer, followed by a 31-cycle PCR amplification (Perkin-Elmer Corp., Norwalk, CT) of the telomeric products. Reagents for TRAP assay were purchased from Boehringer Mannheim (Indianapolis, IN), and oligonucleotide primers were from Bio-gen (Rome, Italy). Analysis of 40 μl of the PCR products was performed on 10% nondenaturating acrylamide gels. Gels were fixed in 0.5 m NaCl, 50% ethanol, and 40 mM sodium acetate (pH 4.2) and then exposed to X-ray film (Kodak, Rochester, NY) at −80°C.

The in vitro direct effect of the antineoplastic agents on telomerase activity was analyzed by adding the drugs directly to the telomerase reaction mixture, before primer addition. Samples were incubated for 1 h at 37°C and processed for the TRAP assay.

A bidimensional densitometry of the six base repetitive ladder bands was performed using a Bio-Rad (Richmond, CA) scanning apparatus (Image densitometer, GS-670; Molecular Analyst software). The signal intensity of each band was measured and corrected for the background. The adjusted signals, expressed in arbitrary units, of the ladder products in each lane were summed and used for quantitative analysis.

**Soft Agar Colony Inhibition Assay.** The colony inhibition test was performed in semisolid agar as described previously (20). Briefly, after drug treatment, cells were suspended in CM containing 0.3% Bacto-agar (Difco Laboratories, Detroit, MI) and seeded (5 × 10⁴/plate, 100-mm plates) in triplicate. The plates were incubated at 37°C in a 5% CO₂ atmosphere. After 12 days in culture, colonies were counted at the inverted microscope, and those containing more than 50 cells were scored.

**RESULTS**

**Limits of Telomerase Activity Detection in Jurkat Cells.** Serial titration of a Jurkat cell extract, corresponding to graded numbers of cells, was performed to define the essential parameters of the assay (i.e., sensitivity and semiquantitative relationship between cell number and ladder band intensity) using our experimental conditions. Fig. 1A shows the results of a representative experiment. Less than 50% reduction of tumor cells, in the range of 1000–31 cells, was easily detected by a substantial decrease of telomerase-produced ladder band intensity after overnight exposure of the gel. Longer exposure of the same gel showed a telomeric ladder in cell extracts corresponding to 15 cells (data not shown). Densitometric analysis (Fig. 1B) of the
ladder indicates a direct correlation between the log of cell number and telomerase activity in the range of 15 to 1000 cells and that as low as 15 cells can be detected. When larger numbers of cells were used, the signal intensity of ladder bands did not increase in a linear fashion (data not shown); thus, all of the other TRAP assays were performed using less than 1000 cells. The specificity of assay is confirmed by demonstrating that preincubation of the cell extracts with RNase abolishes the ladder bands (Fig. 1A).

Analysis of Drug Chemosensitivity of Tumor Cells: Telomerase Activity, MTT Assay, and Cell Count. Jurkat cells were exposed to 30 μM CDDP or 2 μM DOXO for 5 h and then processed for telomerase activity, MTT assay, and cell count as described in “Materials and Methods.” The drug concentrations used correspond to the concentrations capable of inhibiting tumor cell growth by 50% (IC50), evaluated by MTT assay at 24 h. The results of TRAP assay (Fig. 2A) indicate that both drugs induced a marked decline of telomerase activity as early as 24 h after treatment (CDDP, 73% inhibition; DOXO, 63% inhibition). The reduction of telomerase activity was not due to the presence of inhibitors of the PCR reaction in cell extracts obtained from drug-treated cells. In fact, mixing of extracts from CDDP or DOXO-treated cells with extracts from untreated Jurkat cells did not affect telomerase activity (data not shown). A good correlation was found between the results of densitometric analysis of telomeric ladder bands, MTT assay, and cell count at all times (Fig. 2B). However, a slightly higher sensitivity of telomerase activity over that of the other parameters was detected for CDDP at 24 h (Fig. 2B). Moreover, CDDP treatment totally abrogated the clonogenic ability of Jurkat cells, as evidenced by colony counts performed on day 12 after cell seeding (data not shown).

Jurkat cells are characterized by a high level of expression of the DNA repair enzyme OGAT, which specifically removes alkyl adducts at the O6 position of guanine (21). Therefore, these cells are resistant to the cytotoxic effects of the methylating drug triazene (TEMO), with IC50 >1000 μM (21). Jurkat cell growth, evaluated by cell count, was essentially not affected at 48 h or at 72 h following treatment with 100 μM TEMO, a concentration similar to the plasma peak that was achieved in Phase I clinical trials (22). Consistent with the cell count results, telomerase activity did not change at 48 or 72 h after exposure to the drug (Fig. 3A).

In contrast, U937 cells, which do not express OGAT activity (21), are highly susceptible to the cytotoxic effects of TEMO (cell numbers decline at 48 and 72 h; 57 and 90%, respectively). Growth impairment by TEMO was paralleled by a marked decline of telomerase activity (41 and 90% inhibition at 48 and 72 h, respectively; Fig. 3A).

Similar results were obtained using a different model system, represented by the breast carcinoma cell line MCF-7 and its subline resistant to DOXO (Fig. 3B). MCF-7 cell growth was markedly inhibited by treatment with 10 μM DOXO (Fig. 3B, Lane 6). This is the drug concentration used to maintain MCF-7/DOXO in tissue culture under continuous selective pressure of the agent. The results of the MTT assay (66 and 84% inhibition at 48 and 72 h, respectively) were similar to those of the cell counts (67 and 97% inhibition at 48 and 72 h, respectively). However, densitometric analysis of ladder bands showed 94 and 98% inhibition of telomerase activity at 48 and 72 h, respectively, after DOXO treatment (Fig. 3B). In this case, telomerase activity evaluation was markedly more sensitive than the other assays at 48 h. The drug-resistant MCF-7/DOXO subline was essentially not affected by treatment with DOXO, and densitometric analysis of ladder bands indicated only a marginal inhibition (i.e., 10%) at 72 h (Fig. 3B).
Telomerase activity was assayed on days 1, 2, and 3 after exposure of Jurkat cells, admixed with normal endometrial cells, to the antitumor agents under study. The results show (Fig. 4A) that: (a) no telomerase activity was detected in the sample corresponding to normal endometrial cells (Lane 2); (b) telomerase activity detected in Jurkat cells mixed with endometrial cells (Lane 7) was comparable to that of the same number of Jurkat cells incubated alone (Lane 15); (c) no substantial difference in telomerase activity was found between control (Lanes 3, 7, and 11) or Temozolomide-treated Jurkat cells (Lanes 4, 8, and 12); and (d) treatment of Jurkat cells with both CDDP (Lanes 5, 9, and 13) and DOXO (Lanes 6, 10, and 14) at IC_{50} concentrations, resulted in a progressive and substantial reduction of telomerase activity compared to that of untreated controls.

When Jurkat cell extracts were exposed to Temozolomide, CDDP, or DOXO in vitro, these drugs did not substantially inhibit telomerase activity (Fig. 4B). Therefore, a direct influence of these agents on telomerase activity does not seem to be involved in the decline of the enzyme activity observed after drug treatment of tumor cells.

**DISCUSSION**

The results of the present study point out that disappearance of telomerase activity could be successfully used as an indicator of tumor cell death induced by exposure to antitumor agents in vitro, thus providing a possible new approach to examine cell chemosensitivity.

The TRAP assay for telomerase activity easily detects as few as 15 tumor cells (Refs. 11 and 12; Fig. 1B). Thus, the experimental setting adopted in the present study provides a reasonable semiquantitative evaluation of a limited number of viable tumor cells. This prompted our evaluation of whether this method could be used to detect resistance of neoplastic cells to anticancer agents in vitro.

Growth inhibition of Jurkat leukemic cells by IC_{50} concentrations of CDDP or DOXO, evaluated by cell count and MTT...
Fig. 3  Effect of antitumor agents on sensitive (U937) or resistant (Jurkat) cell lines assayed for telomerase activity at 48 and 72 h. A, Jurkat or U937 leukemic cells exposed to TEMO (100 μM). Lanes 1 and 2, cell extract treated with RNase or lysis buffer alone (i.e., negative controls); Lanes 3 and 4, untreated or TEMO-treated Jurkat cells at 48 h after drug treatment; Lanes 5 and 6, untreated or TEMO-treated Jurkat cells at 72 h after drug treatment; Lanes 7 and 8, untreated or TEMO-treated U937 cells at 48 h after drug treatment; Lanes 9 and 10, untreated or TEMO-treated U937 cells at 72 h after drug treatment. CTR, control. B, MCF-7 or MCF-7/DOXO breast cancer cells treated with DOXO (10 μM). Lanes 1 and 2, untreated or DOXO-treated MCF-7/DOXO cells at 48 h after drug treatment; Lanes 3 and 4, untreated or DOXO-treated MCF-7/DOXO cells at 72 h after drug treatment; Lanes 5 and 6, untreated or DOXO-treated MCF-7 cells at 48 h after drug treatment; Lanes 7 and 8, untreated or DOXO-treated MCF-7 cells at 72 h after drug treatment; Lanes 9 and 10, cell extract treated with RNase or lysis buffer alone (i.e., negative controls). CTR, control.

assay, was paralleled by a decline of telomerase activity. Tumor cell suppression was close to 50% as early as 24 h after drug treatment, approaching 100% 1 or 2 days later. This is consistent with the results of clonogenic assays showing that treatment with these agents entirely abrogated colony formation in soft agar (data not shown). Therefore, it is reasonable to consider that a severe and irreversible damage was produced in the majority of Jurkat cells following 5 h exposure to these drugs. This cell damage would explain the telomerase decline pattern detected in this study, whereas the telomerase activity remaining after treatment with antineoplastic agents likely reflects activity from the remaining viable cells.

The problem of testing drug resistance by means of telomerase activity evaluation was approached by using two tumor lines, susceptible or resistant to either TEMO (i.e., U937 and Jurkat cells, respectively) or DOXO (i.e., MCF-7 and MCF-7/DOXO, respectively).

The Jurkat line was found to be highly resistant to TEMO (a DNA-methylating triazene compound; Ref. 11) either in terms of the MTT assay, cell count, or telomerase activity evaluation. This is presumably due to the high levels of the DNA repair enzyme OGAT present in Jurkat cells (21).

On the contrary, profound growth inhibition and telomerase activity impairment was detected in U937 cells, at 48 h, and especially at 72 h after exposure to the triazene. These results are consistent with previous observations indicating that U937 cells express very limited OGAT activity and are highly susceptible to DNA-methylating triazines (21). Similar results were obtained with the MCF-7 breast cancer line and its DOXO-resistant MCF-7/DOXO subline (Fig. 3B), which was selected to survive and proliferate in the presence of 10 μM DOXO (16).

Recent data indicate that telomerase activity can be detected throughout the cell cycle in proliferating immortal cells, whereas it is repressed in quiescent and terminally differentiated cells (23). One report indicates that telomerase activity may be slightly modulated during the cell cycle, gradually increasing...
with progression to the S phase and then decreasing, with the lowest levels at G2-M (24). The decline of telomerase activity observed upon treatment of Jurkat cells with CDDP or DOXO at 24 h is unlikely to be attributed to a G2-M arrest induced by both agents (25, 26), because telomerase activity in leukemic cells does not seem to be cell cycle related (23, 24). Nevertheless, in MCF-7, it cannot be excluded that the reduction of telomerase activity detected at 24 h after drug treatment might also be due to the G2-M block induced by these drugs. However, 48 or 72 h after drug treatment, more than 90% of Jurkat or MCF-7 cells were dead, as determined by the results of the MTT assay or viable cell counts, thus indicating that suppression of telomerase activity is indeed the result of cell death.

One of the main difficulties in testing in vitro sensitivity to drugs of patient’s neoplasia concerns the type of tumor cell preparation available to perform the assay. In many cases, fine needle aspirates, which furnish only a limited number of tumor cells admixed with normal cells and debris, are available. As indicated by several investigators, telomerase activity can be used to detect tumor cells in specimens obtained by fine needle biopsy of breast (27) or hepatocellular carcinomas (28, 29). Differently from MTT assay, which detects an enzymatic activity (i.e., cellular dehydrogenases) present in both normal and tumor cells, a chemosensitivity test based on the TRAP assay allows the measure of an enzymatic activity (telomerase) that is rather selective for tumor cells (11, 12). Therefore, it was anticipated that the extent of telomerase activity could have been used as an indicator of tumor cell survival when a mixture of normal + neoplastic cells are exposed to antitumor agents. The results of the present series of experiments provide direct experimental support for this hypothesis. In this case, Jurkat cells were admixed with normal differentiated, telomerase activity-negative endometrial cells before treatment with the antitumor drugs. In this particular situation, chemosensitivity to CDDP or to DOXO and chemoresistance to TEMO were easily detectable at different times after treatment.

It is possible that false-positive results could be obtained using telomerase activity assay if the antitumor agents under study would provide direct, but reversible, inhibitory effects on telomerase activity itself. The results illustrated in the present experiments (Fig. 4B) suggest that TEMO, CDDP, and DOXO did not directly affect telomerase activity in Jurkat cell extracts, at least at the concentrations used in the assay. In fact, when the agents were added directly to the cell extract, no substantial reduction of telomerase activity was detected. This favors the hypothesis that the impairment of telomerase activity observed following drug treatment of the living tumors should be ascribed

**Fig. 4** Telomerase activity in Jurkat cells exposed in vitro to TEMO (100 μM), CDDP (30 μM), and DOXO (2 μM). A, telomerase activity of Jurkat cells admixed with endometrial (telomerase-negative) cells (1:9 cell ratio), untreated (Lanes 3, 7, and 11), or treated with TEMO (Lanes 4, 8, and 12), CDDP (Lanes 5, 9, and 13), or DOXO (Lanes 6, 10, and 14). TRAP assay was performed 24 (Lanes 3–6), 48 (Lanes 7–10), or 72 (Lanes 11–14) h after drug treatment. Lane 1, Jurkat cell extract treated with RNase; Lane 2, extract from endometrial cells; Lane 15, 400 Jurkat cells alone. B, direct effect of TEMO, CDDP, or DOXO on telomerase activity by exposure of Jurkat cell extracts to the antitumor agents for 1 h. Lane 1, untreated control; Lane 2, TEMO (100 μM); Lane 3, CDDP (30 μM); Lane 4, DOXO (2 μM). In addition, MCF-7 cell extract treated with 10 μM DOXO did not show down-regulation of telomerase activity (data not shown).
to irreversible damage of tumor cells, which eventually die, rather than to direct telomerase activity suppression.

In conclusion, the preliminary results illustrated in the present report encourage further studies to develop chemosensitivity assays in vitro, based on the use of telomerase activity evaluation. This approach would provide the unique opportunity to test the selective effect of antitumor drugs on target cancer cells in a mixed cell population, such as that obtained from tumor biopsies in cancer patients. The test is highly sensitive, does not require that tumor cells are clonogenic or actively proliferate in tissue culture, and could allow detection of the effects of drugs on a minimal number of neoplastic cells. In addition, from a theoretical point of view, measurement of telomerase activity levels may be a surrogate end point marker for failure of chemoprevention trials and as an early marker of chemotherapy failure.

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