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Accelerated loss of telomeric repeats may not explain accelerated replicative decline of Werner syndrome cells

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Abstract The Werner syndrome (WS) is characterized by the premature onset and accelerated rates of development of major geriatric disorders, including atherosclerosis, diabetes mellitus, osteoporosis, ocular cataracts, and various neoplasms. Cultures of WS skin-fibroblastlike cells have been previously shown to undergo accelerated rates of decline of their replicative potentials and to exhibit variegated chromosomal translocations and deletions. Since the replicative decline of normal somatic cells is associated with a loss of telomeric repeats, we investigated the kinetics of telomeric repeat loss in WS cells. The mean length of telomere restriction fragments (TRF) from the earliest passages of WS cells studied was not shorter than those of controls, possibly reflecting selective pressure for subsets of cells with relatively high residual replicative capacity. Statistical evidence indicated an accelerated shortening of TRF length in serially passaged WS cultures, but the mean TRF lengths of WS cultures that had ceased replicating were significantly longer than those of senescent controls. Thus, while accelerated loss of telomeric repeats could potentially explain the rapid decline in proliferation of WS cells, it is possible that WS cells exit the cell cycle via mechanisms that differ from those of replicatively senescent cells from control subjects.

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Introduction

The Werner syndrome (WS; McKusick 277700; McKusick 1994), although affecting between 1–22 cases per million human subjects worldwide (Epstein et al. 1966), has attracted the attention of gerontologists and geriatricians because of its potential to elucidate some underlying processes of aging. Individuals who inherit two copies of the autosomal recessive gene (*WRN*) appear to be perfectly normal at birth and typically do not come to the attention of physicians until adolescence, when they fail to undergo the usual adolescent growth spurt. They then develop premature graying and thinning of the hair, atrophy of skin, regional atrophy of subcutaneous tissues, cataracts, diabetes, osteoporosis, gonadal atrophy, various forms of arteriosclerosis, and a variety of benign and malignant neoplasms. The gene has been mapped to 8p (Goto et al. 1992; Schellenberg et al. 1992) but has not yet been cloned. A report of inherited mutations in the DNA polymerase beta gene of WS patients (Sadakane et al. 1994) has not been substantiated (Chang et al. 1994).

Studies of its pathophysiology have uncovered two interesting and possibly related phenomena. First, compared to biopsies from age-matched controls, cultures of dermal fibroblastlike cells undergo very rapid clonal attenuation of their growth (Martin et al. 1970, 1974). Second, several lines of evidence indicate an increased propensity of WS somatic cells to undergo chromosomal and intragenic mutations, especially translocations and deletions (Salk et al. 1981; Fukuchi et al. 1989, 1990; Rünger et al. 1994). Given the recently reported association between the decline in replicative potential of human somatic cells and the loss of telomeric DNA (reviewed by Martin 1994), we set out to determine if there is a correlation between telomeric length and growth potential in WS. Such observations would be consistent with a causal role for telomere loss in the emergence of the phenotypes of accelerated replicative decline and chromosomal instability. A statistical analysis of our results does indicate an accelerated rate of telomere restriction fragment (TRF) decline during

Table 1 Origins of cell cultures (*WS* Werner syndrome, *F* female, *M* male)

Culture ID[a]	WS registry no.[b]	Diagnosis[c]	Age[d]	Sex	Ethnicity	Biopsy site
MG-1	UH607902	Definite WS	26	F	Caucasian	Thoracic skin
SE-1	CTA60001	Definite WS	51	F	East Indian	Mesial mid upper arm skin
78–82	MCI7882	Definite WS	53	F	Japanese	Abdominal skin (autopsy)
73–26	MCI7326	Definite WS	57	F	Japanese	Abdominal skin (autopsy)
430–26F		Normal control	26	F	Caucasian	Deltoid skin
88–6		Autopsy control[e]	52	M	Caucasian/ Hispanic	Abdominal skin (autopsy)
88–1		Autopsy control[f]	62	F	Caucasian	Abdominal skin (autopsy)

[a] University of Washington Department of Pathology Cell Repository registry number
[b] University of Washington International Registry of Werner syndrome patients
[c] Diagnosis of Werner syndrome according to criteria of Nakura et al. (1994)
[d] Age at time of biopsy or autopsy
[e] Autopsy diagnosis: atherosclerosis
[f] Autopsy diagnosis: cholangiocarcinoma

serial passage of fibroblastlike cells from four WS subjects as compared with those from three matched controls, but there is overlap in the observed rates of decline for controls and patients even among these small samples of subjects. Moreover, the mean TRF lengths of senescent WS cells are significantly longer than those of senescent controls, consistent with previous biochemical evidence suggesting that the mechanisms by which WS cells exit the cell cycle may differ from those of senescent controls (Oshima et al. 1995).

Materials and methods

Werner syndrome patients and controls and cell cultures

Table 1 summarizes the relevant information concerning the origins of the cell cultures utilized for the reported research. Clinical findings in three of the four WS subjects have been reported previously in the literature. Registry numbers MCI7326 and MCI7882 were Japanese sisters originally reported as case 1 and case 3, respectively (Epstein et al. 1966). CTA60001 was published by Tannock and Cook (1988). According to the criteria of Nakura et al. (1994), all four cases could be classified as "definite" Werner syndrome, although, at the time of the original biopsy (age 26 years), UH607902 had not yet had a diagnosis of ocular cataracts. Since the cultures of fibroblastlike cells from the two Japanese cases had been derived from abdominal skin at the time of autopsy, two approximately age-matched controls, with comparable post-mortem time intervals, were also obtained from the autopsy service. Since the cultures from Werner subject CTA60001 had been obtained ante-mortem from the skin of the arm, an additional age-matched control culture was derived from the skin of the arm of a living normal subject (430-26F).

Details of the establishment and cryopreservation of cultures and explants are given in Oshima et al. (1995). For all of the presently reported experiments, cells were grown in Dulbecco's modified Eagle's medium ("high" glucose variant, 4,500 mg/l) with glutamine (Gibco BRL, Gaithersburg, Md.) supplemented with 16.7% (v/v) fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). The first passages were from cryopreserved cells resuspended in 10 ml of growth medium and plated in 75-cm^2 plastic cell culture flasks (Corning, Corning, N.Y.) (1×10^5 to 4×10^5 cells per flask). Cultures were fed twice weekly (complete change of medium) and subcultured at or near confluence using a brief wash with 0.53 m*M* ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered normal saline (PBS; Grand Island BRL, Gaithersburg, Md.) and dissociated with 0.05% trypsin and 0.53 m*M* EDTA in PBS (Gibco). Harvested cell suspensions were mixed 1:1 with growth medium. Aliquots were removed for the determinations of cell concentrations (hemocytometer). Eight to twelve 75-cm^2 flasks were plated at each passage to provide PBS-washed pellets of 1×10^7 to 2×10^7 cells and cells to reseed 75-cm^2 flasks (1×10^5 to 4×10^5 cells) for the subsequent passage. The moist, centrifuged pellets were frozen and stored at –70°C. Population doublings for each of the serial passages were calculated as the ln (harvested/plated)/ln 2.

Extraction of DNA and determination of telomere restriction fragments

DNA was prepared essentially as described (de Lange et al. 1990). Briefly, the cell pellets were resuspended in 3 ml TNE (10 m*M* Tris HCl, pH 7.5, 10 m*M* EDTA). *Hin*dIII-digested lambda DNA (2.5 μg; New England Biolabs, Beverly, Mass.) was added as an internal control for DNA integrity. Proteinase K (50 μg/ml) and sodium dodecyl sulfate (SDS, 0.5%) were added, and DNA was incubated at 55°C for 2–12 h. The DNA was extracted with phenol/chloroform and precipitated with isopropanol. DNA was again incubated with proteinase K, extracted with phenol/chloroform, and precipitated with isopropanol. The precipitate was resuspended in 0.5 ml 10 m*M* Tris HCl (pH 7.5) and 0.1 m*M* EDTA and the DNA quantified by UV spectrophotometry.

For Southern blots, 2 μg of DNA was digested with *Hin*fI and *Rsa*I (New England Biolabs) in 50 m*M* Tris HCl (pH 7.9), 10 m*M* $MgCl_2$, 100 m*M* NaCl, and 1 m*M* dithiothreitol. DNA was run on a 0.7% gel overnight at 35 V. Gels were treated with 0.25 *M* HCl for 15 min and then soaked twice in 1.5 *M* NaCl and 0.5 *M* NaOH for 20 min, followed by soaking in transfer solution (5 × SSC; Salzberg et al. 1977) for 10 min. The DNA was blotted onto Hybond membrane (Amersham, Arlington Heights, Ill.) in transfer solution. After transfer, the DNA was crosslinked to the filter with UV light using a Stratalinker (Stratagene, La Jolla, Calif.). Hybridizations were carried out in 5 × SSC, 50% formamide, 0.5% low-fat powdered milk, 1 mg/ml total yeast RNA (BDH Biochemicals), 1 × Denhardt solution (Denhardt 1966) at 42°C overnight. Blots were washed twice for 15 min at room temperature in 2 × SSC, 0.1% SDS, and then twice at 65°C in 0.1 × SSC and 0.1% SDS. The blots were exposed on a Molecular Dynamics phosphorimager screen (Johnston et al. 1990). Mean telomeric length was analyzed using ImageQuant and Excel software as described by Harley et al. (1990). Briefly, mean telomere length was calculated as $\sum I_x/\sum(I_x/L_x)$, where I_x is the signal intensity at position x and L_x is the length of DNA at position x. This calculation assumes that

the telomere length is proportional to the length of the terminal restriction fragment length. Telomeric restriction fragments from approximately 3–15 kilobases (kb) were analyzed. Each DNA sample was analyzed on three or four different gels. Some DNA samples from normal and WS cells were mixed with bacteriophage λ DNA, run uncut on a 0.7% gel, blotted as described above, and hybridized to lambda and TTAGGG probes. This analysis showed that the lambda and telomeric DNA was undegraded and of high molecular weight.

Statistical analysis

The primary outcome of interest was the comparison of the rate of decline of telomere length in WS cases versus controls. Rate of decline was modeled using a linear random effects model (Laird and Ware 1982; Feldman 1988). In this model, longitudinal trajectories (the slopes and intercepts) are modeled for each subject. The degree of random variability in the trajectory estimates that is accounted for by random variability in measurements (measurement error) is also estimated and is adjusted for in hypothesis testing. Possible assay batch effects were controlled for by balancing cases and controls in each batch and by including batch effects in the model. The null hypothesis of no difference in rate of decline in WS cases versus normal controls was tested using the *F* statistic. TRF length at replicative senescence was compared in WS cases versus normal controls using a random effects model of TRF length at the final culture passage. Assay batch effects were included in the model. All statistical analyses were performed using the SAS statistical package; random effects models were programmed using the SAS procedure MIXED (SAS Technical Report P-229, SAS Institute, Cary, N.C.).

Results and discussion

Figure 1 illustrates Southern blot evidence of a gradual decline in mean TRF length during the serial passaging of the various cell lines. The mean rates of loss are graphically displayed in Fig. 2, and the numerical data, including variance estimates, are summarized in Table 2.

At the initial stages of culture, which presumably reflect most closely the TRF length of fibroblastlike cells in the intact dermis, WS TRF lengths are not, on average, shorter than those of the controls. There are caveats to this interpretation, however. First of all, the WS subjects we studied may have been losing telomeric repeats in their replicating dermal fibroblastlike cells at relatively high rates in vivo, but this phenomenon may have been masked by constitutionally longer subtelomeric DNA sequences (DNA that is proximal to the TTAGGG repeats and that is not cut by *Hin*fI and *Rsa*I). A second interpretation derives from the fact that the methodology for establishing such cell cultures strongly selects for a small subset of cells of a given type that have the greatest replicative potentials. There is major intraclonal variability in the replication of skin-fibroblastlike cells in culture (Martin et al. 1974), and it seems likely that such clonal attenuation also occurs in vivo. Thus, one would initiate cultures with the very "best" WS cells and therefore would not detect evidence of more rapid replicative declines and shorter telomeres in most of the WS patient's skin fibroblasts. Consistent with this interpretation is the report that, in initial cultures, WS cells usually exhibit good replicative potential (Faragher et al. 1993). Thirdly, given the fact that

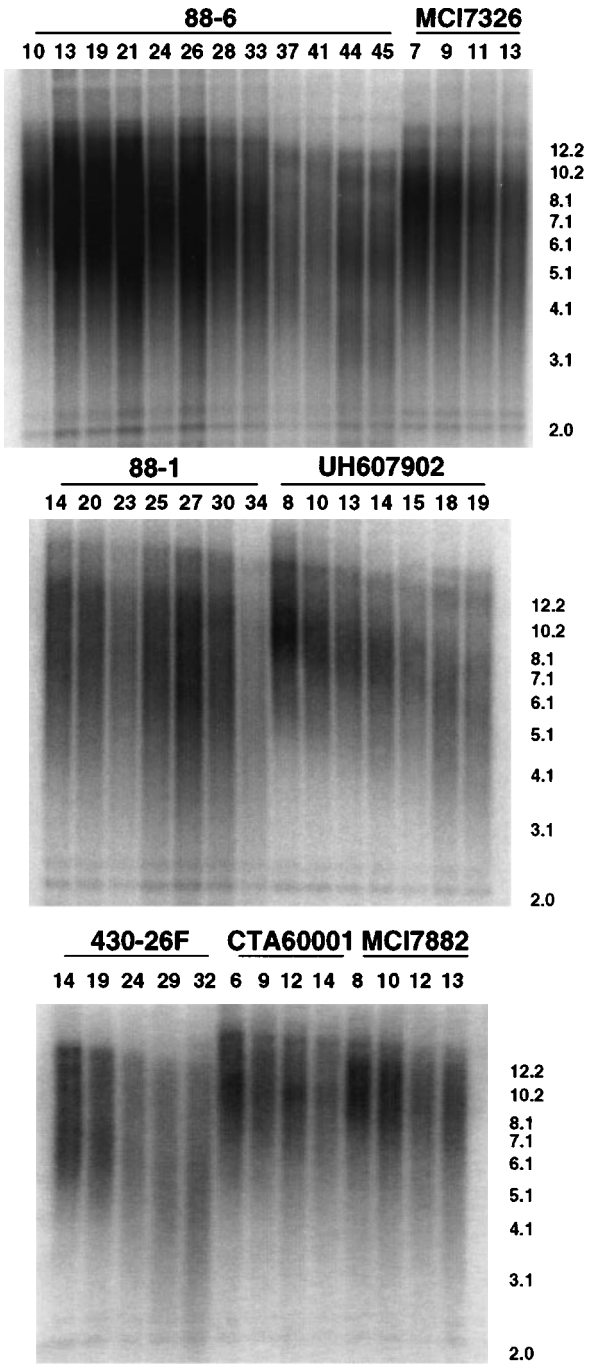

Fig. 1 Southern blots illustrating the telomere restriction fragment length (TRF) of Werner syndrome and control cells. The DNA was isolated, digested, subjected to electrophoresis and blotted as described in Materials and methods. The blots were hybridized to a TTAGGG probe. Positions of DNA size markers are shown at the *right*. Designations of the various cell lines and the respective levels of population doublings at which DNA was extracted are indicated in the *headings* for each series of Southern blots

the skin of patients with WS is variably atrophic, with loss of skin appendages, increases in collagen, and presumably shifts in population heterogeneity, the cultured cells may represent a distinct subset of "fibroblasts" that were rela-

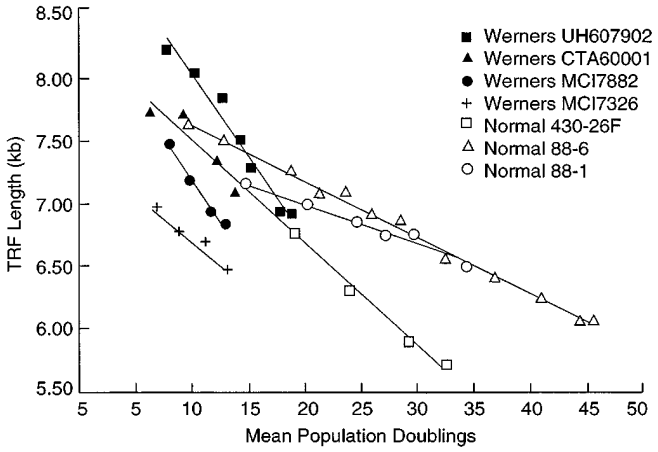

Fig. 2 Rates of decline in TRF length during the serial passaging of skin fibroblastlike cells from subjects with Werner syndrome (*Werners*) and normal controls. See Table 2 for numerical data. The TRF length reported for each DNA sample is the average obtained from analysis of DNA run on three or four independent gels

Table 2 Replicative life spans expressed as maximum achieved population doubling levels (Max PDL) and calculated numbers of nucleotide base pairs per mean population doubling (bp loss/MPD) for serially passaged skin fibroblastlike cells from Werner syndrome (*WS*) patients and controls

Culture ID	Diagnosis	Max PDL	bp loss/ MPD[a]	Standard error
MG-1	WS	22.0	131	9
SE-1	WS	13.7	85	8
78–82	WS	15.0	137	17
73–26	WS	14.3	77	8
Mean		16.3	108	15
430–26F	Normal	38.0	81	8
88–6	Normal	47.6	45	5
88–1	Normal	39.3	32	4
Mean		41.6	53	8.5

[a] The DNA samples from one cell line were run on a gel, and the TRF length was determined for each MPD. The TRF length was plotted versus MPD, and bp loss/MPD was calculated. This analysis was repeated for two or three more gels. The bp loss/MPD reported here is determined by averaging the bp loss/MPD obtained from analysis of three or four gels

tively quiescent until subjected to serum mitogens in culture. Such a phenomenon could also explain the recent report that senesced cultures of WS cells, unlike those of controls, maintain a robust induction of *FOS* upon stimulation with serum mitogens (Oshima et al. 1995). An equally plausible alternative interpretation of that observation, however, is that the mechanism whereby WS cells exit the mitotic cell cycle differs from that of senescing cells from controls. Assuming that the WS cells we have cultivated are indeed representative of the majority of WS fibroblastlike cells in vivo, that alternative interpretation would be supported by our observations that WS cultures cease replicating when mean TRF length is significantly longer than that of senescent controls (mean TRF length 6.795 kb vs 6.079 kb, $F_{1,9} = 8.47$, P –0.017; Fig. 2).

Heterogeneity of cell types could also be a potential explanation for the variation in rate of decline of TRF length in the control cultures. In fact, the normal strain 430–26F shows a rate of decline apparently identical to WS strain SE-1 despite the fact that it can undergo many more population doublings than SE-1 (Table 2). This discrepancy is despite a deliberate matching for gender, exact age of donor, and biopsy from the same region (skin of arm) and from a living subject. Substantial variance in the rate of decline in TRF length in human skin fibroblast cultures from different donors has been described by Harley et al. (1990) and Allsopp et al. (1992). That such variance is largely biologically determined and not merely related to experimental error is supported by twin studies indicating an exceptionally high heritability (78%) for mean TRF length (Slagboom et al. 1994).

A statistical analysis, testing the null hypothesis of no difference in rates of TRF loss in WS versus controls, led to a rejection of that hypothesis. The decline in TRF length in WS cases was significantly faster than in controls ($F_{1,117} = 4.51$, $P = 0.036$). Given the small sample size and the considerable variance in controls (Harley et al. 1990; Allsopp et al. 1992; this study), it would be prudent, however, to reinvestigate this question with a larger cohort of controls and WS subjects.

Only one other study has reported on the telomere length of WS fibroblastlike cells (Kruk et al. 1995). It was concluded that the telomere length of the single strain of WS fibroblasts that was investigated was the shortest among a group of fibroblast cultures from five different donors ranging in age from 2 days to at least 75 years. Since we have shown that the TRF length of WS fibroblastlike cells from early cultures is not demonstrably shorter than those of controls, it is likely that the unusually short TRF length reported by Kruk et al. (1995) was related to the use of WS cells that were in later stages of their in vitro replicative histories.

Allsopp et al. (1992) found that the pooled mean TRF length in five cell strains established from donors with the diagnosis of Progeria (Hutchinson-Gilford syndrome; Brown 1990) was significantly shorter than those of five controls strains ($P < 0.001$). In that study, however, it is also possible that the finding was related to the use of DNA from Progeria cultures that had completed a relatively higher proportion of their replicative life spans, as they were said to have undergone more rapid replicative senescence.

How can our observations elucidate the pathogenesis of WS? The most likely interpretation of the composite results is that, as a result of a more rapid rate of clonal attenuation (the cause of which remains unknown), WS cells lose telomeres at accelerated rates. Another possibility is that WS cells undergo higher rates of cell death, and thus the fewer remaining cells must undergo comparatively larger numbers of doublings, with concomitantly larger losses of telomere repeats. One cannot rule out, however, some “leaky” mutation in either the RNA (McCormick-Graham and Romero 1995) or protein moieties (Collins et al. 1995) of telomerase. If telomere metabo-

lism is indeed of primary significance in the pathophysiology of WS, however, it is more likely that the mutation affects a protein that protects telomeres from degradation, is defective for conventional DNA polymerase replication or repair of telomeres, or alters the process by which telomere loss is translated into an antiproliferative signal. Alternatively, the WS mutation may mediate exit from the cell cycle via a mechanism that is independent of its effect on telomere metabolism.

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