Telomere Erosion Varies during in Vitro Aging of Normal Human Fibroblasts from Young and Adult Donors

Reynel Figueroa,2 Heike Lindenmaier, Manfred Hergenhahn, Kirsten Vang Nielsen, and Petra Boukamp

Divisions of Carcinogenesis and Differentiation [R. F., H. L., P. B.] and Genetic Alterations in Carcinogenesis [M. H.], German Cancer Research Center, D-69120 Heidelberg, Germany, and DAKO A/S, DK-2600 Glostrup, Denmark [K. V. N.]

Abstract
The life span of normal fibroblasts in vitro (Hayflick limit) depends on donor age, and telomere shortening has been proposed as a potential mechanism. By quantitative fluorescence in situ hybridization and Southern blot analysis, we show progressive telomere loss to about 5 kb mean telomere restriction fragment length in fibroblasts from two adult donors within 40 population doublings, whereas in fibroblasts from two infant donors, telomere erosion is reduced, leaving a mean telomere restriction fragment length of ~7 kb at senescence (after ~60 population doublings). Aging immortal cells from both infant and adult donors was not accompanied by chromosomal abnormalities but was correlated with increased telomere repeat-binding factor 2 expression at both the protein and transcriptional level.

Introduction
Telomeres are highly conserved sequences at the ends of the chromosomes. They consist of a large number of tandem repeats that are made up of (TTAGGG)n repeats in humans and other vertebrates (1). Due to the inability of DNA polymerase in replication of the outermost ends of the lagging strand DNA (the “end replication problem”) (2, 3), telomeres are believed to shorten with each round of replication by approximately 50–200 bp. Indeed, telomere shortening has been observed in cultured fibroblasts and in vivo in tissues from donors of different ages (Ref. 4 and the references therein).

Many immortal cell lines and tumor cells, on the other hand, have developed mechanisms to avoid telomere erosion by activation or up-regulation of telomerase activity, the ribonucleoprotein complex that is able to de novo add telomeric sequences (for review, see Ref. 5). In addition, some of the immortal cell lines and a small fraction of tumor cells have developed an alternative and as yet unknown pathway to maintain telomeres (6). These cells lack detectable telomerase activity but nevertheless are characterized by long telomeres.

Olovnikov (7) was the first to propose that progressive shortening of chromosomes in dividing somatic cells eventually leads to irreversible cell cycle exit. This telomere hypothesis of cellular senescence is now widely accepted; however, detailed kinetics of telomere erosion is regulated with aging, and we suggest that this up-regulation is closely linked to the aging process.

Materials and Methods
Culture and Treatment of Human Fibroblasts. Fibroblasts established from human foreskin or trunk skin were routinely cultured in DMEM containing 10% FCS and antibiotics. For passaging, cells were detached by incubation in 0.05% EDTA/0.025% trypsin (37°C, 5 min) and replated at a split ratio of 1:10 at weekly intervals or at a ratio of 1:5 when confluent. For antisense treatment, the cells were plated in 6-well dishes (1 × 10^5 cells/well) and treated with 2 µM TRF-2 antisense oligonucleotide or 2 µM control oligonucleotide for 72 h. The digested products were subjected to electrophoresis on a 0.7% agarose gel, blotted on a positively charged nylon membrane (Boehringer Mannheim, Germany), and neutralized (1.5 M NaCl/0.5 M Tris-HCl and 10 mM EDTA (pH 7.2)) for 30 min at room temperature, and the DNA was cross-linked to the membrane by UV light (Stratalinker; Stratagene, Heidelberg, Germany). The membranes were prehybridized (5× SSC, 0.1% lauryl sarcosine, 0.02% SDS, and 1% 10× blocking reagent; Boehringer Mannheim) at 68°C for 30 min, hybridized for 12–18 h at 68°C with 20 ng/ml digoxigenin-labeled telomeric human DNA probe (Oncor Appligene, Heidelberg, Germany); washed in 4× SSC and 1% SDS for 10 min, washed in 2× SSC and 0.1% SDS for 5 min, and washed twice in 0.1× SSC and 0.1% SDS. Detection was performed using the CDP-Star kit (Boehringer Mannheim).
recorded as a graph with increasing intensity values on the X axis and number of signals on the Y axis. The integrated fluorescence intensity of individual telomeres is expressed in arbitrary units, and all intensity values were ranged into 30 intensity classes.

**Indirect Immunofluorescence.** Cells grown on coverslips were fixed and stained with rabbit antibodies against TRF-1 and TRF-2 (at a dilution of 1:500; generously provided by T. de Lange; Rockefeller University, New York, NY) as described previously (10). The secondary Cy3-labeled antirabbit antibody (Jackson ImmunoResearch Laboratory, Dianova, Hamburg, Germany) was used at a dilution of 1:500 for 20 min at 37°C, followed by 20 min at room temperature. Nuclei were viewed under a Leica microscope (Leitz, Wetzlar, Germany) equipped with epifluorescence optics using a ×100 objective.

**Western Blot Analysis.** Cells were grown to semiconfluence, washed, and trypsinized. To measure protein level on the basis of equivalent cell numbers, 10⁵ cells were lysed in 100 μl of 2× Laemmli buffer, denatured for 5 min at 95°C, and left frozen at −20°C until use. When measured on the basis of equivalent protein concentrations, 2–4 × 10⁵ cells were lysed in 100 μl of radioimmunoprecipitation assay buffer [10 μl of 1 M Tris (pH 7.2); 300 μl of 0.5 M NaCl; 10 μl of Triton X-100; 10 μl of 10% SDS; 10 μl of 10% sodium deoxycholate; 50 μl of 100 mM EDTA; 610 μl of H₂O; 1 μl each of aprotinin, leupeptin, and pepstatin (1 μg/μl); and 5 μl each of vanadate (100 mM) and Pefabloc (1 μg/μl)], and the protein concentration was determined by using BCA Protein Assay Reagent A (Pierce, Rockford, IL). Either 20-μl aliquots or 20 μg of protein in a total volume of 30 μl of 1× Laemmli buffer, respectively, were separated by SDS-PAGE and blotted onto nitrocellulose membranes. Filters were blocked for 2 h in 10% low-fat milk (in PBS with 0.1% Tween 20) and incubated with rabbit anti-TRF-1 and TRF-2, respectively (at a dilution of 1:1,000) for 3 h at room temperature. Filters were washed with blocking buffer, incubated with a horseradish peroxidase-conjugated goat antirabbit antibody (1:10,000; Jackson Immunoresearch Laboratory, Dianova, Hamburg, Germany) was used at a dilution of 1:500 for 20 min at 37°C, followed by 20 min at room temperature. The secondary Cy3-labeled antirabbit antibody (Pierce, Rockford, IL) was applied (1:10,000; Jackson Immunotech, Hamburg, Germany) in blocking buffer for 1 h at room temperature, washed, and developed by using enhanced chemiluminescence reagents (Amersham Buchler, Braunschweig, Germany).

**RT-PCR.** RNA was isolated from early and late passage fibroblasts by using QIAshredders and the RNeasy Qiagen Minikit (Qiagen GmbH, Hilden, Germany). RT-PCR was performed using the Omniscript RT-Kit and PCR with HotStar Taq polymerase (both from Qiagen). Primer sequences for TRF-1 (636 bp) were 5′-TGTGCG-GATGTAGGATGC-3′ and 5′-GGGCTGATTCCAAGGGTGTA-3′, primer sequences for TRF-2 (636 bp) were 5′-AGTCAATCGCTGGTG-GCTCA-3′ and 5′-CCTGGTGCTGGGTGGTATC-3′, and primer sequences for β-actin (244 bp) were 5′-GAAGTGCGTGCACGAC-3′ and 5′-CAACGAGGATGAGCAGT-3′.

**Results and Discussion**

It has been shown previously that the high-affinity telomeric PNA probe is optimally suited for Q-FISH analysis of telomeres (9, 11). In our hands, the Cy3-labeled PNA probe (PNA Telomere FISH Kit K5326; DAKO A/S, Glostrup, Denmark) gave the most intense and reproducible FISH signals when compared with other hybridization probes (data not shown) and was therefore used throughout this study. Several reports indicated that telomeres are stable in telomerase-positive cell lines (for review, see Ref. 12). To confirm this in our laboratory, we first determined the telomere status of the immortal telomerase-positive HaCaT skin keratinocyte cell line (13, 14). Q-FISH analysis demonstrated a rather narrow signal distribution pattern, with most signals being of low intensity (Fig. 1A) and corresponding to a mean TRFL of 4–5 kb as determined by Southern blot analysis (data not shown). This distribution was characteristic for all metaphases within a given population as well as after cloning and long-term passage (see Fig. 1A). Fetal human fibroblasts, on the other hand, reportedly exhibit long telomeres. Also, our investigation of a secondary culture of fetal human fibroblasts by Q-FISH analysis revealed that the majority of telomere signals were of high intensity. In addition, these cells were characterized by a wide range of signal intensities (Fig. 1B), demonstrating a much more heterogeneous distribution of telomere lengths than seen in the HaCaT cells.

To study telomere loss during in vitro propagation and aging, we passaged skin fibroblasts from two adult (25 and 33 years) and two infant donors (both 1 year old) to cellular senescence and analyzed their telomere length at different passages. Senescence was defined by morphological changes, i.e., an increase in cell size, irregular shape, increased levels of β-gal (pH 6.0) staining (Ref. 8; Fig. 2, A and B), and terminal growth arrest. Previous studies suggest that telomeres

![Fig. 1. Telomere length regulation in immortal cells and fibroblasts from donors of different ages.](cancersres.aacrjournals.org)
shorten in normal human somatic cells with continuous passaging at a rate of 50–200 bp with each population doubling, implying an approximately linear loss of telomere sequences during aging in vitro (Ref. 4 and the references therein).

**Significant Telomere Loss in Fibroblasts from Adult Donors.** The two fibroblast strains from the adult donors, AII and AIII, remained proliferatively active for 11 and 14 passages, respectively (35 and 42 population doublings). Q-FISH analysis of early-passage cells revealed a broad distribution of signal intensities (Figs. 1C and 2C). With passaging, the number of high-intensity signals steadily decreased (Fig. 2D), and the number of undetectable telomeres increased from 18 in early passages to >40 in passages close to senescence (see Fig. 1C). Thus, the number of very short telomeres, which were only detectable when the exposure time was extended, increased from about 11% to 22%. This pattern of quite uniform telomere erosion was highly reproducible, as shown by repeat experiments with cells from the same fibroblast strain as well as experiments with fibroblasts from the second donor and confirmed by Southern blot analysis. The final mean TRFL was estimated to be ~5 kb (data not shown).

**Reduced Telomere Loss in Foreskin Fibroblasts from Young Donors.** Whereas fibroblasts from adult donors exhibited a rather short life span in vitro, the fibroblasts from the two infant donors, VH7 and VH12, could be maintained in a proliferatively active state for >20 passages (>65 population doublings). Q-FISH analysis of these cells revealed a heterogeneous telomere profile for early-passage cells similar to that observed for fibroblasts from adult donors (Fig. 1D). However, during passaging, an unexpected “stable” telomere profile was seen (see Fig. 1D and Fig. 2, E and F). The shift of the major signal peak as well as the increase in very low signal

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**Fig. 2. Characterization of aging fibroblasts.** A and B, phase-contrast micrographs after staining of the cells for β-gal (pH 6.0; Ref. 8; blue) in an early-passage (A) and a late-passage (B) fibroblast culture. C–F, FISH analysis of metaphases from fibroblasts hybridized with the PNA telomeric probe (K5326; DAKO). C and D, AIII fibroblasts (33-year-old donor) from passage 2 (C) show signals at all telomeres, whereas when the cells were close to senescence (D, passage 13), significantly fewer telomeres are seen. E and F, VH12 fibroblasts (1-year-old donor) show intense telomere signals at almost all telomeres at passage 4 (E) and passage 20 (F).

**Fig. 3. Differential staining pattern of TRF-1 and TRF-2.** Immunostaining of TRF-1 and TRF-2 in VH12 fibroblasts (1-year-old donor). TRF-1 is detectable as the typical punctated nuclear staining at all passage levels (A; passage 4 cells are shown), whereas the intensity of TRF-2 staining of the same fibroblast strain increases from passage 9 (B) to passage 21 (C). All figures were taken using the same magnification and an exposure time of 4 s. Bar, 10 μm.
intensities was modest, and the number of undetectable telomeres increased only moderately from <10 in early-passage metaphases to ~20 close to senescence, leaving ~90% of the telomeres visible under standard conditions that allowed the detection of all HaCaT telomeres and ~80% of telomeres of the fibroblasts from adult donors (Fig. 2, E and F). In agreement with these results, Southern blot analysis revealed only a minor loss of telomere DNA, leaving a final mean TRFL of ~7 kb (data not shown). This difference in visible telomeres from infant and adult donors is compatible with the results of a previous study in which lymphocytes from young donors and adult donors were compared using the primed in situ labeling method (15). In addition, it was shown by Frenck et al. (16) that the rate of telomere loss varied with age in hematopoietic cells. They reported a rapid loss in very young children, a plateau between the age of 4 years and adulthood, and gradual loss again later in life. Together, these findings lend support to their hypothesis that at different stages of maturation in vivo, the cells may differ quantitatively or qualitatively. With regard to dermal fibroblasts, we suggest that during infancy, i.e., a phase of rapid growth, factor(s) are active that are able to minimize telomere erosion, whereas these factor(s) are ineffective (down-regulated or inhibited) in adults. Whereas this is easily detectable under “forced” proliferation in vitro, it may not be as evident in the dermis in vivo because proliferation of fibroblasts is minimal in adults.

**TRF-2 is Up-Regulated with Aging in Vitro**. In the search for possible candidates involved in telomere stabilization in fibroblasts from young donors, we first examined and were able to exclude reactivation of telomerase activity at any point during fibroblast cultivation by using the telomeric repeat amplification protocol assay (data not shown). Next we investigated the two double-stranded TTAGGG repeat-binding proteins, TRF-1 and TRF-2, because they are believed to be involved in telomere stabilization (10, 17, 18). Whereas TRF-1 is presumed to be a negative regulator of telomere length, TRF-2 is suggested to play a key role in protecting the chromosome ends from end-to-end fusions. Punctated nuclear staining was easily detectable for TRF-1 at all passage levels by immunohistochemistry (Fig. 3A). TRF-2 staining, on the other hand, was faint in early passages (Fig. 3B) but was significantly increased in later passage cells (Fig. 3C), and the increase appeared to parallel the pattern of telomere erosion. This increase was also verified by Western blot analysis. TRF-2, present as a distinct band of Mr 56,000, steadily increased up to about 15-fold in late-passage fibroblasts as compared with early-passage fibroblasts from adult and infant donors, and this was irrespective of whether protein loading was normalized for cell number or protein concentration (Fig. 4A). The increase in protein was also accompanied by an increase in TRF-2 transcription, as verified by semiquantitative RT-PCR analysis (Fig. 4B). Whereas the level of TRF-1 transcription increased only slightly, if at all, in late-passage versus early-passage cells, a clear increase was seen for TRF-2 expression, suggesting transcriptional up-regulation of TRF-2 with aging.

To further substantiate the significance of TRF-2 up-regulation, we investigated the expression pattern of two other proteins: (a) the cyclin-dependent kinase inhibitor p21, which has been shown by other investigators to increase progressively throughout the life span of normal human fibroblasts (19); and (b) the tumor suppressor p53, which showed constant levels (20). Similar to TRF-2, p21 was induced in aged but still-proliferating cells, whereas p53 expression remained largely unchanged (see Fig. 4A). In summary, during the process of replicative senescence, the level of TRF-2 protein increases in human skin fibroblasts and does not appear to be displaced from the telomeres as suggested by the characteristic punctuated pattern also seen in interphase nuclei of late-passage fibroblasts when cells are stained with an antibody against TRF-2 (see Fig. 3C).

It is generally believed that senescence is accompanied by an increased number of chromosome aberrations (dicentric and ring chromosomes, and sister chromatid fusions; for review, see Ref. 21). It has recently been shown that loss of TRF-2 is responsible for such chromosomal changes (10, 22). We therefore hypothesized that due to the increasing amount of TRF-2 in the aging fibroblasts, such aberrations would not occur. In fact, when we screened for aberrant chromosomes, we detected chromosomal changes only rarely in metaphases from normal cells. All metaphases from late-passage fibroblasts analyzed here were numerically and structurally normal (see Fig. 2, D and F).

To determine the initial functional consequences of TRF-2 up-regulation, we sought to decrease the level of TRF-2 protein by treating late-passage VH12 fibroblasts (passage 18 represents ~60
population doublings) with TRF-2 antisense oligonucleotides. After 4 weeks of treatment, the level of TRF-2 protein was reduced by 20–40%, whereas the level of p53 remained relatively unchanged (Fig. 5C). In these cultures, we found an approximately 25% reduction in β-gal (pH 6.0) staining and a slight increase in cell number (data not shown). The most obvious change was in cell morphology. Big, odd-shaped cells characteristic of aging cultures and present in all controls (Fig. 5A) were rare; instead, elongated, more actively proliferating cells were observed (Fig. 5B). Q-FISH analysis of the telomeres from antisense oligonucleotide-treated or control cells (either treated with control oligonucleotides or untreated) showed the same telomere size distribution in all cultures (Fig. 5D). Thus, in contrast to the recent finding that complete inhibition of TRF-2 by a dominant negative TRF-2 construct caused telomeric fusions and rapid growth arrest or even apoptosis in certain cell types (10, 22), a slight reduction of TRF-2 in aging fibroblasts appeared rather to protract the aging process. Future studies will aim to more precisely characterize the role and function of TRF-2 in this process.

In conclusion, our studies demonstrated that the telomeres of the immortal telomerase-positive HaCaT cells were stable and relatively uniform in size, whereas normal human fibroblasts were characterized by a very heterogeneous distribution of telomere lengths and an age-dependent pattern of telomere erosion. Whereas fibroblasts from infant donors showed only minor telomere loss, fibroblasts from adult donors exhibited an approximately linear loss of telomeric sequences. TRF-2 protein levels, which were significantly up-regulated in late-passage fibroblasts, may be controlled by transcriptional regulation. Finally, because reduction of TRF-2 protein levels in late-passage fibroblasts was associated with diminished appearance of the characteristics of aged cells, TRF-2 up-regulation may be important in the aging process.

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References

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