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Erosion of telomeric 3′-overhangs in white blood cells of aged subjects with high frequency of very short telomeres

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ABSTRACT

After extended proliferation, cells enter a state of replicative quiescence that is probably due to progressive telomere shortening. It is supposed that changes in telomere structure eventually expose the chromosome ends to undesired recombination events and thus promote cell senescence. The telomeric 3′-overhang is crucial for efficient chromosome capping, but its specific role in telomere shortening and in triggering the senescence program is uncertain. We have addressed this issue by measuring the 3′-overhangs of a human tissue cells aging *in vivo*. The 3′-overhangs were analyzed in blood samples from 41 individuals aged 91–106 years and 89 individuals ranging from 6 months to 85 years. We found that the overall 3′-overhang length did not significantly change with age, but did, however, find extensively eroded 3′-overhangs in 3 subjects of the 91–106 years cohort and one 61 years old subject affected with Down syndrome. These subjects had 3′-overhang length distributions skewed towards shorter tails, the shortest overall telomere lengths and the highest frequencies of very short telomeres. These data raise the possibility that during ageing very short telomeres with very poor 3′-overhangs can reach a critical point for functional telomeres.

1. Introduction

Aging is associated with a gradual decline in the performance of organ systems due to loss of cell function and reduced tissue regeneration. Such functional decline can result from the inability to replace damaged cells and to sustain cell replication that occurs either to balance normal cell loss or in response to injury. It has been known since the early 1960's that cultures of normal human cells, after a limited number of cell divisions, enter a state called replicative senescence that leads to the accumulation of non-dividing cells (Hayflick and Moorhead, 1961). The cell culture phenomenon was extensively used as a model to study human aging at a molecular and cellular level and many studies support the hypothesis that cellular senescence is due at least in part to the progressive telomere shortening that is observed in cultures of normal cells (Hayflick, 2000).

The telomeres are distinct DNA–protein structures that protect eukaryotic chromosome ends from degradation and inappropriate recombinations or fusions (Blackburn, 1991; Zakian, 1995). Human telomeric DNA is composed of many kilobases of $(TTAGGG)_n$ repetitive hexamers followed by a single-stranded overhang at the 3′-end of the G-rich strand that may be up to about 300 nucleotides long (Makarov et al., 1997; McElligott and Wellinger, 1997; Wright et al., 1997; Cimino-Reale et al., 2001). The telomeres will continuously shorten because of the end replication problem unless telomerase, a special ribonucleoprotein reverse transcriptase that adds TTAGGG repeats to the 3′-ends, stabilizes its length (Cech, 2004). When telomere shortening reaches a critical level, a DNA damage checkpoint mechanism may be activated that induces the cell to stop dividing (de Lange, 2002; d'Adda di Fagagna et al., 2003). Thus, telomere length serves as a biological regulator that participates to limit division potential of human cells (Harley, 1991).

The observation that telomere length does not always correlate with entry into replicative senescence, indicates that the relationships between telomere length, telomerase expression and replicative lifespan are more complex than previously believed (Karlseder et al., 2002; Stewart et al., 2003; Masutomi et al., 2003). It has been suggested that the telomeres can change from a normal

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capped to an abnormal uncapped state that will expose the chromosome end to unwanted recombinational events (de Lange, 2002; Blackburn, 2000). A functional 3′-overhang seems to be crucial to regulate the cell proliferation by promoting third order structure able to form an efficient cap critical for sealing the chromosome end (Blackburn, 2001). Human 3′-overhang is much longer than the expected 9–12 nucleotides that would result from removal of the final primer of the lagging-strand synthesis. A longer and more variable overhang would result if the end of the last priming event did not take place at the very end (Verdun and Karlseder, 2007). A blunt end is expected if leading-strand synthesis proceeds to the very end of the telomere or might leave a 5′-overhang if the replication machinery falls off prematurely (Cimino-Reale et al., 2003a). Because overhangs are present on both chromosome ends, the leading-strand telomere has to be subjected to some form of DNA processing that, if telomerase is inefficient, drives telomere shortening (Gilson and Géli, 2007). Since it was demonstrated that the 5′-ends terminate with ATC, it has been hypothesized that a post-replicative resection mechanism has to operate in order to generate overhangs on the leading-strand (Cimino-Reale et al., 2003b; Sfeir et al., 2005).

It seems then that the cell machinery, in generating functional 3′-overhangs, dictates the rate of telomere shortening and replicative senescence. A proposal has been advanced that their loss could be the real trigger of replicative senescence (Stewart et al., 2003; Hashimoto et al., 2005). However, evidence is mounting that it is a subset of critically short telomeres that determine the cell viability and chromosome stability rather than the overall telomere length (Hemann et al., 2001; Karlseder et al., 2002; Zou et al., 2004). Notwithstanding the considerable advances of knowledge, the role of the 3′-overhang in replicative senescence is far from being clarified and conflicting results are often reported (Stewart et al., 2003; Hashimoto et al., 2005; Chai et al., 2005).

The vast majority of studies addressed to clarify the specific role of the 3′-overhang in telomere shortening and eventually in triggering the senescence program were conducted *in vitro* cultured cells. In order to study 3′-overhang processing *in vivo* in relation to aging, we analyzed the telomeres of leukocytes in a series of subjects with ages varying from 6 months to 106 years. Thus, we have carried out a perspective life-long analysis of telomeric 3′-overhangs in a human tissue like blood and we provide some evidence on the *in vivo* state of the telomeres present in, *bona fide*, senescent cells. We measured the 3′-overhang lengths of peripheral white blood cells in a group of 41 aged subjects ranging from 91 to 106 years old and, for comparison, in a group of subjects (89 subjects) from 6 months to 85 years old. We found that while the length of the 3′-overhang remains quite constant throughout the life span and does not seem to correlate with the well-known telomere shortening, a dramatic drop of its length was observed in some old subjects. In fact, we found considerably eroded 3′-overhangs in 3 subjects of the 91–106 age group. Furthermore we found that the subjects with very short 3′-overhangs had the highest frequency of very short telomeres. Interestingly, we found that one subject of 61 years had very short 3′-tails similar to the oldest subjects. Clinical analysis revealed that this person was affected by Down syndrome, thereby has to be considered very old (Vaziri et al., 1993).

2. Materials and methods

2.1. Sample collection

Blood samples were collected from 130 unrelated subjects. 41 of them were "Oldest Old" individuals, aged 91–106 (mean age: 96.8). Eighty-five years and older is the group considered by gerontologists to be the "Oldest Old" (Suzman et al., 1992). The subjects were selected among the registered residents of Rome according to their year of birth. Their life habits and health status were evaluated with a direct interview, conducted at the subject's residence by physicians with geriatric training. The questionnaire was aimed at various topics of general health and well-being. The comparison group (89 subjects), named "general population" (GP), aged from 6 months to 85 years was randomly selected without regard to health or disease status from our DNA collection. All subjects gave written informed consent.

DNA was extracted according to a protocol for obtaining high molecular weight DNA (Sambrook et al., 1989). DNA preparations were monitored for quality as well as quantified by agarose gel electrophoresis before using in the study. All DNA preparations resulted of good quality.

2.2. Telomeric restriction fragments (TRFs)

Telomere length was determined by TRF Southern blot analysis. DNA aliquots of 2 μg were double digested overnight with 5 U of RsaI and HinfI. Digested DNA fragments were separated in 0.7% agarose gel in 0.5× TBE buffer at 40 V for 40 h and transferred to nylon membrane filters (Hybond N+). The telomeric probe $(CCCTAA)_3$ was [γ ^{32}P]-ATP 5′-end labeled using T4 polynucleotide kinase. The filters were pre-hybridized and hybridized in Quick Hybridization solution (Stratagene) at 50 °C for 1 h, washed in 2× SSC/0.1% SDS for 10 min at room temperature, in 0.2× SSC/0.1% SDS for 20 min at 50 °C and in 0.1× SSC for 5 min at room temperature. After autoradiography the image was analyzed by a 1D Image Analysis (Kodak Digital Science). Both the median, the distribution of telomere lengths (shown as the cumulative fraction curve) and the frequencies of telomeres shorten than 6 kb were calculated as previously described (Ouellette et al., 2000) using the program TELORUN (available at http://www4.utsouthwestern.edu/cellbio/shay-wright/research/sw_lab_methods.htm).

2.3. Telomeric oligonucleotide ligation assay (t-OLA)

t-OLA was performed as previously described with modifications (Cimino-Reale et al., 2001). Telomeric 12-mer oligonucleotides $(CCCTAA)_2$ were end-labeled and completely phosphorylated by the enzyme OptiKinase (USB Corporation) in a forward reaction aimed to reach very high specific activity. A mix of 25 μl containing 5 pmol of oligonucleotide and 10 pmol of [γ ^{32}P]-ATP (10 mCi/ml), 50 mM TRIS pH 7.5, 10 mM $MgCl_2$, 5 mM DTT and 10 U of OptiKinase was incubated for 40 min at 37 °C. In order to obtain the highest phosphorylation possible, 1 mM of ATP was then added and the reaction was continued for 20 min. Oligonucleotides were sodium acetate/ethanol precipitated and dissolved in an appropriate volume of water. Hybridization and ligation reactions were conducted in a volume of 20 μl containing 5 μg of undenatured high molecular weight DNA, 0.5 pmol of labeled oligonucleotide probe, 50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 25 μg/ml BSA, 400 U of T4 DNA ligase. All these ingredients were placed into 0.5 ml Eppendorf tubes and incubated at 34 °C for 12–14 h. Reactions were ended by adding 30 μl of water and by phenol/chloroform extraction. Samples were precipitated with ethanol and dissolved in 6 μl of TE. An aliquot of 3 μl was mixed to 4 μl of 95% formamide stop solution. Samples were heated at 95 °C and immediately quenched in ice before loading 2 μl onto the gel and run on denaturing 6% acrylamide sequencing gels. Electrophoresis was conducted for 40 min at 2000 V in 0.5× TBE buffer. Then, the gel was dried and exposed at −70 °C to autoradiography film. An aliquot of the ligation reaction were run in agarose gel and referred to for normalization. The images were acquired by 1D Image Analysis Software (Kodak Digital Science). Since the intensity of each band depends on both the frequency of the specific tail and on its length, the intensity (background subtracted) of each band of the ladder was divided by the number of concatenated oligonucleotide probes expected to be in the band so that the resulting relative intensities would be proportional to the relative tail frequency. This value was then normalized to the total intensity to obtain a relative frequency for each length of the tails and plotted both as relative frequency and as length distribution of the tails. The sum of all the t-OLA products of each sample, normalized by the DNA loaded in the reaction as visualized by the EtBr stained gel, represents the overall amount of the 3′-overhang.

2.4. Solution hybridization and non-denaturing gel electrophoresis

To validate t-OLA results, all samples were subjected to a simplified method of the conventional non-denaturing hybridization assay. Indeed, t-OLA is based on hybridization of the $(CCCTAA)_2$ probe to native DNA thereby it is expected that the fraction of bound probe is proportional to the amount of the 3′-overhang. In brief, the remaining 3 μl of the reaction were diluted in water and in glycerol loading buffer to a final volume of 10 μl and run in 1% agarose gel for 1 h at 90 V in 1× TAE buffer. To quantify the amount of DNA and the bound probes in each sample, the gels were stained with ethidium bromide, photographed, dried on nylon membrane (Stratagene) and exposed to autoradiography film. The total amount of 3′-overhangs for each sample was obtained by normalizing total hybridization signal to amount of DNA as detected by BrEt stained gel.

2.5. Statistical analysis

The differences in values were assessed for significance with Student's *t*-test. Values ± SEM are indicated. The Pearson coefficient of the linear regression lines was calculated.