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Increased frequency of 6-thioguanine-resistant peripheral blood lymphocytes in Werner syndrome patients

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Summary. The frequency of spontaneous 6-thioguanine (TG)-resistant peripheral blood lymphocytes in five unrelated Werner syndrome (WS) patients was determined using an autoradiographic labeling assay. The average frequency of TG-resistant lymphocytes was eightfold higher in WS patients than in sex- and age-matched normal control donors. This finding and previous identification of increased spontaneous chromosomal rearrangements and deletions in WS cells or cell lines suggest that WS is a human genomic instability or mutator syndrome.

Introduction

Werner syndrome (WS; McKusick catalog number 27770) is a rare, autosomal recessive disorder that has been referred to as a "segmental progeroid syndrome" or caricature of normal aging (Martin 1978; Epstein et al. 1966). WS patients demonstrate several features of premature aging. These features include short stature; premature graying of the hair; early development of cataracts, atherosclerosis, diabetes mellitus, and osteoporosis; and a predisposition to malignancy (Epstein et al. 1966).

The genetic defect in WS has not been characterized. A number of differences between cells from WS patients and normal individuals have been identified, however. For example, skin fibroblasts from WS patients exhibit slow growth and a reduced life span in vitro (Martin et al. 1970; Holliday et al. 1974; Higashikawa and Fujiwara 1978; Thompson and Holliday 1983), and a reduced rate of DNA replication (Fujiwara et al. 1977; Takeuchi et al. 1982a, b) though apparently normal DNA repair (Fujiwara et al. 1977; Higashikawa and Fujiwara 1978; Thompson and Holliday 1983).

WS is a chromosomal or genomic instability syndrome at the cellular level. We and others have observed an increased frequency of both spontaneous and induced chromosomal aberrations in WS cells (Nordenson 1977; Salk et al. 1981; Scapparicci et al. 1982; Gebhart et al. 1988). WS also appears to be a mutator mutation at the molecular level. Simian virus 40 (SV-40)-transformed fibroblast cell lines from WS patients display an increased spontaneous mutation rate at the X-linked hypoxanthine phosphoribosyltransferase (HPRT) locus, and a high proportion (76%) of extensive HPRT gene deletions in independent spontaneous TG-resistant mutants (Fukuchi et al. 1985; Fukuchi et al. 1989).

We have used an autoradiographic labeling assay (Strauss and Albertini 1979) to determine whether the mutator phenotype of WS cells occurs in vivo. The frequencies of TG-resistant and spontaneously cycling lymphocytes in peripheral blood from five unrelated WS patients and control donors were determined using this assay.

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Materials and methods

WS patients and control donors

Peripheral blood samples were obtained by venipuncture from six unrelated WS patients and six control donors. The diagnosis of WS was made on the basis of clinical findings summarized in Table 1 (Epstein et al. 1966; Murata and Nakashima 1982). The parents of WS patients, WS-2, WS-4, and WS-5, were first cousins. None of the WS patients were sibs. Patients, WS-2, WS-4 and WS-5, were receiving no medication at the time of study, and none of the WS patients or controls had previously received therapeutic agents that are known mutagens or carcinogens. Patients WS-1 and WS-3 were receiving medication for skin ulcers and for atherosclero-

Table 1. Clinical features of Werner syndrome (WS) patients[a]

WS patient	WS-1	WS-2	WS-3	WS-4	WS-5	WS-6
Age (years)	52	42	41	37	37	25
Sex[b]	F	M	M	F	F	F
Parental consanguinity[c]	−	+	−	+	+	−
Skin ulcers on legs	+	−	+	−	−	−
Soft tissue calcification	+	+	−	+	−	−
Osteoporosis	+	+	+	−	−	+
Affected sibs	−	+	−	+	−	−

[a] All six patients demonstrated the following clinical findings: short stature, thin limbs with a stocky trunk, premature graying and loss of hair, scleroderma like skin changes, glucose intolerance or diabetes mellitus, cataracts, and a high-pitched or hoarse voice. None of the patients had a documented malignancy
[b] F, female; M, male; +, present; −, absent or not detected
[c] The parents of WS-2, WS-4, and WS-5 were first cousins; the first cousin pairs were not related

tic vascular disease with agents not known to be cytotoxic or mutagenic. Lymphocytes from five patients (WS-1 to WS-5) and matched control donors (N-1 to N-5) were used to determine the frequency of TG-resistant cells in peripheral blood, while lymphocytes from the remaining patient (WS-6) and control donor (N-6) were used to determine the fraction of spontaneously cycling cells in cultures labeled with [^{3}H]-thymidine for 30 min or 12 h.

Autoradiographic method

The variant frequency of TG-resistant peripheral blood lymphocytes in WS patients and control donors was determined by a modification of the method of Strauss and Albertini (1979). Heparinized (10 units/ml) whole blood was mixed with an equal volume of phosphate-buffered saline (PBS) and laid over Lymphocyte Separation Medium (Bionetics) in a 15-ml polystyrene centrifuge tube (Corning). The tubes were centrifuged for 30 min at $400\times g$. The lymphocyte layer was removed, washed twice with PBS, and then resuspended in RPMI 1640 medium containing 12.5% fetal calf serum (previously heat-inactivated at 56°C for 20 min), 100 units/ml penicillin and 50 μg/ml streptomycin. Four identical lymphocyte cultures were set up in 15-ml polystyrene centrifuge tubes. Each culture contained 1.6×10^6 peripheral blood lymphocytes in 2 ml of medium supplemented with 2.5 μl/ml bactophytohemagglutinin P (PHA-P, Difco catalog no. 3110-56-4). Two of the four cultures were supplemented with 1.8×10^{-4} *M* (30 μg/ml) 6-thioguanine (TG) (2-amino-6-mercaptopurine; Wako) prior to growth. A fifth culture containing 1.8×10^{-4} *M* TG but no PHA-P was set up to allow determination of the frequency of spontaneously cycling cells in each patient and control sample. Cultures were grown in a humidified, 37°C 5% CO_2/95% air atmosphere for 40 h, then labeled by the addition of [^{3}H]thymidine (specific activity 25 Ci/mmol; New England Nuclear) to a final concentration of 1 μCi/ml for an additional 12 h of growth.

Cell nuclei were prepared for autoradiography by the addition of 4 vol (8 ml) of 0.1 *M* citric acid to each culture, followed by centrifugation and fixation of the resuspended pellet in 0.4 ml of methanol-acetic acid (5:1) at 4°C for ≥ 3 h. Nuclei were triturated through a 23-gauge, 6-cm needle fitted in a 2.5-ml syringe, then counted and distributed onto 18 mm^2 coverslips fixed to glass slides with Eukitt (Zeiss). Coverslips were air-dried, stained with 1% aceto-orcein for 3 min, rinsed with distilled water, and then dipped in NTB 2 emulsion (Eastman Kodak) diluted 1:1 (v/v) with water. Emulsion-coated coverslips were exposed at 5°C for 1 week prior to development.

Labeled and unlabeled nuclei were counted by light microscopy. Approximately 5,000 nuclei from cultures without TG, and approximately 300,000 nuclei from cultures supplemented with TG, were analyzed for each WS patient and control donor. Counts from duplicate cultures were pooled to calculate variant frequencies.

The frequency of spontaneously cycling cells in five WS patients (WS-1 to WS-5) and five control donors (N-1 to N-5) was determined using 2-ml lymphocyte cultures that had not been PHA-P-stimulated. Cultures were grown for 40 h in the presence of 1.8×10^{-4} *M* TG, then labeled for 20–30 min with 1 μCi/ml [^{3}H]thymidine. Labeled nuclei were determined as described above. A comparable number of nuclei, approximately 3×10^5, were examined from each WS and control culture. TG-supplemented lymphocyte cultures from the remaining WS patient (WS-6) and control donor (N-6) were grown for 40 h in the absence of PHA-P, then labeled with [^{3}H]-thymidine for either 30 min or 12 h and counted as described above to determine whether the frequency of spontaneously cycling cells varied as a function of the length of [^{3}H]thymidine labeling. A comparable number of nuclei, approximately 3×10^5, were examined from WS (WS-6) and control (N-6) cultures at each time point.

The TG-resistant variant frequency of cultures was calculated by dividing the frequency of labeled nuclei in TG-containing cultures by the frequency of labeled nuclei in cultures grown in the absence of TG. The frequency of labeled nuclei in TG-containing cultures was corrected by subtracting the frequency of spontaneously cycling cells in each culture.

Results

Thioguanine-resistant variant frequencies ranged from 1.24×10^{-4} to 3.86×10^{-4} in WS patients, and from 0.12×10^{-4} to 0.31×10^{-4} in control donors. An eightfold difference in mean variant frequency was observed between WS patients ($1.86\pm0.45\times10^{-4}$) and control donors ($0.23\pm0.03\times10^{-4}$; Table 2). The 95% confidence intervals for variant frequencies in each patient and donor were calculated by assuming that the number of labeled nuclei was a Poisson variable and that the proportion of labeled nuclei in control cultures was a binomial fraction (Sylwester and Albertini 1985).

One spontaneously cycling cell was observed in each of two WS patients (WS-2 and WS-5) and two spontaneously cycling cells in one control donor (N-1) in TG-containing cultures grown without PHA-P stimulation (Table 2). The number of spontaneously cycling cells was similar in the WS (WS-6) and control (N-6) cultures labeled for 30 min or 12 h after growth for 40 h in the presence of TG: two cells were detected in the WS (WS-6) and one cell in the control (N-6) culture after 30 min labeling, while one cell was detected in the WS and two cells in the control culture after 12 h of labeling when comparable numbers of nuclei were examined from each culture and time point.

Discussion

We have observed an eightfold elevation in the mean frequency of TG-resistant peripheral blood lymphocytes in WS patients as compared with normal donors using an autoradiographic labeling assay. This difference in variant frequencies is statistically significant ($0.01<P<0.025$, $d=3.23$ with 4 degrees of freedom in a single-tail *t*-test where variances were not assumed equal; Bailey 1981). The control donor variant frequencies we determined (0.12×10^{-4} to 0.31×10^{-4}) are comparable to control donor variant frequencies determined by others using similar methods (Strauss and Albertini 1979; Lange and Pranter 1982). Elevated variant frequencies comparable to those we observed in WS patients (1.24×10^{-4} to 3.86×10^{-4}) have been observed in patients receiving chemotherapy or radiation therapy (Strauss and Albertini 1979; Lange and Pranter 1982), and in patients with Bloom syndrome and Fanconi anemia, two rare, recessive chromosomal instability syndromes (Vijayalaxmi et al. 1983; 1985). Vijayalaxmi et al. (1983, 1985) observed more cycling cells (zero to four cycling cells in 10^4 consecutive nuclei) in Bloom syn-

Table 2. Frequency of thioguanine-resistant cells in peripheral blood lymphocyte cultures from WS patients and controls

	+ Thioguanine[a]		− Thioguanine[a]		Adjusted variant frequency[b] ($\times 10^{-4}$)	95% Confidence interval[c] ($\times 10^{-4}$)
	No. of labeled cells	No. of cells counted	No. of labeled cells	No. of cells counted		
WS patients						
WS-1	15	309,725	1,997	5,341	1.30	0.72–2.14
WS-2	13	265,600	1,940	5,325	1.24	0.64–2.17
WS-3	23	140,859	2,287	5,412	3.86	2.45–5.80
WS-4	13	301,250	2,245	8,345	1.60	0.85–2.76
WS-5	16	263,467	2,248	5,082	1.29	0.72–2.12
Mean ± standard error					1.86 ± 0.45	
Control donors[d]						
N-1	7	485,704	2,371	5,530	0.24	0.078–0.56
N-2	4	476,999	2,288	5,122	0.19	0.047–0.48
N-3	4	346,104	2,405	6,528	0.31	0.085–0.80
N-4	1	197,458	2,193	5,211	0.12	0.003–0.67
N-5	3	321,600	1,775	5,210	0.27	0.056–0.80
Mean ± standard error					0.23 ± 0.03	

[a] PHA-P-stimulated cultures grown in the presence (+) or absence (−) of $1.8 \times 10^{-4} M$ 6-thioguanine (TG)

[b] The adjusted variant frequency for each sample was calculated by subtracting the number of spontaneously cycling cell nuclei, corrected for the number of nuclei counted, from the frequency of labeled nuclei in PHA-P-stimulated TG-containing cultures, then dividing by the frequency of labeled nuclei in PHA-P-stimulated cultures grown in the absence of TG (see Materials and methods)

[c] 95% confidence intervals for adjusted variant frequencies were calculated by assuming that the number of labeled nuclei is a Poisson variable, and that the labeling index (fraction of nuclei labeled) is a binomial fraction (Sylwester and Albertini 1985)

[d] WS patients and control donors were matched for age and sex, with the exception of the ages of patient WS-6 (age 25; female) and control donor N-6 (age 28; female)

drome and Fanconi anemia patients than we did in WS patients. To estimate cycling cells, they incubated peripheral blood lymphocytes immediately after isolation in the presence of [^{3}H]thymidine for 15 min, while we incubated lymphocytes for 40 h in medium containing $1.8 \times 10^{-4} M$ TG prior to [^{3}H]thymidine labeling for 20–30 min. Growth of lymphocytes for 40 h in the presence of TG may have suppressed DNA synthesis in a portion of spontaneously cycling cells in our experiments.

In the autoradiographic assay, labeled nuclei are derived from HPRT-deficient, TG-resistant cells and from "phenocopies," cycling cells that contain HPRT activity and are not stably TG-resistant (Albertini et al. 1981). Variant frequencies obtained using fresh lymphocytes from the autoradiographic assay are parallel to, but 10- to 100-fold higher than, variant frequencies obtained using cryopreserved lymphocyte populations, in which most "phenocopies" are not detected (Albertini et al. 1981, Albertini 1982). Autoradiographic assays using cryopreserved lymphocytes give variant frequencies that are quantitatively similar to those obtained using T-cell cloning assays (Albertini 1985), and thus both assays can be used to quantify TG-resistant lymphocytes in human peripheral blood. Analyses of cell surface antigens, of the structure of T-cell receptor genes, and of HPRT gene alterations in TG-resistant T-cell clones suggest that many of the HPRT-deficient, TG-resistant lymphocytes detected by T-cell cloning and autoradiographic labeling assays are independent mutants (Albertini 1985; Albertini et al. 1985; Turner et al. 1985; Nicklas et al. 1987).

The elevated variant frequencies we observed in WS patients do not appear to be a result of more spontaneously cycling cells or different labeling kinetics in WS patients as compared with control donors. We observed comparable frequencies of spontaneously cycling cells in five WS patients and five control donors after labeling lymphocyte cultures for 20–30 min with [^{3}H]thymidine, and no difference in the frequency of spontaneously cycling cells in one WS patient (WS–6) and one control donor (N–6) after labeling cultures for 30 min or 12 h.

We plan to isolate and characterize TG-resistant lymphocyte clones from WS patients to confirm the presence of an elevated mutation frequency and to rule out other possible causes of elevated variant frequencies in WS patients. For example, cells containing the WS mutation could be more resistant to TG than are control cells. This is unlikely, however, as cell lines derived from WS patients and controls are comparably sensitive to killing by TG (Fukuchi et al. 1989). Other possible explanations for elevated WS variant frequencies in the absence of elevated mutation frequencies are relaxed selection against HPRT-deficient peripheral blood lymphocytes, and clonal amplification of TG-resistant lymphocytes (Nicklas et al. 1988), in WS patients in vivo. Somatic selection against HPRT-deficient cells in vivo is suggested by the much lower than expected frequencies of TG-resistant lymphocytes and erythrocytes, but not fibroblasts, in females heterozygous for HPRT deficiency (McDonald and Kelley 1972; Strauss et al. 1980; Dempsey et al. 1983), and by the rapid decline in elevated variant frequencies associated with cyclophosphamide therapy in multiple sclerosis patients after the cessation of treatment (Ammenheuser et al. 1988). The elevated variant frequencies we observed in WS patients can be explained solely by the elevated spontaneous mutation rate we have observed in WS cells (Fukuchi et al. 1985, 1989), however. Thus preferential clonal expansion of or relaxed selection against TG-resistant lymphocytes in WS patients are unlikely explanations for our results.

The elevated variant frequency of spontaneous TG-resistant peripheral blood lymphocytes and the elevated rate and altered spectrum of spontaneous HPRT mutations we have identified in cells and cell lines from WS patients suggest that WS is a

human genomic instability syndrome or mutator mutation. These results suggest that somatic mutation accumulation, and perhaps the preferential accumulation of deletions, might play pathogenetically important roles in the production of WS and of associated, age-dependent human disease processes such as atherosclerosis and neoplasia (Fukuchi et al. 1985; Benditt and Benditt 1973; Bishop 1988).

Localization and characterization of the genetic defect in WS should aid identification of the biochemical pathways responsible for the mutator phenotype of WS. Identification of the genetic defect in WS may have additional practical importance, as the affected gene or genes appear to modulate the rate of appearance of the human aging phenotype and of several clinically important, age-associated human disease processes.

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