 Doi: 10.1111/j.1474-9726.2007.00330.x

Shared environmental factors associated with telomere length maintenance in elderly male twins

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Summary

During aging, chromosome ends, or telomeres, gradually erode or shorten with each somatic cell division. Loss of telomere length homeostasis has been linked to age-related disease. Remarkably, specific environmental assaults, both physical and psychological, have been shown to correlate with shortened telomeres. However, the extent that genetic and/or environmental factors may influence telomere length during later stages of lifespan is not known. Telomere length was measured in 686 male US World War II and Korean War veteran monozygotic (MZ) and dizygotic (DZ) twins (including 181 MZ and 125 DZ complete pairs) with a mean age of 77.5 years (range 73–85 years). During the entire process of telomere length measurement, participant age and twin status were completely blinded. White blood cell mean telomere length shortened in this elderly population by 71 base pairs per year ($P < 0.0001$). We observed no evidence of heritable effects in this elderly population on telomere length maintenance, but rather find that telomere length was largely associated with shared environmental factors ($P < 0.0001$). Additionally, we found that individuals with hypertension and cardiovascular disease had significantly shorter telomeres ($P = 0.0025$ and 0.002, respectively). Our results emphasize that shared environmental factors can have a primary impact on telomere length maintenance in elderly humans.

Key words: aging; environment; genetics; telomeres.

Introduction

Human telomeres form a protective DNA and protein cap to stabilize chromosome ends, which is essential for genomic stability (Blackburn, 2001; Wright & Shay, 2002; Smogorzewska & de Lange, 2004). Disruption of telomere homeostasis is likely a key cause of age-related disease, especially cancer (Feldser *et al*., 2003; Chin *et al*., 2004; Greider & Blackburn, 2004; Gilley *et al*., 2005). Telomerase, the enzyme responsible for adding telomeric repeat sequences, is lacking or expressed at insufficient levels in most somatic cells, resulting in progressive telomere shortening during each cell cycle. Telomere shortening can eventually lead to telomere dysfunction, resulting in genomic catastrophe (Blackburn, 2000).

The observed increase in human lifespan within the last century is primarily due to changes in environment rather than a rapid evolution of genetic determinants (Christensen *et al*., 2006). Moreover, classic twin studies suggest that normal variation in longevity is largely influenced (~75%) by nongenetic or environmental factors (Herskind *et al*., 1996; Christensen *et al*., 2006). Several recent reports indicate that environmental factors such as stress, smoking, obesity and socioeconomic group correlate with shorter telomere lengths as measured in white blood cells (WBC), likely due to increased cell turnover in response to oxidative stressors and injury (Epel *et al*., 2004; Valdes *et al*., 2005; Cherkas *et al*., 2006).

We studied a group of elderly male twins to further examine the question of how environment and genetic determinants influence telomere length during aging. Monozygotic (MZ) twin pairs share all genes, and same-sex dizygotic (DZ) twin pairs share on average half of their genes. Maximum likelihood methods partition the variance of a specific phenotype (here, telomere length) into three components: (i) genetic (G); (ii) shared or common environmental effects (C), which make siblings more alike than would be expected solely based on shared genes; and (iii) nonshared or unique environmental effects (E), which may make siblings different from one another (Heath *et al*., 1989). Results yield estimates for each of these components, which are often presented as h^2 (heritability), c^2 (shared environment) and e^2 (unique environment). For a trait solely influenced by additive genetic effects, the expected DZ correlation (0.50) would be exactly half that of the MZ correlation (1.0). If MZ twins have a correlation that is greater than twice the correlation in DZ twins, then the MZ pairs likely share environment more than DZ twins. Common environmental effects are also clearly implied when the DZ correlation is greater than the expected correlation for genetic control alone (0.5).

Here, we report evidence suggesting that shared environmental factors are mainly responsible for regulating telomere length in this group of elderly male twin veterans. Additionally, we report that individuals in this population with hypertension and cardiovascular disease had significantly shorter telomeres.

Results

Participants were 686 World War II and Korean War male twins (181 MZ and 125 DZ complete pairs with 74 single cotwins),

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Accepted for publication *6 June 2007*

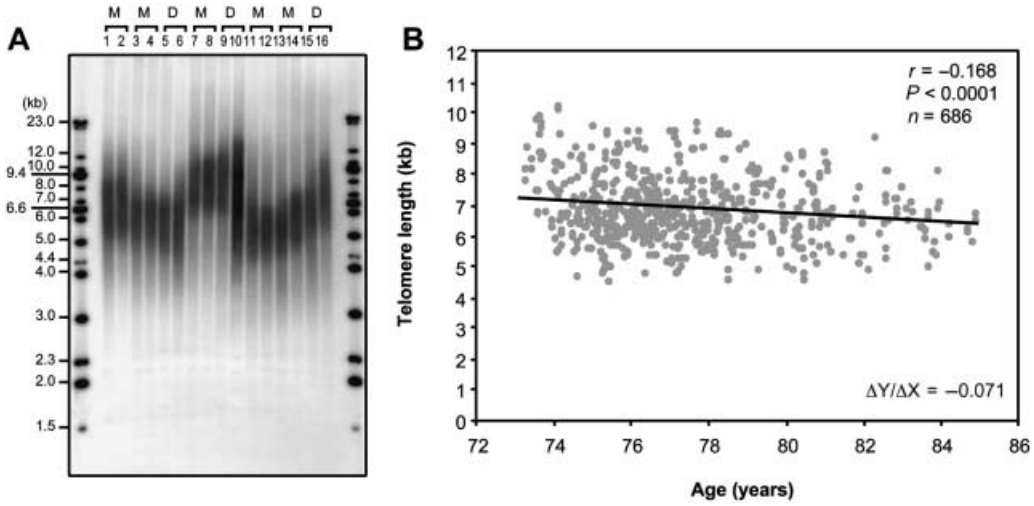

Fig. 1 Relation between telomere length and age. (A) Representative Southern blot of telomeric DNA from selected twin pairs. Samples were initially received from an independent DNA repository coded in a blinded fashion. Only after telomere length was determined in a completely blinded manner with several precautions for sample anonymity (described in Experimental procedures) were samples identified for data analysis. D and M represent monozygotic and dizygotic twins, respectively. (B) All 686 individual mean telomere lengths are plotted as a function of age. Standard linear regression analysis was used to correlate telomere length with age.

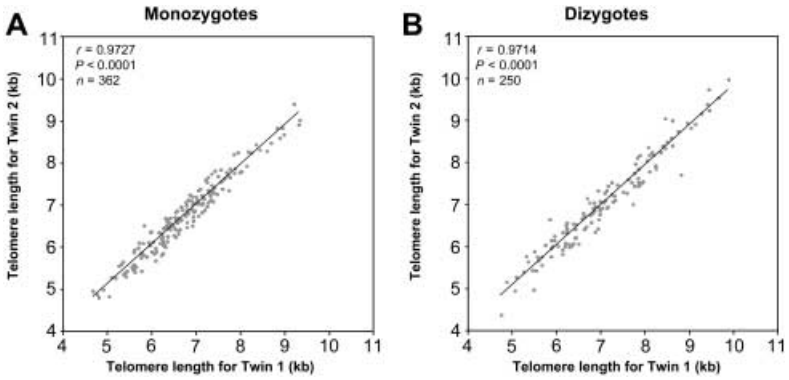

Fig. 2 Monozygotic and dizygotic twin pairs have highly similar telomere lengths. The age-adjusted mean telomere length of first-born (Twin 1) plotted against second-born (Twin 2) twins for: (A) monozygotic and (B) dizygotic twin pairs.

with a mean age of 77.5 years (range 73–85 years). All subjects completed a health history questionnaire and provided a blood sample in a study of genes related to healthy aging (Reed *et al.*, 2004). Thus, the subjects in this select sample generally have better health than comparable aged males in the general population. Individual items regarding the cardiovascular outcomes in the questionnaire were validated in a subset of the sample that had physical examinations and health records (Reed & Dick, 2003).

WBC DNA was isolated from each blood sample and mean telomere length was measured (Herbert *et al.*, 2003; Benetos *et al.*, 2004). During telomere length determination, participant age and twin status were completely blinded for all samples. Mean telomere lengths were calculated as described using a modified Southern analysis (Fig. 1A; Herbert *et al.* 2003). Standard linear regression analysis was used to correlate the telomere length with age (Fig. 1B). WBC telomere length in this elderly male population gradually decreased with age at a mean rate of 71 base pairs (bp) per year ($P < 0.0001$; Fig. 1B). Previous studies have reported that telomere loss per year in WBCs varies dramatically during development (Frenck *et al.*, 1998). Telomeric sequences are lost rapidly by more than 1000 bp per year in young children (Frenck *et al.*, 1998). However, during adult life the rate of telomere shortening slows with reported ranges of losses of 19–59 bp per year (Rufer *et al.*, 1999; Hodes *et al.*, 2002). Our finding that the rate of telomere loss is 71 bp per year in this elderly population may indicate that the rate of shortening increases slightly during late human lifespan.

We observed that telomere lengths were highly similar within each twin pair in this elderly male population (Fig. 2A,B). No significant differences in correlation of average twin pair telomere lengths were noted between MZ and DZ twin pairs (Fig. 2A,B). The intraclass correlation was 0.97 in both MZ and DZ pairs (Fig. 2A,B). For a trait solely influenced by additive genetic effects, the DZ correlation (0.50) would be expected to be exactly half that of the MZ correlation (1.0). If DZ twins have a correlation that is greater than 0.5, the twin pairs likely have

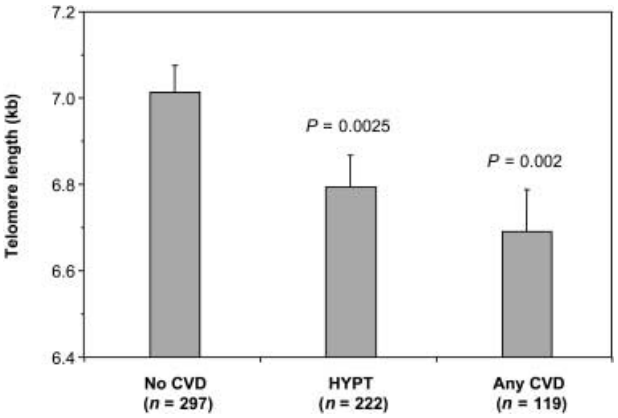

Fig. 3 Relationship between telomere length, hypertension and cardiovascular disease. Data are age-adjusted mean telomere length with standard error. Telomere lengths of individual twins with no cardiovascular disease (CVD) or hypertension (no CVD) (mean telomere length = 7.0 kb), hypertension only with no cardiovascular disease (only HYPT) (mean telomere length = 6.8 kb), and with any form of CVD excluding individuals with only hypertension (mean telomere length = 6.7 kb).

greater shared environment. Therefore, the best fitting model was a nongenetic (C+E) model in which 97% of the variance was due to shared or common (C) environmental effects. Our data strongly indicate that telomere length is primarily maintained by common environmental determinants in this select elderly population of generally healthy male twins.

To test whether this elderly twin group displayed shortened telomere length with certain age-related diseases, we examined both hypertension and cardiovascular disease (heart attack, coronary surgery or stroke). Cardiovascular disease is the leading cause of age-related mortality, and hypertension is a major risk factor for cardiovascular disease (MacMahon *et al*., 1990; Lusis, 2000). Both hypertension and cardiovascular disease have been associated with telomere shortening (Samani *et al*., 2001; Serrano & Andres, 2004). Based on the health history questionnaire, participants were placed into the following groups: (i) no cardiovascular disease and no hypertension; (ii) hypertension but no cardiovascular disease; and (iii) cardiovascular disease (Reed & Dick, 2003). We calculated the mean telomere length of each group to determine whether telomere length inversely correlated with hypertension and cardiovascular disease in this population of elderly male twins. In agreement with previous studies (Serrano & Andres, 2004), individuals without cardiovascular disease and hypertension had significantly longer age-adjusted telomeres than those individuals with hypertension and cardiovascular disease (Fig. 3). Whether WBC telomere erosion is a cause or a consequence of hypertension and cardiovascular disease is not known (Serrano & Andres, 2004). Along with the expected relationship of declining telomere length with age (Fig. 1B), the additional confirmation of previous results with shorter telomeres in those with cardiovascular disease further strengthens our finding that telomere length is remarkably similar regardless of whether twin pairs are MZ or DZ in this select population.

Discussion

Previous twin studies have reported various degrees of heritability responsible for the control of telomere length. The major reason for this variability may be the unique characteristics of the specific populations examined within each study. Focusing on studies that employed Southern analysis, as our study did, these studies used subjects with wide age ranges or excluded elderly subjects. For instance, Slagboom *et al*. (1994) reported a 78% heritability estimate with an intraclass correlation of 0.78 in 59 MZ twin pairs and a correlation of 0.39 in 56 DZ pairs. This analysis pooled three twin pair groups with mean ages of 4, 17 and 44 years (Slagboom *et al*., 1994). Their data set also included eight twin pairs with a mean age of 79, comparable in age to our twin subjects, but these elderly twin pairs were not actually used to calculate heritability (Slagboom *et al*., 1994). In a comparison of 10 MZ and 39 DZ pairs ranging in age from 18 to 44 years, Jeanclos *et al*. (2000) subsumed all sources of familial aggregation, including common environment, with the additive genetic effects and calculated a heritability of 84%. Vasa-Nicotera *et al*. (2005) made a similar subsumption from sib-pair correlations and reported a heritability of 82% in a sample of individuals with heart disease onset prior to age 65 (age range 47–82 years; mean age of 65.8 years). Andrew *et al*. (2006) used pairs of female twins with a mean age of 47.8 years (range 18–80 years) and reported a heritability of 36%. Andrew *et al*. (2006) estimated common environmental effects at 49%. Combining these two causes of familial resemblance results in 85% of the variation in this study. It is of interest that Nawrot *et al*. (2004) reported sib-sib correlations between 0.61 and 0.83 in offspring. The mean age of the persons studied in this report was 51.7 in the parents and 30.2 in the offspring (Nawrot *et al*., 2004). The correlations observed were higher than 0.5, which supports the conclusion that shared environment may influence telomere length maintenance (Nawrot *et al*., 2004). Twin correlations using telomere fluorescence *in situ* hybridization and flow cytometry (flow FISH) to determine telomere length have been reported in two small studies with subjects roughly the same age as our twins (Rufer *et al*., 1999; Graakjaer *et al*., 2004). It is worth noting that granulocyte measurements of telomere length using flow FISH resulted in an intraclass correlation that was not significantly different in MZ and DZ twins (Rufer *et al*., 1999) as found in our population of elderly male twins.

In commenting on the higher estimate of common environmental effects found in their study, Andrew *et al*. (2006) pointed out that their heritability estimate of 36% was probably comparable to earlier studies that did not model common environmental effects. These authors concluded that the large common environmental effect genuinely reflects environmental factors that influence telomere length variation shared between siblings. Twins are the same age and share their environment beginning *in utero*. Our results suggest that shared environmental factors play a fundamental role in telomere length maintenance in this elderly twin population. Our results demonstrate variable telomere length between twin pairs but

highly comparable telomere length within twin pairs indicative of mainly common environmental effects. As noted, our sample is elderly (mean age 77.5), with a relatively narrow age range, and was selected for pairs in which one or more cotwins had absence of cardiovascular disease. Both cotwins survived World War II and were healthy enough to participate in our study over 50 years later through completion of a health questionnaire and willingness to go to their physicians for blood sample collection. Senescent-independent causes of death, which in the case of cardiovascular disease also has been shown to influence telomere length, become less important in determining life expectancy in the elderly. It may be that the narrower age distribution in our sample completely composed of elderly individuals is more selective compared with other heritability studies that employed subjects with wide age ranges. Common environmental effects may include gene by environmental effects. Environmental factors or assaults, including damage at telomeres and other genomic regions, may be cumulative from various sources and amass with age over the course of a lifespan. Also, telomere length may be programmed in early development during fetal growth and/or early childhood at a time when both MZ and DZ twin pairs share a common environment (Barker, 2004). However, as younger twin pairs demonstrate significant heritability, the *in utero* maternal effect may not be manifest until later ages when other causes of early death have occurred. It is of interest to note that there was no correlation between father and sons in the Nawrot *et al*. (2004) study, which found sibling correlations greater than 0.5. Because of a correlation between father and daughters, the favored explanation was that genes on the X chromosome were involved in telomere length.

Presumably, common environmental factors can influence the expression of a complex array of genes involved in telomere length maintenance during human lifespan. Several studies have reported intraclass correlations greater than 0.50 in DZ twins or siblings (Rufer *et al*., 1999; Nawrot *et al*., 2004; Andrew *et al*., 2006), which was also demonstrable in our elderly twin pairs. Our results suggest that in the elderly, shared environmental effects, perhaps as a result of maternal effects *in utero* or gene–environmental interactions, may become more important with aging. Future research is likely to focus on characterizing these nongenetic factors that play a key role in the complexity of telomere length maintenance throughout human lifespan.

Experimental procedures

Participants

Subjects were members of the National Academy of Sciences–National Research Council twin panel. The creation of the registry is described in detail elsewhere (Hrubec & Neel, 1978). Individual participants in this study were 686 World War II and Korean War male twins (181 MZ and 125 DZ complete pairs with 74 single cotwins), with a mean age of 77.5 (± 2.6) years (range 73–85 years). As previously described (Reed *et al*. 2004), all subjects completed a health history questionnaire and provided a blood sample. Twin analysis was performed using an updated version of the program TWINAN90 (Williams *et al*., 1992), which partitions the variance using both maximum likelihood methods and analysis of variance methods to calculate heritability (Williams *et al*., 1992).

Telomere length analysis

Isolated DNA samples from WBCs from an independent DNA repository (Indiana University School of Medicine – Department of Medical and Molecular Genetics DNA Repository), coded in a blinded fashion, were used to determine average telomere lengths as previously described (Benetos *et al*., 2004). Briefly, 2.5 μg of each DNA sample were digested for 12 h with 3 U μg^{-1} each of the tetra-cutter restriction enzymes, *Rsa*I and *Hin*fI. Digested DNA samples were resolved on a 0.8% agarose gel in random order, dried on Whatman 3MM paper under vacuum for 1 h at 55 °C, denatured for 20 min in 0.5 M NaOH/1.5 M NaCl, rinsed in distilled water for 10 min, and neutralized for 30 min in 1.5 M NaCl/0.5 M Tris-HCl (pH 8.0). In-gel hybridization analysis was performed with a ^{32}P-labeled $(TTAGGG)_4$ telomere probe for 16 h at 42 °C in 0.5 M sodium phosphate (pH 7.2), 1 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0), 7% (w/v) sodium dodecyl sulfate (SDS) and 1% (w/v) bovine serum albumin. The gel was then washed once for 20 min in 2× SSC, twice for 15 min each in 0.1× SSC at room temperature, and exposed to a phosphor screen (PhosphorImager; Molecular Dynamics, Sunnyvale, CA, USA).

Mean telomere lengths were calculated from PhosphorImager scans of gels hybridized to kinased $(TTAGGG)_4$ probes using the Excel spreadsheet program TELORUN© as described (Ouellette *et al*., 2000; Herbert *et al*., 2003). A grid of 30 boxes was positioned over each lane, and the signal intensity and size (kb) corresponding to each box was determined. The distribution of telomere lengths within a population of cells was determined by plotting the cumulative fraction of telomeres vs. size. The signal from each of the 30 image quantitation boxes in the grid over each lane in the telomere gel was first divided by the position of each box converted to kb, so that the calculated signal intensity of, for example, a telomere with 6 kb of repeats would be the same as the signal from a telomere with 1 kb of repeats. The results were then divided by the sum of all of the normalized signals, so that each represented a fraction of the total. This simultaneously adjusts for variations in signal intensities due to different amounts of DNA actually loaded or different hybridization efficiencies/probe-specific activities between gels. Finally, the results were added together starting with the smallest to obtain a cumulative fraction of telomeres that were at least a given size. The size at which the cumulative fraction equals 0.5 thus represents the median length, where 50% of the telomeres are longer and 50% are smaller than that size. A different individual who was also totally blinded to any sample identification then performed a second, independent analysis of the blots.

Acknowledgments

We dedicate this manuscript to John Preer and T. M. Sonneborn. We thank Ken Cornetta, Steven Dlouhy, Dan Koller, Marc Mendonca, Virginia Thurston and Christopher Williams for valuable comments during the preparation of this manuscript. We thank the Indiana University School of Medicine, Department of Medical and Molecular Genetics DNA Repository, Amy Breman, Christian Carbe and Jonathan Steinhofer for their valuable assistance. This work was supported by the Indiana University Cancer Center, the American Cancer Society, the Showalter Foundation, the Susan G. Komen Foundation, NIH grant AG18736, and the Indiana Genomics Initiative (INGEN). INGEN of Indiana University is supported in part by Lilly Endowment Inc.

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