Published in final edited form as:

Clin Sci (Lond). 2014 September ; 127(6): 415–421. doi:10.1042/CS20140051.

Cellular aging of skeletal muscle: telomeric and free radical evidence that physical inactivity is responsible and not age

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Abstract

Telomeres play an essential role in maintaining chromosomal integrity in the face of physiological stressors. Although the age-related shortening of TL (telomere length) in highly proliferative tissue is predominantly due to the replication process, the mechanism for telomere shortening in skeletal muscle, which is minimally proliferative, is unclear. By studying TL in both the upper and lower limbs of the young, old-mobile and old-immobile subjects and by virtue of the bipedal nature of human locomotion, which declines with age, it may be possible to elucidate the mechanism(s) responsible for cellular aging of skeletal muscle. With this approach, we revealed that TL (~15 kb) in arm skeletal muscle is unaffected by age. In contrast TL fell progressively in the legs across the young (~15 kb), the old mobile (~13 kb) and old immobile (~11 kb) subjects. Interestingly, there was a reciprocal increase in leg muscle free radicals across these groups that

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AUTHOR CONTRIBUTION

Massimo Venturelli performed the experiments, analysed the data, prepared the Figures, and wrote the paper. Garrett Morgan performed the experiments, analysed the data, and drafted the paper. Anthony Donato interpreted the results of the experiments and drafted the paper. Van Reese, Renato Bottura, Cantor Tarperi, and Chiara Milanese performed the experiments. Federico Schena, Carlo Reggiani, Fabio Naro and Richard Cawthon edited and revised the paper before submission. Russell Richardson edited, revised and approved final version of paper.

was correlated with TL ($r = 0.7$), with no such relationship in the arm ($r = 0.09$). Our results document that chronological age does not affect the cellular aging of skeletal muscle, but reveals that physical inactivity, probably mediated by free radicals, has a profound effect upon this process.

Keywords

aging; free radical; physical activity; telomere length; sarcopenia

INTRODUCTION

Cellular aging, unlike chronological aging, is an overall decline in cellular homeostasis and a reduced ability to adapt to physiological stress [1,2]. Telomeres, terminal sequences of TTAGGG repeats that form the natural end of chromosomes, not only provide a marker of cellular aging [3], but also play an essential role in protecting the end of chromosomes from the end-replication problem and from resembling broken DNA. TL (telomere length) is reduced by successive cell divisions and therefore varies across somatic tissue in proportion to both time and replicative activity, with leucocyte TL becoming shorter quicker than minimally proliferative somatic tissue, such as skeletal muscle [4]. Of note, leucocyte TL in humans is correlated with years of healthy life and longevity [5], with healthy centenarians, the best example of successful human aging [6], exhibiting long TL in leucocytes [7]. Indeed, in this longevous population, TL is associated with health and less age-related disease [7]. In contrast, little is known about skeletal muscle TL with age.

Interestingly, although the link between tissue replicative activity and TL appears robust, it has recently been suggested that, in terms of telomere shortening, there may be a general synchrony of age-related changes across different human tissues [8]. Therefore in skeletal muscle, a relatively quiescent tissue, genotoxic stressors, such as free radicals, rather than replicative activity, would probably play the dominant role in both telomere shortening and functional decline [9]. Indeed, the age-related loss of muscle mass or sarcopenia is associated with a reduction in the capacity of myosatellite cells to proliferate and differentiate [10], resulting in attenuated muscle function. However, the link between cellular aging of skeletal muscle, free radicals and physical function is still poorly understood.

As both aging and physical inactivity have been proposed to be pro-oxidant [11–15], and with recognition of the bipedal nature of human locomotion, which declines with age, studying TL in skeletal muscle in both the upper and lower limbs of young and old people with and without mobility limitations may elucidate the link between cellular aging, physical inactivity and free radicals. Thus we measured skeletal muscle TL and directly assessed the concentration of the mitochondrial ubisemiquinone free radical by EPR (electron paramagnetic resonance) spectroscopy in the vastus lateralis and biceps brachii muscles of 12 young subjects, 12 old-mobile subjects and 12 old-immobile subjects (wheelchair-bound, but not-self propelling the wheelchair).

MATERIAL AND METHODS

Subjects

Twelve young (25 ± 2 years of age), 12 old-mobile (87 ± 3 years of age) and 12 old-immobile (88 ± 4 years of age) sex-matched subjects without a history of smoking and severe cognitive impairment [MMSE (mini-mental state examination) >25] [16] took part in the study. The old-mobile and old-immobile subjects were also matched for age. The young subjects were normally physically active college students. The old-mobile subjects were all community dwelling and were able to walk independently [17]. The old-immobile subjects were all institutionalized and unable to stand up from a chair and walk independently [17], but it is important to note that these subjects were not self-propelling their wheelchairs and thus arm use was, as with the other groups, related only to activities of daily living.

The study procedures were approved by the University of Verona Institutional Review Board, and both subjects and/or family caregivers gave written informed consent.

Muscle mass

The assessment of body fat and thigh and arm lean mass was determined by dual-energy X-ray absorptiometry (QDR Explorer W; Hologic; fan-beam technology, software for Windows XP version 12.6.1).

Daily energy expenditure

Each participant was fitted with an Actiheart device (CamNtech) allowing heart rate and acceleration data to be simultaneously recorded for 24 h/day for 7 consecutive days. Energy expenditure recorded with the Actiheart was calibrated with indirect calorimetry measured with a telemetric gas analysis system (K4b²; Cosmed) [18].

Muscle biopsies

Skeletal muscle tissue was obtained with a 14-gauge tru-cut needle [19] from the vastus lateralis and biceps brachii. Muscle samples were immediately (30 s) immersed in a 2-methylbutane solution immersed in liquid nitrogen, and subsequently stored at -80°C .

Mean telomere length

A sequence-independent multiplex qPCR (quantitative real-time PCR) technique using a SYBR Green master mix with 0.625 unit of AmpliTaq Gold 360 DNA polymerase (Life Technologies) was utilized to determine mean TL and the telomere signal was normalized to cell number, as described previously by Cawthon [20]. It is important to note that muscle myosatellite nuclei constitute only ~4% of muscle nuclei [21], therefore these analyses were heavily weighted towards the non-replicative muscle tissue.

DNA isolation and purification

Total genomic DNA was isolated and purified from blood sample buffy coats using QIAamp mini DNA kit (Qiagen), according to the manufacturer's protocol.

EPR analysis of muscle biopsy samples

EPR scans were run on the muscle tissue samples using a Bruker EMXmicro 6/1 EPR spectrometer system. The tissue sample was positioned in a quartz glass finger dewar positioned by two blocks of frozen saline. The EPR scan was run with a centre field at approximately $g = 2.004$ and the resulting peak height was recorded [22].

Statistical analysis

Two-way ANOVA was used to establish differences between groups and tissues. Following these analyses, where indicated, a Tukey post-hoc test was used to determine group differences. A Pearson correlation was used to examine the relationship between variables. Significance was set at an α level of 0.05, and results are presented as means $\pm$ S.D.

RESULTS

Subject characteristics

The characteristics of young, old-mobile and old-immobile subjects are shown in Table 1. As reflected by the differences in daily energy expenditure, the young subjects were normally active, the old-mobile subjects were relatively inactive, and, being wheelchair-bound for 24 ± 4 months, the old-immobile subjects had lower limbs that were extremely inactive. This graded level of leg inactivity from young to old-mobile to old-immobile subjects was reflected by a progressive fall in thigh muscle mass in these three groups (Figure 1). In contrast with the lower limbs, the lean mass of the arms was not different between the young, old-mobile and old-immobile subjects and was unrelated to daily energy expenditure (Figure 1).

Telomere length

Mean skeletal muscle TL of the legs fell across the three groups from 15.0 ± 1.6 kb in the young subjects, to 13.3 ± 1.1 kb in the old-mobile subjects, to 10.9 ± 1.0 kb in the old-immobile subjects (Figure 2a). In contrast, arm skeletal muscle TL was unchanged with age and the mean TL of the arm skeletal muscle was not different across the three groups (Figure 2d). Skeletal muscle TL in the young subjects was not different between the arms and legs, whereas TL of the active legs of the old-mobile subjects and the inactive legs of the old-immobile subjects was significantly reduced compared with the TL of their arms (Figures 2a and 2d). Leucocyte TL was affected by age, with both the age-matched old-mobile and old-immobile subjects exhibiting equally greater telomere shortening than the young subjects (Figure 3).

Free radicals

The free radical EPR signal from the skeletal muscle of the legs increased across the three groups, with the lowest signal in the young subjects and a 21 ± 2 and $58 \pm 11\%$ greater signal in the old-mobile and old-immobile subjects respectively (Figure 2c) ($P < 0.05$). There was no difference in the EPR signal in the skeletal muscle of the arms in the young, old-mobile and old-immobile subjects (Figure 2e). The EPR signal was significantly greater in the skeletal muscle of the legs compared with the arms in old-mobile ($45 \pm 14\%$) and old-

immobile ($51 \pm 12\%$) subjects, whereas the EPR signal in the arm and leg muscle of the young subjects was not different. There was a strong correlation between TL and the peak EPR signal attained from the leg skeletal muscle ($r = -0.71$, $P < 0.05$; Figure 2c), but such a relationship was not evident in the arm skeletal muscle ($r = -0.09$, $P > 0.1$; Figure 2f).

DISCUSSION

As the link between cellular aging of skeletal muscle, free radicals and physical function is still poorly understood, we employed the innovative approach of examining TL and free radical concentration in the skeletal muscle of both the upper and lower limbs of young, old-mobile, and old-immobile subjects. Several major findings resulted from the present work. Specifically, in direct contradiction of the concept that telomeres of a given tissue shorten at a similar rate, the results of the present study revealed that there is an asynchrony in telomere shortening between the skeletal muscles of the upper and lower limbs.

Acknowledging the differences in limb-specific activity across the lifespan, these findings suggest that the rate of telomere shortening in skeletal muscle is exacerbated by increasing muscle inactivity and not by chronological age. Likewise, the results of the present study have revealed that the skeletal muscle free radical concentration is not a consequence of chronological aging, but is also likely to be a product of inactivity. Recognition that the cellular aging of skeletal muscle is not simply age-dependent, but is the consequence of inactivity and the subsequent increase in free radicals is important as this highlights the importance of maintaining physical activity across the lifespan and raises the potential role for antioxidants as a counter-measure.

Evidence of limb-specific differences in physical activity

The graded level of inactivity from young to old-mobile to old-immobile subjects was reflected by a progressive fall in thigh muscle mass in these three groups (Figure 1). In contrast with the lower limbs, the lean mass of the arms was not different between the young, old-mobile and old-immobile subjects and was unrelated to daily energy expenditure (Figure 1). With the accepted link between physical inactivity and muscle wasting [23,24], these findings support the current experimental premise that, with increasing age and with immobility superimposed upon this process, the predominant impact of increased inactivity is reflected in the legs and not the arms.

Asynchrony in skeletal muscle TL shortening and physical inactivity

Mean skeletal muscle TL of the legs fell progressively across the three groups from young, old-mobile, to old-immobile subjects (Figure 2a). In contrast, building upon the finding of Renault et al. [25] that arm skeletal muscle TL was unchanged with age, in the present study mean TL of the arm skeletal muscle was not different across the three groups (Figure 2d). Interestingly, skeletal muscle TL in the young subjects was not different between the arms and legs, whereas TL of the active legs of the old-mobile subjects and the inactive legs of the old-immobile subjects was significantly reduced compared with the TL of their arms (Figures 2a and 2d). Thus, in contrast with the recently published conclusion of Daniali et al. [8], indicating that there is an equivalent rate of telomeric shortening in somatic tissue, the present findings reveal an asynchrony in telomere shortening even between the skeletal

muscle of the upper and lower limbs. Additionally, in support of the link between these skeletal muscle limb differences and inactivity, there was a strong positive correlation between TL and muscle mass in the leg ($r = 0.81$, $P < 0.05$), but not in the arm ($r = -0.17$, $P > 0.05$). Therefore, supported by both the limb-specific differences in the relationship between muscle mass and overall activity (Figure 1) and the TL data, the results of the present study suggest that the rate of telomere shortening in skeletal muscle is exacerbated by increasing muscle inactivity and not by chronological age.

Role of free radicals in TL shortening

To better understand the mechanism responsible for this apparent inactivity-related asynchrony in human skeletal muscle telomere shortening we utilized EPR spectroscopy to assess the ubisemiquinone free radical concentration in both the arm and leg muscle. Again, and supporting a causal link between free radicals and TL, there was a clear continuum across the three groups in terms of the free radical EPR signal from the skeletal muscle of the legs, with the old-mobile subjects exhibiting a greater peak than the young subjects, while, in turn, the old-immobile subjects displayed an even greater peak than the young subjects (Figure 2b). In stark contrast, there was no difference in the EPR signal in the skeletal muscle of the arms in the young, old-mobile and old-immobile subjects (Figure 2e). However, of note, the EPR signal was significantly greater in the skeletal muscle of the legs compared with the arms in old-mobile and old-immobile subjects, whereas the EPR signal in the arm and leg muscle of the young subjects was not different. These findings reveal that skeletal muscle free radical concentration is not augmented by chronological age, but is actually a product of inactivity. Such a conclusion is supported by a strong correlation between TL and the peak EPR signal attained from the leg skeletal muscle (Figure 2c), which was characterized by vastly different levels of inactivity and the fact that such a relationship was not evident in the more uniformly active arm (Figure 2f). Although currently somewhat speculative, this greater free-radical-mediated genotoxic stress in legs compared with the arms may be a consequence of an initially greater metabolic dependence on oxidative phosphorylation in the skeletal muscle of the legs. This difference may principally result from positive adaptations due to the use of the lower limbs for locomotion, but, as inactivity sets in, such metabolic differences in the arms and legs may have negative consequences. In combination, these findings suggest that in skeletal muscle, a tissue with a low proliferative rate, the genotoxic stress of an increase in free radical concentration triggers the erosion of telomeres and this marker of cellular aging is clearly linked to physical inactivity and not simply chronological age itself. This link between TL shortening in skeletal muscle and free radicals raises the potential for the use of antioxidants as a counter-measure to combat the age-related decline in activity and subsequent increase in oxidative stress.

Leucocyte TL shortening and chronological age

The results of the present study confirm that, in highly proliferative somatic tissues, in this case leucocytes, TL is shorter (~7.5 kb) than in a relatively quiescent tissue such as skeletal muscle. However, unlike TL in leg skeletal muscle, which differed between the young, old-mobile and old-immobile subjects, leucocyte TL was unable to discern mobility from immobility, but there was an impact by chronology, with both the age-matched old-mobile

and old-immobile subjects exhibiting equally greater telomere shortening than the young subjects (Figure 3). Moreover, in support of the idea of asynchrony in telomere shortening across differing tissue, there was no correlation between leucocyte TL and skeletal muscle TL in the leg ($r = 0.18$; $P = 0.32$) and skeletal muscle TL in the arm ($r = -0.09$, $P = 0.41$). Thus these findings corroborate the well-documented observation of age-related telomere shortening in highly proliferative tissue and highlight the importance of the specificity of intracellular stress-induced changes in TL, and subsequent cellular aging, in more quiescent tissue.

Conclusions

On the basis of these multifactorial analyses, we propose that not only is telomere shortening different between highly proliferative and more quiescent tissue, but that in skeletal muscle, a minimally replicative tissue, physical inactivity and the subsequent increase in free radicals plays a major role in cellular aging (Figure 4). This phenomenon results in limb-specific cellular aging of skeletal muscle as progressive physical inactivity with age occurs predominantly in the legs whereas, in contrast, the arms are consistently utilized for the activities of daily living across the lifespan [26]. As this inactivity-driven cellular aging of skeletal muscle strikes muscles that are essential for human ambulation, the result is likely to be a vicious cycle of accelerated cellular aging and reduced muscle function that ultimately threatens independence in the elderly population. Recognition that the cellular aging of skeletal muscle is not simply age-dependent, but is the consequence of inactivity and the subsequent increase in free radicals, highlights the importance of maintaining physical activity across the lifespan and raises the potential role for antioxidants as a counter-measure.

CLINICAL PERSPECTIVES

- Both aging and physical inactivity have been proposed to be pro-oxidant. With recognition of the bipedal nature of human locomotion, which decreases with age, studying TL in skeletal muscle in both the upper and lower limbs of young and old people with and without mobility limitation, has clarified the link between cellular aging, physical inactivity and free radicals.
- Indeed, of major clinical significance, our findings suggest that the rate of telomere shortening, an index of cellular aging, in skeletal muscle is exacerbated by muscle inactivity and not by chronological age. Moreover, skeletal muscle free radical concentration is not a consequence of chronological aging, but is likely to be a product of inactivity.
- Therefore, with the backdrop of an ever-growing elderly population, the results of the present study highlight the importance of physical activity and the potential role for antioxidants as a counter-measure in the clinical battle against sarcopenia.

Acknowledgments

We greatly appreciate the time and effort expended by the subjects who participated in the present study.

FUNDING

Clin Sci (Lond). Author manuscript; available in PMC 2016 February 17.

This work was supported, in part, by the National Institutes of Health PPG [grant number PO1 HL, 09830], a VA Merit grant [grant number E6910R] and the Mons Mazzali Foundation.

Abbreviations

EPR	electron paramagnetic resonance
MMSE	mini-mental state examination
TL	telomere length

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Figure 1. Relationship between daily energy expenditure and muscle mass in the arms and legs of young (Y), old-mobile (OM) and old-immobile (OI) subjects

Figure 2. Skeletal muscle TL and free radical concentration in the arms and legs of young (Y), old-mobile (OM) and old-immobile (OI) subjects
(a) Individual and mean (horizontal bars) TL for leg skeletal muscle. **(b)** Individual and mean level of free radicals (EPR peak) from leg skeletal muscle. **(c)** Relationship between the level of free radicals and TL in the leg skeletal muscle. **(d)** Individual and mean TL from arm skeletal muscle. **(e)** Individual and mean level of free radicals (EPR peak) from arm skeletal muscles. **(f)** Relationship between the level of free radicals and TL in arm skeletal

muscle. *Significantly different from young subjects; †significantly different from old-mobile subjects; §significantly different from the arm.

Figure 3. TL in leucocytes of young (Y), old-mobile (OM) and old-Immobile (OI) subjects
Individual and mean (horizontal bars) TL from leucocytes. *Significantly different from young subjects.

Figure 4. Conceptual diagram of telomere shortening in somatic tissues with differing proliferative activity and intracellular stress

During childhood, a period of rapid growth, it is likely that all somatic tissue experiences telomeric shortening [27]. During adulthood, leg skeletal muscle (double line), characterized by low proliferative activity, experiences greater inactivity with increasing age. This inactivity increases intramuscular free radicals, which erode telomeres. With a severe increase in leg skeletal muscle inactivity as a consequence of becoming wheelchair-bound (double broken line), intramuscular free radical levels are augmented, resulting in a greater genotoxic stress and exacerbated telomere erosion. Owing to the bi-pedal nature of human locomotion, arm skeletal muscle (triple line), also characterized by low proliferative activity, does not experience an increase in inactivity across the human lifespan. Thus intramuscular free radicals are not increased and telomere shortening across adulthood is avoided. In contrast, the highly proliferative leukocytes (single line) are not influenced by physical activity, but experience rapid telomere shortening as a consequence of time and replicative activity, with leukocyte telomeres becoming shorter quicker than minimally proliferative somatic tissue such as skeletal muscle.

Table 1

Subject characteristics

Parameter	Young subjects (<i>n</i> = 12)	Old-mobile subjects (<i>n</i> = 12)	Old-immobile subjects (<i>n</i> = 12)
Age (years)	25 ± 2	87 ± 3 [*]	88 ± 4 [*]
Sex (<i>n</i>) (female/male)	9/3	9/3	9/3
Balance test (0–16)	16 ± 0	16 ± 0	2 ± 1
Gait test (0–12)	12 ± 0	12 ± 0	0 ± 0
BMI (kg·m ⁻²)	23 ± 4	21 ± 3	22 ± 4
Body fat (%)	22 ± 4	33 ± 5 [*]	38 ± 6 ^{*†}
Thigh muscle mass (kg)	5.38 ± 0.27	4.47 ± 0.17 [*]	3.49 ± 0.29 ^{*†}
Arm muscle mass (kg)	1.19 ± 0.09	1.18 ± 0.08	1.17 ± 0.03
Daily energy expenditure (kcal/day)	1985 ± 76	1605 ± 88 [*]	1369 ± 56 ^{*†}
Glucose (mg·dl ⁻¹)	86 ± 3	8 ± 95 [*]	96 ± 7 [*]
RBC (10 ⁶ ·μl ⁻¹)	4.2 ± 0.1	4.0 ± 0.2	3.9 ± 0.3
Haemoglobin (g·dl ⁻¹)	13.6 ± 0.4	11.6 ± 0.5 [*]	11.2 ± 0.8 [*]
HDL-cholesterol (mg·dl ⁻¹)	51 ± 8	52 ± 9	54 ± 6
LDL-cholesterol (mg·dl ⁻¹)	96 ± 10	107 ± 11 [*]	110 ± 14 [*]
SBP (mmHg)	118 ± 4	135 ± 5 [*]	133 ± 6 [*]
DBP (mmHg)	82 ± 4	86 ± 6	87 ± 8
Cognitive function MMSE (0–30)	–	26 ± 3	24 ± 3
Co-morbidity (<i>n</i>)			
Cardiovascular diseases	–	2 (17 %)	1 (8 %)
Diabetes	–	2 (17 %)	2 (17 %)
COPD	–	1 (8 %)	1 (8 %)
Pharmacological treatments (<i>n</i>)			
Trazodone	–	4 (33 %)	3 (25 %)
Thiazolidinedione	–	2 (17 %)	3 (25 %)
Bisoprolol	–	1 (8 %)	1 (8 %)
Etofylline	–	1 (8 %)	1 (8 %)
Etinil-oestradiol	4 (33 %)	–	–

^{*} Significantly different from young subjects;

[†] significantly different from old-mobile subjects. 1≈kcal 4.184 kJ. BMI, body mass index; RBC, red blood cells; HDL high-density lipoprotein; LDL, low-density lipoprotein; SBP, systolic blood pressure; DBP, diastolic blood pressure; COPD, chronic obstruction pulmonary disease.