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Telomere shortening during aging of human osteoblasts in vitro and leukocytes in vivo: lack of excessive telomere loss in osteoporotic patients

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Abstract

We have compared the telomere length, as assessed by Southern analysis, of telomere restriction fragments (TRFs) generated by *Rsa*I/*Hin*fI digestion of genomic DNA in: (i) in vitro cultured human trabecular osteoblasts undergoing cellular aging; and (ii) peripheral blood leukocytes (PBL) obtained from three groups of women: young (aged 20–26 years, $n = 15$), elderly (aged 48–85 years, $n = 15$) and osteoporotic (aged 52–81 years, $n = 14$). The mean TRF length in human osteoblasts undergoing aging in vitro decreased from an average of 9.32 kilobasepairs (kb) in middle-aged cells to an average of 7.80 kb in old cells. The rate of TRF shortening was about 100 bp per population doubling, which is similar to what has been reported for other cell types, such as human fibroblasts. Furthermore, there was a 30% decline in the total amount of telomeric DNA in senescent osteoblasts as compared with young cells. In the case of PBL, TRF length in the DNA extracted from young women was slightly longer (6.76 ± 0.64 kb) than that from a group of elderly women (6.42 ± 0.71 kb). A comparison of TRFs in the DNA extracted from the PBL from osteoporotic patients and from age-matched controls did not show any significant differences (6.47 ± 0.94 versus 6.42 ± 0.71 kb, respectively). Therefore, using TRF length as a marker for cellular aging in

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vitro and in vivo, our data comparing TRFs from osteoporotic patients and age-matched controls do not support the notion of the occurrence of a generalized premature cellular aging in osteoporotic patients. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Aging; Telomere; Osteoblasts; Osteoporosis

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1. Introduction

The cellular and molecular basis of bone loss associated with aging and with involutional osteoporosis is not completely understood. Histomorphometric studies performed on human bone biopsies have demonstrated a decrease in bone formation during aging (Lips et al., 1978; Cohen-Solal et al., 1991). Furthermore, this reduction in bone formation occurs to a higher extent in osteoporotic patients as compared with age-matched controls (Eriksen et al., 1990; Cohen-Solal et al., 1991). Possible pathogenetic factors responsible for this phenomenon could be premature loss of proliferative capacity and accelerated cellular aging in osteoporotic patients.

During the past few years, several studies have demonstrated that the loss of proliferative capacity of various types of normal human cells is related to random shortening of their telomeres during repeated cell division (Harley et al., 1990; Harley, 1991; Allsopp et al., 1992). To recount, telomeres are nucleoprotein complexes at the ends of chromosomes, consisting of tandem arrays of TTAGGG nucleotide repeats of up to 10–15 kilobasepairs (kb). They are essential for chromosomal stability and for preventing degradation and abnormal chromosomal recombinations (Blackburn, 1994; Greider, 1994). During each cell division, the replication of linear chromosomal DNA by DNA polymerase necessarily leads to a loss of some of the terminal telomeric sequences due to the intrinsic biochemical nature of the DNA replication process (Levy et al., 1992). Although this loss of telomeric repeats can be compensated by de novo synthesis, either involving telomerase enzyme or by non-telomerase-dependent pathways, it has been observed that normal diploid cells generally lack telomerase activity and progressively lose telomeres on each round of cell division both in vitro and in vivo (Harley, 1991; Blackburn, 1994). In contrast, most of the immortal cancer cell lines have been reported to have telomerase activity, by virtue of which, they maintain their telomere length indefinitely (Harley, 1991; Levy et al., 1992). Therefore, it has been suggested that shortened telomeres signal a growth check-point that triggers irreversible cell-cycle arrest, manifested as proliferative senescence (Harley, 1991; Levy et al., 1992). This was recently supported by studies showing that the maintenance of telomeres by transgenic telomerase activity delays cellular aging (Bodnar et al., 1998). Furthermore, it has been suggested that some of the genes responsible for cellular senescence may be located in the subtelomeric region, and that they are activated when the telomere region has reached a certain critical length (Gottschling et al., 1990; Sprung et al., 1996).

Recently, we have characterized a system of serially passaged normal human trabecular osteoblasts in culture that exhibited a typical Hayflick phenomenon of cellular aging (Kassem et al., 1997). The phenomenon of cellular aging in vitro is a very well established experimental model system to study various cellular, biochemical and molecular aspects of aging (Rattan, 1995; Campisi, 1996). We have reported that cultured osteoblasts exhibited a limited proliferative lifespan and showed several characteristics of cellular senescence, including decreased rates of RNA and DNA synthesis and osteoblast-specific proteins (Kassem et al., 1997). We have also demonstrated that osteoblast cell strains established from an osteoporotic patient had a severely reduced proliferative capacity in vitro (Kassem et al., 1997). Here, we report the results of our analysis of the mean telomere length in normal human trabecular osteoblasts undergoing aging in vitro. Furthermore, we have also tested the hypothesis of the occurrence of accelerated cellular aging in osteoporotic patients as compared with age-matched controls (Lips et al., 1978; Eriksen et al., 1990; Cohen-Solal et al., 1991). As the phenotype of accelerated aging may be observable in terms of an excessive shortening of telomeres in all proliferative cell pools, we employed peripheral blood leukocytes (PBL), in which an age-related loss of telomeres is a well established phenomenon (Hastie et al., 1990; Oexle et al., 1997), and compared telomere length in a group of osteoporotic women with a group of age-matched controls.

2. Materials and methods

2.1. Cell culture

The human trabecular osteoblast cell strain used in this study was the same as described previously (Kassem et al., 1997). This cell strain of normal diploid osteoblasts, designated K73, was established from a 73-year-old normal woman. Establishment of primary cultures was performed as previously (Kassem et al., 1993). In brief, trabecular bone samples were washed extensively in phosphate buffered saline (PBS), minced and digested with crude collagenase IV (1 g/l) in Dulbecco's minimal essential medium (DMEM) for 2 h at 37°C. These fragments were cultured in DMEM supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were allowed to grow out from the bone explant and to form a confluent monolayer. At confluence, the cells were passaged by trypsinization at a split ratio of 1:2. The number of cells in confluent cell layers was counted using a Coulter Counter (Coulter Electronics, UK) and the number of population doublings (PDs) was calculated as $\log N/\log 2$, where N is the number of cells in a confluent cell layer divided by the initial number of cells seeded. This cell strain was maintained for 451 days, during which it underwent 31 cumulative PDs (Kassem et al., 1997). In order to make comparisons among different cell strains, percentage lifespan completed was calculated from the proliferative lifespan (cumulative population doubling level, CPDL) attained by the cell strain.

2.2. Cytochemical staining for β-galactosidase

Cells were washed in PBS, fixed for 3–5 min (room temperature) in 2% formaldehyde/0.2% glutaraldehyde. The cells were incubated at 37°C with fresh β-galactosidase stain solution (1 mg of 5-bromo-4-chloro-3-indolyl β-D-galactoside per ml, 40 mM citric acid/sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM $MgCl_2$). Staining was evident in 2–4 h and was maximal in 12–16 h (Dimri et al., 1995).

2.3. Extraction and restriction enzyme digestion of genomic DNA

Genomic DNA was extracted from in vitro serially passaged human osteoblasts at seven different points covering the entire lifespan of the cells, using a commercial kit (Puregene, Gentra Systems, USA). Blood samples were obtained from 15 normal young women (20–26 years), from 15 normal elderly women (48–85 years) and from 14 osteoporotic patients (52–81 years). Osteoporosis was defined as the presence of at least one non-traumatic vertebral fracture. Moreover, lumbar bone mineral density (BMD) (g/cm^2) and femoral neck BMD in the group of osteoporotic women was 0.614 ± 0.095 and 0.572 ± 0.093, respectively, compared with 0.926 ± 0.187 and 0.755 ± 0.057 in the group of age-matched controls. Total leukocyte DNA was extracted using a standard phenol/chloroform method. Undigested DNA was quantified by absorption measurements and then digested simultaneously with *Hin*fI/*Rsa*I (1–3 units/μg DNA) for 3–24 h at 37°C. Integrity of the DNA and completeness of the digestion was monitored before and after digestion by agarose gel electrophoresis.

2.4. Southern blot hybridization

Electrophoresis of 1 μg digested genomic DNA was performed in 0.5% agarose gels at 20 V for 24 h. Hybridization of oligonucleotide probes to dried gels was based on a modification of the procedure described by Mather (1988). In brief, gels were dried under vacuum at 60°C for 45–60 min, denatured in 0.5 M NaOH and 1.5 M NaCl for 10 min and then neutralized in 0.5 M Tris, pH 8, and 1.5 M NaCl for 10 min at room temperature. Gels were then immersed in a standard hybridization solution (5 × Denhardts solution, 5 × SSC, 0.1 M Na_2HPO_4, 0.01 M $Na_4P_2O_7$, 33μg of salmon sperm DNA per ml, and 0.1 μM adenosine triphosphate) which contained the telomeric probe, [^{32}P]-labeled $(CCCTAA)_3$, (250000 cpm/ml). Hybridization was performed at 37°C for 12–16 h. Finally, gels were washed in 0.24 × SSC at room temperature. Mean telomere restriction fragment (TRF) length was determined from densitometric analysis of autoradiograms as described by Harley et al. (1990), or the dried gels were directly analyzed by a phosphor imager (KEBO Lab).

2.5. Statistical analysis

Differences between groups were analyzed using the unpaired Student's t-test. The relationship between TRF length and osteoblast age in vitro, and between TRF length and in vivo bone mass measurements, were performed using simple regression analysis.

3. Results

3.1. Telomere shortening during serial passaging

Cultured trabecular osteoblasts exhibited a characteristic phenotype of mature osteoblasts, including production of alkaline phosphatase, osteocalcin and high levels of type I collagen. Serial passaging of the cells showed a typical Hayflick phenomenon of cellular aging together with a wide variety of cellular, biochemical and molecular alterations, as described in detail previously (Kassem et al., 1997). Furthermore, as β-galactosidase activity has been shown to be a marker of cellular senescence in vitro for fibroblasts (Dimri et al., 1995), we stained early and late passage osteoblast cultures for the presence of this enzyme (Fig. 1). A comparison of young and old cells showed that less than 10% of cells in the young culture were β-galactosidase-positive, while more than 95% of cells in the old culture exhibited positive blue staining, confirming that β-galactosidase activity can also be used as a marker of senescent osteoblasts (Fig. 1).

After demonstrating that osteoblasts underwent in vitro aging, we examined mean TRF length in human trabecular osteoblasts in relation to their in vitro age. Genomic DNA obtained from serially passaged osteoblast cultures, at seven points covering their replicative lifespan (CPDL 31), was analyzed for the size distribution of TRFs by Southern blot analysis (Fig. 2). The mean TRF length decreased as a function of PD level, from an average of 9.32 kb in middle-aged cells (52% lifespan completed) to an average of 7.80 kb in old cells (97% lifespan completed). On per cell division basis, the rate of telomere shortening was about 100 bp per PD (Fig. 3). Furthermore, the total amount of telomeric DNA, as measured by the signal intensity, was also found to decrease significantly with increasing cellular age in the culture, reaching a value of about 70% in senescent cells, as compared with the amount of telomeres in young cells, indicating a true loss of DNA and not just rearrangements of restriction sites in the telomeric region.

3.2. Telomere length in PBL from osteoporotic patients and age-matched controls

Having established that mean TRF length is a useful marker of osteoblast aging, we examined TRFs in PBL from osteoporotic patients and age-matched controls. Fig. 4 shows the Southern blot analysis (Fig. 4a) and the quantitative analysis (Fig. 4b) of mean TRF length for each of the three groups of women: young, normal elderly and age-matched elderly osteoporotic patients (clinical criteria are described

in Section 2.3). Mean TRF length in PBL from young women was 6.76 ± 0.64 kb (mean $\pm$ standard deviation) as compared to 6.42 ± 0.71 kb in the group of elderly women, which is equal to a small and not statistically significant decrease of about 100 bp per decade of life. Comparing TRFs from osteoporotic patients and age-matched controls showed no statistically significant difference between the two groups (6.47 ± 0.94 versus 6.42 ± 0.71 kb, respectively; Fig. 4). Furthermore, no statistically significant correlations were found between bone mass measurements at various sites (spine, femoral neck, total body) and TRF length in normals or osteoporotic patients (data not shown).

4. Discussion

In this study, we have employed serially passaged normal human trabecular osteoblasts as an in vitro model for cellular aging. In addition to the typical

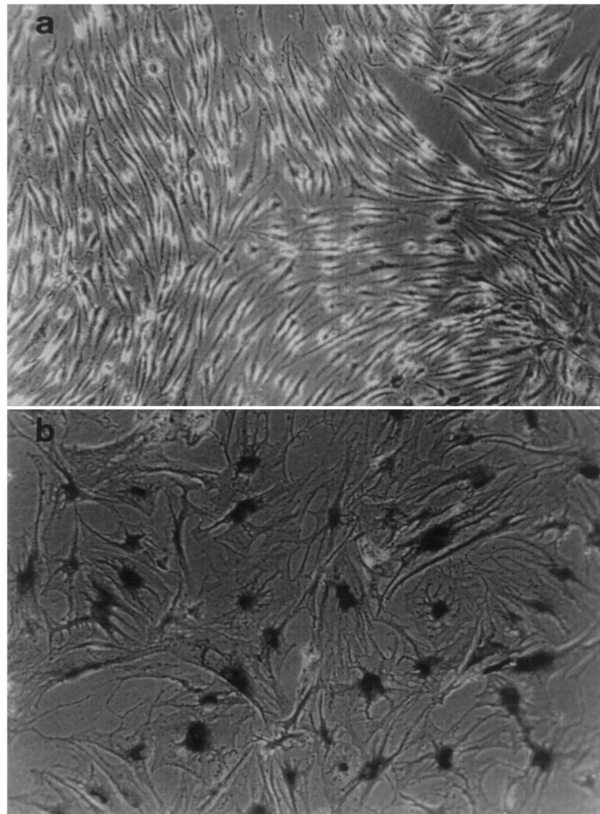

Fig. 1. Staining for β-galactosidase activity in (a) young osteoblasts (CPDL 12, 40% lifespan completed) and (b) old osteoblasts (CPDL 28, 90% lifespan completed). Microscopic magnification, $50\times$.

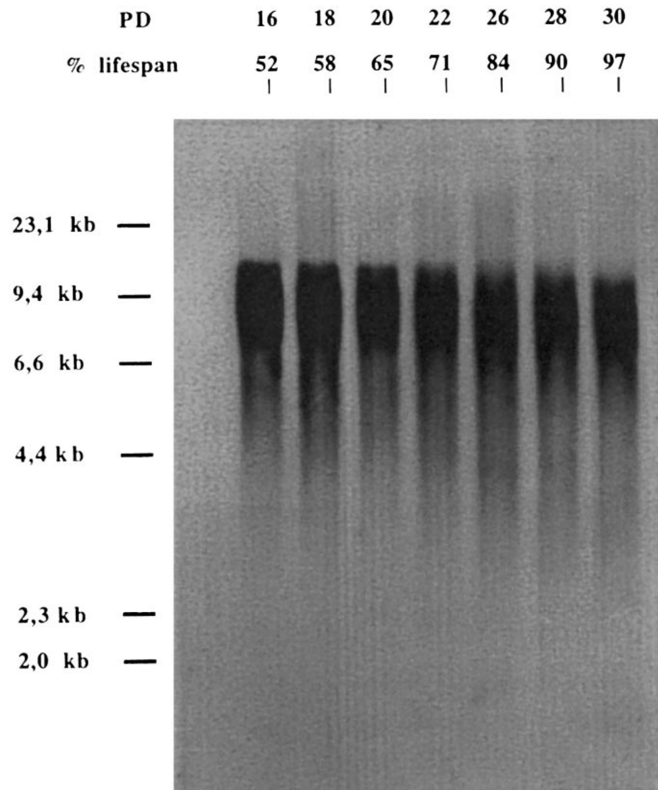

Fig. 2. Telomere restriction fragment length in cultured normal trabecular osteoblasts. Genomic DNA was analyzed at the indicated PD levels and the corresponding percentage lifespan completed. DNA was digested, electrophoresed and hybridized with a telomeric probe. The size (kb) and position markers are indicated.

characteristics of in vitro aging, including a loss of proliferative capacity, and a decline in DNA, RNA and protein synthesis reported previously (Kassem et al., 1997), we have observed an increased number of β-galactosidase-positive cells with increased cellular age in vitro. While β-galactosidase is a recently described biomarker of aged cells in vivo and in vitro (Dimri et al., 1995), its function in aged cells is still not known. However, the increased enzymatic activity of neutral (pH 6) β-galactosidase in senescent cells indicates that senescence is a phenotype quite distinct from a state of growth arrest which is generally fully reversible under appropriate growth conditions (Rattan, 1995; Campisi, 1996).

The validity of using the system of serially passaged cultures of trabecular osteoblasts for studies on cellular aging and senescence is further strengthened by our observations on the loss of telomeres. We have found a progressive shortening of TRF length as a function of PD during in vitro aging of human osteoblasts. The rate of telomere shortening was about 100 bp per population doubling, which is

similar to what has been found in other human somatic cell types (Harley et al., 1990; Allsopp et al., 1992). The total loss of 1.5 kb in senescent osteoblasts (97% lifespan completed) as compared with middle-aged cells (52% lifespan completed) is similar to what has been reported (≈ 2 kb loss) in human fibroblasts (Harley et al., 1990). Thus, our results support the view that TRF length is a useful marker of replicative senescence of normal diploid cells grown in culture.

An association between osteoporosis and the phenotype of excessive generalized aging has been suggested in the literature. For example, women with low bone mass have an increased risk of early mortality, especially from stroke (Browner et al., 1991). Similarly, an association between osteoporosis and aggressive atherosclerosis has been demonstrated as osteoporotic patients had a higher incidence of aortic calcification compared to age-matched controls (Frye et al., 1992). As regards the relationship between telomere shortening and premature aging, excessive telomere shortening has been found in fibroblasts from patients with premature aging syndromes, such as Werner's syndrome and Hutchinson–Gilford progeria (Allsopp et al., 1992; Kruk et al., 1995), in human endothelial cells in relation to atherosclerosis (Chang and Harley, 1995), and in PBL in patients with ataxia telangiectasia (Metcalfe et al., 1996). Therefore, it is reasonable to assume that if a disease has a phenotype of generalized accelerated aging, then one could expect to see an excessive loss of telomeres in a variety of proliferative cell types in vivo and in vitro. However, our results show that the mean TRF length decreased only slightly in PBL with age, and there was no additional and excessive loss in telomeres between a group of osteoporotic patients and age-matched controls. In addition, there was a large heterogeneity in the TRF lengths of PBL from different donors, which is a reflection of both inter-donor genetic variation and the inter- and intra-donor variable proliferative history of PBL in vivo (Rufer et al., 1998).

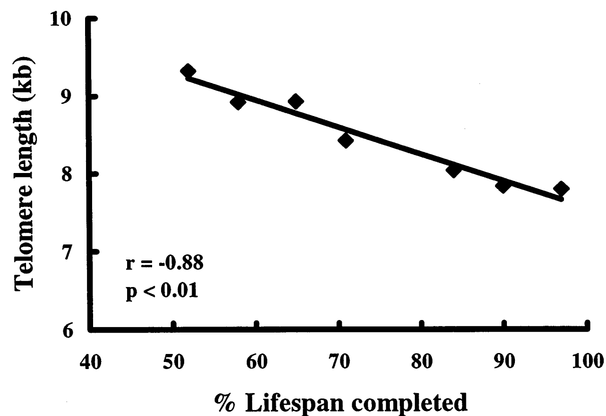

Fig. 3. A decrease in telomere length during replicative aging of cultured normal human trabecular osteoblasts. The mean telomere length is shown as a function of in vitro age. Each point represents DNA of osteoblasts isolated at a certain age level of in vitro lifespan.

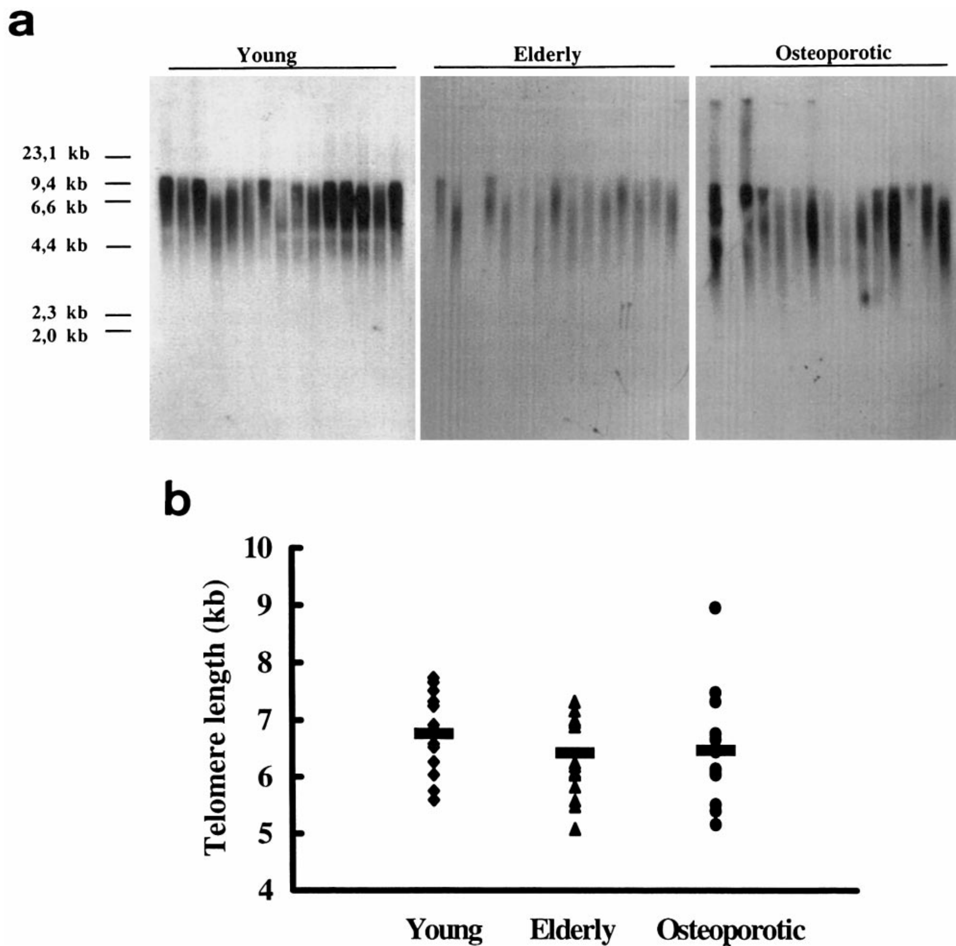

Fig. 4. Southern blot analysis (a) and quantitative analysis (b) of individual mean telomere restriction fragment length for peripheral blood leukocytes (PBL) from a group of young women (aged 20–26 years, $n = 15$), a group of elderly normal women (aged 48–85 years, $n = 15$), and a group of age-matched osteoporotic women (aged 52–81 years, $n = 14$).

This lack of excessive telomere shortening in the PBL from osteoporotic patients may have several explanations. First, decreased bone formation in osteoporosis may be related primarily to the defect in bone forming osteoblasts without any generalized defect in other proliferative cell pools. Therefore, TRFs may be different between PBL and osteoblasts, and direct measurement of TRF length in osteoblasts is necessary. However, no method is currently available to measure in-situ mean TRF length in bone biopsies. Second, decreased bone formation in osteoporosis may be related to a decreased number of osteoblasts recruited during bone formation, rather than a decreased proliferative capacity of mature osteoblasts (Parfitt, 1988). Therefore, the telomere length may remain very similar even in

osteoblasts from normal and osteoporotic patients. There is further evidence to support the view that there may not be significant differences in various characteristics of cultured osteoblasts isolated from normal and osteoporotic individuals, as we have previously reported that osteoblast cultures established from normal and osteoporotic patients in short-term cultures had similar cellular proliferative responses to growth hormone and serum growth factors (Kassem et al., 1994).

In conclusion, TRF length is a useful marker for monitoring cellular aging and proliferative potential in vitro and in vivo. Since TRF length is not excessively shortened in the PBL from osteoporotic patients, it suggests that osteoporosis is not a disease of a generalized accelerated aging, and that other pathogenetic mechanisms are involved in the etiology of this disease, which is distinctly age dependent.

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