Journal of Gerontology: BIOLOGICAL SCIENCES
Cite journal as: J Gerontol A Biol Sci Med Sci. 2011 November;66A(11):1163–1168
doi:10.1093/gerona/glr136

Published by Oxford University Press on behalf of The Gerontological Society of America.
Advance Access published on August 22, 2011

Telomere Dynamics in Rhesus Monkeys: No Apparent Effect of Caloric Restriction

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The role of telomere attrition in limiting the replicative capacity of cells in culture is well established. In humans, epidemiologic evidence suggests telomere length (TL) in leukocytes is highly variable at birth and inversely related to age. Although calorie restriction (CR) significantly increases life span in most rodent models, its association with TL is unknown. Using linear regression analysis, TLs (as measured by Southern blot analysis) of skeletal muscle (a postmitotic tissue that largely represents early development TL), fat, leukocytes, and skin were tested for effects of age, sex, and diet in 48 control and 23 calorie restriction rhesus monkeys. After controlling for the individual's muscle mean TL, differences between leukocytes muscle and skin muscle were significantly associated with age ($p = .002$; $p = .002$) and sex ($p = .003$; $p = .042$), but not calorie restriction ($p = .884$; $p = .766$). Despite an age-dependent shortening of TL in leukocytes and skin, calorie restriction did not significantly affect TL dynamics in these samples.

Key Words: Telomere—*Macaca mulatta*—Aging—Caloric restriction—Rhesus monkeys.

Received February 18, 2011; Accepted July 11, 2011

Decision Editor: Rafael de Cabo, PhD

CALORIC restriction (CR), the reduction of calories while maintaining adequate nutrition, is the single most effective environmental modality shown to extend the life span of short-lived organisms, ranging from worms to mice to dogs and possibly to longer lived animals, that is, nonhuman primates (1–3). CR produces favorable metabolic, hormonal, and tissue effects that diminish the likelihood of aging-related diseases (4,5) and ostensibly attenuates immune senescence in a number of species, including the rhesus monkey (6–8). Although most mammalian CR research has utilized rodent models of aging, ongoing studies in rhesus monkeys, *Macaca mulatta*, are corroborating many previous findings, suggesting that CR may work by similar mechanisms across species. Although health benefits and disease prevention have clearly been observed, whether CR will increase life span, particularly maximum life span, of the nonhuman primate is still unclear (3,9).

In humans, leukocyte telomere length (TL) is apparently influenced by oxidative stress and inflammation and considered a biomarker of aging (10). Leukocyte TL is inversely related to the BMI (11–13) and insulin resistance (11,14), and shortened leukocyte TL is associated with multiple aging-related diseases, principally atherosclerosis (10,15). Moreover, studies using same-sex twins demonstrated that the co-twin with the shorter leukocyte TL was more likely to die first (16,17).

Although genetic knockouts in mice have provided data to better understand telomere biology, substantial differences exist between mice and humans in aging, diseases of aging, and in TL dynamics. Nonhuman primates, including rhesus monkeys, provide a more relevant model for human aging and TL dynamics (12,18) because the periods of growth, development, and overall life span of rhesus monkeys are approximately 1/3 that of humans, compared with mice that scale at 1/30 that of humans. Both rhesus monkeys and humans share common patterns of disease development and responses to nutritional interventions. Therefore, rhesus monkeys provide a relevant model for human aging and aging-related traits and disease (18).

In the present project, we explored TL dynamics in rhesus monkeys from the long-term nonhuman primate aging study being conducted by the National Institute on Aging in which monkeys subjected to 30% CR are compared with control fed animals. As considerable interindividual variations in

Table 1. Mean Telomere Length by Tissue Type*

Sex	Diet	Muscle†	Fat†	Leukocyte†	Skin†
Males	CON (n = 24)	15.74 (1.39)	15.55 (1.57)	14.92 (1.58)	14.76 (1.45)
	CR (n = 10)	15.70 (0.96)	15.39 (1.00)	14.67 (0.88)	14.41 (0.89)
Females	CON (n = 24)	16.12 (1.26)	15.92 (1.32)	14.96 (1.36)	14.98 (1.24)
	CR (n = 12)	16.43 (1.70)	16.32 (1.70)	15.12 (1.63)	15.07 (1.63)

Notes: CON, control; CR, caloric restriction.
*Mean (*SD*). UMDNJ#1 removed.
†Kilobases.

TLs are present among both humans (10,12,19,20) and monkeys (12) from birth onward, a model was developed that was built on the following two central premises: (i) TL is approximately synchronized (equivalent) in different tissues of the fetus and newborn (19,21) and (ii) skeletal muscle is a predominantly postmitotic tissue (22), with TL attrition considerably lower in comparison with proliferative tissues, such as the hematopoietic system and the skin. Therefore, in each animal, skeletal muscle TL may serve as a proximal reference of early development TL. In this way, at any time during the animal's life span, the difference between TL in skeletal muscle and leukocytes (ΔTL), for instance, reflects leukocyte TL shortening. This model provides a better account of age-dependent leukocyte TL shortening than a cross-sectional analysis of leukocyte TL versus age, which usually requires numerous subjects due to the considerable interindividual variation in TL at birth and afterwards. We hypothesized that ΔTL for leukocyte and skin would be related to age and that CR would attenuate the increase in ΔTL for leukocytes.

Methods

Animals and Tissues

Specimens were acquired from rhesus macaques, *Macaca mulatta*, from the National Institute on Aging's nonhuman primate study of aging and CR (23). As described previously, the CR (30%) diet was vitamin and mineral supplemented. Blood work for all monkeys was monitored biannually, and general health/wellness of the monkeys was routinely monitored, with no difference in the incidence of infection or acute conditions between the two diet groups.

In total, 48 control monkeys (24 males: age [mean ± *SD*] 17.63 ± 6.83 years and 24 females: age 15.58 ± 6.39 years) and 23 CR monkeys (11 males: age 22.4 ± 1.26 years and 12 females: age 19.83 ± 3.81 years) were biopsied for samples to measure TL (Table 1). A single biopsy sample was acquired from the upper thigh, resulting in samples from skeletal muscle (vastus lateralis, ~0.5 g), white adipose tissue (subcutaneous above the muscle, ~0.5 g), and skin (~0.5 g) under anesthesia (Ketamine 7–10 mg/kg or Telazol 3–5 mg/kg, intramuscular), along with heparinized whole-blood samples for leukocytes. Tissue samples were frozen in liquid nitrogen and stored at −80°C until use. All samples were acquired during routine physical examinations in 2007.

Measurements of TL by Terminal Restriction Fragment Length

Telomere restriction fragment length measurements were determined by Southern blot analysis as described (24). Measures of TL that included within-subject mean, median, mode, and 50th or 25th percentiles were quantified as described previously (24). The coefficient of variation was determined for all within-subject measures of TL based on replicate measures from different gels (mean ± *SD*: 1.12% ± 0.85%). Data are reported as mean ± *SD* for TL.

Statistical Analysis

TLs of skeletal muscle, fat, leukocytes, and skin were tested for effects of diet, age, and sex. Linear regression models evaluated the effects of age, sex, and diet on TL in each tissue and the differences for leukocyte–muscle and skin–muscle TL comparing mean, median, mode and 50th or 25th percentile measures using SAS (v. 9.1, Cary, NC). Results were considered statistically significant when $\alpha < .05$ (two tailed). One outlier (UMDNJ#1) was identified by examination of Cooks' *D*, and analyses performed with or without this sample are noted.

Results

TL in Various Tissues

Descriptive statistics are provided in Table 1. The mean telomere length (mTL) of different tissues was highly variable among the different animals (Figure 1A). Despite this range of variability, muscle TL was significantly longer than any other sampled tissues (Table 1 and Figure 1A) in both male and female monkeys (Figure 1B). Additionally, the mTL of fat was greater than skin or leukocytes, in line with the concept that fat cells are much less proliferative than those in the hematopoietic system and skin (Table 1 and Figure 1A).

Synchrony of TL Between Tissues of the Same Animal

Although considerable variability of mTL among animals was observed, the mTL of each tissue was highly correlated across tissues (r^2: muscle versus fat = .89, versus leukocytes = .83, and versus skin = .84) (Figure 2A–C).

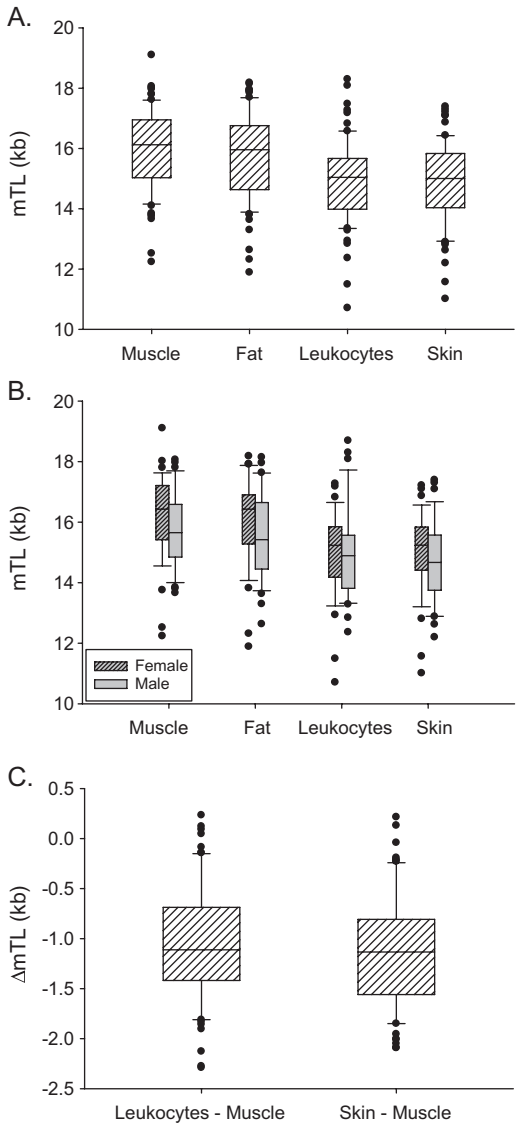

Figure 1. mTL in various tissues. (**A**) mTL from muscle, fat, leukocytes, and skin from all samples as measured by Southern blot. (**B**) mTL for muscle, fat, leukocytes, and skin by sex. (**C**) mTL difference between leukocyte–muscle and skin–muscle to estimate interindividual telomere attrition after controlling for "early development" TL. Box plots showing the 25th and 75th percentiles as the upper and lower half of each box along with the 10th and 90th percentiles as upper and lower error bars plus individual outliers.

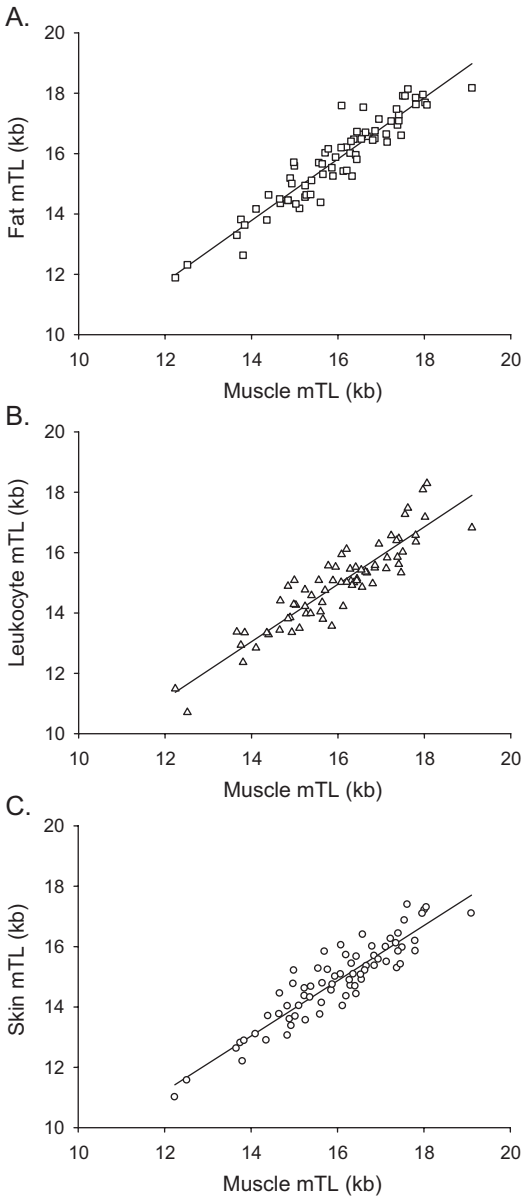

Figure 2. Relationship between mTL of muscle and other tissues. Muscle mTL is high correlated with (**A**) fat ($r^2 = .89$, $p < .001$), (**B**) leukocytes ($r^2 = .78$, $p < .001$; $r^2 = .83$ excluding UMDNJ#1), and (**C**) skin ($r^2 = .84$, $p < .001$) mTL within individuals.

TL and Age

Within each of the four tissues (muscle, fat, leukocytes, and skin), mTL was not significantly related to age (Table 2). However, using muscle mTL, as a surrogate of early development TL, the differences of TL between leukocytes–muscle and skin–muscle (ΔTL) were significantly associated with age ($p = .002$, $p = .002$; Table 2 and Figure 3A and B).

TL and Sex

Sex was not significant for mTL measures in muscle, fat, or skin, but was marginally significantly in leukocytes ($p = .046$; Table 2). Again, comparing the ΔTL leukocyte–muscle mTL and skin–muscle mTL to control for individual TL variation revealed that sex was significantly associated with the attrition rate with females showing greater attrition compared with males ($p = .003$, $p = .042$; Table 2 and Figure 3A and B), yet females still exhibited longer telomeres than males due to the length across all tissues (Figure 1B).

TL and Diet

CR did not significantly influence the mTL of skeletal muscle, fat, leukocytes, or skin in either sex (Table 2). Diet was

Table 2. Potential Determinants of Telomere Shortening by Tissue Type in Rhesus monkeys

Tissue Type	Age at Biopsy β (p value)	Sex β (p value)	Diet β (p value)
Muscle	0.998 (0.774)	0.482 (0.144)	0.148 (0.686)
Fat	−0.012 (0.706)	0.467 (0.189)	0.271 (0.495)
Leukocytes*	−0.016 (0.302)	0.357 (0.046)	0.105 (0.601)
Skin	−0.033 (0.259)	0.236 (0.472)	0.109 (0.762)
Leukocytes–muscle*	0.037 (0.002)	−0.396 (0.003)	0.021 (0.884)
Skin–muscle*	0.041 (0.002)	−0.246 (0.042)	0.040 (0.766)

*UMDNJ#1 removed.

not significant for ΔTL leukocyte–muscle mTL and skin–muscle mTL ($p = .884$, $p = .766$; Table 2 and Figure 3C and D).

Discussion

The present work offers a proof of concept supporting the validity of the model that the difference between muscle TL and TL of proliferative tissues (that is leukocytes or skin) provides information over and above that of cross-sectional analysis of age-dependent TL shortening in proliferating tissues. That said, the present work shows no evidence that CR in this study of rhesus monkeys influences age-dependent TL shortening in leukocytes (or skin cells). Although the mechanism underlying CR benefits is still not fully understood, these results do not support a link between TL dynamics in leukocytes and CR in rhesus monkeys. With the recent publication from the nonhuman primate CR study being conducted at the University of Wisconsin that demonstrated mortality reduction with CR (3), it will be interesting to determine whether National Institute on Aging colony monkeys subjected to CR, from which the samples were acquired, also display reduced mortality risk.

Results of the current study confirm earlier findings of synchrony in TL within cells and tissues of humans and macaques at birth and later in life (12,19,25). Moreover, they underscore the considerably longer telomeres in the rhesus monkey than in humans (12,26,27). With such long telomeres and a comparatively shorter life span, which amounts to ~25 years on average and ~40 years for maximum life span in captivity (3,18,28), it is uncertain if telomere-mediated replicative aging would be a determinant in inter-individual variations in longevity and age-related changes among macaques. Similarly, TL in most established mouse strains is considerably longer than that of humans, although in two wild-derived strains, *CAST Ei* and *Mus speretus*, TL is approximately 19 and 8 kb, respectively (29–31). However, even in these strains, TL is relatively long in relation to their life span, which is ~30 times shorter than that of humans (32). Although TL in the mouse is evidently not calibrated to play a role in its life span, the genetically engineered telomerase-deficient mice indicates that short TL can

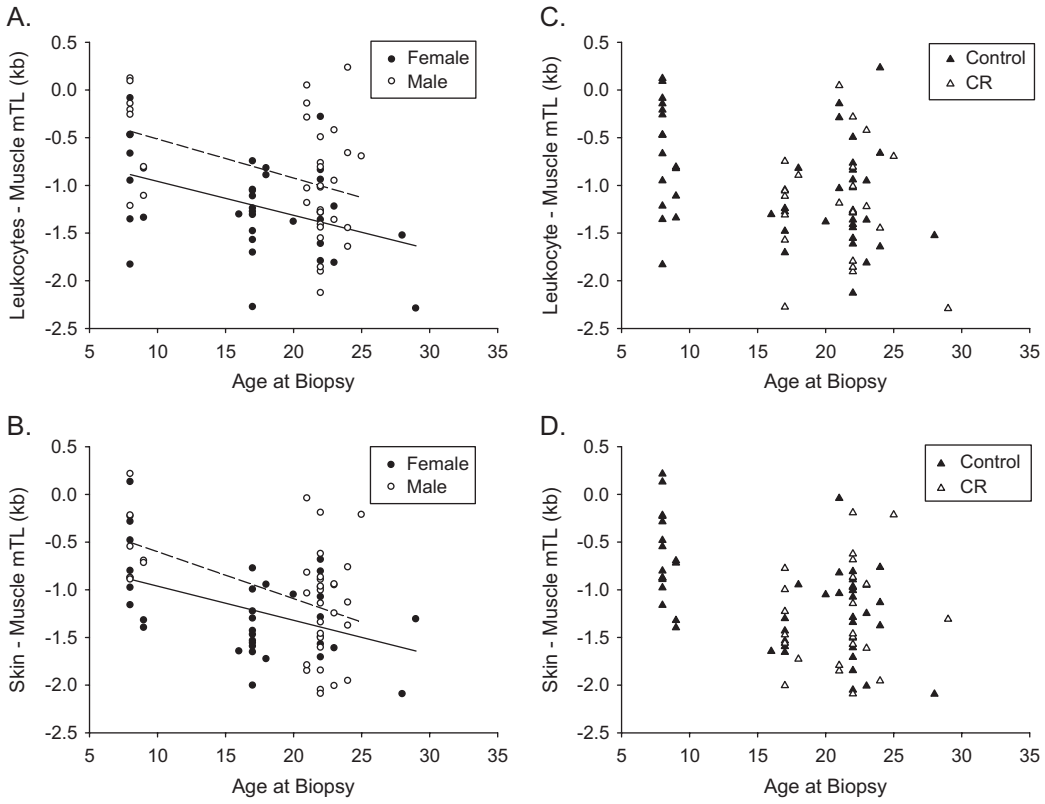

Figure 3. ΔTL in leukocytes and skin with age, sex, and diet. Effect of age and sex on ΔTL of (**A**) leukocytes and (**B**) skin after controlling for muscle as an "early development" telomere surrogate (male, dashed line; female, solid line), and no effect of diet on (**C**) leukocyte or (**D**) skin ΔTL.

impose a limit on the life span of a mammal (33). We note, however, that given that the shortest TL is the determinant of replicative senescence in cultured cells (34,35), in principle, even in short-lived mammalian species the shortest telomeres rather than the mean length of all telomeres might impact the animal's life span.

TL in leukocytes at birth and its shortening afterwards corresponds to the respective variables in hematopoietic stem cells (10). Therefore, leukocyte TL dynamics (birth leukocyte TL and its age-dependent shortening) would mirror variations with age in the rate of replication and the amount of telomeric DNA repeats lost with each replication of hematopoietic stem cells. Chronic inflammation increases replicative demand on hematopoietic stem cells to accommodate the inflammatory response, whereas an increase in oxidative stress augments the loss of telomere repeats per replication (36,37). The associations of shortened leukocyte TL with age-related diseases in humans have been attributed in part to these processes (10,15,38), with exceptions (39,40). We anticipated, therefore, that CR animals would display attenuation of leukocyte TL shortening if CR diminished inflammation and oxidative stress burdens. However, there was no apparent evidence of attenuation of leukocyte (and skin) telomere shortening by CR.

One of the limitations of this study is that the youngest monkeys were all controls, resulting in the CR animals grouping around ~15 years of age or older. This is due to the longevity study design for the primary outcome measures but prevents a broad-scale age group in the CR animals. Similarly, only a limited sample size of CR animals is available. Although all monkeys were routinely monitored for nutritional and health status, with no difference between the diet groups, we cannot exclude the possibility that the limited numbers have reduced the sensitivity of detection for any CR-related difference. However, the fact that both sex- and age-related differences were observed in the TL measures provides confidence in the null findings for diet.

Leukocyte TL attrition is very rapid during early life (41–43). This was shown not only in humans (41,42) but also in a longitudinal study in baboons (43). More TL measurements from younger and older CR monkeys, along with controls, would have provided more precise information of TL dynamics. Additionally, although the "standardized" age-dependent TL shortening can be estimated from the ΔTL, longitudinal measures from the same animal would be preferred to reduce potential interindividual variation.

We have focused the results and discussion on the mean TL but have also tested the median, mode, and 50th or 25th percentiles and observed similar outcomes, namely that although age and sex significantly affect TL shortening in leukocytes and skin after controlling for muscle TL, diet was not significantly related to TL dynamics.

Although mortality may be considered the ultimate outcome related to TL, other related outcomes of health (eg, insulin resistance, inflammatory indices, etc.) and disease patterns of specific organs might be of interest in relationship to TL dynamics in CR macaques and their controls.

In conclusion, TL is synchronized among different tissues/cells in rhesus monkeys. Controlling for a surrogate early development TL with skeletal muscle, both skin and leukocytes showed age- and sex-related effects on TL attrition. However, CR did not significantly attenuate leukocyte and skin TL attrition in CR macaques involved in this long-term study.

Funding

This work was supported by National Institutes of Health (NIH) grants (T32 DK062710, P30 DK056336, AG 021593, and AG 020132); and the Intramural Program of the National Institute on Aging, NIH.

Acknowledgment

The opinions expressed herein are those of the authors and not necessarily those of the National Institutes of Health or any other organization with which the authors are affiliated.

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