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TELOMERE SHORTENING WITH AGING IN HUMAN ESOPHAGEAL MUCOSA

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ABSTRACT

Progressive telomere shortening with aging was studied using normal esophageal mucosal specimens from 177 human subjects aged between 0 and 102 years (yrs). We observed age-related shortening of the telomere, at a rate of 60 base pairs (bp) per year (yr). The mean telomere length of 12 neonates was 15.2 kilobase pairs (kbp) and that of 2 centenarians was 9.3 kbp. Mean (± SD) telomere lengths were 14.9±1.3, 14.0±1.8, 10.1±3.7, 10.4±3.3 and 9.5±3.1 kbp for the age groups less than 2 yrs, 2-20 yrs, 21-60 yrs, 61-80 yrs and 81-102 yrs, respectively. The variation in telomere length among individuals in the same age group was greater for the 3 older groups than for the 2 younger groups, as shown by the SDs. Furthermore, older individuals had greater telomere length variation than younger individuals, based on the lengths of DNA digested smears. Although the telomere length decreased significantly with aging at the rate of 60 bp per yr, differences in the mean telomere lengths between the 3 older age groups were not significant. Rapid shortening occurred in the young generations and there was no further substantial decrease in the esophageal mucosa after 60 yrs of age. Compared to the very rapid renewal rate of the esophageal epithelial cells, the annual reduction rate in telomere length was very low. These findings support the hypothesis that germ cells in the esophageal epithelium have a mechanism to lengthen telomeres.

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INTRODUCTION

Normal human cells exhibit a limited capacity to proliferate in culture, as stated in Hayflick's hypothesis,[1] and the finite replicative lifespan of normal cells in culture is frequently used as a model of human aging. This phenomenon is considered to be associated with reduction in telomere length as an indicator of the number of cell divisions undergone.[2] The telomeres, regions of DNA located at the ends of eukaryotic chromosomes, are considered to protect chromosomes against degeneration, reconstruction, fusion and loss[3] and also to ensure the complete replication of DNA molecules.[4] Telomeric DNA has a very simple structure, with hundreds to thousands of TTAGGG repeats, in humans and other vertebrates.[5] Although it has been known for many years that these repeats are shortened by about 50-150 base pairs (bp) at each cell division in human fibroblasts[6] and lymphocytes in vitro,[7] telomere shortening with aging in many other human cell types in vivo has not been studied. Reduction rates of human telomere shortening per year have only been reported for skin fibroblast-like cells,[6] peripheral lymphocytes,[7] epidermal cells,[8] peripheral blood cells in vivo,[2] mucosa of the large and small intestines,[9] large bowel mucosa[10] and vascular intimal tissues.[11] We have conducted systemic studies to measure the telomere length from human tissue of all types, including the esophageal mucosa. In the present study we measured the lengths of HinfI digested terminal restriction fragments (TRFs), which are mainly composed of repeats of TTAGGG, to estimate the telomere length in normal epithelial cells of esophageal specimens obtained from 177 patients at autopsy examinations. We then calculated the annual rate of telomere loss.

MATERIALS AND METHODS

Autopsy specimens of normal esophageal mucosa were obtained from 177 patients (87 males and 90 females, aged between 0 and 102 yrs). This study group included 12 neonates (0-4 weeks), 4 infants (4 weeks - less than 2 yrs), 6 children and youths (2-20 yrs) and 2 centenarians (> 99 yrs). Only one each could be examined in their 20s and 30s, and only three in their 40s.

Autopsy examinations were performed within 5 h of death at the Tokyo Metropolitan Geriatric Hospital and the Japanese Red Cross Medical Center. None of the patient histories or examination findings suggested evidence of esophageal carcinoma. All samples of esophageal mucosa were stored at -80°C until use.

Genomic DNA was prepared from each sample by treating the samples with proteinase K and sodium dodecyl sulfate (SDS) followed by repeated phenol-chloroform extraction. Five microgram aliquots of DNA were digested with the restriction enzyme HinfI (Boehringer Mannheim Biochemica, Germany). Complete cutting by the enzyme was confirmed by electrophoresis of the DNA digests on 0.8% agarose gels. Fractionated DNA fragments were transferred to nylon membranes (Hybond-N+, Amersham, UK) by an alkaline transfer technique using capillary blotting. The membranes were incubated for 12 h at 50°C in hybridization buffer (6 × SSPE [1 × ;0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4], 1% SDS) containing a $(TTAGGG4)_4$ probe that had been labeled with [γ-^{32}P]ATP (Amersham) at the 5' end using T4 polynucleotide kinase (Toyobo, Japan). The membranes were washed first in 2 × SSC (NaCl 17.55 g/l, sodium citrate 8.82 g/l) at room temperature, and then in 6 × SSC, 0.1% SDS at 50°C for 15 min with shaking, then dried with filter papers and used to expose to x-ray film and Fuji Imaging Plates (Fuji Photo Film Co. Ltd., Japan) for 3 h at room temperature. The Fuji Imaging Plates were subsequently analyzed with a BAS-2500 Mac image analyzer (Fuji Photo Film Co. Ltd.) using the programs Image Reader (version 1.1, Fuji Photo Film Co. Ltd.) and Mac Bas (version 2.4, Fuji Photo Film Co. Ltd.). We estimated mean TRF length at the peak position of hybridization signal in each lane in comparison with the molecular size markers and TRF length was recorded for simplicity as telomere length.[2,12] Differences in mean values were analyzed by Student's t-test and correlations were analyzed by the Fisher's test.

RESULTS

The smears of telomeric DNA of the esophageal mucosa on the Southern blotting radiographs were very short for the 2 younger groups and long for the 3 older groups, indicating greater variation in telomere length among the cells from an individual subject in the older groups (Figure 1).

Figure 2 shows the telomere lengths for all of the subjects, plotted as a function of donor ages. Fisher's test confirmed conspicuous age-related shortening ($r = -0.468$, $p < 0.001$), despite the long telomere lengths occasionally recognizable in older individuals, and the mean rate of telomere shortening was calculated to be 60 bp per yr.

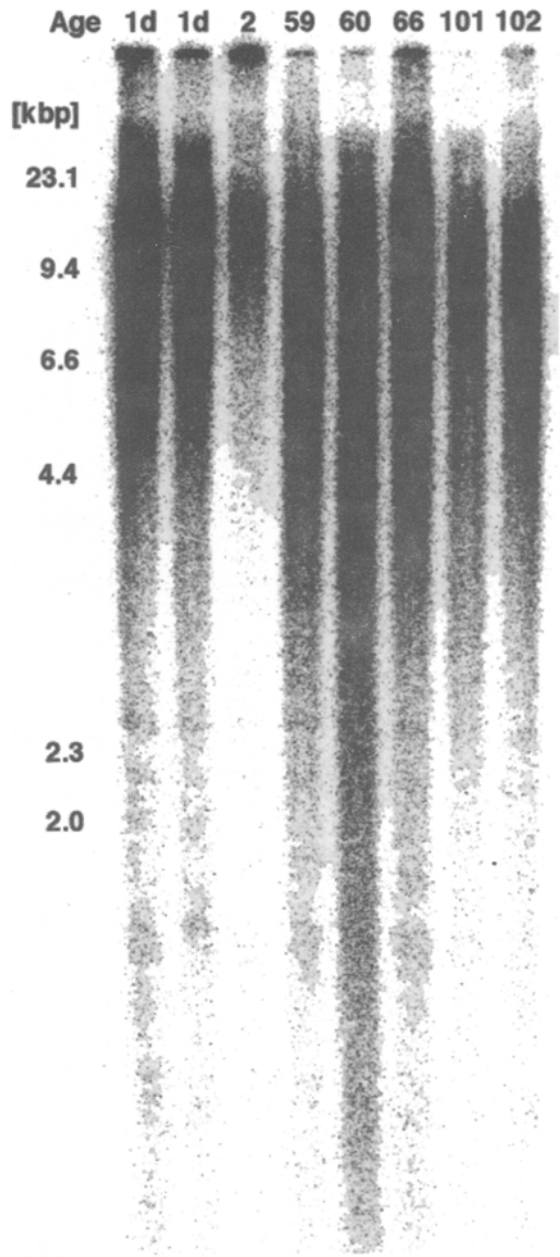

Figure 1: Representative autoradiogram showing Southern blot analysis of telomeric DNA from the esophageal mucosa of subjects aged 0 to 102 yrs. Sizes are indicated on the left. Radiographic results for subjects aged 1 day (2) and 2, 59, 60, 66, 101, and 102 yrs old are shown. The terminal restriction fragment lengths for these subjects were 15.1, 17.2, 14.9, 8.9, 9.4, 13.7, 8.3, and 10.3 kbp respectively. The smears of telomeric DNA of the esophageal mucosa on the Southern blotting radiographs are short for 2 neonates and an infant and long for the 5 older subjects, demonstrating greater variability among cells in the latter. Note that long telomeres could still be occasionally observed in older subjects.

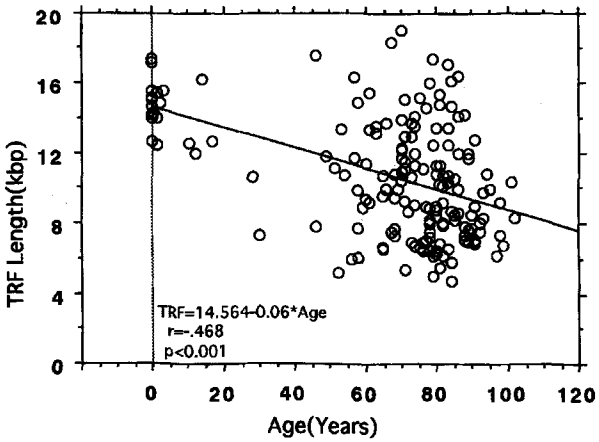

Figure 2: Telomere lengths of individuals. The mean terminal restriction fragment length for each individual, determined by quantitative analysis, is plotted against age. The slope (-60 bp per yr) of the linear regression line is significantly different from 0 ($p < 0.0001$).

The mean telomere length of 12 neonates was 15.2 kilobase pairs (kbp) and that of 2 centenerians was 9.3 kbp). The mean (± SD) telomere lengths by age group were 14.9±1.3, 14.0±1.8, 10.1±3.7, 10.4±3.3 and 9.5±3.1 kbp for the age groups less than 2 yrs, 2-20 yrs, 21-60 yrs, 61-80 yrs and 81-102 yrs old, respectively (Figure 3). Telomere lengths were not significantly different between the 3 older age groups (21-60, 61-80 and 81-102 yrs) (Figure 3). However, telomere shortening was found to be significant when the data for the 2 younger groups were compared to the data for the 3 older groups.

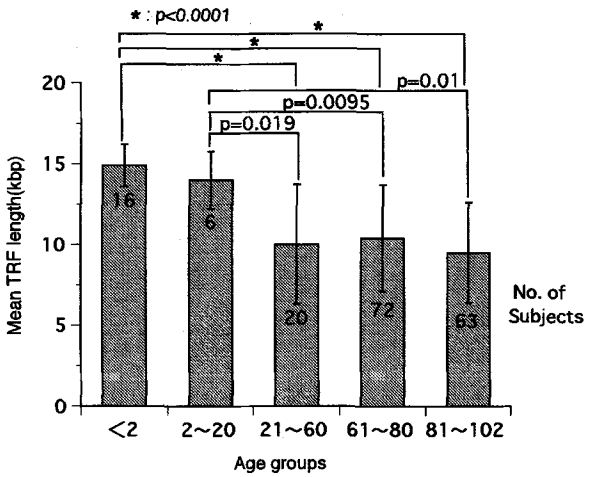

Figure 3: Mean telomere lengths in the five age groups. Only two autopsy cases were 21-40 yrs old and these two and those in the 41-60 yrs age category were considered together (21-60 yrs). Telomere lengths were not significantly different between the 3 older age groups (21-60, 61-80, and 81-102 yrs). However, shortening was found to be significant when the data for the 2 younger groups were compared to data for the 3 older age groups.

Subjects with telomere lengths more than 14 kbp occurred with frequencies of 81% (< 2 yrs), 50% (2-20 yrs), 15% (21-60 yrs), 13% (61-80 yrs) and 13% (81-102 yrs). Values for 10 up to 14 kbp were 19% (< 2 yrs), 50% (2-20 yrs), 35% (21-60 yrs), 40% (61-80 yrs) and 29% (81-102 yrs), for 6 up to 10 kbp were 0% (< 2 yrs), 0% (2-20 yrs), 40% (21-60 yrs), 44% (61-80 yrs) and 50% (81-102 yrs), and for less than 6 kbp were 0% (< 2 yrs), 0% (2-20 yrs), 10% (21-60 yrs), 3% (61-80 yrs) and 8% (81-102 yrs)(Figure 4). No subjects in the 2 younger age groups had short telomeres less than 10 kbp.

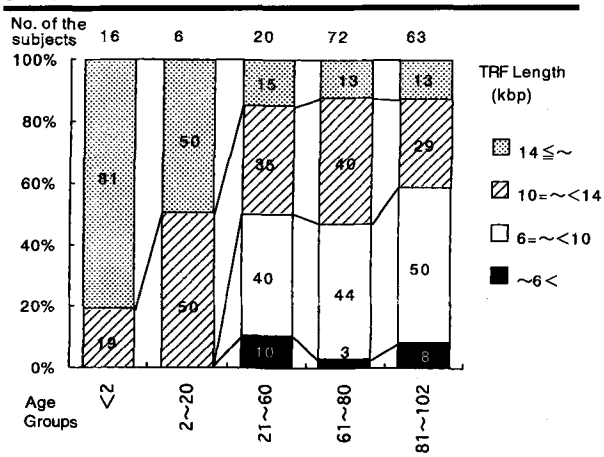

Figure 4: Telomere length as a function of subject age. No subjects in the 2 younger age groups had short telomeres less than 10 kbp. About half of the telomere lengths of subjects older than 21 yrs were less than 10 kbp.

DISCUSSION

Telomeres shorten after every cell division, with the exception of immortal, carcinomatous and germ cells.[3,13] Telomere shortening has been reported in skin fibroblast-like cells,[6] peripheral lymphocytes,[7] epidermal cells,[8] peripheral blood cells,[2] mucosa of the large and small intestines,[9] large bowel mucosa[10] and vascular intimal tissues[11] of humans. However, there has hitherto been no information documented about esophageal mucosa. In the present study we established that telomere shortening occurs at the rate of 60 bp per yr in the esophagus of Japanese. The telomere lengths were similar in the 3 older groups (21-60, 61-80 and 81-102 yrs), but significantly shorter when compared with the 2 younger groups. Suggesting rapid shortening occurred in the young patients and at some point between 21 and 60 years, substantial telomere shortening ceased. It proved difficult to collect enough esophageal samples from patients within the 21-60 yr age span to estimate when this may occur. Reported rates of telomere shortening are 41 bp per yr in human peripheral lymphocytes,[7] 19.8 bp per yr in human epidermal cells,[8] 33 bp per yr in human peripheral blood cells,[2] 42 bp per yr in human intestinal mucosa[9] and 67 bp per yr in human large bowel mucosa.[10] These reduction rates are all less than the generally assumed rate of 100 bp per cell division. However, turnover times of esophageal and colonic mucosal epithelia are very rapid, about 3-7 days,[14] and the proliferative zone of the esophageal epithelium is a basal layer that sits on the basement membrane on the lamina proper mucosae.[14,15] Therefore, the germ cells in the basal layer of the esophageal

epithelium may divide more than 50 times a yr. Based on a simple calculation, this suggests that the telomere length should reduce by 5000 bp per yr (100 bp lost per cell division × 50 cell divisions per yr), whereas we observed a reduction of only 60 bp, compatible with only one cell division per yr. These results suggest that either not all germ cells divide and only a very small number divide very frequently, or germ cells in the esophageal epithelium have a mechanism to lengthen telomeres.

In 1965 Marques-Pereira and Leblond[15] reported that after a cell division in the epithelium of esophageal mucosa either 1 of the 2 daughter cells stays in the germinal area and the other goes up to the epithelial surface, or both daughter cells stay in the germinal area, or both go up to the epithelial surface. Their results may suggest why we observed greater variation in telomere length in individuals in the older age groups than in individuals in the younger groups.

Although we have reported in a previous paper that 22 of 92 normal esophageal mucosae had detectable telomerase activity,[12] that is of a ribonucleoprotein that synthesizes telomeric DNA onto chromosomal ends by using a segment of its RNA component as a template,[16] none was evident in the present study. However, our data on the distribution of hybridization signals in the older groups and wide variation of the telomere lengths among individuals in the three groups might suggest that only small numbers of germ cells are involved, introducing a sampling variable.

Strong telomerase activity in humans has been detected in germline cells and carcinoma tissues as well as in established cultured cell lines,[17] but, in contrast, not in most normal human somatic cells. However, keratinocytes of the skin epidermis[18] and endothelial cells[19] have been reported to have weak telomerase activity when maintained in culture. Moreover, it has recently been reported that non-neoplastic peripheral leukocytes,[20] gastric mucosa[21] and colonic mucosa[10] of man are positive. We reported earlier that the human esophageal mucosa has telomerase activity that is detectable by telomeric repeat amplification protocol assay,[12] in line with results from other groups using the TRAP assay and in situ hybridization, respectively.[22, 23] In the latter case squamous esophageal epithelium demonstrated weak positive signals limited to basal cells.[23] Our data and the data of others support the idea that a telomerase maintenance mechanism, perhaps telomerase, in the stem cells of the basal layer in the esophageal mucosa is able to prevent significant telomere shortening as compared to other somatic cells without telomerase.

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