Elevated Superoxide Dismutase in Bloom’s Syndrome: A Genetic Condition of Oxidative Stress

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ABSTRACT

We have found that Bloom's syndrome (BS) cells exhibit elevated levels of superoxide dismutase activity. Since SOD activity has been shown to reflect the intracellular superoxide (O2·−) content, these results indicate that BS cells exhibit oxidative stress which ultimately results in DNA damage. Elevated sister chromatid exchange, the major cytological characteristic of BS, and superoxide dismutase induction were simulated in normal lymphoblastoid cells by treatment with compounds that increase the steady-state concentration of O2·−. The sister chromatid exchange response of a BS lymphoid cell line was modulated through the control of the endogenous O2·− content. We therefore suggest that a major biochemical defect resulting from this genetic disorder is chronic overproduction of the superoxide radical anion. The consequence of high O2·− levels concomitant with induced superoxide dismutase activity is the formation of enormous amounts of H2O2, which can apparently inactivate the enzymes responsible for its elimination. The inefficient removal of peroxide can result in high rates of sister chromatid exchange and chromosomal damage in BS cells and in normal cells treated with oxidation-reduction cycling compounds through the formation of highly reactive intermediary forms of active oxygen.

INTRODUCTION

BS is a rare, autosomal recessive disorder exhibiting numerous clinical features including sensitivity to sunlight, growth retardation, immunological disorders, and a predisposition to cancer at a relatively young age. Cytogenetically, BS is characterized by a high level of spontaneous chromosomal aberrations and a 10-fold increase in SCE (1). BS exhibits one of the clearest examples known of a hereditary disorder that features chromosomal instability and an increased risk of malignant tumor formation. It has been suggested that the chromosomal instability associated with this disease predisposes BS patients to the higher-than-normal incidence of cancer (2). Interestingly, there is no consistent pattern in the occurrence of the cancer type or location. Though several biochemical abnormalities have been proposed (3–12), none can explain the divergence of phenotypes associated with this disorder.

Emerit and Cerutti hypothesized that abnormal oxygen metabolism is taking place in BS and have shown that addition of exogenous SOD can prevent the clastogenic action of BS-conditioned medium while having only a minimal effect on DNA damage. Elevated sister chromatid exchange, the major cytological characteristic of BS, and superoxide dismutase induction were simulated in normal lymphoblastoid cells by treatment with compounds that increase the steady-state concentration of O2·−. The sister chromatid exchange response of a BS lymphoid cell line was modulated through the control of the endogenous O2·− content. We therefore suggest that a major biochemical defect resulting from this genetic disorder is chronic overproduction of the superoxide radical anion. The consequence of high O2·− levels concomitant with induced superoxide dismutase activity is the formation of enormous amounts of H2O2, which can apparently inactivate the enzymes responsible for its elimination. The inefficient removal of peroxide can result in high rates of sister chromatid exchange and chromosomal damage in BS cells and in normal cells treated with oxidation-reduction cycling compounds through the formation of highly reactive intermediary forms of active oxygen.

Received 11/18/87; revised 4/24/89; accepted 6/22/89.

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1 The abbreviations used are: BS, Bloom's syndrome; SCE, sister chromatid exchange; SOD, superoxide dismutase; Mn-SOD, manganese-superoxide dismutase; Cu/Zn-SOD, copper/zinc-superoxide dismutase; Cu-DIPS, copper(II) diisopropyl sulfoxycarbamate; O2·−, superoxide radical; OH·, hydroxyl radical; DDC, diethyldithiocarbamate.

Materials. Paraquat, menadione, diethyldithiocarbamate, cytochrome c (type III), xanthine, xanthine oxidase (Grade I), and 5-bromo-2-deoxyuridine were obtained from Sigma Chemical Co., St. Louis, MO; Hoechst 33258 from Calbiochem, La Jolla, CA; and RPMI-1640 medium and fetal bovine serum from GIBCO, Grand Island, NY. Cu-DIPS was provided by Dr. John Sorenson, University of Arkansas for Medical Sciences, Little Rock, AK. The BS cell lines (GM1492, GM3498, GM3510, and GM3403), control cell lines (GM130, GM131, GM4408, and GM1526), and an ataxia telangiectasia cell line were obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository, Camden, NJ. YBL, a BS fibroblast cell line, was obtained from Dr. Malcolm Paterson, Chalk River Nuclear Laboratory, Ontario, Canada, and a control lymphocyte cell line, BD/1, was obtained from Dr. Tin Han, Roswell Park Memorial Institute, Buffalo, NY. Lymphocytes (JG) were also obtained from a young male BS patient.

Cell Cultures. Lymphoid and normal fibroblast cells were cultured in RPMI-1640 medium and supplemented with 10% fetal bovine serum, 100 μg/ml of penicillin, and 50 μg/ml of streptomycin. BS fibroblasts were supplemented with 20% fetal bovine serum for optimal growth conditions.

Cell Extracts. Cells were collected and washed in 0.9% NaCl solution at 0°C, centrifuged at low speed (1000 rpm), resuspended in 0.9% NaCl solution, and sonicated twice for 10 s at 50 W in a Branson sonifier. These conditions resulted in complete disruption of cells as observed by light microscopy. Triton X-100 was added to the sonicate (0.1%), and the mixture was allowed to stand for 15 min. The sonicate was centrifuged for 30 min at 105,000 × g, and this supernatant was utilized in the SOD assay. Total protein concentration was determined by the method of Lowry et al. (18).

Superoxide Dismutase Assay. SOD activity in cellular extracts was determined by measuring the decrease in the rate of superoxide-dependent reduction of cytochrome c (19). Superoxide was generated by the xanthine/xanthine oxidase reaction, and the enzymatic activity was determined in 0.02 M carbonate at pH 10.0 and 0.1 mM EDTA, 50 μM xanthine, and 6 μM ferricytochrome c in a 1.0-ml total reaction volume.

The rate of cytochrome c reduction was monitored at 25°C using a thermostatted Cary 15 spectrophotometer at 417 nm. One unit of SOD activity is defined as the amount of enzyme required to inhibit the rate of cytochrome c reduction by 50%. Units are expressed as units/mg of protein.
protein of the prepared cell extract. Inhibition of Cu/Zn-SOD by cyanide allows for the differential quantitation of both Cu/Zn-SOD and Mn-SOD. Using the inhibition constant, Cu/Zn-SOD activity was calculated. Mn-SOD activity was obtained by subtracting Cu/Zn-SOD activity from the total uninhibited activity. Statistical analysis was performed by the Student t test.

SOD Activity Staining Assay. Starch gel electrophoresis of cell extracts was carried out in 9.0% gels prepared in sodium barbital buffer (pH 7.5, 12.3 g/liter) for 18 h at 4°C. Equal amounts of cell homogenates (70 μg) were loaded in 0.05 M Tris-HCl buffer at pH 7.5. Gels were stained with 25 mg of nitroblue tetrazolium and 3 mg of phenazine methosulfate in 100 ml of phosphate buffer at pH 7.5 for one-half h in the dark. The gel was then transferred to a light box until an achromatic region localizing the dismutase activity emerged against a dark background (2 h). The presence of SOD prevents the conversion of nitroblue tetrazolium to the blue-black formazan precipitate, allowing the SOD bands to be localized.

Sister Chromatid Exchange. Cells were incubated with bromodeoxyuridine (5 μg/ml) and the compound to be tested for approximately 2 cell cycles (46 h), followed by mitotic arrest with Colcemid (0.15 μg/ml) for 2 h. After harvesting, the cells were treated in hypotonic KCl (0.075 M) for 20 min at 37°C, then fixed, and washed 3 times in methanol:glacial acetic acid (3:1). Slides were prepared at least 24 h prior to staining. Chromosomes for SCE analysis were stained using a modified fluorescence plus Giemsa technique (20) and compared them to normal controls. The results (Table 1) show a 200% increase in total SOD activity in BS fibroblasts and lymphocytes when compared to their respective control cell lines. In fibroblasts, Mn-SOD demonstrated a higher level of inducibility than did Cu/Zn-SOD. Mn-SOD was not detectable in lymphocytes, and therefore all the activity in these cells was attributed to Cu/Zn-SOD. A revertant BS lymphoid cell line (GM4408), shown to have normal SCE levels by the Cell Repository, was also shown to possess normal SOD activity. Another cell line of ataxia telangiectasia origin, GM1526, was also shown to have SOD activity within the normal range. These results were substantiated in fibroblast cell lines by using starch gel electrophoresis to separate the two enzymes, which were then localized by the nitroblue tetrazolium activity staining assay (Fig. 1). Both Cu/Zn-SOD and Mn-SOD activities were elevated in BS fibroblasts compared to control cells. The conditions used in this assay differ from the cytochrome c reduction assay, rendering it more sensitive to Mn-SOD relative to Cu/Zn-SOD. This accounts for the relatively light Cu/Zn-SOD bands observed.

In both normal human fibroblasts and lymphocyte cell lines treated with the O₂•-generating compound paraquat, SOD activities were found to be inducible. A dose-dependent increase in both Cu/Zn-SOD and Mn-SOD activities was demonstrated in the normal human fibroblast cell line GM 3348, with Mn-SOD possessing a greater responsiveness (Fig. 2). At 1.0 mM paraquat, there was a remarkable similarity in the degree of induction of SOD activities in normal fibroblasts compared to the mean SOD activities of BS fibroblasts (Fig. 2). In the control experiments, however, Mn-SOD was not inducible, whereas Cu/Zn-SOD was only weakly inducible. In BS fibroblasts, Mn-SOD was inducible but not Cu/Zn-SOD. CYTOCHROME C REDUCTION

Table 1 Study of superoxide dismutase activities in Bloom's syndrome and controls

<table>
<thead>
<tr>
<th></th>
<th>Fibroblast SOD activities</th>
<th>Lymphocyte SOD activities</th>
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<tbody>
<tr>
<td></td>
<td>Cu/Zn-SOD</td>
<td>Mn-SOD</td>
</tr>
<tr>
<td>Normal</td>
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<td></td>
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<tr>
<td>GM316</td>
<td>125.3</td>
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<td>GM408</td>
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<td>GM3348</td>
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<td>GM2987</td>
<td>142.2</td>
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<td>BS</td>
<td>132 ± 3.5*</td>
<td>22.8 ± 4.9</td>
</tr>
<tr>
<td>GM1492</td>
<td>205.5</td>
<td>63.9</td>
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<tr>
<td>GM3498</td>
<td>232.7</td>
<td>114.7</td>
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<td>GM3510</td>
<td>238.0</td>
<td>56.9</td>
</tr>
<tr>
<td>YBL</td>
<td>186.0</td>
<td>61.6</td>
</tr>
<tr>
<td>BS</td>
<td>218.0 ± 10.2</td>
<td>74 ± 27.1*</td>
</tr>
</tbody>
</table>

* Mean ± SE.
† Denotes a statistically significant difference (P < 0.01) compared to controls.
° ND, not detectable.
treated BS cells and in paraquat-treated control cells concomitant with elevated SOD activity is an apparent discrepancy. Either the induced enzyme is insufficient to dismute the high rates of $O_2^-$ generated or, more likely, the high amounts of hydrogen peroxide ($H_2O_2$) generated by superoxide dismutation are not concurrently removed. To test these possibilities we added the SOD mimetic compound, Cu-DIPS (23), to BS cells and monitored its effect on SCE rates. A dose-dependent increase in SCEs was observed with Cu-DIPS treatment (Fig. 4). Presumably, increasing superoxide dismutation through the addition of Cu-DIPS resulted in still higher SCE rates. Whether other mechanism(s) of Cu-DIPS are responsible for this increase is not presently known. However, a copper(II) control was examined for its effect on SCE, and our results show that Cu(II) at $10^{-8}$ and $10^{-7}$ causes a slight decrease in the SCE level in BS cells.

To further probe into the mechanism of oxygen radical-induced SCEs, we attempted to modulate the SCE response in BS lymphocytes by decreasing the level of oxy-radical formation with the lipophilic antioxidant, $\alpha$-tocopherol, which acts as a chain reaction terminator of lipid peroxidation reactions (24). The results show a modest 10% to 15% decrease in SCE levels in BS lymphocytes treated with $\alpha$-tocopherol (Fig. 4). Using a 3-day pretreatment protocol of cells from $10^{-8}$ to $10^{-3}$ M $\alpha$-tocopherol, the SCE rate dropped 17% to 30%. Therefore, pretreatment of cells doubles the observed decrease in SCEs at a high tocopherol concentration and dramatically lowers the concentration required to observe a measurable decrease in SCE compared to the nonpretreated cells. These observations indicate that lipid hydroperoxide products can contribute to SCE formation either directly or indirectly through the additional formation of $O_2^-$. Additional evidence of oxy-radical mechanisms in SCE formation was obtained through the inhibition of Cu/Zn-SOD in BS cells by DDC. The 5-log range of inhibitor concentration used in the BS lymphocyte cell line yielded a biphasic curve (Fig. 5). The first part of the curve demonstrated a small (20%) but statistically significant decrease in the SCE response of these cells. Inhibition of Cu/Zn-SOD at concentrations above $10^{-7}$ M DDC resulted in a dose-dependent increase in SCEs by decreasing the level of oxy-radical formation.

FIG. 4. SCE frequency per metaphase chromosome of the BS lymphoid cell line CM 3403, treated for 2 cell cycles with Cu-DIPS (●), $\alpha$-tocopherol without pretreatment (○), or $\alpha$-tocopherol with a 3-day preincubation period prior to SCE analysis (△).
overproduction of O$_2^\cdot$. Confirmation of this hypothesis rests primarily on our observation that all five BS cell lines examined were found to possess elevated SOD activity compared to control cell lines (Table 1). Also, we have previously shown that treatment of Don cell line fibroblasts with paraquat, a compound known to result in increased levels of endogenous O$_2^\cdot$, causes a dose-dependent increase in chromosomal aberrations, SCEs, and SOD activity (14). In the present study, this correlation was extended to normal human fibroblasts and lymphoid cell lines. Treatment of control cells with paraquat, menadione, and DDC resulted in dose-dependent increases in SCEs and SOD activity.

Finally, we have modulated the SCE response directly in a BS lymphoid cell line through the control of the dismutase activity and therefore of the intracellular O$_2^\cdot$ content (Figs. 4 and 5). Addition of the SOD mimetic compound, Cu-DIPS, to a BS cell line resulted in a dose-dependent increase in SCE levels. Treatment of cells with the SOD inhibitor, DDC, resulted in a biphasic curve, first decreasing and then increasing the SCE level. The initial decrease in SCEs was presumably due to the diminished H$_2$O$_2$ formation which resulted from the inhibition of Cu/Zn-SOD. Above $10^{-7}$ M DDC, O$_2^\cdot$ can accumulate until it reaches a sufficient concentration for its non-enzymatic breakdown to occur (25) and leading to other forms of reactive oxygen. These data suggest that H$_2$O$_2$ is the more proximal species to SCE formation; however, the rates of O$_2^\cdot$ formation and dismutase contribute to SCE rates, since they control a major pathway for H$_2$O$_2$ generation. Furthermore, treatment of a BS lymphoid cell line with α-tocopherol resulted in a decrease of the SCE level. Though α-tocopherol indirectly affects oxy-radical concentrations through the inhibition of lipid peroxidation, its depletion in animals is linked to an increase in SOD activity (26).

Given the elevated level of O$_2^\cdot$ production concomitant with the high SOD activity observed in BS cell lines, abnormally high rates of H$_2$O$_2$ formation would be expected. However, a recent analysis of one BS cell line reported to have a low catalase activity (27). Whether this decreased activity is a general phenomenon in BS is not presently known; however, fluxes of O$_2^\cdot$ have been shown to inhibit both catalase (28) and glutathione peroxidase (29). When the H$_2$O$_2$ concentration becomes sufficiently high, SOD can also be inactivated (30). A relationship analogous to that presently described in BS between O$_2^\cdot$ levels and antioxidant enzymatic activities is shown to exist in guinea pigs exposed to hyperoxic conditions (31). Neutrophils and macrophages derived from these hyperoxic animals, as expected, have elevated O$_2^\cdot$ concentrations and demonstrate an induction of SOD activity simultaneously with a decrease in catalase and glutathione peroxidase activities. Similarly, it has been shown that cells transfected with plasmids containing the Cu/Zn-SOD gene and shown to have elevated dismutase activity demonstrated enhanced resistance to paraquat, while at the same time showing elevated paraquat-induced lipid peroxidation levels compared to the parental cell line (32). Without a corresponding induction of catalase and peroxidases, these cells would be unable to maintain a normal endogenous H$_2$O$_2$ concentration. Under these conditions, the excessive H$_2$O$_2$ results in the formation of the highly reactive hydroxyl radical (OH$\cdot$) by Fenton-type reactions. Alternatively, high O$_2^\cdot$ levels can directly inactivate susceptible enzymes. Thus, the enzymes acting on O$_2^\cdot$ and H$_2$O$_2$ are uniquely complimentary to each other. When this balance is upset, the relatively unreactive O$_2^\cdot$ is converted to the highly reactive OH$\cdot$, which is capable of nonspecific damage to cellular components (33). One can speculate that this pervasive abnormality can explain the plethora of disturbances described to occur in BS cells. These disturbances include an elevated spontaneous mutation rate (34, 35), delayed DNA fork progression rates (3-5), altered regulation of uracil-DNA glycosylase (6-8) and O$^\cdot$-methyl guanine methyltransferase repair functions (9), and reduced topoisomerase II activity (10). Recently, several reports have confirmed the presence of a defective DNA ligase I activity in three of four BS cell lines examined (11, 12). While it is unlikely that multiple genetic abnormalities exist in BS, it is conceivable that these disturbances are linked under the umbrella of oxidative stress. Chronic overproduction of O$_2^\cdot$ can lead to the peroxidation of lipids, depletion of glutathione, formation of mixed disulfides (15), and oxidative damage to cellular macromolecules (33), including the inactivation of the enzymes responsible for the maintenance of a stable cellular oxidation reduction state (28-30). Analysis of antioxidant enzyme-specific activities in BS is necessary to further verify the existence of a prooxidant state, and simulation of this condition in normal cells can determine whether nuclear enzyme functions can be altered. Given the diverse potential for cellular toxicity, it is not surprising that fluxes of O$_2^\cdot$ have been shown to be both clastogenic (14, 36) and mutagenic at specific gene loci (36-38). Conditions of oxidative stress, though only beginning to be understood, can therefore have wide-ranging consequences, ultimately giving rise to a markedly enhanced susceptibility for the development of neoplasia (39).

ACKNOWLEDGMENTS

The authors wish to thank Dr. Carl Barrett for his careful reading of this manuscript.

REFERENCES


Fig. 5. SCE frequency per metaphase of the BS lymphoid cell line, GM 3403, treated with the Cu/Zn-SOD inhibitor DDC for two cell cycles. All points analyzed were found to differ from the control in a statistically significant manner at $P \leq 0.001$ by the Student t test (*)


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