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CELL KINETIC EVIDENCE SUGGESTS ELEVATED OXIDATIVE STRESS IN CULTURED CELLS OF BLOOM'S SYNDROME

MARTIN POOT and HOLGER HOEHN

Department of Human Genetics, University of Würzburg, 8700 Würzburg West Germany

THOMAS M. NICOTERA

Department of Biophysics, Roswell Park Memorial Institute, Buffalo, NY 14263, USA

HUGO W. RÜDIGER

Department of Occupational Medicine, University of Hamburg, West Germany

Bromodeoxyuridine/Hoechst flow cytometry was used to analyse disturbed cell proliferation of fibroblasts and lymphoblastoid cells from Bloom's syndrome (BS). Fibroblasts show poor activation, arrest in the G2 phase of the cell cycle along with a prolongation of the G1 phase. This pattern of perturbed cells proliferation is akin to that elicited in normal fibroblasts by 4-hydroxy-nonenal, a breakdown product of lipid peroxides. Treatment with vitamin E improved growth of BS fibroblasts more strongly than growth of normal fibroblasts. Lymphoblastoid cells from BS, to the contrary, experience only a minor arrest in the G2 phase after one round of bromodeoxyuridine incorporation, but are strongly inhibited during and after the second S phase. Thus, their cell cycle arrest is dependent upon BrdU incorporation, as has been found previously in normal cells exposed to elevated concentrations of oxygen or paraquat, a superoxide generating compound. These results suggest that BS cells may suffer from an elevated, endogenous generation of oxygen free radicals.

KEY WORDS: Bloom's syndrome, vitamin E, cell kinetics, bromodeoxyuridine, Hoechst-33258 dye, bivariate flow cytometry, cell superoxide, DNA damage, proliferation.

INTRODUCTION

Bloom's syndrome (BS) is an autosomal, recessive disorder characterised by stunted growth, telangiectatic erythema of the face, elevated occurrence of infections in the upper respiratory tract and a high frequency of cancer.[1–3] Cultured cells from BS patients show an elevated frequency of sister chormatid exchanges (SCEs),[4] the presence of quadriradial figures,[5] and an increased prevalence of micronuclei[6] and clonal chromosome aberrations.[7] Upon fusion with normal cells the rate of SCE is normalised in BS nuclei.[8–10] A deficiency in DNA repair systems has been suggested to account for these cytogenetic abnormalities, but no consistent defect in DNA repair enzymes has been found in BS cells.[11] Other hypotheses include deficiencies in

Correspondence: Martin Poot, Department of Human Genetics, Koellikerstrasse 2, 8700 Wuerzburg, West Germany, tel: +49-931-31298.

DNA topoisomerase II,[12] DNA ligase I[13–15] and an elevated rate of generation of superoxide.[16,17] This oxygen free radical is able to induce DNA damage (which can be enhanced with bromodeoxyuridine[18,19]) and is known to provoke lipid peroxidation.[20] Each of the two proposed enzyme deficiencies and the free radical induced cell lesions will lead to specific disturbances of cell proliferation. In order to differentiate between the proposed mechanisms for the BS phenotype we analysed the pattern of disturbed cell proliferation in fibroblasts and lymphoblastoid cells from BS with a novel, highly informative, cell kinetic assay, BrdU-Hoechst flow cytometry.[21,22]

MATERIALS AND METHODS

Materials

4-Hydroxy-nonenal (4-HNE) was a kind gift of Dr. H. Esterbauer (University of Graz, Austria). It was dissolved into distilled water, diluted to the appropriate concentration with phosphate buffered saline, and filter sterilised before use (0.22 μm, Millipore).

Cells and culture

Diploid skin fiboblast like (HDFL) cells[23] from three patients with Bloom's syndrome and from two healthy donors were cultured in Minimal Essential Medium (MEM) supplemented with 10% of pre-tested and heat inactivated foetal bovine serum (FBS). Confluent cultures were rendered quiescent by treatment with 0.1% FBS during 48 hours. Subsequently, cells were trypsinised, resuspended in MEM supplemented with 10% FCS and 65 μM of bromodeoxyuridine (BrdU) and deoxycytidine (CdR) each, and plated in 80 cm^2 cell culture flasks (Nunc).

Epstein-Barr virus transformed lymphoblastoid (EBL) cell lines from two patients with BS and two healthy subjects were cultured as suspensions in RPMI 1640 medium supplemented with 10% FBS. These cells reach quiescence if they are left unfed for four days. Quiescent cultures of EBL cells were centrifuged (5 min 800 $\times$ g) and cell pellets were resuspended at a density of 50,000 cells per ml in RPMI 1640 medium supplemented with 10% FBS and 100 μM of each BrdU and CdR. Cell suspensions were distributed among 25 cm^2 culture flasks (Nunc), which were maintained upright.

All experiments were done in incubators equipped with sensors regulating carbon dioxide and oxygen supply (Heraeus, Hanau, F.R.G.). Carbon dioxide was kept constant at 5% (vol/vol), and in some experiments air was replaced by nitrogen to lower oxygen concentration to 5% (vol/vol). At 22 to 96 hours after plating cells were harvested by trypsinisation and stored at -20°C in MEM supplemented with 10% FCS and 10% dimethylsulfoxide. To avoid any exposure to light of short wavelengths all flasks were wrapped in aluminium foil and harvesting was under illumination with red light only.

Cell staining and flow cytometry

After thawing, the cell pellets were stained with a buffer containing 1.2 μg Hoechst 33258 and 2.0 μg ethidium bromide per ml of buffer.[21] Flow cytometric analysis was carried out with an ICP 22 instrument (Ortho Diagnostic Systems, Raritan, NJ).

Bivariate data sets were analysed by framing, rotation and automated curve-fitting of each cell cycle as described.[22]

RESULTS AND DISCUSSION

Figure 1 displays bivariate cytograms of fibroblasts from a healthy subject and a BS patient after 72 hours of culture with BrdU and analysed with bivariate Hoechst/ethidium bromide flow cytometry. Due to incorporation of BrdU into the DNA during the S phase the fluorescence of Hoechst stained DNA is quenched, which allows to resolve three consecutive cell cycles. Note the difference in abundance of cells in the G2 cluster of the first cell cycle after exit from G0/G1 in control and BS fibroblasts (arrows).

By electronic framing, rotation of the axis, deconvolution and fitting of the data to a cell cycle model, the relative abundancies of cells in each compartment of three cell cycles can be obtained. Plotting these cell cycle distributions against time of culture in the presence of BrdU gives the exit kinetics of a particular cell culture. Figure 2 shows the exit kinetics of fibroblasts from a healthy subject and from three patients with BS cultured at 5% (vol/vol) oxygen. The subsequent curves denote the transit from the G0/G1 compartment into the S phase (i.e. the release from quiescence), transit from the S to the G2 phase of the first cycle, transit from G2 to the G1 phase of the second cycle (termed G1′), and so on. The distance between the plateaus of two subsequent curves at the right hand side of each panel indicates the fraction of cells permanently arrested in a cell cycle compartment. As noted previously,[7] cells from BS strain GM 1492 grow much better than cells from any other BS fibroblast strain. In our experiment a non-cycling fraction as low as that of a control cell strain was obtained (12.2% in BS vs. 10.3% in control). All cultures exhibit a certain degree of cell arrest in the G2 phase of the first cell cycle, although the extent of this arrest is much larger in cells from the three BS patients (Figure 2).

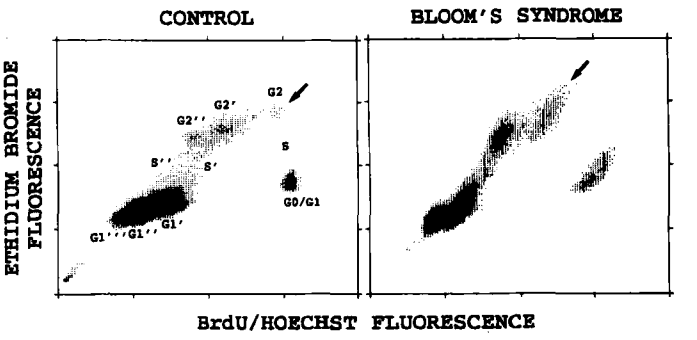

FIGURE 1 Bivariate cytograms of fibroblasts from a healthy subject and a patient with BS cultured during 72 hours with BrdU. The abscissa displays BrdU-quenched Hoechst fluorescence and the ordinate shows unquenched ethidium bromide fluorescence. Due to BrdU quenching of the Hoechst fluorescence the second and third cycle after serum stimulation appear progressively shifted leftwards from the first cell cycle. Note the difference in relative abundance of cells in the G2 cluster of the first cell cycle (arrows).

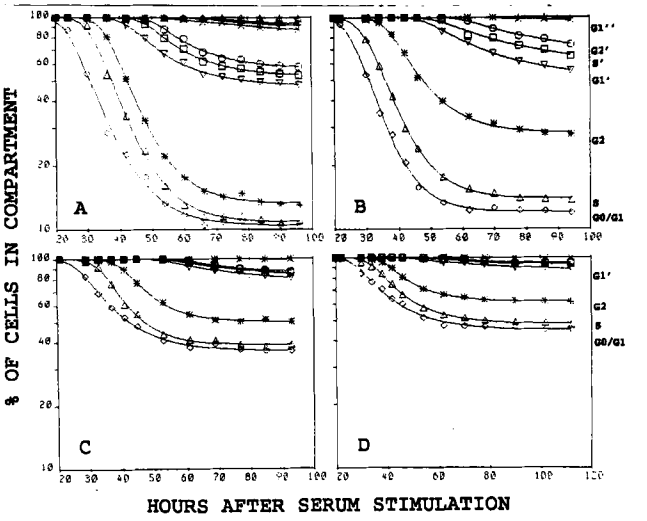

FIGURE 2 Exit kinetics of fibroblasts cultured at 5% oxygen. Panel A control cells, Panels B, C and D cells from three patients with BS. The subsequent curves denote exit from G0/G1 (◇), exit from S (△), exit from G2 (*), exit from G1′ (▽), and so on. The distance between the second and the third curve at the right hand side of the panels gives the fraction of cells irreversibly arrested in the G2 phase of the first cycle. The distance between X axis intercepts of the third and the fourth curve at the top of each panel gives the minimal duration of the G1 phase of the second cycle (G1′). Both the arrest in the G2 phase, and the minimal duration of the G1′ phase are elevated in BS cells relative to control cells.

The computed fractions of cells arrested in G2 are listed in Table 1. Fibroblasts from BS kept at 20% (vol/vol) oxygen show an elevated arrest in G2, which is not diminished by culture at 5% oxygen. In control cells, however, the arrest in G2 elicited by culture at 20% oxygen can be reversed by culturing at 5% (vol/vol) oxygen (the approximate oxygen concentration of venous blood).

The distance between the X-axis intercepts, obtained by extrapolation to 100% cells, of two subsequent curves represents the minimal duration of each cell cycle compartment. BS fibroblasts show an elevated minimal duration of the G1 phase of the second cycle (Figure 2 and Table 2), which, again, is not reversed by lowering the oxygen concentration to 5% (vol/vol). The failure of BS fibroblasts to improve cell cycle transit in response to lowered oxygen is important as it suggests that the system(s) mediating the toxic effect of oxygen upon cell cycling are already saturated at 5% (vol/vol) oxygen in BS fibroblasts.

The pattern of prolongation of the G1 phase of the second cell cycle after serum stimulation with concomitant arrest in the preceeding G2 phase is reminiscent of the cell kinetic disturbance induced in normal cells by 4-hydroxy-nonenal (HNE),[24] a breakdown product of lipid peroxides.[25,26] HNE, and other products of oxygen free radical induced cell damage, elicit elevated rates of sister chromatid exchanges,[27,28] which can be abolished by culturing cells with vitamin E and other antioxidants.[28] In order to test the hypothesis that the disturbed cell proliferation in BS cells results from free radical induced lipid peroxidation, we incubated cells from two healthy donors with 4-HNE. Tables 1 and 2 highlight the parameters of perturbed cell kinetics thus obtained and show clear similarity between the BS pattern and that elicited by HNE in normal fibroblasts.

TABLE I

Fraction of cells arrested in the G2 phase of the first cell cycle of fibroblasts from BS and healthy subjects cultured at 5 and 20% oxygen (in percent of total cells).

	oxygen concentration	
cell type	5%	20%
Controls		
Strain 1	2.4 ± 0.1	9.8 ± 0.4
Strain 2	2.1 ± 0.3	10.4 ± 0.9
BS		
GM 1492	14.2 ± 0.4	14.9 ± 0.5
GM 3498B	9.6 ± 0.5	11.9 ± 0.4
BS 3	13.6 ± 0.2	14.5 ± 0.1
Controls + 10 μM 4-HNE		
Strain 1	14.0 ± 0.6	ND
Strain 2	15.8 ± 0.5	ND

ND = not determined

All data are mean and standard deviations as obtained by fitting the cell cycle distributions from a time sequence of samples out of a single kinetic experiment to the modified cell cycle kinetic model as described.[22]

TABLE II

Minimal duration of the G1 phase of the second cell cycle after release from quiescence of fibroblasts from BS and healthy subjects cultured at 5 and 20% oxygen (in hours).

	oxygen concentration	
cell type	5%	20%
Controls		
Strain 1	6.5 ± 0.7	5.1 ± 1.9
Strain 2	7.2 ± 0.7	6.4 ± 0.6
BS		
GM 1492	12.2 ± 0.4	15.0 ± 0.4
GM 3498B	12.7 ± 0.5	17.5 ± 0.4
BS 3	9.8 ± 0.3	17.3 ± 0.2
Controls + 10 μM 4-HNE		
Strain 1	14.4 ± 0.5	ND
Strain 2	12.4 ± 0.5	ND

ND = not determined

All data are mean and standard deviations as obtained by fitting the cell cycle distributions from a time sequence of samples out of a single kinetic experiment to the modified cell cycle kinetic model as described.[22]

To further analyse a putative role of lipid peroxidation in the cell kinetic phenotype of BS fibroblasts, these cells were pre-treated with 1 IU of vitamin E per ml of culture medium for 72 hours, rendered quiescent in the presence of vitamin E, and serum stimulated to growth with the same concentration of vitamin E. The growth improvement by this treatment amounts to 27.7 ± 2.0% (GM 1492) and 34.4 ± 1.1% (GM 3498 B) in BS fibroblasts, and to 17.1 ± 1.9% (strain 1) and 19.1 ± 1.6% (strain 2) in control fibroblasts (all data are mean and standard deviations from four

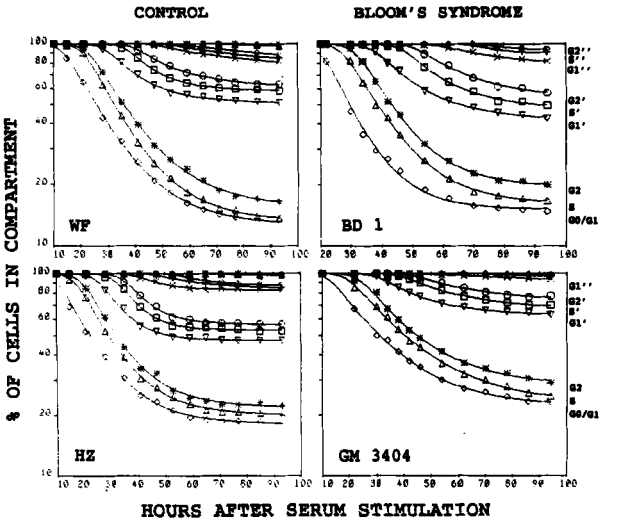

FIGURE 3 Exit kinetics of EBL cells cultured at 5% oxygen. Left row cell lines from two healthy subjects (top: line WF; bottom: line HZ). Right row cell lines from two BS patients (top: line BD 1; bottom: line GM 3404). For explanation see legend to Figure 2.

independent determinations). Thus, BS fibroblasts respond more strongly than normal fibroblasts with growth improvement to vitamin E treatment, which suggests a greater oxidative stress in BS fibroblasts versus their normal counterparts.

A putative oxidative stress as the cause of the BS phenotype is further analysed by determining the cell kinetic pattern of EBL cell lines from two BS patients and from two healthy subjects. Contrary to the results with fibroblasts, only minor quantitative differences between the BS and the normal EBL cells can be seen in Figure 3. Neither a striking arrest of cells in the first G2 phase nor a clear prolongation of the G1 phase of the second cycle occur in EBL cells from BS. On the other hand, a prolongation of the S phase of the second cycle is conspicuous, and the arrest of cells in the second G2 phase is slightly elevated. Table 3 shows the minimal duration of the G1 and S

TABLE III

Minimal durations of the G1 and S phase of the second cell cycle after release from quiescence of EBL cells from BS and healthy subjects cultured at 5% oxygen (in hours), and fraction of cells arrested in the G2 phase of the first and second cell cycle (in % of total cells).

	Minimal durations		Arrest fractions	
	G1 phase	S phase	first G2	second G2
Controls				
Line WF	6.1 ± 0.4	7.6 ± 0.2	2.3 ± 0.2	2.7 ± 0.6
Line HZ	5.8 ± 0.4	6.5 ± 0.3	1.9 ± 0.4	3.6 ± 0.3
BS				
GM 3404	6.1 ± 0.5	9.0 ± 0.8	3.6 ± 0.4	7.4 ± 0.7
BD 1	5.1 ± 0.6	9.7 ± 0.6	3.5 ± 0.3	6.0 ± 1.0

All data are mean and standard deviations as obtained by fitting the cell cycle distributions from a time sequence of samples out of a single kinetic experiment to the modified cell cycle kinetic model as described.[22]

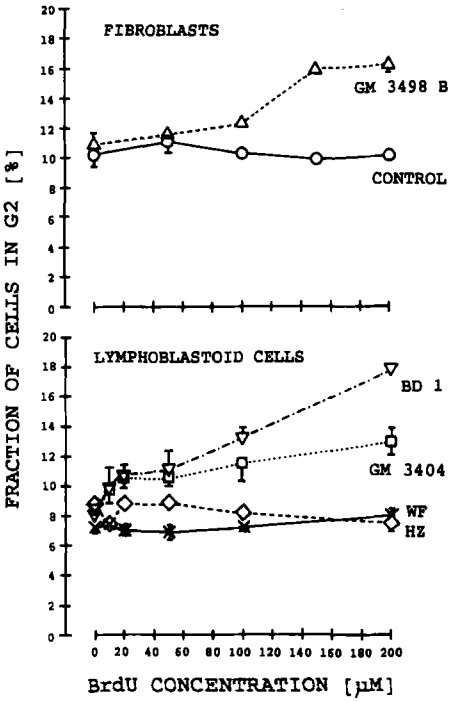

FIGURE 4 BrdU dependency of G2 arrest in fibroblasts and EBL cells. Cells were cultured during 72 hours at 5% oxygen in the continuous presence of a concentration series of BrdU.

phases of the second cell cycle, which indicates that the G1 phase has a normal duration, whereas the S phase is clearly prolonged. The arrest in the first G2 phase is slightly higher in BS cells than in normal cells, but the arrest in the second G2 phase is distinctly elevated in BS cells (Table 3). This pattern of a prolonged S phase in the second cell cycle along with an arrest in G2 is reminiscent of the cell kinetic disturbance induced by 35% oxygen in the presence of BrdU in normal cells.[18] To investigate a possible Brdu dependency of the G2 arrest in BS cells we cultured fibroblasts and EBL cells from BS at 5% oxygen with a concentration series of BrdU. Both EBL cells and fibroblasts from BS show a clear BrdU dependent increase of G2 (Figure 4). This result is in keeping with the BrdU depedency of the levated rate of SCE found in BS cells.[29] Both a BrdU dependent G2 arrest and an increased rate of SCE formation can be induced in normal cells by exposure to paraquat, a generator of superoxide radicals.[19,30]

A deficiency in DNA ligse I has been described in a line of EBL cells from BS, but not in the fibroblast strain GM 1492,[14] which indicates that this enzyme deficiency is not consistently expressed in all cell types of BS patients. Our cell kinetic data also show distinct patterns of perturbed cell cycling in different cell types. These observations are at variance with the hypothesis of a single enzyme deficiency as the prime cause of the cellular BS phenotype. Elevated oxidative stress, on the other hand, can manifest itself differently in different cell types depending upon the distribution of

targets within the cell. For instance, fibroblasts, having a relatively large lipid content, are likely to experience lipid peroxidation in response to oxidative stress, whereas the EBL cells with their small cytoplasm to nuclear volume ratio are more likely to suffer DNA damage. The BrdU dependency of G2 arrest described above links the cell kinetic disturbance of fibroblasts and EBL cells from BS to elevated DNA attack by superoxide radicals.[16,17,19] Superimposed on this cell kinetic disturbance, BS fibroblasts may experience lipid peroxidation, which can be mitigated with antioxidants such as vitamin E (Figure 2). In conclusion, elevated oxidative stress may explain all cytogenetic and biochemical findings reported to date in fibroblasts and EBL cells from patients with Bloom's syndrome.

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