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RAPID COMMUNICATION

Telomere Reduction in Human Liver Tissues with Age and Chronic Inflammation

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Telomere shortening in human liver with aging and chronic inflammation was examined by hybridization protection assay using telomere and *Alu* probes. The reduction rate of telomere repeats in normal liver (23 samples from patients 17–81 years old) was 120 bp per year, which is in good agreement with the reported reduction rate in fibroblasts of 50–150 bp at each cell division and replacement rate of human liver cells, once a year. Mean telomere repeat length shortened to about 10 kbp in normal livers from 80-year-old individuals. The number of telomere repeats in chronic hepatitis (26 samples) and liver cirrhosis (11 samples) was significantly lower than that in normal liver of the same age ($P < 0.01$). Telomere length in all these chronic liver disease samples, other than two exceptions, was not reduced shorter than 5 kbp, which was assumed to give a limit of proliferation (Hayflick's limit) to untransformed cells.

Key Words: **hybridization protection assay (HPA); telomere reduction; human liver; aging; chronic inflammation.**

INTRODUCTION

Human normal somatic cells have a limited proliferative life span (Hayflick's limit) when serially cultured *in vitro* [1, 2]. The maximum number of cumulative population doublings is inversely proportional to the age of the donor [3, 4]. Fibroblasts from patients with premature aging syndrome have a very short proliferative life span *in vitro* as compared with those from normal counterparts of the same age [5, 6]. Telomere DNA of human somatic cells shortens by about 50–150 base pairs (bp) at each cell division, which determines, as a mitotic clock, a finite proliferative capacity of human somatic cells. When the telomere repeats, measured as a length of *Hin*fI-digested terminal restriction fragment (TRF), shorten to about 5–6 kilobase pairs (kbp), human fibroblasts cease proliferation as senescent cells. When the human fibroblasts are cultured in hyperoxic conditions, they prematurely senesce, with accelerated TRF shortening to about 4–5 kbp. A causal relation between telomere shortening and cellular senescence has been supported by showing that transfection of the telomerase reverse transcriptase gene into various human mortal somatic cells results in elongation of telomere length and extension of the *in vitro* replicative life span [7–10]. The idea that the limit of proliferative life span by telomere shortening of human somatic cells is one of determinants of human senescence and human life span *in vivo* has been supported by several lines of evidence, including loss of telomere size and proliferative capability of somatic cells with aging in normal individuals [11, 12] and abnormal telomere loss in patients with heritable disorder of premature aging [11, 13] or accelerated telomere shortening by active oxygen species, which are assumed to be one of the major environmental causes of human aging [14]. However, human skin fibroblasts from 100-year-old individuals still have a TRF length of 6–7 kbp and retain proliferative capability of about 20 doublings [11], and, therefore, they do not reach the limit of proliferative capacity or of telomere shortening even at this age. The TRF length of peripheral leukocytes decreases with human age and shortens close to about 5 kbp in some individuals after age 60 but no cases are found in which the mean TRF length is shorter than 5 kbp even in individuals 100 years old [13, 15]. If some kinds of lymphocytes lose their proliferative activity by telomere shortening to 5 kbp, it may be a possible cause of reduced or abnormal immunological response in aged individuals. Systematic quantitative measurement of TRF length as a function of age has not been

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TABLE 1

T/A Ratio in Normal Liver[a]

Case	Age	Sex	Background	*T/A* ratio
1	17	F	FNH	0.0871
2	18	F	FNH	0.0984
3	31	F	FNH	0.0952
4	31	M	ET	0.0646
5	34	M	HL	0.0825
6	49	F	HM	0.0931
7	55	M	GC	0.083
8	57	M	HP	0.0476
9	61	F	BC	0.0687
10	62	F	CC	0.0804
11	62	F	CC	0.058
12	62	M	CCC	0.0769
13	63	F	CS	0.0748
14	63	M	CCC	0.0628
15	66	F	CC	0.0653
16	68	F	M	0.0661
17	73	F	CCC	0.0437
18	73	F	CC	0.0431
19	73	M	CCC	0.0667
20	76	M	CC	0.0555
21	77	F	CC	0.0504
22	78	M	CC	0.0551
23	81	M	CC	0.0672

[a] Abbreviations: FNH, focal nodular hyperplasia; ET, endocrine tumor; HL, hepatolith; HM, hepatic hemangioma; GC, gallbladder cancer; HP, hepatophyma; BC, breast cancer; CC, colon cancer; CCC, cholangiocellular carcinoma; CS, colon sarcoma; M, mesothelioma.

studied in other human tissues and, therefore, more data on other tissues are required to support or negate the idea that telomere shortening in human tissues is a possible cause of human aging. We are interested in seeing whether significant telomere shortening occurs even in very slowly replacing tissues. For this purpose, we chose liver tissue because hepatocytes are known to be very slowly replacing, once a year, under normal conditions, retaining a very high ability of regeneration [16]. Several papers have been published reporting that TRF length in liver tissues with chronic inflammation was shorter than that in normal liver, but these reports did not consider the donor age and telomere reduction with age [17–19]. There was one paper reporting a reduction of TRF length with age in normal and chronic hepatitis livers, but this paper used only five normal liver samples, which is insufficient for any quantitative conclusion [20]. While it is assumed that the reduction rate of telomere repeats is 50–150 bp per cell division in cultured human fibroblasts [21, 22] and that human liver cells are replaced once a year [16], it should be clarified whether the TRF length significantly shortens with age in normal liver, whether the TRF length shortens to the limit of proliferation in aged people, and whether the TRF length shortens more extensively in liver with chronic hepatic diseases. We report here for the first time that telomere repeats were reduced in human normal liver tissues by approximately 120 bp per year but were still longer than 5 kbp in 80-year-old patients and were shorter in liver with chronic diseases than in age-matched normal liver.

MATERIALS AND METHODS

Tissue Samples

All tissue samples were obtained at Hiroshima University Hospital with informed consent. Twenty-three normal liver (NL) specimens with various background were obtained by surgical resection from patients from 17 to 81 years of age (Table 1). All normal tissue specimens were histopathologically confirmed to contain no malignant cells. Twenty-three chronic hepatitis (CH) and three liver cir-

TABLE 2

T/A Ratio in Chronic Liver Disease[a]

Case	Age	Sex	Disease	Virus	*T/A* ratio
24	34	F	CH	HCV	0.0333
25	43	M	CH	HCV	0.0389
26	43	M	CH	HCV	0.0325
27	46	M	CH	HCV	0.0368
28	46	M	CH	HCV	0.0399
29	46	M	CH	HCV	0.0497
30	47	M	CH	HCV	0.0686
31	47	M	CH	HCV	0.0377
32	50	M	CH	HCV	0.0413
33	54	M	CH	HCV	0.0462
34	55	M	CH	HCV	0.0565
35	56	F	CH	HCV	0.0491
36	58	M	CH	HCV	0.0322
37	61	F	CH	HCV	0.0523
38	61	M	CH	HCV	0.0408
39	61	F	CH	HCV	0.0408
40	62	F	CH	HCV	0.0347
41	63	F	CH	HCV	0.0466
42	63	F	CH	HCV	0.0279
43	67	F	CH	HCV	0.0407
44	68	F	CH	HCV	0.044
45	68	M	CH	HCV	0.0318
46	72	M	CH	HCV	0.0352
47	51	M	LC	HCV	0.0336
48	66	M	LC	HCV	0.0319
49	69	M	LC	HCV	0.0399
50	62	M	CH*	HCV	0.0467
51	71	M	CH*	HCV	0.0296
52	77	M	CH*	NBNC	0.0177
53	77	M	LC*	NBNC	0.039
54	43	M	LC*	HBV	0.0248
55	46	F	LC*	HCV	0.0159
56	54	M	LC*	NBNC	0.0262
57	60	M	LC*	HCV	0.0404
58	67	F	LC*	HBV	0.0376
59	67	M	LC*	HCV	0.0369
60	68	F	LC*	HCV	0.0373

[a] Abbreviations: CH, chronic hepatitis; LC, liver cirrhosis; HBV, hepatitis B surface antigen positive; HCV, hepatitis C antibody positive; NBNC, hepatitis B surface antigen negative and hepatitis C antibody negative; CH* and LC*, samples obtained from tissues surrounding HCC.

TABLE 3

T/A Ratio in HCC[a]

Case	Age	Sex	Background	Virus	*T/A* ratio	Histology	Tumor size (mm)
61	43	M	LC	HBV	0.0318	M	32
62	46	M	LC	HBV	0.0819	P	65
63	54	M	LC	NBNC	0.021	M	130
64	60	M	LC	HCV	0.0495	M	43
65	66	M	CH	NBNC	0.0367	M	25
66	67	F	LC	HBV	0.03	M	18
67	67	M	LC	HCV	0.0191	M	20
68	68	F	LC	HCV	0.0731	M	30
69	71	M	CH	HCV	0.0222	P	51
70	77	M	CH	NBNC	0.0238	M	90

[a] Abbreviations: CH, chronic hepatitis; LC, liver cirrhosis; HBV, hepatitis B surface antigen positive; HCV, hepatitis C antibody positive; NBNC, hepatitis B surface antigen negative and hepatitis C antibody negative; W, well-differentiated HCC; M, moderately differentiated HCC; P, poorly differentiated HCC.

rhosis (LC) specimens were obtained by needle biopsy from patients who had no evidence of hepatocellular carcinoma (HCC) by imaging procedures and by liver biopsy under ultrasonographic guidance (Table 2). Three CH and eight LC specimens were also obtained from tissues surrounding HCC by surgical dissection from 11 patients (Table 2). HCCs (Table 3) were obtained from 10 patients by surgical resection. Histological grades of HCCs were classified into well, moderately, and poorly differentiated HCCs according to the Edmondson–Steiner grading system [23]. All specimens were immediately frozen and stored at −80°C until used.

Preparation of Tissue Lysate and Assay of Telomere Repeats

Lysates of tissue samples were prepared for direct measurement of telomere repeats and *Alu* sequence using acridinium ester labeled probes [22]. About 8 mm^3 of tissue sample was cut in liquid nitrogen and was put into 200 μl of hybridization buffer (0.1 M lithium succinate buffer, pH 4.7, containing 20% lithium lauryl sulfate, 1.2 M lithium chloride, 20 mM EDTA, and 20 mM EGTA) and homogenized. Released genomic DNA was extensively sheared by pipetting. The hybridization protection assay (HPA) procedure was used for quantification of telomere repeats in lysates [22]. To normalize the amount of DNA, the amount of *Alu* sequence was also measured by HPA, and the relative light unit (rlu) ratio of telomere repeats to *Alu* sequence (*T/A* ratio) was calculated. A *T/A* ratio of 0.01 corresponded to ~2 kb of mean TRF length by Southern blotting [22].

Statistical Analysis

The data were analyzed by the χ^2 test, the Fisher exact probability test, and the Mann–Whitney *U* test. A probability of $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

TRF length examined by Southern blotting is generally used for measuring telomere repeats but has several problems as discussed previously [22]. We developed a new method to quantitatively measure the amount of telomere repeats in genomic DNA in cell or tissue lysates by direct hybridization with acridinium ester labeled telomere probe [22]. The amount of genomic DNA in the assay system was normalized by the amount of *Alu* sequence measured by the chemiluminescence-labeled *Alu* probe. The ratio of the amount of telomere repeats to that of *Alu* sequence (*T/A* ratio) of normal liver (NL), chronic hepatitis (CH), liver cirrhosis (LC), and hepatocellular carcinoma (HCC) was compared (Fig. 1). Mean *T/A* ratios of NL, CH, and LC were 0.0690 ± 0.0162, 0.0405 ± 0.0103 ($P < 0.001$ to NL), and 0.0331 ± 0.0078 ($P < 0.05$ to CH), respectively. It was clear that the *T/A* ratios of CH and LC were significantly smaller than that of NL, consistent with previously reported data by Southern blotting [17–19]. However, telomere reduction of normal liver with age was not considered in Fig. 1 or in previous papers [17–19]. *T/A* ratios of HCC (most donors were over 60 years old) showed wide variation, probably because of various extents of telomere elongation by

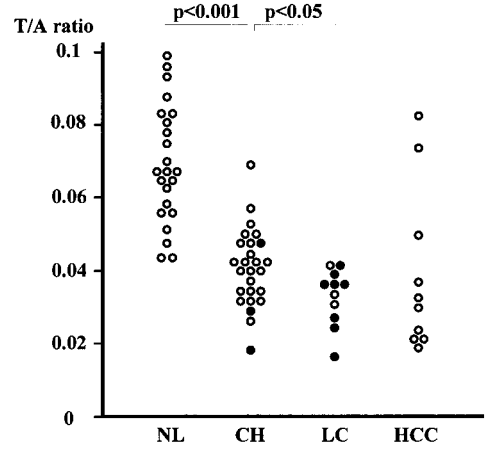

FIG. 1. *T/A* ratio of normal liver (NL), chronic hepatitis (CH), liver cirrhosis (LC), and hepatocellular carcinoma (HCC). Closed symbols in CH and LC were specimens from tissues surrounding HCC.

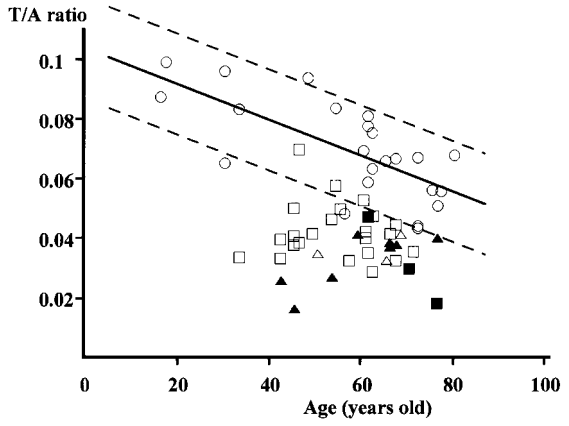

FIG. 2. Changes in *T/A* ratio with donor age in NL, CH, and LC. The regression line (solid line) in NL (○) was obtained by simple regression analysis ($r = 0.71$, $P < 0.001$). The dotted lines show the standard deviation interval. CH (□, ■) and LC (△, ▲) are also presented. Open symbols represent specimens from HCC-free liver and closed symbols are those from HCC-carrying liver.

induction of telomerase activity at various levels [17, 19, 24].

As presented in Fig. 2, the regression analysis revealed that the *T/A* ratio in normal liver decreased with age ($r = 0.71$, $P < 0.001$). The regression equation was $Y = 0.1039 - 0.000604\ X$ ($Y = T/A$ ratio, X = donor age). The reduction rate of telomere repeats in normal liver (0.000604 per year) corresponded to 120 bp per year assuming that $T/A = 0.01$ corresponded to TRF length = 2 kbp as estimated from human fibroblasts [22]. This reduction rate is only a rough approximation but is in quite good agreement with the generally accepted assumption that the reduction rate of telomere repeats is 50–150 bp at each cell division [21, 22] and the human liver cells are replaced once a year [16]. This annual reduction rate is roughly the same as observed in skin fibroblasts [11] and peripheral lymphocytes [15], though we do not know whether this coincidence has some biological meaning in the human body or is simply an occasional phenomenon. If the liver cells ceased proliferation when the TRF length shortened to around 5 kbp ($T/A = 0.025$) as reported for human fibroblasts [11, 22, 25, 26], they would retain proliferative capability even in 80-year-old individuals because the TRF length estimated from the *T/A* ratio was still around 10 kbp. In the previous paper using five normal liver samples, the TRF length of normal liver from one 70-year-old patient was reported to be around 8–9 kbp [20]. These results suggest that liver cells in aged people still have the ability to regenerate. However, since the TRF length varies widely in individuals of the same age, telomere length of normal liver in some old individuals is possibly reduced to the limit of proliferation. If it occurred, it could be one of the possible causes of the undesired prognosis of liver transplantation from aged donors [27, 28].

Figure 2 also shows that the *T/A* ratio in CH was significantly lower than that in NL of the same age ($P < 0.01$). The *T/A* ratio in LC was lower than that in NL ($P < 0.01$) and than that in CH ($P < 0.05$). Telomere length in CH (*T/A* ratio) appeared to shorten with age, but it was insignificant. Variation of telomere length in CH appeared to be wider in younger patients than in aged patients (for example, compare samples before age 60 with those after age 60). Since hepatic cells in CH of younger patients would regenerate and lose telomere repeats more extensively than those in older patients, TRF length in CH varied more in younger patients. In contrast, telomere length in LC was relatively constant among samples from patients of different ages, and shortening of telomere length in LC liver, as compared with telomere length in CH liver, was more obvious in younger patients than in aged patients. It was also observed that telomere length in these CH and LC samples, with two exceptions, did not reduce shorter than 5 kbp ($T/A = 0.025$). Our results are different from those in an earlier report [20], in which shortening of TRF length with age was clearly observed in both CH and LC. However, it was consistent in both sets of data that there were no samples, with two exceptions in our data, with TRF length shorter than 5 kbp. These data are consistent with the assumption that human somatic cells whose telomere length is shortened to about 5–6 kbp are withdrawn from the cell cycle and cease proliferation as senescent cells [11, 22, 25, 26]. If so, patients with a short telomere length of around 5 kbp would suffer impaired regeneration of liver cells and have a poor prognosis. Though prognosis of chronic liver disease is no doubt influenced by many exogenous and endogenous factors, a limit of proliferative life span of hepatocytes could be one of the intrinsic factors.

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