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Telomerase Activity, Telomere Length, and Chromosome Aberrations in the Extension of Life Span of Human Embryo Cells Induced by Low-dose X-rays

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We examined whether the shortening of telomere structure is related to *in vitro* cellular aging after multiple low-dose irradiation. We used three strains of HE cells (HE23, HE31, and HE40) exhibiting different levels of telomerase activity and irradiated these cells twice a week with a dose of 2 cGy or 4 cGy of X-rays until they senesced. The cells were in total exposed to doses of 52–208 cGy of X-rays. Only the HE31 cells, which had no telomerase activity, experienced an increase in the number of cell divisions, reaching a maximum of 120–124% of the non-irradiated controls. However, in two strains which did exhibit telomerase activity in an early passage in culture, no extension of cell life span was found. Telomerase-positive cells completely lost all telomerase activity when the cells were subcultured several times without irradiation. In the HE31 cells where the life span was extended, the ratio of cell having a long telomere was higher than those of the other two cells (HE23 and HE40). Cytogenetic analysis revealed that the life span extension due to multiple low-dose irradiation which was observed in HE31 cells did not correlate with specific chromosome alterations.

Our results suggest that the telomerase activity remaining in the cells at an early passage does not correlate with the extension of life span *in vitro* by X-irradiation. The factor other than telomerase activity may play an important role in the regulation of telomere length and the extension of life span.

INTRODUCTION

Cultured human embryo (HE) cells have a limited proliferative capacity, undergo senescence, and do not spontaneously immortalize[1,2]. In recent years, a clear correlation between telomere shortening and cellular aging in human cells has been demonstrated[3–6]. A function of the

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telomere located at the end of all eukaryotic chromosomes is to prevent aberrant recombination and degradation of the ends of the chromosomes[7]. The telomere acts as a form of biological clock, and the length of telomeric DNA decreases with increasing cell divisions in somatic cells such as fibroblasts, keratinocytes, epithelial cells, peripheral blood leukocytes, and endothelial cells both *in vivo* and *in vitro*[8–14]. However, the outcome of telomere shortening is not always senescence. The telomere length of some cell types can be increased through the activity of the reverse transcriptase containing an RNA template-dependent DNA polymerase, telomerase. Kim et al.[15] have shown a close correlation between the expression of tumorigenic phenotypes and increased telomerase activity, suggesting that telomerase may play a critical role in the progression of the malignant state.

In a previous study, we showed that multiple low doses of γ-rays have a stimulative effect of extending life span of cultured human embryo cells[16,17]. If maintenance of the telomere structure during cell divisions is related to the immortalization, and transformation of a cell, it may be easier to extend the life span of cells with a high level of telomerase activity. Therefore, in the present study, we investigated telomerase activity, telomere length, and chromosome constructions of cells during long-term culture with multiple irradiations using low doses of X-rays.

We report here that the telomerase activity existing in the cells at an early passage in culture is not related to life span extension by low-dose irradiation.

MATERIALS AND METHODS

Cells and cell culture

The cells were obtained from carcass of 7–8 week-old human embryos, as described previously[16]. The cells were cultured in Eagle's Minimum Essential Medium (MEM) supplemented with 0.2 mM serine, 0.2 mM aspertate, 1 mM pyruvate, 10 mM 2-[4-(hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES), 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere with 5% CO_2 and 95% air. 10^6 cells were inoculated in a 75 cm^2 plastic flask and subcultured every 7 days.

Immunocytochemical staining

To determine the tissue origin of the cells used, the cells were stained with several antibodies to intermediate filament proteins by the indirect immunocytochemical fluorescent staining method described previously[18]. 5×10^4 cells were grown on cover glass slips (22×22 mm) and placed in a plastic dish (35 mm ϕ) for 24 h. The cells were washed once with PBS (–), and then fixed with cold absolute methanol for 5 min. Fixed cells were rinsed with PBS (–) five times and immunostained for 120 min with a primary antibody to desmin (Sanbio BV, Uden, Netherlands), vimentin (Amersham International plc., Bucks, UK), cytokeratin (Progen Biotechnik GmbH, Heidelberg, Germany), glial fibrial acidic protein (GFAP) (Sanbio BV, Uden, Netherlands), and neurofilament (Sanbio BV, Uden, Netherlands). Subsequently, the cells were stained for 120 min with fluorescein isothiocyanate (FITC) conjugated anti-mouse *IgG* (Amersham International plc., Bucks, UK) as a secondary antibody.

X-irradiation

Cells were irradiated with a X-ray machine (M-150WE; Softex, Osaka, Japan) operated at 150 kVp and 15 mA with a 0.1 mm Cu filter. 10^6 cells were inoculated into a 75 cm^2 flask and irradiated on the 3rd and 6th day during one passage of 7 days. A dose of each irradiation was 2 or 4 cGy at a dose rate of 0.10 Gy per min. This procedure was repeated until the cells had received 52–208 cGy of X-rays. The cells were cultured until senesced.

Chromosome analysis

Chromosome preparations were made by a standard air-drying method and were banded according to Giemsa banding method as previously described[19]. For analysis of chromosome numbers and karyotypes, at least 100 metaphases and 20 karyotypes were analyzed, respectively.

TRAP assay for telomerase activity

Cell lysates were prepared from cells at 70–80% confluence in culture. Human fibrosarcoma cells (HT1080) were used as a positive control. Cell suspensions were washed once with 1 ml PBS (–) (pH 7.4), and the cells were pelleted by centrifugation at 6,000 rpm for 6 min at 4°C. 10^5 cells were added in 200 μl of ice-cold lysis buffer (10 mM Tris-HCl; pH 7.5, 1 mM $CaCl_2$, 1 mM EGTA, 8.13 mM CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate), 10% glycerol, 5 mM β-mercaptoethanol, and 0.1 mM AEBSF (4-(2-aminoethyl) benzenesulfonyl fluoride hydrochlorine)). The cell lysate was placed on ice for 30 min and then centrifuged at 15,000 rpm. Telomerase activity in the supernatant was assayed by the PCR-based telomere repeat amplification protocol (TRAP) technique[15] with a minor modification. A 2 μl portion of the extracts, adjusted in a quantity of protein equivalent to that of 10^3 cells, was added to the PCR reaction mixture which contained 50 μM each dNTP (PCR Nucleotide Mix, Boehringer Mannheim, Mannheim, Germany), 0.1 μg of TS primer, 2 units of *Taq* polymerase, and 74×10^4 *Bq* of [α-^{32}P]-dCTP (110 TBq/mmol, Amersham International plc., Bucks, UK) in a microcentrifuge tube. The reaction mixture was incubated for 30 min at 20°C for telomerase-mediated extension of the TS primer. After the reaction mixture was heated at 90°C, 0.1 μg of CX primers were added to each reaction mixture. Samples were then subjected to PCR amplification with 31 cycles at 94°C for 45 sec, 50°C for 45 sec, and 72°C for 60 sec. After amplification, 12.5 μl of PCR products were separated by electrophoresis with 12% polyacrylamide nondenaturing gels. The gel was dried at 56°C for 1 h and exposed to a X-ray film (XAR-50; Kodak, Rochester, NY, USA) with an intensifying screen at –80°C.

Determination of the length of terminal restriction fragments (TRFs)

Genomic DNA was purified by the salt-out method described by Miller et al.[20]. Ten micrograms of DNA was digested with 10 units of *Hinf* I and *Rsa* I overnight at 37°C. The digested DNA was then precipitated with 250 μl of ethanol containing 10 μl of 3M sodium acetate (pH 4.8). DNA was dissolved in 20 μl of Tris EDTA buffer and 4 μl of 6 × gel-loading buffer (50% glycerol, 0.1M Na_2-EDTA (pH 8.0), 0.25% bromophenol blue (BPB), 0.25% xylene cyanol) was added, followed by electrophoresis in a 1 × Tris-acetate-EDTA (TAE) buffer on 0.7% agarose gel. After electrophoresis, the DNA in the gel was denatured by soaking in 0.5M NaOH/1.5M

NaCl for 30 min, transferred to a nylon membrane Hybond N^+ (Amersham International plc., Bucks, UK) in 0.4N NaOH, and baked at 80°C for 2 h. Digestion and transfer of DNA were confirmed by staining a gel with ethidium bromide. The membrane was hybridized with ^{32}P-labeled telomeric oligonucleotide probe [γ-^{32}P-$(TTAGGG)_4$] (110 Tbq/mmol, Amersham International plc., Bucks, UK) for 18 h at 37°C in 5 × SSC with 1% SDS. After hybridization, the filter was washed twice in 2 × SSC with 0.1% SDS for 5 min at 37°C and exposed to a X-ray film (XAR-5; Kodak, Rochester, NY, USA) with an intensifying screen. The TRFs signal was analyzed with a photoimage analyzer (FUJIX BAS1000; Fujiphoto Film, Tokyo, Japan). Mean TRF length was defined as

$$\Sigma(\mathrm{OD}i) / \Sigma(\mathrm{OD}i / \mathrm{L}i)$$

where ODi is the densitometer output and Li is the length of the DNA at position i[8]. The sums were calculated over the range of 2.8–27.5 kb pairs.

RESULTS

Cellular characteristics

We examined the telomerase activity of 41 strains of HE cells and found that these cells were positive for telomerase activity in approximately 39% (16 out of 41) of the cell strains at passage 1. Therefore, we selected three strains of HE cells (HE23, HE31, and HE40) with different levels of telomerase activity for the present study. As shown in Fig. 1, telomerase activity was positive in both the HE23 and HE40 cells, but negative in the HE31 cells. The activity was higher in the HE23 cells than in the HE40 cells. Because telomerase activity exists in stem cells, we stained the cells with specific antibodies to intermediate filament proteins to determine the tissue origins of the cells exhibiting telomerase activity. All cells were stained with the antibody to vimentins of fibroblasts. Simultaneously, 14–18% of the cells were stained with the antibody to cytokeratins of epithelial cells at passage 2. Those cytokeratin-positive cells disappeared at late passages. There were very few or no cells stained with antibodies to the desmin filaments of muscle cells, the glial filaments of astrocytes and Schwann cells, and neurofilaments of nerve cells. There is no difference in expression of intermediate filament proteins between telomerase-positive and negative HE cell strains.

Growth of cells repeatedly irradiated with low doses of X-rays

Figure 2 shows the cumulative population-doubling numbers (PDN) in the non-irradiated and irradiated HE cells. When cells were repeatedly irradiated with 2 cGy twice a week, only the HE31 cells had accumulated doses of 52–148 cGy were slightly expanded over that of a control, but the other two cell strains did not. For example, when the HE31 cells had accumulated 148 cGy of X-rays, the maximum PDN reached 124% of the non-irradiated controls (Table 1). However, when cells were repeatedly irradiated with 4 cGy twice a week, all cells were not extended their life span by any irradiation schedule, and irradiated cells ceased proliferation and became senescent the same as non-irradiated cells. To avoid the effects of different cellular sensitivities

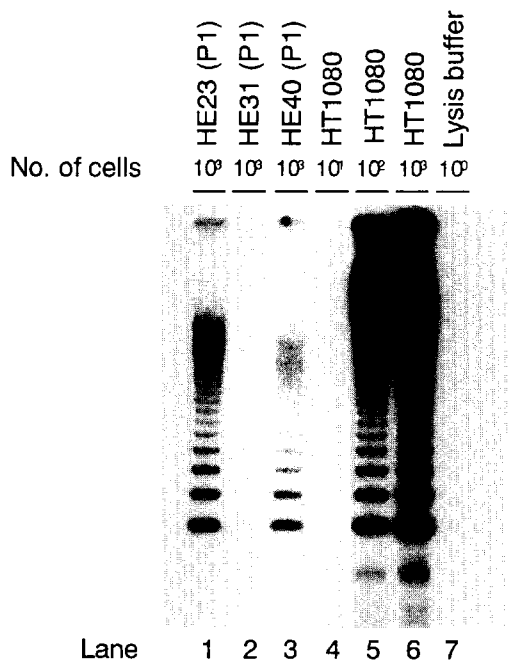

Figure 1. Telomerase activity in human embryo (HE) cells measured by TRAP assay. The names of cell strains and number of cells applied to analysis are shown at the top. Lysis buffer without cell extract and HT1080 cells are used as negative and positive controls, respectively.

to radiation on cell growth, we determined the radiation sensitivity of the three strains of HE cells. The 37% survival doses (D_0) for the HE23, HE31, and HE40 cells were 0.85 Gy, 0.83 Gy, and 0.86 Gy, respectively, showing that the maximum difference in radiosensitivity was 1.04 times, occurring between the HE31 and HE40 cells. Because there are no difference in radiosensitivity among three cell lines, selection of the radioresistant cell populations by X-irradiation may not be responsible for life span extension.

Telomerase activity and telomere length

We examined the change in telomerase activity during *in vitro* passage. As shown in Fig. 3, telomerase activity in the HE23 and HE40 cells drastically decreased with increasing passages, and there was a complete loss of telomerase activity by passage 4. In all three cell strains used, telomerase activity never revived after undergoing multiple X-irradiations before senescence.

Telomere length was assayed by a Southern blot analysis of the TRFs. The average telomere length of HE31 cells at passage 3 (13.2 kb) is clearly long compared to cells of the other two strains (11.2 kb). As shown in Fig. 4, telomere lengths shortened gradually with increasing passages in culture in all cells. There was no significant difference in the rate of telomere shortening between the non-irradiated and irradiated cells in all strains until passage 20. However, in the multiple-irradiated HE31 cells whose life span was extended, telomere shortening was stopped at passage 20 (46 PDN level), and the telomere length did not change during the last 10 passages

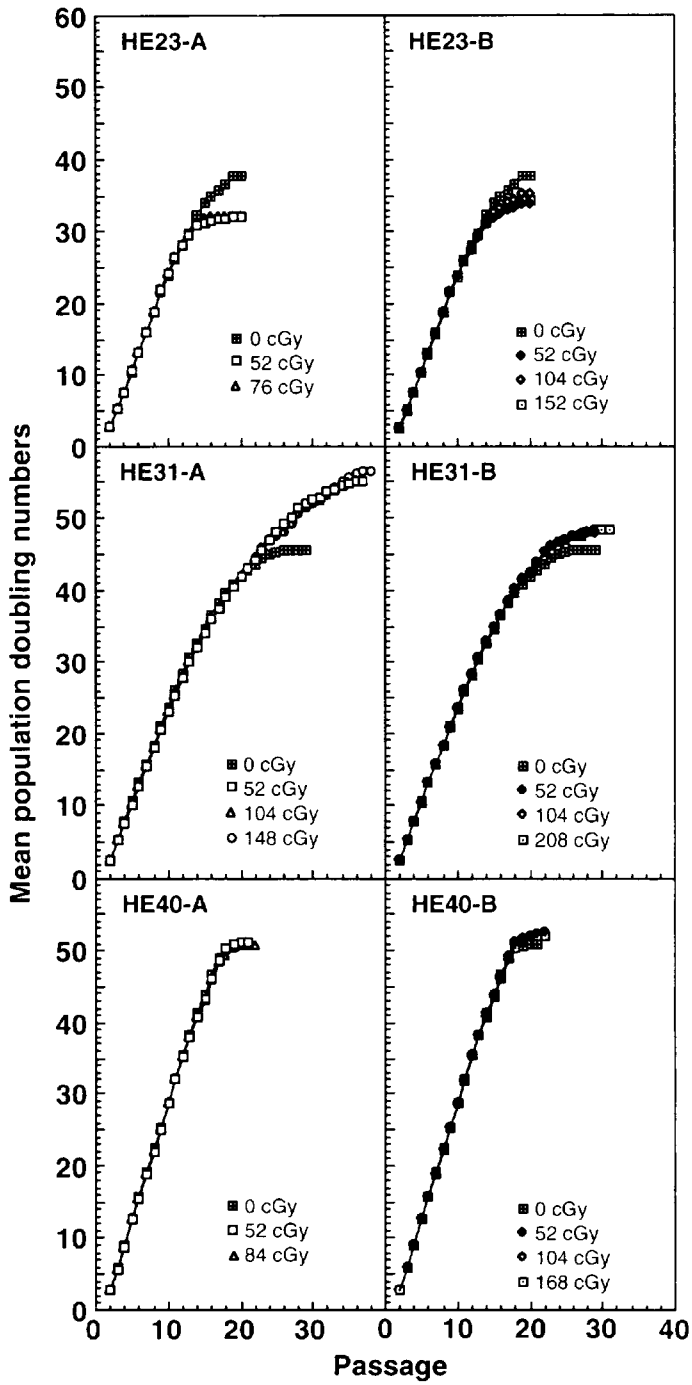

Figure 2. Mean population doubling numbers (MPDN) in non-irradiated and irradiated HE cells. Cells were irradiated on the 3rd and the 6th day during one passage of 7 days. Each irradiation was performed with 2 cGy (A) or 4 cGy (B) at a dose rate of 0.10 Gy per min. Irradiation was repeated until accumulated doses reached 52–208 cGy. The cells were cultured until they senesced.

Table 1. Changes of maximum population doubling numbers of irradiated HE cells

Cell	Radiation dose (cGy)	No. of irradiation	Acummulated doses (cGy)	Passage number at senescence	Maximum PDN[a]	Relative PDN[b]
HE23	0	0	0	20	37.73	1.00
HE23	2	26	52	20	32.15	0.85
HE23	2	38	76	20	32.06	0.85
HE23	4	13	52	20	33.71	0.89
HE23	4	26	104	20	35.14	0.93
HE23	4	38	152	20	34.39	0.91
HE31	0	0	0	28	45.68	1.00
HE31	2	26	52	37	54.96	1.20
HE31	2	52	104	37	55.04	1.20
HE31	2	74	148	38	56.48	1.24
HE31	4	13	52	29	47.97	1.05
HE31	4	26	104	29	47.76	1.05
HE31	4	52	208	31	48.32	1.06
HE40	0	0	0	21	50.93	1.00
HE40	2	26	52	21	51.30	1.01
HE40	2	42	84	22	50.71	1.00
HE40	4	13	52	22	52.67	1.03
HE40	4	26	104	22	52.52	1.03
HE40	4	42	168	22	52.10	1.02

[a] maximum PDN represents population doubling numbers at a passage where cells become senescence.
[b] Relative PDN is obtained by dividing maximum PDN of an irradiated culture by maximum PDN of a non-irradiated culture.

(between 46 and 56 PDN levels) before reaching senescence (Fig. 5). The telomere length did not shorten uniformly, and in some parts of the HE31 cell population, a long telomere length was retained after telomere shorting in both the HE23 and 40 cells.

Chromosome aberrations

We performed chromosome analyses of the cells exposed to multiple X-irradiations using the Giemsa banding method. The cells showed numerical chromosome abnormalities during subculture rather than structural abnormalities such as rearrangements and deletions. In the case of HE31 and HE40 cells, differences in the changes in chromosome number were not observed between the control cultures and irradiated cultures (Fig. 6), suggesting that the numerical chromosome changes are associated with cell divisions in culture rather than the effect of X-ray irradiation. However, X-irradiation accelerated loss of diploid cells during culture in HE23 cells. Non-disjunctions during mitosis would result in the production of cells with both increased and decreased chromosome numbers simultaneously. Surprisingly, we observed cells with decreased chromosome numbers (hypodiploid cells) more frequently than cells with increased chromosome numbers (hyperdiploid cells) (Fig. 7). Furthermore, karyotype analyses revealed that the

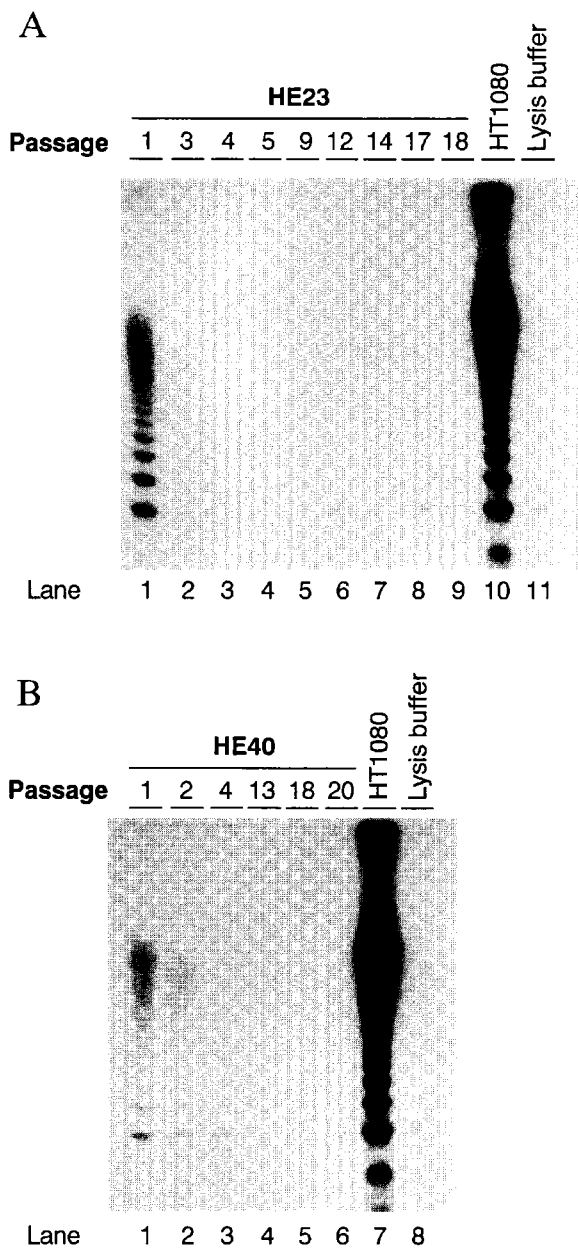

Figure 3. Changes in telomerase activity in human embryo (HE) cells during successive passages in culture measured by TRAP assay. Passage numbers at sampling are shown at the top. 10^3 cells were applied to analysis. Lysis buffer without cell extract and HT1080 cells are used as negative and positive controls, respectively.

missing chromosomes were non-random, and the loss of specific chromosomes such as chromosomes 8, 9, 15, and 20 were more frequent in the irradiated cells than in others (Fig. 7). In Table

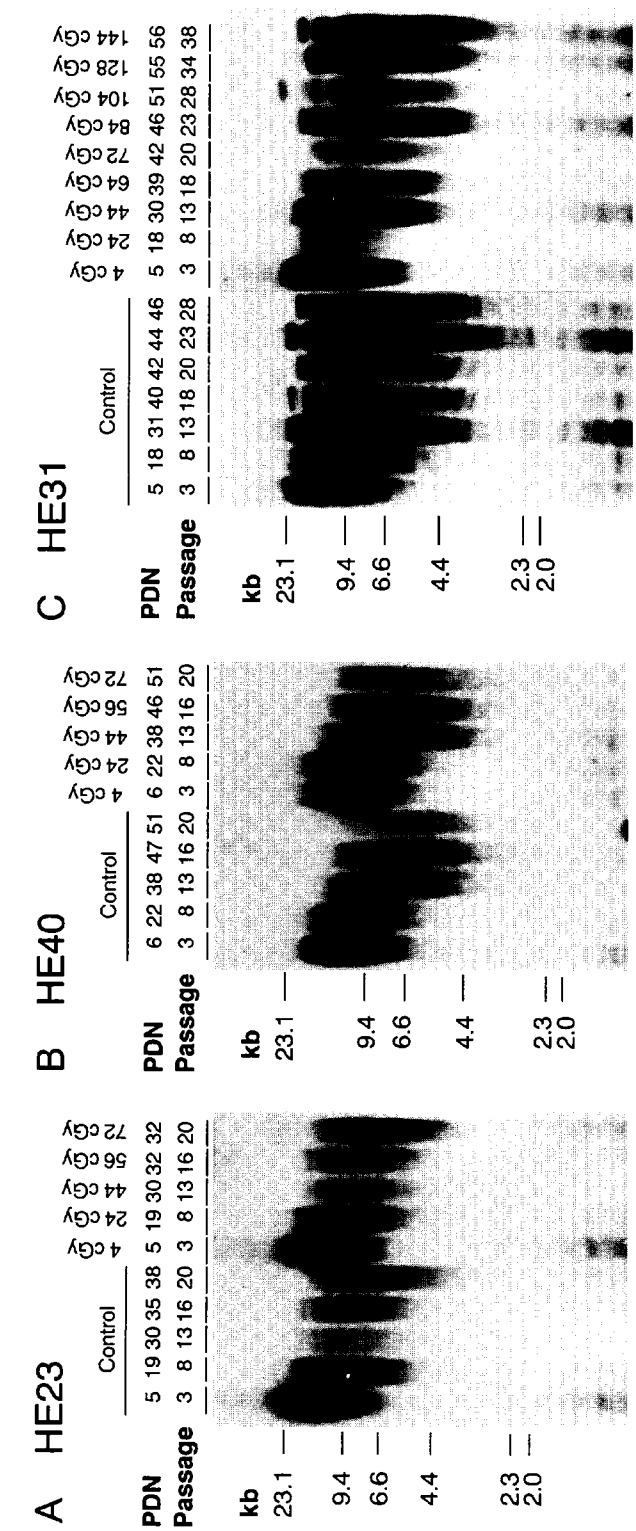

Figure 4. Southern blot analysis for terminal restriction fragments (TRFs) in non-irradiated (control) and irradiated (2 cGy; twice a week) HE cells during successive passages in culture. Passage numbers, PDN and accumulated doses at sampling are shown at the top. (A) HE23, (B) HE40, (C) HE31.

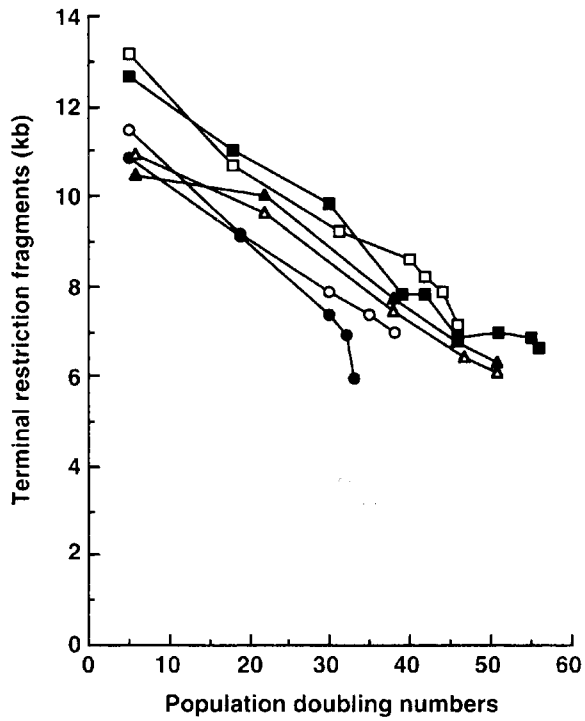

Figure 5. Changes in TRF lengths in non-irradiated (control) and irradiated (2 cGy; twice a week) HE cells during successive passages in culture. The mean TRF lengths were determined from the signals of Southern blot as described in Materials and Methods. ○ and ●, HE23; □ and ■, HE31; △ and ▲, HE40. Open symbols, Non-irradiated control; closed symbols, repeatedly irradiated with 2 cGy of X-rays.

2, we show the frequency of abnormal karyotypes in HE23, HE31, and HE40 cells following multiple irradiations. Results showed that structural aberrations such as translocations and deletions occurred infrequently. No specific aberration associated with life span extension by low dose irradiation was observed.

DISCUSSION

In the present study, we showed that multiple X-irradiations of 2 cGy have a stimulative effect on cell divisions, extending cellular life span in one (HE31) out of three HE cell strains. This confirms our previous reports that showed life span extension of human embryo cells by low dose of γ-irradiation[16,17]. We used two different doses per irradiation, 2 cGy and 4 cGy, in the present study. Results clearly show that multiple irradiations with 2 cGy are more effective than 4 cGy (Table 1). The result also suggests that total accumulated dose is not critical for the stimulative effect on cell divisions (Table 1). To understand a mechanism of this stimulative effect of low dose radiation on cellular life span *in vitro*, we investigated telomerase activity, telomere

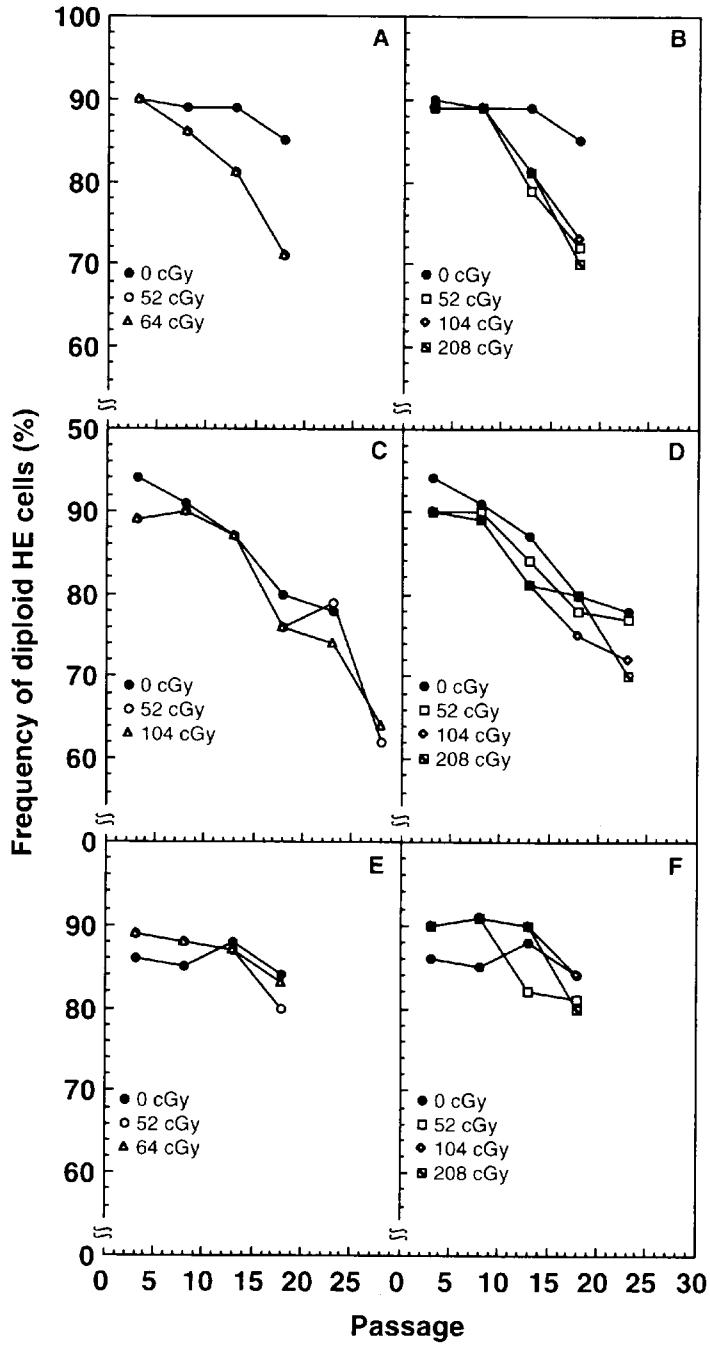

Figure 6. Changes in frequency of diploid cells of non-irradiated (control) and irradiated (2 cGy; twice a week) HE cells during successive passages in culture. A, C and E, non-irradiated HE cells and repeatedly irradiated HE cells with 2 cGy of X-rays; B, D and F, non-irradiated HE cells and repeatedly irradiated HE cells with 4 cGy of X-rays. A and B, HE23 cells; C and D, HE31 cells; E and F, HE40 cells.

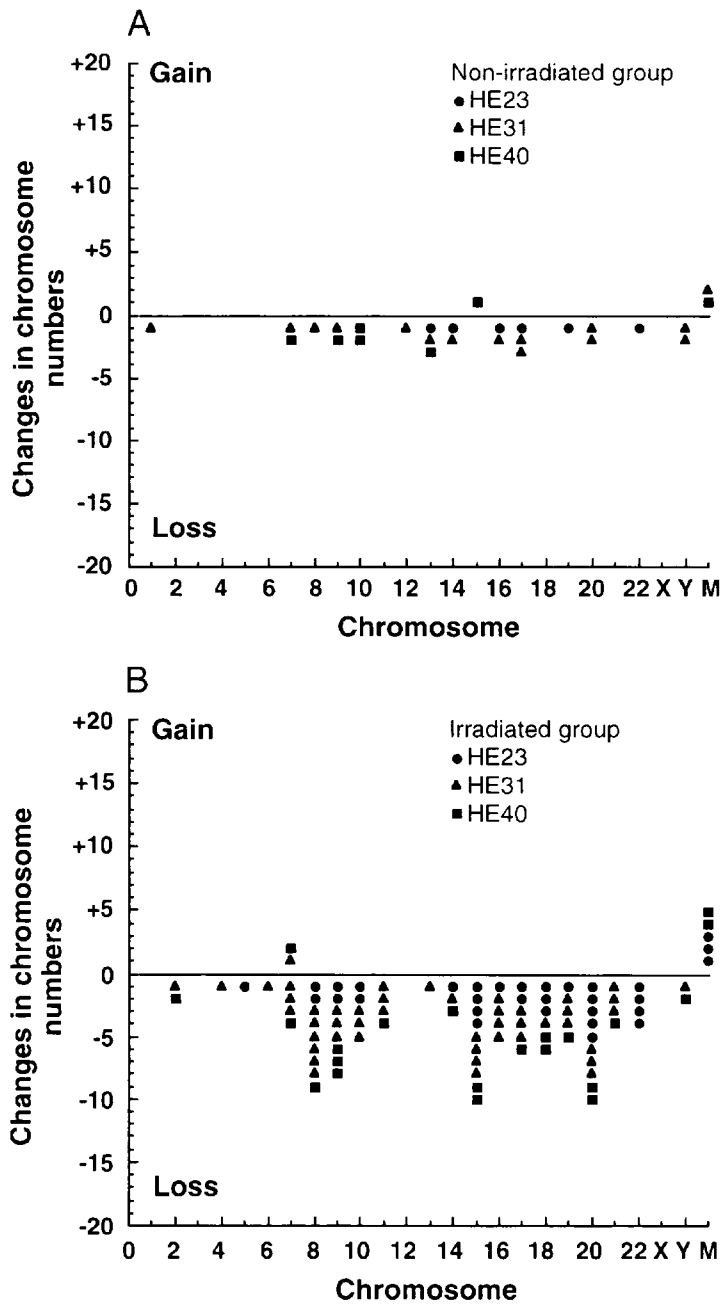

Figure 7. Changes in chromosome numbers observed in HE cells. The ordinate represents number of gain (+) or loss (−) of chromosomes. M represents marker chromosomes. All data were taken from Table 2. A, non-irradiated cells; B, irradiated cells.

length and chromosome constructions in cells during long-term culture.

Because telomeres progressively shorten with each cell division, it has been proposed that

Table 2. Frequencies of abnormal karyotype in HE23, HE31 and HE40 cells following multiple irradiations

Passage	Dose (cGy)	Number of abnormal karyotypes per 20 metaphases[a] (Abnormal karyotype)[b,c]		
		HE23	HE31	HE40
3	0	0	2 (–19, 20); (–8)	0
	2 × 2	2 (–16); (–22)	0	1 (+ M)
	4 × 2	3 (–10, –19); (–9); (–18, + M)	1 (–10)	0
8	0	1 (–14)	1 (–16)	2 (–9); (–10)
	12 × 2	1 (–18)	1 (–19)	2 (–11); (–15)
	12 × 4	2 (–20); (–22)	0	2 (–8); (–19)
13	0	2 (–16); (–19)	0	3 (–7); (–13); (–10, + 15q–)
	22 × 2	2 (–15); (–22)	0	4 (–2); (–17); (–21); (+ 7, –Y)
	13 × 4	2 (–15); (–22)	0	1 (–18)
	22 × 4	0	0	2 (–9); (–18)
18	0	2 (–13, –22); (–17)	2 (–17); (–17, + M)	0
	26 × 2	2 (–15); (–20)	1 (–20)	2 (–7); (–20)
	32 × 2	2 (–17); (–9, + M)	3 (–9); (–17); (–21)	0
	13 × 4	3 (–8, –20); (–5); (1p–, + F)	3 (–4, –11, –15); (–8, –16); (–17, –18)	1 (1p–, –9, + M)
	26 × 4	6 (–8, –10); (–14, –20); (–15); (–17); (–20); (6p–)	8 (–8, –17); (–15, –16); (–7); (–7); (–19); (–20); (–20); (–21)	2 (–9, –14, –15); (–20)
	32 × 4	1 (–18)	8 (–8, –10, –13); (–8, –9); (–10); (–11); (–16); (–16); (–19); (–14, +7)	0
23	0		5 (–1, –9, –12); (–13, –Y); (–7); (–20, –Y); (–14)	
	26 × 2		1 (–8)	
	52 × 2		1 (–Y)	
	13 × 4		3 (–7, –11, –15); (–2, –6); (–8)	
	26 × 4		1 (–15)	
	52 × 4		1 (–9, –21)	

[a] Normal karyotype: HE23, 46, XX; HE31, 46, XY; HE40, 46, XY.

[b] Chromosome abnormalities observed in a cell is represented in parenthesis.

[c] M and F represent a marker chromosome and a fragment, respectively.

telomere shortening is the molecular measure of the remaining proliferative capacity in cells[5,8,9,11]. Re-expression of telomerase and the prevention of telomere shortening occur in most tumors, and these event are probably critical for continuous tumor cell growth[15]. Many tumor cells and immortalized cells that undergo unlimited cell divisions *in vitro* show high telomerase activity[15]. Therefore, we would expect that a cell having a high level of telomerase activity should be easier to immortalize than cells having low or no telomerase activity. In the present study, we examined whether the presence of telomerase activity is related to the extension of life span of cells due to X-ray irradiation.

Until now, many investigators have reported that only a limited number of cells, such as germ cells, have telomerase activity in normal cell strains derived from human organs[21–26]. Very little is known about changes in telomerase activity during human development. We here show that telomerase activity also exists in primary fibroblast cells derived from 7–8 week-old human

embryos. These cells express vimentin filaments (100%) which are specific for intermediate proteins for fibloblasts. In addition, 14–18% of the cells simultaneously express cytokeratins which are specific for proteins in epithelial cells. This suggests that human primary embryo cells at 7 to 8 weeks still contain a fraction of cells which are unspecialized. It is likely that such immaturely differentiated cells may retain telomerase activity in HE cells. We examined the telomerase activity of 41 strains of HE cells and found that these cells were positive for telomerase activity in approximately 39% (16 out of 41) of the cell strains at passage 1. Therefore, we used three strains of HE cells (HE23, HE31, and HE40) with different levels of telomerase activity to examine whether multiple low-dose X-ray irradiations cause a change in life span. As shown in Fig. 2, we found that only one (HE31) out of the three strains of HE cells extend their life span *in vitro* due to multiple low-dose X-ray irradiations. HE31 cells have no telomerase activity at the start of the culture. Although the HE23 and HE40 cells had a high level of telomerase activity, they showed a dramatic decline in activity which finally became undetectable after a few passages *in vitro*. This decline of the activity was observed in both non-irradiated and irradiated cells. This is consistent with a previous report by Wright et al.[21]. However, in the case of rodent embryonic cells, such as hamster, rat, and mouse, telomerase activity is decreased gradually and then up-regulated again in association with immortalization (data not shown). These results indicate that the regulation mechanism for telomerase in humans cells is different from that in rodent cells.

Telomere stability may be an important factor for determining the life span of cells *in vitro*. When telomere becomes unstable, fusion between the telomeres happens. In fact, the end fusion of chromosomes occurs in cells at advanced *in vitro* passage in culture. Counter et al.[27] have reported that the number of dicentric chromosomes drastically increases in senescent cells. However, we did not observe an increased frequency of dicentric chromosomes in either the non-irradiated or the irradiated cells (Table 2). The abnormalities observed were numerical abnormalities rather than structural abnormalities, especially the decrease in chromosome number as reported[28]. Karyotype analysis revealed that specific chromosomes such as chromosomes 8, 9, 15, and 20 were missing in irradiated cells. Mukhetjee and Thomas[29] have reported that chromosomes 1, 4, 6, 8, 10, and 15 show significantly higher frequencies of aneuploidy than the other chromosomes in cells from older individuals studied. Although the significance of relatively high levels of aneuploidy for certain chromosomes associated with aging remains unclear, it is intriguing to note that some of these chromosomes such as chromosomes 1, 4, 6, and 7 have already been determined to harbor senescence genes[30–33]. In addition, the gene for Werner syndrome characterized by premature aging is mapped on chromosome 8[34]. It is conceivable that the degree and type of aneuploidy specific for a certain chromosome might have some gene-dosage effects in controlling cellular proliferation and selection for life span extended cells during aging. However, in the present study, we did not find any specific chromosome changes associated with life span extension.

The evidence that telomerase activity in HE31 cells never revived after undergoing multiple X-irradiations before senescence supports the idea that life span extension is independent of telomerase ability. Recently, Bryan et al.[35] reported that a large proportion of immortal cell lines were negative for telomerase and had very long and heterogeneous telomeres, suggesting that

these cells have been able to overcome telomere shortening through a novel mechanism. This possibility is under investigation. Because a part of the HE31 cell population retained telomeres of long size during an expanded period (46–56 PDN) (Fig. 5), we can not ignore the possibility that a continuous existence of the cells having long telomere in HE31 cell populations is a cause of extension of life span by low-dose X-ray irradiation. It may be presumed that life span of a cell having a longer telomere may be long compared that of a cell having a short telomere. However, we have no evidence that X-ray acts as leaving a cell having longer telomere. It cannot reasonably be assumed that a cells having longer telomere exist without cell growth during an expanded period.

An alternative model is that some subtelomeric restriction sites were not digested due to DNA modification such as methylation, and the TRF dynamics described were caused by changes in such modification. However, this is unlikely since there is no difference in the proportion of cells having longer telomere between non-irradiated cells and irradiated cells. The mechanism of life span extension due to multiple low-dose X-ray irradiations still remains unclear.

In summary, we conclude that telomerase activity is not directly related to the extension of life span in human embryo cells. The involvement of the mechanism for telomere stabilization, which is not associated with telomerase, may be a regulator of cellular life span *in vitro*.

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