Mutation Research 740 (2012) 13–20

Contents lists available at SciVerse ScienceDirect

Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis

journal homepage: www.elsevier.com/locate/molmut
Community address: www.elsevier.com/locate/mutres

Telomere loss, not average telomere length, confers radiosensitivity to TK6-irradiated cells

F. Berardinelli[a,b,1], D. Nieri[a,b,1], A. Sgura[a,b], C. Tanzarella[a,b], A. Antoccia[a,b,*]

[a] Dip. Di Biologia, Università "Roma Tre", Rome, Italy
[b] INFN – "Roma Tre", Rome, Italy

ARTICLE INFO

Article history:
Received 12 June 2012
Received in revised form
20 November 2012
Accepted 22 November 2012
Available online 7 December 2012

Keywords:
TK6
Telomere
Radioresistance
Telomerase
Telomere loss
Ionizing radiation

ABSTRACT

Many and varied are the proposed mechanisms that lead to resistance to ionizing radiation treatment. Among them, an inverse relationship between telomere length and radioresistance has been recently advanced. Investigating such a relationship in TK6 lymphoblasts, we found that clones originating from cells survived to 4 Gy of X-rays showed a significantly higher telomere length when compared with clones grown from untreated cells. The lengthening observed was not attributable to a radiation-induced increase in telomerase activity, as demonstrated by TRAP assay performed in the dose range of 1–10 Gy. Given the evidence that TK6 whole population was characterized by heterogeneity in cellular mean telomere length and telomere loss, we tested the hypothesis that a process of selection may favour cells with longer telomeres (more radioresistant cells) following exposure to irradiation. In order to do this 15 independent TK6 clones were selected and characterized for telomere length and loss on the basis of q-FISH and flow-FISH analysis. Among the screened clones four characterized by long telomeres and four characterized by short telomeres were tested for their radiosensitivity by means of clonogenic assay. The results obtained showed that, in our experimental conditions (cellular model, radiation doses) no significant correlation was observed between radiosensitivity and mean telomere lengths, whereas a positive correlation was observed with respect to telomere loss. Overall, these results indicate that telomere loss and not mean telomere length plays a critical role in the phenomenon of radiosensitivity/radioresistance.

1. Introduction

A major factor in the failure of radiotherapy is inherent or induced cellular radioresistance [1]. This is a characteristic of many different tumour types which is also retained in cultured cells. Radioresistance could develop in cancer cells by several possible mechanisms. Among the possibilities differences in the amount of initial DNA damage related to chromatin conformation and capacity to repair DNA double-strand breaks (DSBs) generally have been considered the most obvious mechanisms. Many previous papers have provided us with evidence of a relationship between cell survival and rejoining of DNA lesions [2]. Other factors, such as apoptosis failure and altered expression of genes taking part in the regulation of cell cycle checkpoints, have been proposed as bases for radioresistance [3–6]. In addition, a possible relationship between radiosensitivity/radioresistance and telomere dysfunctions/telomere length has been reported [7–11] leading to the possible use of telomere length as a marker for chromosomal radiosensitivity/radioresistance.

Telomeres, the nucleoprotein complexes located at the ends of linear chromosomes, perform at least two essential functions in cells. First, telomeres, which consist of tandem TTAGGG repeats and associated proteins, counterbalance the incomplete replication of terminal DNA that occurs as a result of the incapability of conventional polymerases to complete the replication of linear DNA ends. In most organisms such a problem is overridden by the action of a specific enzyme termed telomerase, a specialized reverse transcriptase that extends terminal DNA making use of internal specific RNA template [12,13]. Telomerase is reactivated in most human tumours, whereas a minor part of them override the end replication problem trough the activation of recombination based mechanism termed Alternative Lengthening of Telomeres (ALT). The second essential function of telomeres is chromosome capping. In fact, telomeres protect chromosomes ends, allowing cells to distinguish natural chromosome termini from DNA DSBs [7] and thus avoid degradation and chromosome fusion events [14].

In this respect the loss of telomere sequences is a dramatic event that in normal cells results in cellular replicative senescence or

* Corresponding author at: Università "Roma Tre", V.le Marconi 146, 00146 Rome, Italy. Tel.: +39 06 57336336; fax: +39 06 57336321.
E-mail address: antoccia@uniroma3.it (A. Antoccia).
[1] These authors contributed equally to the work.

0027-5107/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.mrfmmm.2012.11.004

apoptosis [15]. Moreover, the telomere loss has been associated with genomic instability [16] supporting the notion that telomere loss plays a central role in cancer transformation, especially during the crisis phase [17]. Even cancer cells commonly show telomere loss despite the expression of telomerase. Although not as frequent as the telomere loss occurring during crisis, this lower rate of telomere loss is tolerated by the cell, and can therefore result in chromosome rearrangements that can continue to occur throughout the lifetime of the tumour [17]. In addition to chromosomal instability generated as a direct response to excessive telomere shortening, it should be pointed out that such telomeric modification alters the kinetics of DNA damage response in irradiated human fibroblasts, thus contributing also indirectly to genomic instability [18]. Interestingly, primary cells carrying mutations in genes devoted to DNA repair as *DNA-PKcs*, *Ku70*, *Ku80*, *NBS1*, *MRE11*, *RAD50*, *ATM*, *FA*, *BRCA1* and *WRN* display accelerated telomere shortening [19–21] and increased sensitivity to irradiation. Overall, these studies indicate that an inverse relationship between telomere length and the degree of individual sensitivity to ionizing radiation exists in primary cells with an absent or insufficient level of telomerase [14,22–24,11]. However, it should be pointed out that such a relationship is still a matter of debate, in that controversial data are available in the literature. A 7–8 fold reduction in telomere length was observed in radiosensitive murine lymphoma cells L5178Y-S compared with the radioresistant parental cells L5178Y, possessing 7 kb and 48 kb, respectively [14]. On the other hand, in a more recent paper comparing the same mouse lymphoma cell lines belonging to different stocks, the authors found an uncoupling between telomere length and radioresistance [25]. Again McIlrath et al. reported an inverse correlation between telomere length and radiation-induced cytogenetic damage in a survey carried out in lymphocytes drawn from 24 breast cancer patients and 5 normal individuals [14]. On the contrary, the analysis of LCLs established from 33 cancer patients who suffered significant tissue damage in the irradiated area (radiosensitive patients) showed that a subset of them had abnormally long telomeres, even longer than telomeres observed in 18 LCLs from individual with normal tissue response after radiotherapy [26]. Recently, Zongaro et al., immortalizing human fibroblast clones by ectopic expression of telomerase, showed that in the presence of an active enzyme, even short telomeres do not cause an increase in radiosensitivity [27].

Overall, these conflicting results show that further investigation is needed to strength the link between telomere length and sensitivity to ionizing radiations. In addition, it should be pointed out that in the studies above reported, based on Telomere Restriction Fragment (TRF), q-FISH or f-FISH analysis, principally the mean telomere length was investigated, whereas the role of telomere loss was almost totally neglected.

For this purpose, a detailed analysis of mean telomere length and telomere loss and their correlation with radiosensitivity was carried out in radiation-surviving clones. In particular we analyzed whether or not radiation-induced telomere length modulation could be attributable to a selection process taking part in exposed cells. To test this hypothesis the clonogenic capability of isogenic TK6 clones with different telomere lengths was evaluated after X-rays exposure.

2. Materials and methods

2.1. Cell culture conditions

The human B-lymphoblast cell line TK6 was maintained in RPMI 1640 supplemented with 15% heat-inactivated horse serum, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. Cells were kept at the density of 1×10^5 ml^{-1} to maintain them in exponential growth.

2.2. Low density seeding and clone selection

In order to obtain monoclonal cell colonies, TK6 cells were seeded at very low density in 96-well microtitre plates using a Costar® Transtar-96® System (1 cell/10 ml in order to obtain about 2 positive wells per plate). In this condition less than 2% of the wells showed positive growth and the probability for each colony of not being monoclonal was less than 1.5%. About 800 wells were seeded in order to harvest 15 clones. After isolation, each clone was sub-cultured in T-25 tissue culture flask for about 10 days and then characterized for telomere length using quantitative-FISH (q-FISH) and flow-FISH (f-FISH) techniques. Among the 15 clones thus obtained, four long telomere (named L1, L2, L3 and L4) and four short telomere (named S1, S2, S3, S4) clones were selected for assessing their radiosensitivity.

2.3. Clonogenic assay

Cells were exposed to 0.25, 0.5, 1, 2 and 4 Gy of X-rays (Gilardoni apparatus 250 kV, 6 mA, with a dose rate of 0.57 Gy/min) in T-25 flasks (up to 1×10^6 cells/dose) and then seeded into 96-well microtitre plates at 1–120 cells/well to determine the surviving fraction. Control cultures were treated in an identical manner except for the exposure to X-rays. Colonies were scored after 10–15 days of growth, and relative surviving fractions were calculated according to standard methods [28,29]. For each clone tested at least three independent experiments were performed. For each clone D_0 values were calculated from the surviving fraction experiments as the dose necessary to reduce the surviving fraction by the 37%.

2.4. Collection of chromosome spreads

Chromosome spreads were obtained following a 30-min incubation in calyculin-A (30 μM; Wako, Germany), a protein phosphatase inhibitor, which induces chromosome condensation irrespectively of cell-cycle phase [30]. Spreads of these prematurely condensed chromosomes (PCC) were prepared by a standard procedure consisting of treatment with hypotonic solution (75 mM KCl) for 20 min at 37 °C, followed by fixation in freshly prepared Carnoy solution (3:1, v/v, methanol/acetic acid). Cells were then dropped onto slides, air dried and utilized for FISH analysis.

2.5. Quantitative-FISH analysis (q-FISH)

q-FISH staining was performed as previously described [31]. Briefly, chromosome spreads were hybridized with fluorescent PNA telomeric probes and chromosome 2 centromeric PNA probe (DAKOCytomatation), used as references for telomere lengths quantification. Images were captured with an Axio Imager M1 (Carl Zeiss, Germany) equipped with a CCD camera, and the telomere size was analyzed with ISIS software (MetaSystems, Germany). The software calculates telomere lengths as the ratio between the fluorescence of each telomere signal and the fluorescence of the centromere of chromosome 2, used as the internal reference in each metaphase analyzed. Data were expressed as percentage (T/C%) [31,32].

Telomere loss has been calculated as the sum of telomeric signals with a T/C% lower than 1.5% for each sample analyzed. The 1.5 T/C% threshold value was chosen considering the mean background value of all the q-FISH experiments performed.

Student's *t*-test with "two-tails" was used to calculate the statistical significance of the observed differences. For Student's *t*-test the differences were considered significant for $p < 0.05$.

2.6. Real time quantitative-telomerase repeat amplification protocol assay (RTQ-TRAP)

The SYBR green RTQ-TRAP assay was conducted as described elsewhere [33] with minor modifications. In short, the reaction was performed with cell extracts (1000 cells), 0.1 μg of telomerase primer TS, and 0.05 μg of anchored return primer ACX, in 25 μl with SYBR Green PCR Master Mix (Biotools, Spain). Primer sequences were described by Kim and Wu [34]. Using the RotorGene 6000 Thermal cycler (Corbett), samples were incubated for 20 min at 25 °C and amplified in 35 PCR cycles with 30 s at 95 °C and 90 s at 60 °C (two step PCR). The threshold cycle values (C_t) were determined from semi-log amplification plots (log increase in fluorescence vs cycle number) and compared with standard curves generated from serial dilutions of TK6 cell extracts (2000, 1000, 500, 100 cells). Each sample was analyzed at least in duplicates. Telomerase activity was expressed relative to TK6 1000 cells untreated sample extract (RTA, Relative Telomerase Activity).

2.7. Flow-FISH (f-FISH)

Flow-FISH experiments were performed following standard protocols [35]. For each sample, 10^6 cells were washed in PBS then twice in PBS/BSA 0.1% and split in two different tubes. Cells in the first tube were resuspended 0.5 ml hybridization solution (deionized formamide, 70% Tris–HCl 20 mM, BSA 1%) added with PNA-A488 Telomeric Probe (0.3 μg/ml) while cells in the second one were resuspended in the same solution without PNA-probe. Chromosomal DNA were denatured in the dark on a thermomixer (80 °C, 500 rpm) and then hybridized for 2 h at room temperature. After washing twice with 1 ml of washing buffer I (deionized formamide 70%, Tris–HCl pH 7 10 mM, BSA 1%, Tween 20 0.1%) and once with 1 ml of washing buffer