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Accelerated telomere shortening in Fanconi anemia fibroblasts – a longitudinal study

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Abstract Fanconi anemia (FA) is a fatal inherited disease displaying chromosomal instability, disturbances in oxygen metabolism and a high burden of intracellular radical oxygen species. Oxygen radicals can damage DNA including telomeric regions. Insufficient repair results in single strand breaks that can induce accelerated telomere shortening. In a longitudinal study we demonstrate that telomeric DNA is continuously lost at a higher rate in FA fibroblasts compared to healthy controls. Furthermore, we show that this loss is caused rather by an increased shortening per cell division in regularly replicating cells than by apoptosis.

Key words: Fanconi anemia; Telomere shortening; Fibroblast; Cell culture

1. Introduction

Fanconi anemia (FA) is an autosomal recessive disease of childhood that typically leads to death as a result of progressive hypoplastic pancytopenia and bone marrow failure. Abnormalities of the skeleton and of inner organs, and an increased risk of developing cancer are additional features of this disease [1]. The most prominent cellular aberration is a spontaneous chromosomal instability that is augmented by the use of alkylating crosslinkers such as mitomycin C, diepoxybutane [2] and reactive oxygen species (ROS) [3,4]. A characteristic cell cycle delay in G2 [5] serves as a further diagnostic tool.

FA comprises at least seven complementation groups, A–G [6]. While all FA genes except *FANCB* and a subgroup of *FANCD* have been cloned [7–12] only the gene product of *FANCG* could be associated with a known protein, XRCC9, which is thought to participate in DNA repair or control of the cell cycle checkpoint [9].

The exact molecular defect of FA is not known, though defective DNA repair and deficiencies in the redox cycling activities have been investigated and reviewed by Pagano and Korkina [13]. An elevated level of ROS has been measured in FA cells [3]. ROS can induce lasting damage to telomeres leading to accelerated telomere shortening in replicating cells [14].

Since aging of cells is thought to be accompanied by shortening of telomeres [15] and since aging is accelerated by ROS [16,17], it was surmised that, if FA is a disorder of ROS metabolism, telomere shortening should be accelerated in FA patients. Indeed, it was recently demonstrated [18,19] that despite a high individual variance, cells from FA patients have shorter telomeres in peripheral blood mononuclear cells (PBMC) compared to controls. Moreover, the rate of telomere shortening was claimed to be accelerated in six FA patients when measured sequentially at two different ages. It was suggested that the short telomere length in PBMC might reflect a high turnover of hematopoietic stem and progenitor cells, necessitated as compensation for an increased rate of apoptosis and cell cycle delays in FA. Such stress-induced replication would lead to shorter telomeres even with a constant rate of shortening per cell division [19].

This explanation might be valid for a whole category of diseases, such as acquired aplastic anemias, in which telomere shortening could be demonstrated [18]. FA, however, is an inherited disease characterized by chromosomal instability. In FA telomere shortening may not only be a disastrous event in hematopoietic cells accelerating pancytopenia but may also be the operative defect underlying the disease. Were this to be the case then, in fact, telomere shortening should be found in cells other than hematopoietic cells of affected individuals.

The results reported here demonstrate that telomere shortening is accelerated in cultured primary FA fibroblasts when measured periodically during a long period of their lifespan. Since neither apoptosis nor cell loss was intensified, telomere shortening may be due to oxidatively damaged telomeric DNA rather than to stress-induced replication.

2. Materials and methods

2.1. Cell lines and culture conditions

Fibroblast cell lines are described in Table 1. Cells were grown in minimum essential medium (Biochrom, Berlin, Germany) containing 10% fetal calf serum (Greiner, Germany) in air plus 5% CO_2 at 37°C

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Abbreviations: FA, Fanconi anemia; ROS, reactive oxygen species; PBMC, peripheral blood mononuclear cells; PD, population doubling; TRAP, telomere repeat amplification protocol

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Table 1
Cell lines

Cell line	Race, sex, age	Phenotype	Origin
FLB	Caucasian female, 13 years	FANCG	M. Hirsch-Kauffmann, Innsbruck, Austria
GM1309	Black male, 12 years	FANCA	Human Genetic Mutant Cell Repository, Camden, NJ, USA
1424	Caucasian male, 10 years	FANCG	K. Sperling, Berlin, Germany
F71	Arabian male, 14 years	FANCA?	R. Voss, Israel
GM368	Black male, 8 years	FANC	Human Genetic Mutant Cell Repository, Camden, NJ, USA
FISG	Caucasian male, 1 year	FANC	M. Hirsch-Kauffmann, Innsbruck, Austria
KoHe	Caucasian male, 25 years	control	M. Hirsch-Kauffmann, Innsbruck, Austria
KoDo	Caucasian male, 28 years	control	M. Hirsch-Kauffmann, Innsbruck, Austria
KoHi	Caucasian female, 38 years	control	M. Hirsch-Kauffmann, Innsbruck, Austria
KoKr	Caucasian male, 28 years	control	M. Hirsch-Kauffmann, Innsbruck, Austria

and 90% humidity. Cultures were free of mycoplasma. Cells were passaged at confluency 1:2. One passage equals one population doubling (PD).

2.2. Telomere length

Samples for telomere length determination were taken every third to fifth PD. Two or three independent cultures were examined for each cell line. For preparation of highest quality genomic DNA, cells at subconfluency were embedded in 0.65% low-melting agarose plugs at a density of 10^6 cells/ml before deproteination by proteinase K treatment [20,21]. DNA was completely digested by *Hin*fI (60 U per plug, Boehringer, Mannheim, Germany) at 37°C. Plugs were analyzed in a 1% agarose gel by pulsed field gel electrophoresis (Bio-Rad, Hercules, CA, USA). Gels were blotted to Hybond N^+ membranes and hybridized with the telomeric probe $(TTAGGG)_4$, conjugated directly to alkaline phosphatase (Promega, Madison, WI, USA). A chemiluminescence signal was recorded on film within the linear range and analyzed in an imaging densitometer (Bio-Rad). The average telomere length was calculated as weighted mean of the optical density as described [20]. Average telomere shortening rates were calculated by linear regression.

2.3. Apoptosis

Cells were trypsinized, stained with 2 μg/ml propidium iodide for 5 min on ice in the dark and the fluorescence in 2×10^4 cells was measured in a flow cytometer (Partec, Münster, Germany) using blue excitation. Cells showing red propidium iodide fluorescence indicating a damaged plasma membrane were gated out (1–2% of all cells). Apoptotic cells were defined by their lower forward and higher sideward scatter intensity [22].

2.4. Growth rate

The PD time was estimated for all cells listed in Table 1 by at least three independent experiments per cell line as described by Weirich-Schwaiger et al. [23]. Essentially, cells were seeded on slides. Cytokinesis was blocked by cytochalasin and after 48 h nuclei were stained. A thousand cells were scored for mono- and binucleate cells and the relation taken as an indicator of cell cycle duration. In addition, when cells were cultivated, days between splitting of confluent cultures at a 1:2 ratio and reach of confluency were recorded as one PD.

2.5. TRAP assay

For the telomerase measurements the semiquantitative telomere repeat amplification protocol (TRAP) (Intergen) was used. Lysates equivalent to 2000 cells were analyzed in a telomerase reaction for 20 min, followed by 27 PCR cycles (30 s at 95°C, 45 s at 60°C). Gels were scanned in a phosphorimager (Bio-Rad).

3. Results

For the longitudinal study of telomere length in primary fibroblasts, control cells were grown under basal culture conditions for up to 50 PDs, FA fibroblasts for about 40 PDs. Telomere shortening was measured by Southern blotting at every third to fifth PD.

In Fig. 1A regression curves demonstrate the decline of telomere lengths in cells of two controls and two representative FA strains. Fig. 1B gives the average telomere loss in bp per PD including standard deviation for the controls and four FA fibroblast lines. In both graphs the accelerated shortening of telomeres in FA fibroblasts is noted.

In order to test whether this phenomenon might be due to an increased rate of apoptosis in FA fibroblasts leading in turn to an increased rate of replication in the remaining cells,

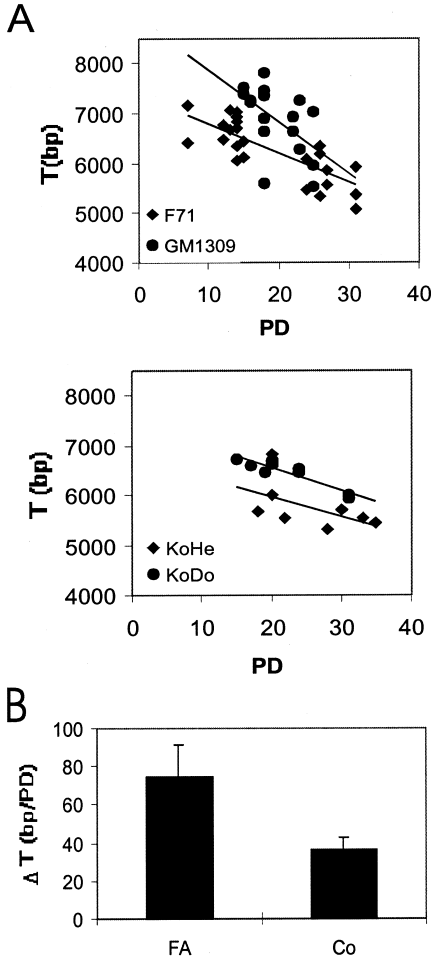

Fig. 1. Telomere shortening in FA and control fibroblasts. A: Average telomere length T (in bp) vs. PD for two FA fibroblast lines (top) and two control lines (bottom). Regression slopes are F71: −59 bp/PD, GM1309: −127 bp/PD, KoDo: −22 bp/PD, KoHe: −31 bp/PD. B: Mean telomere shortening (ΔT) in bp/PD in FA and control fibroblasts. The telomere shortening was measured in F71, 1424, FLB and GM1309 and in controls. There is a significant difference between the FA group and the control group with $P<0.05$.

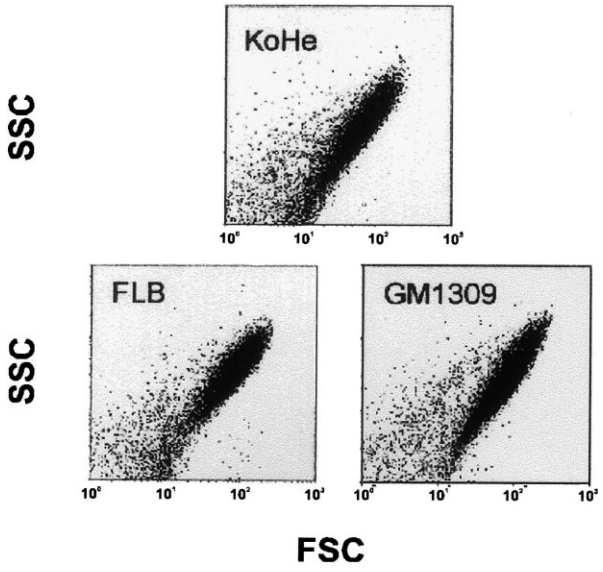

Fig. 2. Apoptosis in FA and control fibroblasts. Forward/sideward (FSC/SSC) scattergrams of control cells (KoHe) and two different FA lines (FLB and GM1309). Apoptotic cells are smaller (lower FSC) and more granular (higher SSC) than normal cells.

we measured the fraction of apoptotic cells in two of the FA strains and in one control strain (Fig. 2). No significant increase in the fraction of the apoptotic cells could be detected in any of the cells. This result could be confirmed by cell counting (data not shown).

With respect to the growth rate of the cells, FA cells tend to grow more slowly than fibroblast lines derived from healthy control individuals. Throughout the entire observation period growth was linear and the growth rate was estimated by linear regression (Fig. 3). Interestingly enough there was no evidence for an increased drop-out of FA cells from the cycling cells that would lead to forced replication and telomere loss in the remaining fraction as measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test and in the trypan blue assay (data not shown).

In FA PBMC, a higher telomerase activity than in control PBMC has been found [19]. Normal human fibroblasts are devoid of telomerase. In order to ensure that telomere shortening in FA fibroblasts is not modified by telomerase, we measured its activity using the TRAP assay (Fig. 4). Telomerase activity was not measurable in the four FA strains tested.

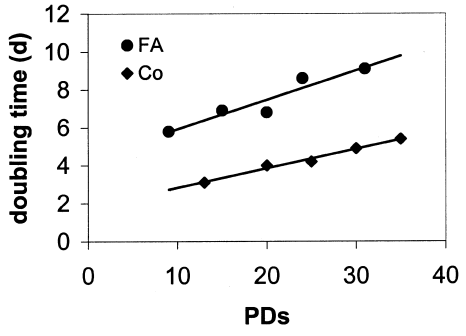

Fig. 3. Growth rate of FA and control fibroblasts. Growth (in PD) vs. time (in days) in five FA lines (●) and four control fibroblast lines (◆). Values are means of at least three independent experiments at clusters of five PDs over the entire observation period.

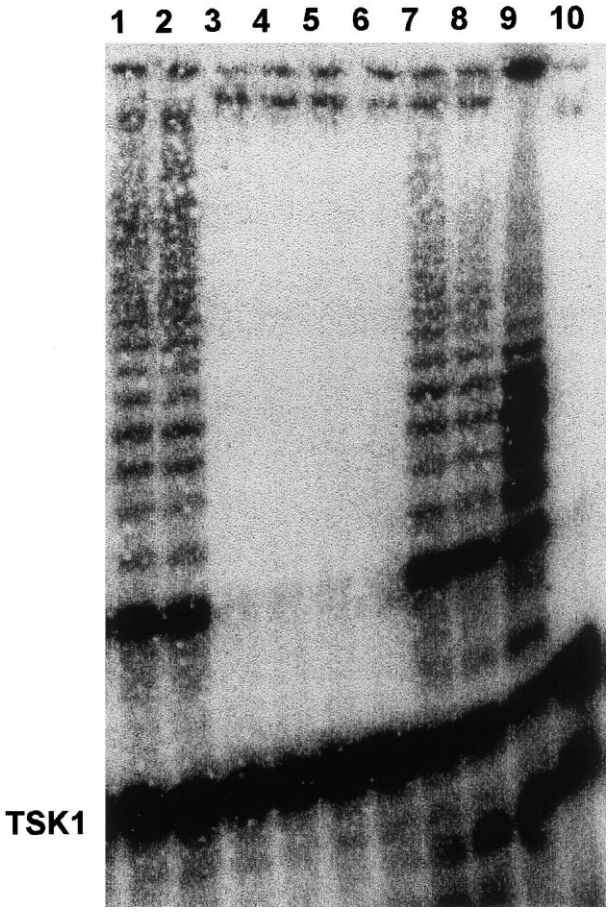

Fig. 4. TRAP assay. The intensity of the ladder pattern in relation to that of the TSK1 amplification standard is a semiquantitative indicator of telomerase activity. Lanes 1 and 2: Human BJ6te fibroblasts overexpressing the hTERT gene resulting in strong telomerase activity [36]. Lane 3: F71; lane 4: 1424; lane 5: FLB; lane 6: GM1309; lane 7: F71 mixed 1:1 with BJ6te; lane 8: GM1309 mixed 1:1 with BJ6te; lane 9: intensity standard (R8); lane 10: negative control.

That this negative result might have occurred due to the presence of a PCR inhibitor could not be confirmed by mixing experiments with fibroblasts expressing exogenous telomerase (Fig. 4, lanes 7 and 8).

4. Discussion

Our data show for the first time that FA fibroblast telomeres shorten faster than control fibroblast telomeres during in vitro growth. Since fibroblasts in culture recapitulate the lifespan of the individual from whom they were extracted [24,23] they are most suitable for a longitudinal study of telomere shortening. Without a corresponding change in the amount of telomere shortening per cell division, the accelerated rate of telomere shortening in FA PBMC might be ascribed to cell loss and cycle exit alone [19]. However, cultures of fibroblasts show no indication for further accelerated cell loss, neither was the rate of apoptosis increased nor was other evidence of cell decay observable.

The slower growth of FA fibroblasts compared to their normal counterparts (Fig. 3) is probably the result of spontaneous chromosomal breaks inducing a G2 delay [25,26]. Telomere loss in turn signals cell cycle arrest and chromosomal

instability as has been shown in yeast [27,28]. These findings emphasize that the accelerated telomere shortening rate seen in FA fibroblasts is not artifactual – that is, it does not occur as a result of cell loss from the cycling pool.

Our data suggest that the faster telomere shortening seen in FA fibroblasts reflects an accelerated telomere loss per cell division due to the underlying pathology of FA. Telomere shortening rates in somatic human cells depend on the interaction of oxidative stress and antioxidative defence. Increased oxidative stress accelerates telomere shortening [14,29,30]. Such an imbalance in oxidative metabolism was postulated for trisomy 21 [31] in which an accelerated telomere shortening was found in lymphocytes [32]. The relationship between oxidative stress and telomere length is most likely a result of a deficiency in telomeric base excision repair [20]. This results in the accumulation of single strand breaks in telomeres before DNA replication and the transfer of these breaks into shorter telomeres during replication [21,33].

The fibroblast cell culture system permitted, for the first time, the accelerated rate of telomere shortening in FA cells to be measured directly by consecutive analysis. Prior to this, leukocytes could be analyzed only at non-consecutive time periods. Moreover, in contrast to the analysis of blood cells drawn from patients (with the inherent time lapse), we were able to closely observe our cells in culture continuously. No significant signs of senescence were seen during the observation period nor was a substantial cell loss noticeable with viability assays. Excluding stress replication, the continuous acceleration of telomere shortening in FA fibroblasts strengthens the arguments discussed above, that factors such as oxidative stress may influence the telomere shortening process.

In FA, chromosomal instability and cell cycle delay may result from a series of events aggravating each other in a vicious circle: elevated ROS damages DNA, the repair of which may be impaired [34]. The resulting single strand DNA breaks accelerate telomere loss. The DNA breaks and telomere loss lead to cell cycle delay. Shortened telomeres with or without induction of chromosomal aberrations may cause malignancy and, at a critical length, may lead to cell death and pancytopenia [35]. This cascade of events occurring within the hematopoietic system may be responsible for the fatal outcome of this disease.

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