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Telomere Shortening With Aging in Human Liver

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Progressive telomere shortening with aging was studied in the normal liver tissue of 94 human subjects aged between 0 and 101 years old to determine the rate of telomere loss in 1 year. Telomere length demonstrated age-related shortening with reduction of 55 base pairs (bp) per year. The mean telomere length in five neonates was 12.9 ± 2.6 kilobase pairs (kbp), and that in one centenarian was 8.3 kbp. Mean telomere lengths by age group were 13.2 ± 2.0 kbp (≤8 years; 10 subjects), 7.8 ± 1.9 kbp (40–79 years; 29 subjects), and 7.5 ± 2.0 kbp (≥80 years; 53 subjects), with reduction thus appearing to show slowing on the attainment of middle age. The difference of mean telomere lengths for two groups with or without advanced malignancies of other than liver origin was not significant in the older two groups. Despite the slow turnover of liver tissue, the overall reduction rate of telomere length decrease in 1 year was almost the same as that of digestive tract mucosa, with its very rapid renewal.

NORMAL human cells exhibit a limited capacity to proliferate in culture (1), and this finite replicative life span is frequently used as a model of human aging. The phenomenon is considered to be associated with reduction in telomere length as an indicator of the number of cell divisions undergone (2,3). Human fibroblasts in culture usually stop proliferating with a telomere length of approximately five to six kilobase pairs (kbp); this is known as cellular senescence (4,5). Telomeres are located at the ends of eukaryotic chromosomes, where they are considered to protect against degeneration, reconstruction, fusion, and loss (2) and to ensure complete replication of DNA molecules (6). Telomere DNA is very simple in structure, comprising hundreds to thousands of TTAGGG repeats in humans (7) and other vertebrates. Although it has been known for more than 10 years that these repeats are shortened by 50–150 base pairs (bp) at each cell division in human fibroblasts and lymphocytes in vitro (8,9), telomere reduction with aging in humans has not been clarified for most tissues. We have therefore conducted systemic studies to assess the telomere length from human tissues of all types (10,11), including the liver. To our knowledge, there has hitherto been no systematic measurement of telomere length in the normal human liver. Southern blot analysis has revealed large differences among individuals (11), so many subjects must be examined for optimal results. For the present study we measured terminal restriction fragments (TRFs), containing tandem repeats of TTAGGG, by Southern blotting in normal liver tissue obtained from 94 autopsied patients with no metastatic carcinoma, hepatocellular carcinoma, liver cirrhosis, or hepatitis.

Materials and Methods

Samples of normal hepatic tissue were obtained from the left lobe of the liver from 94 individuals without metastatic carcinoma, hepatocellular carcinoma, liver cirrhosis, or hepatitis (46 males and 48 females between 0 and 101 years of age), including five neonates (0–4 weeks), two children (1–2 years) and one centenarian (more than 99 years of age), who underwent autopsy within 5 hours after death at the Tokyo Metropolitan Geriatric Hospital or the Japanese Red Cross Medical Center. Only two subjects could be examined who were in their 20s and 30s. The causes of death in the five neonates were congenital obstruction of the small intestine, congenital abdominal wall defect, sepsis, holoprosencephaly, and Bochdalek's hernia. The causes of death in the two children were cardiomyopathy and transposition of the great vessels. All samples were stored at −80°C until use. The study protocol was approved by the Tokyo Metropolitan Institute of Gerontology Ethical Committee.

Preliminarily, for five of the 94 subjects, hepatocytes were isolated from hepatic tissue by collagenase perfusion through the portal vein as previously described (12). Briefly, fresh tissue blocks from autopsied livers were perfused with collagenase from the portal veins, and total liver cell suspensions were centrifuged at 50 g for 1 minute. The supernatant was further centrifuged at 50 g for 3 minutes to pellet hepatocytes.

From the whole liver tissues from the 94 subjects and from the isolated hepatocytes from the five subjects, genomic DNA was prepared by using proteinase K and sodium dodecyl sulfate (SDS) followed by repeated phenol-chloroform extractions. Five-microgram aliquots were digested

with the restriction enzyme *Hin*f I (Boehringer Mannheim Biochemica, Germany), with complete cutting being 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, followed by hybridization for 12 hours at 50°C in an appropriate solution [6× SSPE (1×; 0.15 M of NaCl, 10 mM of sodium phosphate, and 1 mM of ethylenediamine tetra-acetic acid; pH 7.4), 1% SDS] to a $(TTAGGG)_4$ probe labeled with [γ-^{32}P]ATP (where ATP is adenosine triphosphate; Amersham) at the 5′ end using T4 polynucleotide kinase (Toyobo, Japan). The membranes were washed in 2× SSC (17.55 g/l of NaCl, and 8.82 g/l of sodium citrate) at room temperature, and then in 6× SSC, 0.1% SDS at 50°C for 15 minutes while being shaken. They were then dried with filter papers and exposed to Fuji Imaging Plates (Fuji Photo Film Co. Ltd., Japan) for 3 hours at room temperature. Analysis was with a BAS-2500 Mac image analyzer (Fuji Photo Film), with the use of the programs Image Reader (Version 1.1, Fuji Photo Film) and Mac Bas (Version 2.4, Fuji Photo Film). TRF lengths were determined by comparing the position of the maximum radioactivity in each lane with molecular size markers. Differences in mean values were assessed for significance with the Student's *t* test and correlations by Fisher's test.

Results

In the preliminary experiment, the differences in telomere lengths between whole tissue samples and isolated hepatocytes for the five subjects were from −0.2 to +0.6 kbp, without significant difference (Figure 1). Accordingly, we regarded telomere lengths of whole liver tissues as those of hepatocytes. The smear widths on Southern blot analysis of telomeres of hepatic tissue increased with age (Figure 2).

Figure 3 summarizes telomere lengths for all 94 subjects, plotted as a function of subjects' age. According to the Fisher's test in all 94 subjects, age-related shortening was highly significant ($r = -.6$, $p < .0001$), with average telomere length shortening by 55 bp per year. With the same test in the 82 subjects more than 40 years old, average telomere length shortening by 12 bp per year was recognizable, but this was not significant (data not shown).

Mean telomere length in five neonates was 12.9 ± 2.6 kbp, and the value for one centenarian was 8.3 kbp. Mean

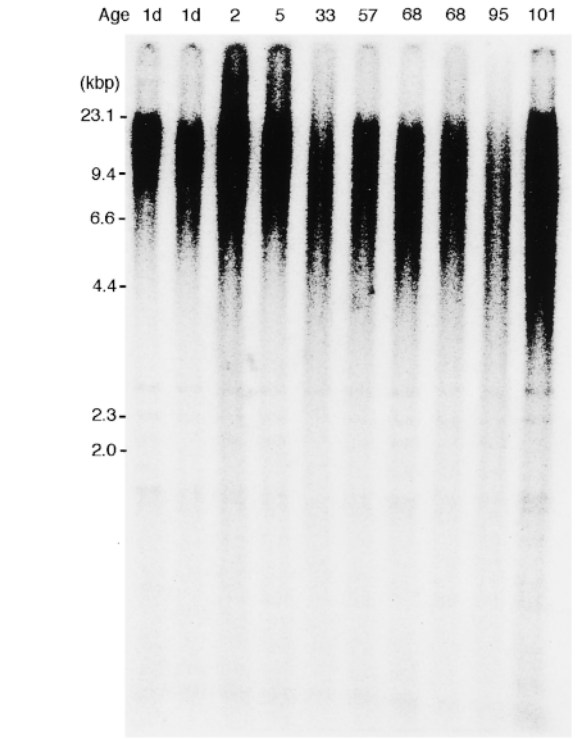

Figure 2. Southern blot analysis of telomeres of human hepatic tissue from subjects aged from 0 to more than 100 years old. Sizes are indicated on the left. Radiograms for samples from 1-day-old individuals (two subjects) and individuals aged 2, 5, 33, 57, 68 (two subjects), 95, and 101 years are shown. Terminal restriction fragment lengths are, respectively, 17.1, 13.2, 14.9, 13.6, 9.2, 10.6, 8.6, 9.9, 6.4, and 8.3 kbp.

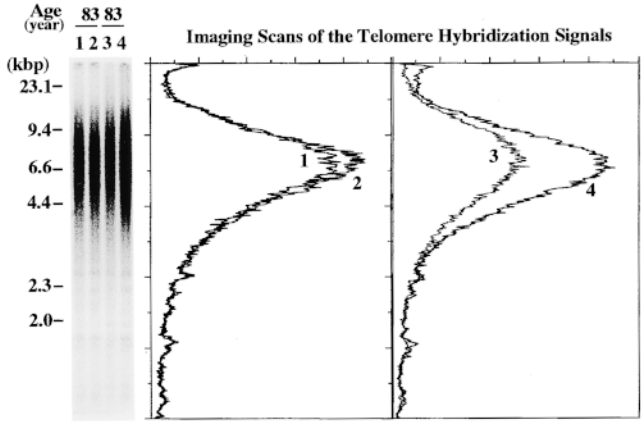

Figure 1. Southern blot analysis of telomeres of the whole liver tissues and isolated hepatocytes from two 83-year-old men (left), and imaging scans of these telomere hybridization signals (middle and right). Lanes 1 and 3 show whole liver tissues and lanes 2 and 4 show isolated hepatocytes. Telomere lengths of lanes 1 and 2 are 7.0 and 7.2 kbp, and those of lanes 3 and 4 are 7.5 and 6.9 kbp, respectively.

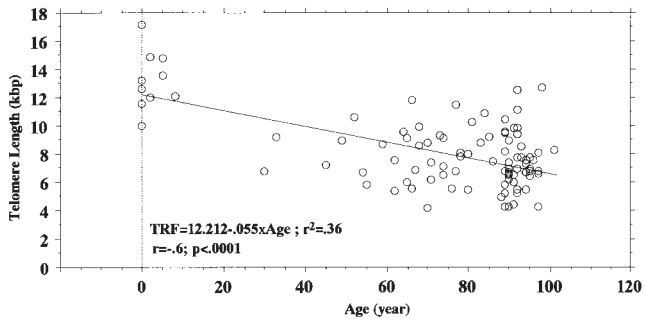

Figure 3. Loss of telomere DNA in human hepatic tissue as a function of subject age. Mean terminal restriction fragment (TRF) lengths from a quantitative analysis of autoradiograms, as described in the Materials and Methods section, were plotted as a function of subject's age. The slope (−55 bp/y) of the linear regression line is significantly different from zero ($p < .0001$).

telomere lengths for three age groups were 13.2 ± 2.0 kbp (≤8 years; 10 subjects), 7.8 ± 1.9 (40–79 years; 29 subjects), and 7.5 ± 2.0 kbp (≥80 years; 53 subjects). The difference between the youngest and oldest groups was significant ($p < .0001$). However, this was not the case for the oldest two age groups.

Mean telomere lengths for two groups, those with subjects 40–79 years old with (17 subjects) or without (12 subjects) advanced malignancies of other than liver origin, were 8.0 ± 2.2 and 7.6 ± 5.7 kbp; difference was not significant. Mean telomere lengths for two groups with subjects above 80 years of age (17 subjects) or without (36 subjects) advanced malignancies were 7.3 ± 4.1 and 7.6 ± 4.1 kbp, again without significant difference.

Subjects with telomere lengths more than 14 kbp were observed in 30.0% (≤8 years), 0% (40–79 years), and 0% (≥80 years). Telomere lengths of 11–14 kbp were observed in 60.0% of those aged ≤8 years, 6.9% of those aged 40–79 years, and 5.7% of those ≥80 years. Respective figures for lengths of 8–11 kbp were 10.0%, 37.9%, and 30.2%, and those for less than 8 kbp were 0%, 55.2%, and 64.2% (Figure 4). The two subjects aged 9–39 years were excluded from Figure 4.

Discussion

Telomeres from all human tissues are generally considered to shorten on cell division, except in carcinomatous and germ cells (2,3,6,8), although this has not been confirmed for many cases. Reduction has been reported for peripheral lymphocytes in vivo (41 bp/y, ages 0–107 years) (9), skin fibroblasts in vitro (15 bp/y, ages 0–93 years) (4), epidermal cells in vivo (19.8 bp/y, ages 0–92 years) (13), peripheral blood cells in vivo (33 bp/y) (8), human mucosae from large and small intestines (42 bp/y, ages 0–89 years) (14), human large bowel mucosa from surgical and autopsy specimens (67 bp/y, ages 0–97 years) (10), esophageal mucosa (60 bp/y, ages 0–102 years) (11), and vascular intimal tissues (15). Here we obtained data pointing to an average reduction rate in human hepatic tissue of 55 bp/y. Although

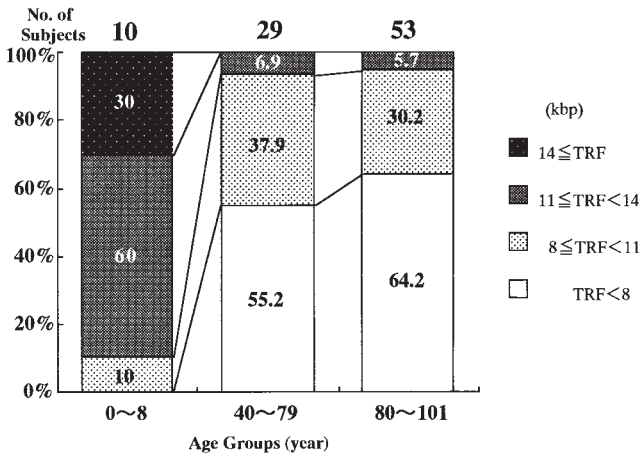

Figure 4. Distribution of telomere lengths in three different age groups. The two subjects aged 9–39 years were excluded (TRF; terminal restriction fragment).

a renewal time for human hepatocytes has not been reported, that for mouse hepatocytes was found to be 480–620 days (1.3–1.7 years) in an autoradiographic study (16). Telomere repeats are shortened by 50–150 bp at each cell division in human fibroblasts in vitro and lymphocytes in vitro (4,5,8). In contrast, turnover of esophageal and colonic mucosal epithelia is very rapid at approximately 3–7 days (17,18), and we previously revealed reduction of only 60 and 67 bp respectively in 1 year in these tissues (10,11). Although the turnover time is very different among the tissues, annually telomere reduction rates among them are not very different. Thus, the rate of telomere shortening of hepatic tissue in 1 year is almost the same as those for esophageal and colonic mucosae, suggesting that germ cells in the latter have a mechanism for lengthening telomeres.

As to the telomere length of the hepatic tissue, hepatocellular carcinomas demonstrated a shorter telomere length than the corresponding noncancerous liver tissues in 20 cases, with respective mean values of 5.4 and 8.8 kbp (19). Although many different research groups (20,21) have also measured the telomere lengths of hepatocellular carcinomas and corresponding noncancerous liver tissues, the noncancerous tissues were not in a normal condition but rather demonstrated chronic hepatitis or liver cirrhosis. There has been no systematic study of telomere length in normal human liver. Kojima and colleagues (20) have reported the mean telomere length in eight normal livers (43–68 years) to be 7.3 ± 1.4 kbp, and Miura and coworkers (21) described a value for four normal livers (13–79 years) of 8.9 ± 1.2 kbp. Urabe and associates (22) have reported telomere lengths for 13 normal autopsied livers (one was from a 14-year-old individual, and the remaining 12 were from individuals aged more than 40 years; the mean age was 64.1 years) but could not demonstrate a significant decrease with aging. According to Fisher's test, in the 82 subjects more than 40 years old, age-related shortening was not significant. Moreover, there was no significant difference between the oldest two age groups (40–79 years and ≥80 years).

It has occasionally been reported that rapid telomere loss occurs in the young, and only slow reduction is typical in the elderly. Thus Frenck and colleagues (23) reported that rapid loss of telomeres from peripheral blood cells in the young contrasted with a much slower rate of loss in older adults. Esophageal mucosa also has been suggested to exhibit rapid reduction of telomere length in the young (11). These data suggest the possibility that rapid shortening occurs in the young and only a slower reduction occurs after 40 years of age, not only in the liver but also in many other human organs or tissues. However, in human fibroblasts no rapid reduction has been reported (4), so tissue or cell-specific differences might also be expected.

Our results indicate that telomere length in the hepatic tissue is not influenced by malignant disease originating in other organs.

Telomerase is a ribonucleoprotein that synthesizes telomere DNA onto chromosomal ends by using a segment of its RNA component as a template (24). Strong telomerase activity has been detected in human germline cells and carcinoma tissues, and in established cell lines in culture (25).

In contrast, it appears to be lacking in most normal human somatic cells. Keratinocytes of the skin epidermis (26) and endothelial cells (27) in culture have been reported to demonstrate weak telomerase activity. Moreover, it has been recently shown that nonneoplastic peripheral leukocytes (28), gastric mucosa (29), esophageal mucosa (30,31), and colonic mucosa (14) have detectable telomerase activity. However, there has been no such report for hepatic tissue.

Although human liver tissue consists of hepatocytes, sinusoidal and vessel endothelial cells, Kupffer stellate cells, Ito cells, bile duct and capillary cells, and inflammatory cells, hepatocytes constitute 79% of the volume (32). In another report hepatocytes accounted for 78% of hepatic tissue volume (33). Ogawa and colleagues (34) described the relative numbers of these four cellular components in hepatic tissue to be 100 for hepatocytes, 27.5 for endothelial cells, 15.8 for Kupffer cells, and 10.1 for Ito cells. Accordingly, approximately 64% of all cells in the liver are hepatocytes. Our data for telomere length in the hepatic tissue point to a mean reduction rate of 55 bp/y for all these cells mixed together. However, our preliminary experiment comparing the telomere lengths between the whole liver tissues and isolated hepatocytes demonstrated very similar values. We therefore conclude that telomere lengths of mixed cells can be regarded to reflect those of hepatocytes themselves.

In the present study, telomere shortening predominantly occurred before 40 years of age, as in previous investigation of leukocytes (23) and esophageal mucosa (11). The reason for this age-dependent shortening requires explanation, but presumably the slowing down with age may be related to a gradual reduction in cell turnover with advancing years.

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