

Telomerase activity in peripheral blood mononuclear cells after whole body irradiation

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Abstract. Telomerase activity *in vitro* represents a radiation-inducible function. To test this effect after irradiation *in vivo*, we measured telomerase activity in peripheral blood mononuclear cells (PBMC) of 25 patients with leukemia or lymphoma before and 1 h after whole body irradiation. Telomerase activity of the patients was compared to telomerase activity in PBMC of 15 healthy volunteers. Peripheral blood of the patients was taken before and 1 h after the first fraction of whole body irradiation (2 Gy). Blood samples of the volunteers were irradiated *ex vivo* with 2 Gy. After Ficoll-Paque density gradient centrifugation telomerase activity of 10^4 PBMC per sample was measured using the telomerase PCR ELISA method. No age-dependence of telomerase activity was detected for both the volunteer and the patient group. Telomerase activity in patients was not statistically significantly increased compared to healthy individuals, and this parameter was also no prognostic factor for patient survival. After whole body irradiation an induction of telomerase activity was observed for only 7 patients (28%), or in PBMC of 2 volunteers (13%), respectively. In patients with radiation-inducible telomerase activity, a slightly better survival was indicated, but this difference did not reach statistical significance. The feasibility to assess *in vivo* radiation-induction of telomerase activity in PBMC of leukemia or lymphoma patients was demonstrated. An unexpectedly low number of whole body irradiated patients displayed this phenotype, and the treatment impact of telomerase upregulation in PBMC upon radiation exposure needs to be further analyzed.

Introduction

Telomerase is a reverse transcriptase that adds DNA repeats at chromosome ends. Human somatic cells usually lack telomerase activity and telomeres shorten with each division

until the cells reach replicative senescence (1). By contrast, high levels of telomerase are expressed in the majority of human cancers (2). Stabilization of telomere length by telomerase, or less frequently by an alternative recombinational (ALT) pathway, appears to be a prerequisite for tumor cells to proliferate indefinitely (2). Some actively proliferative somatic cells, for example, hematopoietic stem cells (3), intestinal crypt cells (4) and basal cells in the epidermis (5), have weak but detectable telomerase activity. Telomerase activity is also detectable in human peripheral blood mononuclear cells (PBMC) and its activity is increased by antigen processing (6,7). PBMC possess the capability for indefinite proliferation and the mechanism of clonal expansion to maintain sufficient immunoreactivity to antigens over lifespan. Telomerase activity in PBMC was reported to vary with age: the level of telomerase activity in PBMC of individuals aged <20 years was higher compared with that in individuals aged ≥ 20 years (8). Furthermore, elevated telomerase activity in PBMC of leukemia patients compared to healthy controls has been shown, and was even related to disease progression (9-12).

Among other classes of DNA damage, ionizing radiation induces DNA double-strand breaks (DSB), which can lead to chromosome aberrations and cell death. Cells respond to DSB through a cascade of proteins ranging from sensors, which recognise the damage, through mediator proteins to a series of effectors that induce cell-cycle arrest, repair by homologous or nonhomologous mechanisms or cell death by apoptosis (13). In addition to telomere maintenance, telomerase can add telomeric sequences onto chromosome breaks at non-telomeric locations, a process termed chromosome healing (14,15). Ionizing radiation-induced DSB provide a substrate for chromosome healing, but this appears to be a relatively rare event in mammalian cells (15). Nevertheless, a role of telomerase in the cellular response to radiation damage is supported by the observations from several laboratories that telomerase activity represents a radiation-inducible function (16-18).

Changes of telomerase activity after irradiation have been studied so far under *in vitro* conditions. To investigate telomerase activity after irradiation *in vivo* we measured telomerase activity in PBMC of 25 patients with leukemia or lymphoma undergoing whole body irradiation before bone marrow or blood stem cell transplantation. Telomerase activity of the patients was compared to telomerase activity in PBMC of 15 healthy volunteers. The PBMC of the volunteers were

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irradiated *ex vivo*. We evaluated changes of telomerase activity with age, differences between patients and healthy volunteers, induction of telomerase activity after irradiation, and telomerase activity as a possible prognostic factor for patients undergoing whole body irradiation.

Materials and methods

Heparinized peripheral blood (3x10 ml) was obtained by venopuncture from 15 healthy volunteers aged from 25 to 65 years old (median age: 34 years). The blood samples were irradiated *ex vivo* with 0 Gy (sham-treated) and 2 Gy using a medical linear accelerator operating in 6-MV X-ray mode at a dose-rate of 2.5 Gy/min. One hour after irradiation PBMC were isolated by Ficoll-Paque (Sigma Inc, St. Louis, USA) density gradient centrifugation. The PBMC were washed twice with phosphate-buffered saline (PBS) and stored at -80°C until measurement of the telomerase activity.

To evaluate telomerase activity after irradiation *in vivo* 25 patients with leukemia or lymphoma undergoing whole body irradiation before bone marrow or blood stem cell transplantation were studied. All patients had received chemotherapy before the whole body irradiation, but never radiotherapy. The whole body irradiation is an established method in the preparation of patients to bone marrow or blood stem cell transplantation. The combination of an intensified chemotherapy and the whole body irradiation eliminates the malignant cell clone and suppresses the immune system to prevent a repulsion of the graft. The median age of the patients was 45 years, range 22 to 66 years. After agreement of the patients 10 ml of heparinized peripheral blood was taken before the first irradiation. PBMC were purified on a Ficoll gradient, washed with PBS and stored at -80°C. Patients receiving whole body irradiation were irradiated twice daily with 2 Gy (medical linear accelerator, 6-MV X-ray mode) on three days to a whole dose of 12 Gy. One hour after the first fraction (2 Gy) 10 ml of heparinized peripheral blood was obtained. PBMC were purified on a Ficoll gradient, washed with PBS and stored at -80°C. Overall survival was calculated using the Kaplan-Meier method. Survival distributions were compared using the log-rank test (Statistica, StatSoft Inc, Tulsa, USA).

Telomerase activity was measured using the Telomerase PCR ELISA kit (Roche Diagnostics, Mannheim, Germany). The assay allows a nonradioactive detection of telomerase activity. In principle, telomerase activity, if present in cell lysates, adds telomeric repeats to a biotin-labeled primer. The elongation products are then amplified by PCR. An aliquot of the PCR product is denatured and hybridized to a digoxigenin-labeled, telomeric repeat-specific detection probe. This hybrid is immobilized by the biotin-labeled primer to a streptavidin-coated microtiter plate. Finally, the digoxigenin labels are detected with an anti-digoxigenin peroxidase. The probe is visualized by virtue of peroxidase-metabolizing tetramethyl benzidine to form a colored reaction product. Using a microtiter plate reader, color intensities are measured at 450 nm with a reference wavelength of 690 nm. Samples of 10⁴ PBMC were analysed by the Telomerase PCR ELISA method, and derived as mean optical density from three separate determinations.

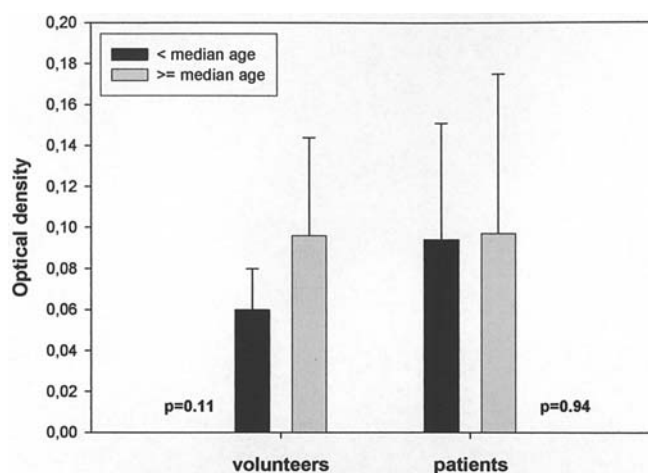


Figure 1. Mean telomerase activity of 10⁴ peripheral blood mononuclear cells of the volunteer and patient groups 'age < median age' and 'age ≥ median age'. Telomerase activity of the volunteers seemed to increase with age, but the difference was not significant (p=0.11). Telomerase activity of the patients was equal (p=0.94).

In previous *in vitro* experiments telomerase activity of unirradiated cells was used for an internal calibration curve, and the telomerase activity of the irradiated cells was converted to the cell equivalent of unirradiated cells (18). For the *in vivo* experiments of this study it is not possible to use an internal calibration curve. The levels of telomerase activity of the PBMC before and after *in vivo* irradiation have to be compared directly. To investigate the variability of the Telomerase PCR ELISA telomerase activity of 10⁴ PBMC of four blood samples from one volunteer, taken in the morning and afternoon on two following days, was measured: the standard deviation was 9% of the mean value. Therefore an induction of telomerase activity after irradiation was defined as an elevation of at least 20% compared to the level before irradiation.

Results

To evaluate changes of telomerase activity with age the 15 healthy volunteers were divided into two groups: age <34 years (median age), and age ≥34 years. Mean telomerase activity of 10⁴ PBMC of the two groups is given in Fig. 1. Mean optical density, as a measure of telomerase activity, of 10⁴ PBMC was 0.060 for the group 'age <34 years', and 0.096 for the group 'age ≥34 years'. Telomerase activity seemed to increase with age, but the difference between the two groups was not significant (p=0.11). After *ex vivo* irradiation with 2 Gy an induction of telomerase activity was observed for two individuals (13%).

Patient and treatment characteristics are shown in Table I. Of the 25 patients receiving whole body irradiation 11 patients had acute myeloid leukemia, 5 patients had acute lymphocytic leukemia, 4 patients had aggressive lymphoma, one patient had chronic myeloid leukemia, one patient had chronic lymphocytic leukemia, one patient had myelodysplastic syndrome, one patient had stage IV indolent lymphoma and one patient had stage IV Hodgkin lymphoma. The volunteer patients were divided by median age into two groups: age <45 years (median age), and age ≥45 years. Mean optical density, as a measure

Table I. Patient and treatment characteristics.

Characteristic	No.	%
Age (years)		
Median	45	
Range	22-66	
Sex		
Male	13	52
Female	12	48
Primary diagnosis		
AML	11	44
ALL	5	20
Aggressive lymphoma	4	16
CML	1	4
CLL	1	4
MDS	1	4
Indolent lymphoma, stage IV	1	4
Hodgkin lymphoma, stage IV	1	4
Remission status before RT		
PR	9	36
CR	16	64
Transplantation		
Autologous	9	36
Allogeneic	16	64

AML, acute myeloid leukemia; ALL, acute lymphocytic leukemia; CML, chronic myeloid leukemia; CLL, chronic lymphocytic leukemia; MDS, myelodysplastic syndrome; RT, radiotherapy; PR, partial remission; CR, complete remission.

of telomerase activity, of 10^4 PBMC was 0.094 for the patient group 'age <45 years', and 0.097 for the patient group 'age ≥ 45 years' ($p=0.94$) (Fig. 1). The telomerase activity of the patients in comparison with the volunteers is shown in Fig. 2. No difference between these two groups was detected ($p=0.43$). Remission status before radiotherapy (partial response vs. complete response to chemotherapy) had no statistically significant influence on telomerase activity ($p=0.61$). After whole body irradiation with 2 Gy, 7 patients (28%) showed an induction of telomerase activity in PBMC. This did not relate to preirradiation activity levels (three of these 7 patients had elevated telomerase activity before irradiation compared to the healthy volunteers). The patients with telomerase induction were not from one single leukemia group (5 patients had acute myeloid leukemia, one patient had chronic myeloid leukemia, and one patient had myelodysplastic syndrome).

The mean follow-up time of the patients was 9 months. A total of 7 patients (28%) died. The 1-year survival rate was 65%, the 2-year survival rate 55% (Fig. 3). Remission status before radiotherapy had no impact on survival ($p=0.44$). Patients who had high telomerase activity levels (≥ 0.090 , median optical density) showed no statistically significant difference of survival rates compared to patients with low

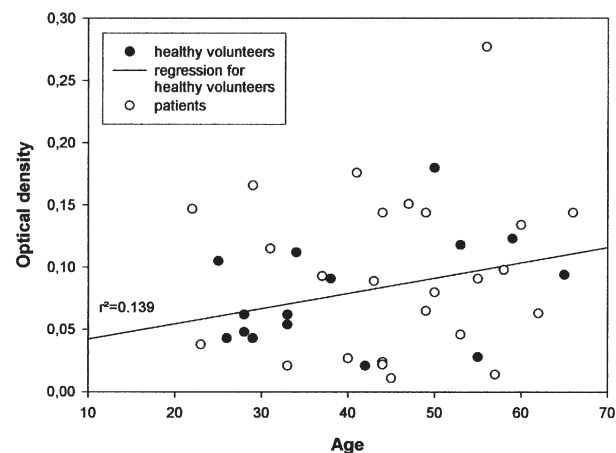


Figure 2. Telomerase activity of the 15 volunteers (black circles) and the 25 patients (white circles). To compare the telomerase activity of the patients with the telomerase activity of the volunteers the regression line of the telomerase activity levels of the volunteers was calculated ($r^2=0.139$).

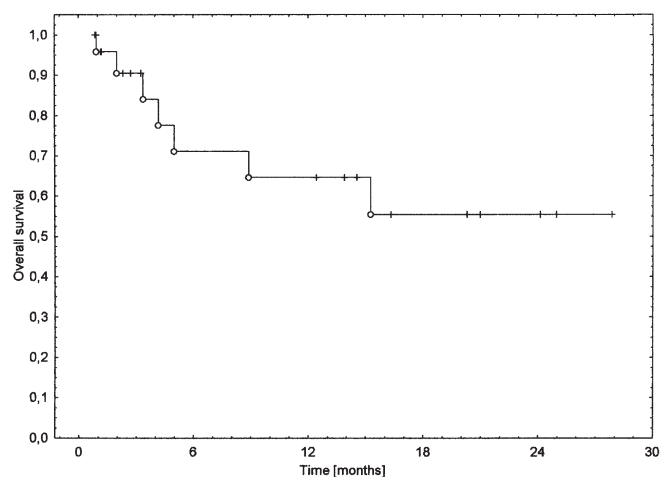


Figure 3. Overall survival of the 25 patients receiving whole body irradiation before bone marrow or blood stem cell transplantation. The 1-year survival rate was 65%, the 2-year survival rate was 55%.

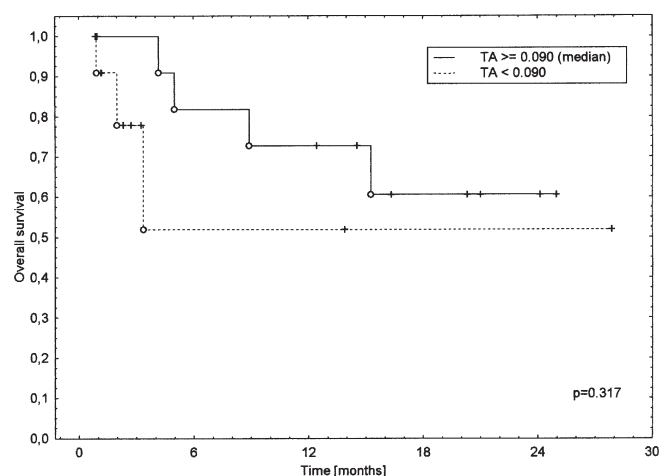


Figure 4. Overall survival of patients regarding level of telomerase activity before irradiation (telomerase activity ≥ 0.09 vs. telomerase activity < 0.09 ; median telomerase activity, 0.09). There was no statistically significant difference in survival ($p=0.317$).

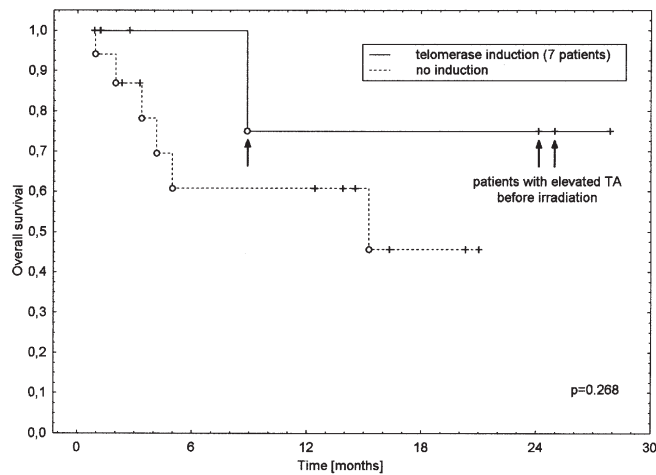


Figure 5. Overall survival of patients regarding induction of telomerase activity after 2 Gy whole body irradiation (telomerase induction vs. no telomerase induction). In patients with radiation-inducible telomerase activity, a slightly better survival was indicated, but this difference did not reach statistical significance ($p=0.268$). Three of the 7 patients with induction of telomerase activity had elevated telomerase activity before irradiation.

telomerase activity levels ($p=0.317$, Fig. 4). In patients with radiation-inducible telomerase activity, a slightly better survival was indicated, but this difference did not reach statistical significance ($p=0.268$, Fig. 5).

Discussion

In the present study, no age-dependence of telomerase activity in PBMC of healthy individuals (25 to 65 years old) was demonstrated, and the slight difference of mean telomerase activity between the groups 'age <34 years (median)' and 'age ≥ 34 years' was not statistically significant. This result is contrary to those of previous studies: Iwama *et al* reported a decrease of telomerase activity in PBMC with age (8). However, their study contained younger individuals and higher telomerase activity in PBMC was observed for individuals aged <20 years compared to individuals aged ≥ 20 years. Katayama *et al* also showed that telomerase activity declined with age in healthy individuals (aged 24-78 years), but not in patients with systemic lupus erythematosus (19). Telomerase activity in PBMC of patients with leukemia or lymphoma before undergoing whole body irradiation was also not dependent on patient age. Thus, it is presently not clear whether a general age-dependence of telomerase activity can be presumed.

A more important aspect of the present investigation was the possible elevation of telomerase activity in PBMC of patients with leukemia or lymphoma compared to healthy controls, which was reported for both adults (9-12) and children (20-22). An increase of telomerase activity was also inferred to relate to disease state, as a reflection of a progressively acquired capacity of tumor cells to sustain proliferation by this mechanism and/or of higher relative tumor cell burden (9,10,12). It is still unclear, however, whether elevated telomerase activity is a prognostic factor for shortened survival with leukemias. For patients with adult T-cell leukemia high telomerase activity was associated with poorer prognosis

(23). Verstovsek *et al* showed that, for pediatric patients with acute myeloid leukemia, telomerase activity overexpression was a significant prognostic indicator for shortened survival (24). In contrast Ohyashiki *et al* found no correlation between telomerase activity and survival for adult patients with acute myeloid leukemia (10). For children with acute lymphoblastic leukemia telomerase activity was not associated with clinical outcome (25). In patients who achieved remission telomerase activity levels decreased to normal but were significantly higher at the time of recurrence or disease progression compared with telomerase activity levels at the time of diagnosis (9,10,12). In our study, telomerase activity in leukemia or lymphoma patients did not exhibit a statistically significant increase compared to healthy controls, and this parameter was also no prognostic factor for the survival of patients. Considering the substantial interindividual variation of telomerase activity in the patients as well as in the healthy volunteers with large overlap between these groups, a delineation of a difference between them, if in fact present at all, would certainly require a much higher number of individuals being tested. Similarly, the significance of elevated telomerase activity as putative prognostic factor may only become detectable with much larger cohorts of patients, but with respect to the present result of a close similarity of such activity levels in leukemia patients with those of the healthy controls, the prognostic value of this parameter, even if explored to reach statistical significance in a large cohort, remains questionable.

One major focus of the present investigation was, for the first time, to assess a possible induction of telomerase activity in PBMC upon (whole body) irradiation *in vivo*. Several *in vitro* studies revealed that telomerase activity represents a radiation-inducible function (16-18), even at low radiation doses and persisting over many cell generations (18). Based on the above-mentioned reports of a possible relation between telomerase activity levels and leukemia patient survival, we reasoned, as a working hypothesis, that i) telomerase activity might also be induced in PBMC due to 2 Gy whole body irradiation, and ii) that this induction could impact on disease progression when cancers recur from surviving cancer cells with telomerase activity being elevated. Contrary to our expectation, radiation induction of telomerase was detectable in only 28% (7 out of 25) of the patients and 13% of the healthy individuals (criterion: increase >2 standard deviations of the interexperimental variability; see Materials and methods), and the magnitude of telomerase upregulation was in all these cases much lower than the respective results with diverse *in vitro* systems [e.g. ≤ 4 -fold in human lymphoblastoid cells (18)]. One could speculate that induction of telomerase activity occurred in patients that had low baseline activity (i.e. prior to irradiation), but 3 out of the 7 patients with radiation-induced activity already had preirradiation levels above the mean. This failure to detect a more frequent (among individuals) upregulation of telomerase in PBMC obviously prevented a survival analysis in relation to the extent of telomerase activation (in this subgroup). Also the survival of the radiation-inducible group was not statistically significantly decreased compared to the non-responders, whereby this limited data set rather showed a slightly better survival of the patients with radiation-inducible telomerase activity. Further study will be necessary to clarify this important question. In this respect, the still

unknown biological role of radiation induction of telomerase activity needs to be addressed. The ability of telomerase to add telomeric sequences at nontelomeric locations has conceivably been demonstrated (14,15). This process could represent an important cofactor in the cellular DNA damage response by converting radiation-induced DNA double-strand breaks into stable chromosome breaks (chromosome healing), thus preventing DSB rejoining (14), or more importantly, mis-rejoining that could give rise to chromosomal rearrangements. While such considerations are still speculative, this type of damage response could potentially enhance the efficacy of radiation treatment aimed at eradicating tumor cells.

This study investigated for the first time telomerase activity in human PBMC after irradiation *in vivo*. The feasibility to assess *in vivo* radiation-induction of telomerase activity in PBMC of leukemia or lymphoma patients was demonstrated. An unexpectedly low number of whole body irradiated patients displayed this phenotype, and the treatment impact of telomerase upregulation in PBMC upon radiation exposure needs to be further analyzed.

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