Human Reproduction, Vol.24, No.5 pp. 1206–1211, 2009
Advanced Access publication on February 6, 2009 doi:10.1093/humrep/dep007

human reproduction

ORIGINAL ARTICLE *Reproductive genetics*

Telomere length and reproductive aging

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BACKGROUND: Rate of reproductive aging may be related to rate of biological aging. Thus, indicators of aging, such as short telomere length, may be more frequent in women with a history suggestive of premature reproductive senescence.

METHODS: Telomere-specific quantitative PCR was used to assess telomere length in two groups of women with evidence of reproductive aging: (i) patients with idiopathic premature ovarian failure (POF, $N = 34$) and (ii) women with a history of recurrent miscarriage (RM, $N = 95$); and two control groups: (1) women from the general population (C1, $N = 108$) and (2) women who had a healthy pregnancy after 37 years of age (C2, $N = 46$).

RESULTS: The RM group had shorter age-adjusted mean telomere length than controls (8.46 versus 8.92 kb in C1 and 9.11 kb in C2, $P = 0.0004$ and $P = 0.02$ for C1 and C2, respectively), although short telomeres were not confined to subsets of this group known to have experienced single or multiple trisomic pregnancies. Although sample size is limited, mean telomere length in the POF group was significantly longer than that in C1 (9.58 versus 8.92 kb, $P = 0.01$).

CONCLUSIONS: Women experiencing RM may have shorter telomeres as a consequence of a more rapid rate of aging, or as a reflection of an increased level of cellular stress. Longer telomere length in the POF group may be explained by abnormal hormone exposure, slow cell division rates or autoimmunity in these women. Despite small sample sizes, these results suggest that different manifestations of reproductive aging are likely influenced by distinct physiological factors.

Key words: telomere length / premature ovarian failure / recurrent miscarriage / trisomic pregnancy / reproductive aging

Introduction

Female fertility declines with age due to the combined effects of both a decrease in the rate of conception and an increase in the rate of pregnancy loss due to aneuploidy (Dorland *et al*., 1998a). Age-related changes in the human ovary, including depletion of ovarian follicles (Faddy *et al*., 1992; Faddy, 2000) and a decline in oocyte genomic stability leading to aneuploidy (Hassold and Hunt, 2001), may contribute to this phenomenon. The rate of female reproductive aging displays a large amount of inter-individual variability. This is reflected in the variability in age of reproductive senescence (menopause) that typically occurs anytime between 40 and 60 years of age (Kato *et al*., 1998; te Velde and Pearson, 2002), as well as in the individual variability in risk of conceiving a trisomic pregnancy (Warburton *et al*., 2004; Nicolaides *et al*., 2005). This natural variation in reproductive aging may be the result of environmental and genetic factors that affect individual rates of cellular aging.

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Both animal models and human epidemiological studies support the suggestion that longevity is associated with an increase in reproductive lifespan. Mice and flies selectively bred for reproductive longevity have an overall increase in total lifespan when compared with unselected controls (Hutchinson and Rose, 1991; Nagai *et al*., 1995). Human population studies have reported that higher total fecundity (Manor *et al*., 2000; Muller *et al*., 2002), later age at last reproduction (Doblhammer, 2000; Muller *et al*., 2002; Smith *et al*., 2002; Helle *et al*., 2005) and older age at menopause (Snowdon *et al*., 1989; Cooper and Sandler, 1998; Jacobsen *et al*., 1999) are positively correlated with longevity. A study of female centenarians found that women living to at least 100 are greater than four times more likely to have had a child while in their 40s than women living at age 73 (Perls *et al*., 1997). There are several possible explanations for the relationship between longevity and age at menopause: (i) prolonged estrogen exposure associated with later menopause may have a positive influence on life expectancy (Perls *et al*., 1997), (ii) effective age of the ovary could directly affect longevity (Hsin and Kenyon, 1999; Cargill *et al*., 2003) or (iii) selective pressures to maximize a woman's reproductive years by slow reproductive aging may have positively selected for women with slower rates of cellular aging (Perls and Fretts, 2001; Perls *et al*., 2002).

Telomeres are a marker for cellular aging as their length declines with each cell division. Telomere length exhibits considerable inter-individual variation (Hastie *et al*., 1990) and may contribute to the observed variability in reproductive aging. Telomere variability may be due to differences in telomere length at conception, telomerase activity during early development, rate of cell division and rate of telomere loss per cell division. Shorter telomeres may limit the mitotic capacity of primordial germ cells during fetal development and therefore restrict the size of the follicular pool (Keefe *et al*., 2006). Studies examining telomere length and reproductive aging in humans have produced contradictory results in which telomere length has been both positively and negatively associated with different measures of reproductive aging (Dorland *et al*., 1998a; Aydos *et al*., 2005; Keefe *et al*., 2007).

Given the links between reproductive aging and biological aging, and the potential influence of telomere length on oocyte quality, we hypothesized that women who display evidence of premature reproductive aging will have a shorter average telomere length than control women. The objective of this study was to assess telomere length in peripheral blood leukocytes in two groups of women with evidence of premature reproductive aging: (i) patients with idiopathic premature ovarian failure (POF) who experienced menopause before 40 years of age and (ii) women with a history of recurrent miscarriage (RM), and two control groups: (1) women from the general population not selected on the basis of reproductive history and (2) women who had had a healthy pregnancy after the 37 years of age and had not experienced any pregnancy loss. This latter group may represent women with potentially slower rates of reproductive aging, as they have not experienced difficulties conceiving or maintaining pregnancy despite a relatively advanced reproductive age.

Materials and Methods

Samples

Women with RM ($N = 95$), defined as three consecutive miscarriages of <20 weeks of gestation, were ascertained through the Recurrent Pregnancy Loss Clinic at the Women's Health Centre of British Columbia. These 95 women had a total of 458 miscarriages, and of those, 167 were karyotyped. Karyotyped miscarriages consisted of 72 diploid losses, 71 aneuploid losses and 24 other anomalies, including polyploidy, sex chromosome aneuploidies and translocations. Of those women with aneuploid losses, there were 32 women who had a single trisomic miscarriage (ST) and 17 women who had multiple trisomic miscarriages (MT). POF patients ($N = 34$) with idiopathic secondary amenorrhea were ascertained from the POF Clinic at the Women's Health Centre of British Columbia. POF diagnosis was made based on the absence of menses for at least 3 months and two serum FSH results of >40 mIU/ml obtained more than 1 month apart, prior to 40 years of age. Two control groups were used in this study: Control group 1 ($N = 108$) consisted of healthy women of reproductive age, ranging from 17 to 55 years, and Control group 2 ($N = 46$) consisted of women who have had a healthy pregnancy over 37 years of age with no history of infertility or miscarriage. DNA was obtained by standard salt extraction from $\sim$5 ml of blood collected in EDTA tubes. The collection of the DNA samples for this study was obtained after informed consent and approved by the University of British Columbia Clinical Ethics Review Board, approval number CO1-0460.

Telomere length

Average relative telomere length was determined by quantitative PCR (qPCR) (Cawthon, 2002). Amplification of the telomeric repeat region was expressed relative to amplification of *36B4*, a single copy housekeeping gene on chromosome 12. This telomere to single copy (T/S) ratio is proportional to the average telomere length of the sample, due to the amplification being proportional to the number of primer binding sites in the first cycle of the PCR reaction (Cawthon, 2002). The protocol was performed as described by Cawthon (2002) with several modifications; amplifications were carried out in 20 μl reaction with $\sim$5 ng genomic DNA, 0.5 μM ROX Reference Dye (Invitrogen, Carlsbad, USA) and 0.2× SYBR Green I nucleic acid gel stain in dimethylsulfoxide (Invitrogen). Samples were run in triplicate on 96-well plates containing a standard curve constructed with reference DNA serially diluted to concentrations from 10 to 0.625 ng. A no-template control and both short- and long-telomere reference samples were run on each plate as quality controls. Dissociation melting curves were run after each sample to ensure amplification of a single species. Replicates of each plate were done to ensure reliable values were ascertained. The values between both runs were significantly correlated, with a correlation coefficient of $r = 0.49$ ($P < 0.0001$). To improve the accuracy of our estimates for each individual blood sample, we averaged the values of the two independent measurements. When values were discrepant between the two runs by more than 0.2 SDs, subsequent runs were done and an average of all values was used in further data analysis.

The telomere-specific qPCR assay was validated using DNA extracted from leukocyte cell pellets following flow fluorescence *in situ* hybridization (FISH) ($N = 12$) (Baerlocher *et al*., 2006). There was a strong correlation between the qPCR T/S ratio and the flow-FISH telomere lengths ($r = 0.96$). The strong correlation obtained validates the use of an average measurement of T/S values as an accurate reflection of telomere length. T/S values were converted to kilobases (kb) using the linear equation from this correlation ($y = 7.25\,x + 2.50$). As expected the *y*-intercept is at 2.5 kb since the flow-FISH assay was calibrated using Southern blot telomere restriction fragment lengths, which includes $\sim$2.5 kb of subtelomeric repeat (Baerlocher *et al*., 2006).

Statistical analysis

Rate of telomere decline was determined by linear regression analysis, and one-tailed *t*-tests were used to determine the significance of the regression because of the *a priori* hypothesis that telomere length was associated with age. Yearly rates of telomere decline were compared using two-tailed *t*-tests for comparison of regression slopes. Mean telomere length comparisons between sample groups were determined using pairwise analysis of covariance (ANCOVA) tests to adjust for differences in ages between sample groups.

Results

Telomere length in Control group 1 significantly declined with age ($P = 0.001$, one-tailed *t*-test) at a rate of 40 bp per year [95% confidence interval (CI) = 14–66 bp], although there was significant variability in telomere length at any given age ($R^2 = 0.081$, Table I, Supplementary Fig. 1). There was also a weak ($R^2 = 0.161$) but significant negative association between telomere length and age in POF patients ($P = 0.01$, one-tailed *t*-test), but not in Control group 2 or the RM group as a whole. Subsets of the RM group who have experienced ST or MT are of particular interest, as incidence of trisomic pregnancy increases with age, contributing to the age-related increase in RM. There was a weak ($R^2 = 0.130$) but significant relationship between telomere length and age in the ST subset of the RM group ($P = 0.02$, one-tailed *t*-test) but not the MT subset (Table I, Supplementary Fig. 2). However, in no sample group was the rate of telomere decline significantly different from that of Control group 1 (two-tailed *t*-tests for comparison of regression slopes), thus ANCOVA was used to adjust for age effects on mean telomere length for further comparisons of mean telomere length between groups.

Mean telomere length and age-adjusted mean telomere length for each sample group are shown in Table II. Although women in Control group 2 had longer age-adjusted mean telomere lengths than those in Control group 1, this difference was not significant. The RM group had shorter age-adjusted mean telomere length than Control group 1 (8.46 versus 8.92 kb, $P = 0.0004$) and this was also apparent in comparison with Control group 2 (9.11 kb, $P = 0.02$). However, short telomeres were not specifically confined to the subset of this group that had had either a single trisomy or multiple trisomic pregnancies. Contrary to expectation, age-adjusted mean telomere length in the POF patient group was longer than that in Control group 1 (9.58 versus 8.92 kb, $P = 0.01$), although this was not significant in comparison with Control group 2.

Discussion

Telomere-specific qPCR was used to assess telomere length in groups of women with a reproductive history suggestive of premature

Table I Rate of telomere loss per year in women with evidence of premature reproductive aging and controls

Sample group	N	Age range (years)	Rate of telomere decline (base pairs/year)			R^{2a}	*P*-value[b]
			Mean	Lower 95% CI	Upper 95% CI		
Control group 1	108	17–55	−40	−66	−14	0.081	0.001
Control group 2	46	37–54	26	−56	107	0.009	0.26
POF patients	34	21–50	−98	−178	−17	0.161	0.01
RM	95	24–45	−3	−40	40	0.000	0.44
Single trisomy	32	24–44	−56	−110	−2	0.130	0.02
Multiple trisomy	17	33–44	−23	−150	105	0.010	0.35

CI, confidence interval; POF, premature ovarian failure; Control group 1, women from the general population; Control group 2, women who had a healthy pregnancy after 37 years of age.
[a]R^2 is a measure of the goodness of fit of the regression.
[b]*P*-values are based on a one-tailed test for significance of the regression based on the *t*-distribution.

Table II Raw and age-adjusted mean telomere length

Sample group	*N*	Mean age (years)	Mean telomere length		*P*-value[a,b]
			Raw data (± SD) (kb)	Age-adjusted (kb)	
Control group 1	108	36.3	8.98 ± 1.15	8.92	
Control group 2	46	41.5	8.99 ± 1.03	9.11	0.36
POF patients	34	35.4	9.61 ± 1.38	9.58	0.01, 0.32
RM	95	35.8	8.47 ± 0.92	8.46	0.0004, 0.02
Single trisomy	32	36.3	8.80 ± 0.78	8.80	0.39, 0.26
Multiple trisomy	17	39.3	8.42 ± 0.69	8.52	0.11, 0.06

[a]*P*-values in comparison with Control groups 1 and 2, respectively.
[b]Analysis of covariance ($k = 2$ in comparison with Control group 1 or 2) was used to adjust raw telomere length data by age in comparisons between groups.

reproductive senescence to determine whether telomere length is associated with reproductive aging. As hypothesized, women experiencing RM had a shorter age-adjusted mean telomere length than control women, although this effect was not specifically confined to women with trisomic pregnancies. In contrast, POF patients had a longer age-adjusted mean telomere length than that of controls. The high variability in telomere length at any given age and the rate of telomere length decline with age have been previously reported in many control populations (Hastie *et al*., 1990; Slagboom *et al*., 1994; Benetos *et al*., 2001). In this study, the relationship between telomere length and age was not significantly different than zero in all sample groups, perhaps reflecting the limited age range in some groups. Regardless, none of the groups had a significantly different rate of telomere decline than that of controls.

The observed shorter average telomere length in women with RM and the trend of longer telomere lengths in women in Control group 2, who have had viable pregnancies late in their reproductive life, are consistent with the hypothesis that telomere length is a determinant of the rate of reproductive aging in women. Previous studies have reported that telomere length is a strong predictor of developmental potential of sister oocytes from women undergoing IVF (Keefe *et al*., 2007) and is also correlated with reproductive lifespan in women (Aydos *et al*., 2005). Short telomere length in telomerase-deficient mice is associated not only with premature aging but also with reduced fecundity leading to sterility (Liu *et al*., 2002). These mice exhibit impaired oogenesis and mimic the human age-related decline in oocyte quality, with increased rates of apoptosis of the oocytes, impaired chromosome synopsis and recombination and increased likelihood of non-disjunction and aneuploidy (Liu *et al*., 2004). Young mothers of children with Down's syndrome have normal telomere lengths (Dorland *et al*., 1998b), suggesting that predisposition to non-disjunction may not be the explanation for the finding of shortened telomeres in women with RM. Although, the telomere length of peripheral blood cells may not necessarily reflect that of oocytes or embryos. Psychological stress indicated by physiological stress markers has also been shown to negatively influence telomere length (Epel *et al*., 2004) due to an increased rate of cell turnover and increased exposure to reactive oxidative species. The shorter telomere lengths in women with RM may therefore reflect higher levels of psychological and physiological stress and/or constitutionally shortened telomeres.

The finding of increased telomere length in POF patients does not support the hypothesis that these women have accelerated cellular aging. As this is a relatively small sample size and the findings were not highly significant, it is possible that these results are due to a type 1 error. Although care must be taken in conclusions drawn from this data, and the necessity for additional study in this area must be emphasized, these findings are nonetheless intriguing. One explanation for the increase in telomere length observed in the POF cohort may be a constitutional genetic tendency toward an overall slower rate of cell division, perhaps by predisposing toward a prolonged cell cycle. A slower cell division rate in the developing ovary could lead to the establishment of a reduced follicular pool during early embryonic development, whereas fewer cell divisions in hematopoietic stem cells could result in longer telomeres measured in peripheral blood. If longer telomeres in blood reflect fewer mitotic divisions in the initial germ cell pool, this could explain a smaller follicular pool and early menopause in POF patients (Dorland *et al*., 1998a). A second possibility is that longer telomeres in the POF patients are a result of autoimmunity in these women. Autoimmune destruction of the ovaries is a common cause of POF (Goswami and Conway, 2005), and autoimmunity could conceivably alter blood cell composition to a cell type with longer telomeres. However, the limited existing evidence on telomere length and autoimmunity suggests that autoimmune conditions are associated with shorter rather than longer telomeres (Jeanclos *et al*., 1998), making this a less likely explanation.

Alternatively, longer telomeres in the POF patient group may be the result of abnormal hormone exposure in these women. POF patients in our study may have been exposed to elevated estrogen levels as a result of recruitment of large cohorts of oocytes in menstrual cycles occurring prior to POF onset. Premature follicular pool exhaustion resulting from the continual recruitment of large cohorts of follicles prior to menopause has been proposed as one mechanism for POF (Pal and Santoro, 2002). Estradiol is secreted from developing follicles (Havelock *et al*., 2004) and stimulated estrogen level is correlated with the size of the antral follicle cohort (Scheffer *et al*., 2003). If abnormally large follicular cohorts were recruited while POF patients were still cycling, this could lead to elevated estrogen levels with a positive influence on telomere length. This positive influence on telomere length prior to the onset of POF may be reflected later in life in the form of long telomeres after POF diagnosis. On the other hand, maintenance of telomere length may be a recent phenomenon in these women, resulting from hormone replacement therapy following POF diagnosis. Although we lack the clinical details to assess this possibility in our POF population, this hypothesis is supported by the finding that long-term hormone replacement therapy in post-menopausal women slows the rate of telomere attrition (Lee *et al*., 2005). Two mechanisms by which estrogen may positively regulate telomere length have been proposed: (i) estrogen may ameliorate the negative effects of reactive oxygen species (Aviv *et al*., 2005) which reduce telomere length by inducing single strand breaks (von Zglinicki, 2000) and (ii) estrogen may stimulate telomerase activity (Aviv *et al*., 2005). Ovarian telomerase activity is reportedly low in POF patients with follicular depletion, but high in POF patients with ovarian dysfunction and normal follicle counts (Kinugawa *et al*., 2000). Since follicle count is correlated with circulating estrogen level (Vital-Reyes *et al*., 2006), this supports the suggestion that telomerase activity is influenced by estrogen exposure, at least at the ovary.

There are several limitations to this study that must be considered. Telomere length varies among blood cell types (Lansdorp, 2006); therefore variability between individuals in telomere length measured in peripheral whole blood may be a consequence of differences in blood cell composition. Furthermore, telomere length measured in peripheral blood may not necessarily reflect telomere length in the ovary or developing embryo. However, there is a strong correlation between telomere lengths from tissues of a single individual (Butler *et al*., 1998) suggesting that telomere lengths measured in whole blood may be an accurate proxy for telomere length at the ovary. Small sample sizes and limited clinical details (including incomplete karyotype information on the losses of RM group and no details on reproductive history of the Control group 1) restrict the ability to subdivide sample groups into more homogeneous phenotypes, and negatively impact the power of these analyses.

RM, trisomic pregnancy and POF have all been considered measures of premature reproductive aging. However, the observation

that RM and POF showed opposite associations with telomere length, and trisomic pregnancy showed no evidence of an association, suggests that these different types of reproductive aging are likely influenced by unique factors. Further studies are necessary to confirm these findings in larger more precisely defined populations, examine the physiological mechanisms that influence both telomere length and reproductive aging and investigate the molecular mechanisms responsible for longer telomere lengths in the POF population.

Supplementary Data

Supplementary data are available at http://humrep.oxfordjournals.org.

Acknowledgements

We thank the patients and control women for their voluntary participation in this research. We thank Dr Peter Lansdorp for advice on the manuscript and contribution of flow-FISH samples for validation of the telomere qPCR technique.

Funding

C.W.H. and K.L.B. were funded by the Interdisciplinary Women's Reproductive Health training program, a Canadian Institutes of Health Research (CIHR) funded strategic training program at the Child and Family Research Institute in Vancouver, British Columbia, Canada. K.L.B. was supported by CIHR and the Michael Smith Foundation for Health Research. This project was funded by a CIHR operating grant to W.P.R.

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Submitted on September 29, 2008; resubmitted on December 15, 2008; accepted on December 28, 2008