Telomerase and apoptosis in human hematopoietic cell lines: modulation of the radiation response by pharmacological inhibition of DNA repair enzymes

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Abstract. Telomeres play an important role in genome stability maintenance and have been related to radiation sensitivity. Telomerase is a ribonucleoprotein involved in telomere maintenance and cell survival. DNA-repair enzymes might be selective targets for enhancing radiation sensitivity of tumor cells. We investigated the effect of wortmannin and 3-aminobenzamide (3-AB) on telomerase activity (TA) and apoptosis in two human leukemia cell lines. MOLT-4 (p53-wild type) and KG1a (p53-null) cells were irradiated with γ-rays (3 Gy at 1.57 Gy/min). Cell cultures were treated with 1µM wortmannin, an inhibitor of phosphatidylinositol-3 kinase (PI 3-K) and 10mM 3-AB, a poly(ADP-ribose) polymerase (PARP) inhibitor. TA was measured by PCR and the expression of hTERT, hTR and TP1 was assessed by RT-PCR. Apoptosis was evaluated by fluorescence microscopy and flow cytometry, which also allowed to analyze cell cycle distribution.. A radiation-induced up-regulation of TA was observed from 4h-post-irradiation (p.i) in both cell lines. This up-regulation was abrogated by wortmannin and 3-AB. TA was maximal 24h p.i., coinciding with an accumulation of hTERT mRNA. Apoptosis and G2/M arrest were evident from 4h p.i. in MOLT-4 cells. KG1a cells exhibited G2/M block at 24h-p.i. and apoptosis increased thereafter. Three-AB abolished G2/M blockage and enhanced radiation-induced apoptosis in both cell lines. While wortmannin increased early apoptosis in MOLT-4 cells, it did not radiosensitize KG1a cells. This study demonstrates that ionizing radiation induces a transient up-regulation of TA in MOLT-4 and KG1a cell lines. Our findings indicate the participation of post-transcriptional mechanisms in the regulation of TA during the first hours p.i., whereas transcriptional activation of hTERT seems to be contributing to the peak of TA observed later. We provide evidence that, besides their known roles as PI3K and PARP inhibitors, wortmannin and 3-AB also inhibit both constitutive and up-regulated TA, with different consequences on the radiation induced apoptotic cell death of MOLT-4 and KG1a cells.

1. Introduction

Eukaryotic chromosomes are capped by telomeres, structures composed by proteins and a tandem repeat of a guanine-rich sequence that protect them from DNA degradation and prevent illegitimate recombination[1] (Greider and Blackburn 1996, Preston 1997, Blackburn 2001, Poole et al. 2001) . Telomeres progressively shorten in each round of DNA synthesis. A specialized polymerase called telomerase catalyzes the synthesis and extension of telomeric DNA thereby compensating for this telomere loss (Blackburn 2001). Telomeres have been related to radiation sensitivity [2-3]. In a previous study we observed a radiation induced up-regulation of TA in KG1a cells which was influenced by the dose, dose-rate and radiation quality [4]. Disregulation of apoptotic cell death may contribute to the abnormal expansion of malignant cells and may account for cell resistance to radio and chemotherapy. It has been suggested that telomerase could play an anti-apoptotic role and that down-regulation of hTERT increases apoptosis in mammalian cells [5].

Wortmannin is a fungal metabolite that potentiates cellular radiosensitivity by its highly specific inhibition of phosphatidylinositol-3 kinase (PI3K), a cytoplasmic signal transducer involved in the cellular response to genotoxic stress [6]. It also inhibits PI3K-related proteins, such as DNA-dependent protein kinase (DNA-PK) and ataxia-telangiectasia-mutated (ATM). Wortmannin has been widely used for in vitro cell radio and chemosensitization [7].

Three-aminobenzamide (3-AB) is a potent poly(ADP-ribose) polymerase (PARP) inhibitor which retarded the joining of DNA strand breaks rendering cells more sensitive to genotoxic stress. The clinical utility of PARP inhibitors as adjuvant therapeutics for the treatment of various forms of cancer has been proposed [8].
The purpose of the present study was to assess the effects of wortmannin and 3-AB on telomerase activity (TA) and apoptosis following gamma-irradiation of two human leukemia cell lines differing in their p53-status.

2. Material and methods

2.1 Cell lines and cultures
MOLT-4 is a cell-line derived from a human acute lymphoblastic T-cell leukemia that exhibits increased levels of p53 protein after exposure to ionizing radiation. These cells were grown in RPMI 1640 medium (Gibco™-BRL) supplemented with 10% fetal bovine serum (Gibco™-BRL), at 37°C in a humidified atmosphere containing 5% CO₂.

KG1a is a p53-deficient subclone of the KG1 cell-line isolated from a human acute myelogenous leukemia, with an immune phenotype very similar to that of primitive hematopoietic stem cells (Clave et al. 1996). These cells were grown in Iscove’s modified Eagle’s medium (Gibco™-BRL) supplemented with 10% fetal bovine serum (Gibco™-BRL), at 37°C in a humidified atmosphere containing 5% CO₂.

2.2 Irradiation of cell cultures
Exponential-phase growing cells were suspended in normal growth medium and irradiated with 3 Gy at a dose rate of 1.5 Gy/min at room temperature, at least in three separate experiments, using a teletherapy gamma unit (60Co). One equilibrium-thickness of water-equivalent material was included in order to provide secondary electron equilibrium. Control samples were sham-irradiated.

2.3 Evaluation of telomerase activity and telomerase-related gene expression
TA was determined at least in three separate experiments, using a PCR-based telomeric repeat amplification protocol (TRAPeze Intergen™ C #87700-Kit) as previously described [4]. TA in the irradiated samples was expressed as relative to the control.

Telomerase is composed of two proteins, a catalytic subunit (human telomerase reverse transcriptase, hTERT), a telomerase-associated protein (TP1) and an internal RNA template (hTR). The mARN level of these telomerase-related genes was evaluated by RT-PCR as previously described (Pérez et al. 2003). The thermal cycling protocols applied for each PCR-assay are presented in table 1. The results in irradiated samples were expressed relative to the control.

Table 1 Thermal cycling conditions applied for PCR amplification of hTERT, hTR, and TP1

<table>
<thead>
<tr>
<th></th>
<th>hTERT</th>
<th>hTR</th>
<th>TP1</th>
<th>β-actine</th>
</tr>
</thead>
<tbody>
<tr>
<td>General conditions</td>
<td>94ºC/5 min + «n»cycles [94ºC/30 sec; 60ºC/40 sec; 72ºC/40 sec] + 72ºC</td>
<td>94ºC/5 min + «n»cycles [94ºC/30 sec; 64ºC/40 sec; 72ºC/40 sec] + 72ºC</td>
<td>94ºC/5 min + «n»cycles [94ºC/30 sec; 58ºC/40 sec; 72ºC/40 sec] + 72ºC 7 min</td>
<td>94ºC/5 min + «n»cycles [94ºC/30 sec; 58º-64°C (*) /40 sec; 72ºC/40 sec] + 72ºC 7 min</td>
</tr>
<tr>
<td>Nº of cycles KG1a</td>
<td>32</td>
<td>32</td>
<td>28</td>
<td>18</td>
</tr>
<tr>
<td>Nº of cycles MOLT4</td>
<td>26</td>
<td>24</td>
<td>28</td>
<td>18</td>
</tr>
</tbody>
</table>

(*) adapted to temperature conditions of each primer

2.4 Evaluation of apoptosis and cell-cycle distribution
Samples were collected at different time-points and assessed for apoptosis and cell-cycle distribution by flow cytometry (FACStar Plus Becton Dickinson™). Briefly, 1x10⁶ cells previously fixed in 70% ethanol were washed and resuspended in PBS containing RNase 50µg/ml and propidium iodide (IP) 40 µg/ml. Apoptotic cells were identified in a DNA histogram as a sub-G₁ hypodiploid population.

In order to verify flow cytometric findings apoptosis was also evaluated by fluorescence microscopy. A mixture containing 2µg/ml of Hoechst 33342 (H), 15µg/ml of fluorescein diacetate (FDA) and 5 ug/ml of IP was prepared in PBS pH7.4. Ten µL of this fluorescent mix was added to 500µL aliquots of cell suspensions (3 x 10⁶ cells/ml). After 5 min incubation at 37°C and one wash, samples were dropped onto slides and observed by fluorescence microscopy (Carl Zeiss™ MC 80 DX). Early
(living) and late (dead) apoptotic cells were distinguished by the presence of nuclear condensation and of spotted blue (H) or red (IP) bodies, respectively.

2.5 Incubation with chemical agents
Wortmannin (Sigma®), was dissolved in anhydrous dimethyl sulfoxide (DMSO) at a stock concentration of 10mM and stored at -80°C until use. A working solution was prepared in culture medium, sterilized by filtration, and added to cell cultures at a 1µM final concentration 60 min prior to irradiation. Dilutions of 3-AB (Sigma™) were prepared in culture medium, sterilized by filtration, and added to cell cultures 30 minutes prior to irradiation, at a final concentration of 10mM. The media containing these products were replaced by simple culture medium 24h-p.i. Optimal conditions for assays were chosen after testing cell viability at different final concentrations and incubation times.

3. Results

3.1 Time-course of telomerase activity after irradiation
The time-course of TA following irradiation with 3 Gy at a dose-rate of 1.5 Gy/min was evaluated over a 72-hour period. As shown in figure 1, TA early (4h-p.i.) increased in MOLT-4 and KG1a cells. A similar temporal pattern was observed thereafter for both cell-lines: maximal activation up-to around 4-fold control values at 24h-p.i., followed by a decline toward basal values by 72h-p.i.

![FIG. 1. Time-course of telomerase activity following gamma-irradiation.](image1)

3.2 Effect of wortmannin and 3-AB on radiation-induced telomerase activation
We next evaluated the effect of pre-treatment with PI3K and PARP inhibitors on TA for a 24-h time period, corresponding to maximal radiation-induced up-regulation of TA. As seen in figure 2, wortmannin and 3-AB inhibited radiation induced up-regulation of TA in both cell lines. Constitutive TA was reduced by about 50% in sham-irradiated samples following 24-hour-incubation with wortmannin and 3-AB (data not shown).

![FIG. 2. Effect of PI3K and PARP inhibitors on telomerase activity, following gamma-irradiation](image2)
3.3 Telomerase-related gene expression after irradiation

To determine whether the radiation-induced increase in TA was due to changes in telomerase-related gene expression, hTERT, hTR, and TP1 mRNA levels were quantified by RT-PCR for 24 hours following irradiation, the time-period during which TA increased maximally. As seen in figure 3, an accumulation of hTERT mRNA was observed at 24h-p.i. in both cell-lines. The hTR mRNA level did not reveal a significant alteration in the response to ionizing radiation in either of the two cell-lines during the period studied. While the level of TP1 mRNA did not significantly change in MOLT-4 cells, a decrease was observed during the first 8h-p.i. in KG1a cells.

![Graph showing the time-course of telomerase-related gene expression following gamma-irradiation](image)

**FIG. 3.** Time-course of telomerase-related gene expression following gamma-irradiation

3.4 Effects of wortmannin and 3-AB on telomerase-related gene expression

We next evaluated the effect of PI3K and PARP inhibitors on telomerase-related gene expression at 24h-p.i. As shown in figure 4, wortmannin abolished radiation-induced hTERT mRNA accumulation only in MOLT-4 cells, without modifying hTR or TP1 mRNA levels in either of the two irradiated cell-lines. PARP inhibition by 3-AB did not modify hTERT, hTR, and TP1 mRNA levels in irradiated cell-lines.

![Graph showing the effect of PI3K and PARP inhibitors on telomerase-related gene expression](image)

**FIG. 4.** Effect of PI3K and PARP inhibitors on telomerase-related gene expression 24 hours p.i.

3.5 Kinetics of apoptotic cell-death and the cell-cycle upon irradiation

The kinetics of the appearance of apoptotic cells was followed by flow cytometry and fluorescence microscopy over a 72-hour-period after irradiation. As seen in figure 5, the time-course of radiation-induced apoptosis differed in the two cell-lines. The induction of apoptosis following irradiation of MOLT-4 cells, already evident at 4h-p.i., attained maximal values within the first 48h-p.i. In contrast, KG1a cells did not die by apoptosis during the first 24h-p.i. However, apoptotic cell death became evident later, between 48h and 72h-p.i. Fluorescence microscopy confirmed these flow cytometry findings. The kinetics of G2/M accumulation following irradiation is presented in figure 6. In MOLT-4 cells, early G2/M blockage was already evident by 4h-p.i., and reached a maximum at 8h-p.i., decreasing thereafter. Exposure of KG1a cells to ionizing radiation induced a G2/M arrest at 24h-p.i. that disappeared at 48h-p.i.

![Graph showing the kinetics of apoptotic cell-death and the cell-cycle](image)

**FIG. 5.** Kinetics of apoptotic cell-death and the cell-cycle upon irradiation

**FIG. 6.** Kinetics of G2/M accumulation following irradiation.
3.6 Effect of wortmannin and 3-AB on apoptosis and on the G2/M checkpoint
The effect of wortmannin and 3-AB on apoptotic cell-death following irradiation was studied over the same 72-h period. To determine whether these pharmacological products were cytotoxic per se, apoptosis was also evaluated in control sham-irradiated cells treated under the same conditions. No cytotoxic effects were observed following treatment with wortmannin alone, whereas the apoptotic cell fraction of both cell types increased in non-irradiated cells treated with 3-AB. As shown in figure 7, PI3K inhibition by wortmannin induced a significant increase in the radiation-induced apoptotic cell fraction during the first day p.i. in Molt4 cells, whereas it did not modify radiation-induced apoptotic cell-death in KG1a cells. PARP inhibition by 3-AB enhanced radiation-induced apoptosis in both cell lines, as seen in figure 8. While this effect was observed between 8 to 24h-p.i. in Molt4 cells, it was evident later (after 24h-p.i.) in KG1a cells.

We next evaluated the effects of 3-AB and wortmannin on radiation-induced G2/M arrest. PARP inhibition by 3-AB abolished radiation-induced G2/M blockage in both cell-lines. Cell cycle flow cytometric profiles corresponding to one representative experiment are presented in figure 9.
FIG. 8. Effect of 3-aminobenzamide (3-AB) on apoptotic cell-death, following gamma-irradiation

FIG. 9. Apoptotic cell fraction and cell cycle redistribution following gamma-irradiation
4. Discussion

4.1 Radiation-induced up-regulation of TA
We found that ionizing radiation up-regulated TA in both cell lines during the first days p.i., indicating a transient radiation-dependent modulation. The present report represents the first observation of TA regulation by ionizing radiation in MOLT-4 cells. The 24h-peak of TA coincided with an accumulation of hTERT mRNA, which was not observed at earlier time-points. These findings suggest the participation of post-transcriptional mechanisms in the regulation of TA during the first hours p.i., whereas transcriptional activation of hTERT seems to be contributing to the peak of TA observed later. Several pathways for radiation-induced post-transcriptional regulation of TA may be proposed. Protein kinase B (Akt/PKB), one of the downstream targets of PI3K, activates hTERT by phosphorylation and the PI3K/Akt signal transduction pathway is activated by ionizing radiation. Ionizing radiation may also modulate TA by inducing translocation of telomerase within the cell [9]. Concerning the radiation-induced transcriptional activation of TA, the hTERT promoter contains several binding sites for ionizing radiation-inducible transcription factors involved in telomerase activation, such as c-myc, NF-kB, and Ap1[10].

4.2 Inhibition of telomerase by wortmannin and 3-AB
The inhibition of PI3K could account for the early TA decrease observed within the first 15 minutes p.i. after pre-treatment with wortmannin, which abolishes Akt-dependent hTERT activation by phosphorylation. When MOLT-4 cells had been previously incubated with wortmannin, the radiation-induced hTERT mRNA accumulation did not occur. In contrast, wortmannin did not modify the time-course of hTERT mRNA in KG1a cells following irradiation. Thus, the inhibition of TA by wortmannin occurred both at the transcriptional and post-transcriptional levels in MOLT-4 cells, whereas in KG1a cells it seems to be only a post-transcriptional phenomenon. Our results provide evidence that the decrease of TA by 3-AB is not mediated by transcriptional down-regulation of hTERT in either MOLT-4 or KG1a cells. The inhibition of TA by 3-AB may result from post-transcriptional modifications, a hypothesis reinforced by the fact that 3-AB inhibits protein kinase C (PKC), an enzyme that participates in the regulation of telomerase [11-12].

4.3 Effects of wortmannin and 3-AB on apoptosis and cell cycle kinetics
The timing of radiation-induced apoptosis initially coincided with G2/M blockage in MOLT-4 cell-line. Apoptotic cell death occurred in KG1a cells after they were released from G2/M blockage as observed in other p53-deficient cell-lines. Our findings demonstrate that in spite of their different p53 status, both MOLT-4 and KG1a cells exhibited G2/M arrest following exposure to ionizing radiation indicating that this effect may be p53-independent. Wortmannin enhanced early radiation-induced apoptosis in MOLT-4 cells. Although wortmannin has been reported to sensitize cells to ionizing radiation, it is noteworthy that in the present study it did not radiosensitize KG1a cells. Similar results were recently observed in a human glioblastoma cell line [13]. Because of its involvement in cell recovery from DNA damage, PARP operates as a survival factor. In the presence of PARP inhibitors, the DNA repair process is not completely blocked, but is very much slower. Indeed, we found that 3-AB enhanced radiation-induced apoptosis in both cell lines. We also found that 3-AB abolished radiation-induced G2/M blockage in both cell lines. It has been demonstrated that 3-AB down-regulates genes involved in cell cycle control. The lack of radiation-induced G2/M arrest was accompanied by an increase in apoptosis, a finding previously described [14].

4.4 Telomerase and apoptosis
By stabilizing broken DNA-ends by chromosome healing, telomerase could allow cells to survive for a short time. Since chromosome healing does not provide legitimate DNA repair, it may lead to chromosomal aberrations which could trigger apoptosis at later time-points. Our study demonstrated that in MOLT-4 cells the radiation-induced up-regulation of TA temporally coincided with the beginning of apoptosis, indicating that maintenance of higher levels of TA does not avoid triggering of apoptotic cell death in this cell line. Although wortmannin and 3-AB inhibited TA and significantly enhanced apoptosis within the first 24-p.i. in MOLT-4 cells, these results do not
allow to conclude that TA plays an anti-apoptotic role in this cell line. Methods to regulate specifically hTERT should be applied in order to confirm this hypothesis. Regarding KG1a cells, the present study showed that radiation-induced apoptosis was triggered once TA had returned toward basal values. However, the inhibition of TA by wortmannin did not increase radiation-induced apoptotic cell death thus precluding the involvement of TA in the modulation of late apoptosis in this cell line.

4.5 Concluding comments
We provide evidence that, besides their known roles as PI3K and PARP inhibitors, wortmannin and 3-AB also inhibited both constitutive and up-regulated TA, with different consequences on radiation-induced apoptotic cell death of MOLT-4 and KG1a cells. This study also demonstrated that ionizing radiation induced a transient up-regulation of TA in MOLT-4 and KG1a cell lines. DNA-repair enzymes might be selective targets for enhancing radiation response of tumor cells provided that the pharmacological agents to be applied were carefully selected according to the specific cell type. Further investigations should be carried out in order to determine the criteria which could guide such selection.

5. References
6. Acknowledgements

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