Abnormal Response of Xeroderma Pigmentosum Cells to Bleomycin1

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ABSTRACT

The repair of bleomycin-damaged DNA was examined in human fibroblasts isolated from patients having the disease xeroderma pigmentosum (XP). In normal fibroblasts, the appearance of low-molecular-weight DNA was observed in the presence of increasing amounts of the drug. The studies in XP fibroblasts produced results which differed from those obtained in normal cells in two ways. (a) Prelabeled XP cells from most complementation groups contained more low-molecular-weight DNA than observed in the other human fibroblasts examined. (b) When XP cells were exposed to low doses of bleomycin, the low-molecular-weight DNA disappeared, suggesting induction of a repair process. If the XP cells were exposed to bleomycin in the presence of hydroxyurea and 1-beta-D-arabinofuranosylcytosine, the disappearance of low-molecular-weight DNA was not observed; instead, a normal dose response to the drug was observed. Our results suggest that XP cells show an "induced" repair response following bleomycin treatment and that blocking DNA chain elongation uncovers normal incisions in bleomycin-treated DNA.

INTRODUCTION

XP4 is an autosomal recessive disease of humans, characterized biochemically as a DNA repair-deficient disease and clinically by enhanced sensitivity of the skin to sunlight and subsequent appearance of carcinomas in exposed skin areas. Cells isolated from patients with the disease show defective repair of UV light-induced damage to DNA (8). There are 2 classes of XP patients according to their response to UV damage. Patients whose cells are defective in excision repair of UV light-induced damage to DNA are classified as excision deficient. Patients whose cells show abnormal postreplication repair but possess the normal excision repair functions are called XP variants (3, 16). Those patients whose response to UV damage is defective in excision repair are not a homogeneous group. Cell strains isolated from these patients vary in the extent of the loss of the excision repair response to UV damage. They have been assigned to complementation groups designated A to H, based upon restoration of UV responsiveness following cell fusion (15). There have been reports of various repair deficiencies in these complementation groups; e.g., some show excision defects, and others do not. The explanation for these results may be a repair pathway with many common steps, capable of responding to various insults.

We have used the drug BLM, a glycopeptide antibiotic currently used in cancer chemotherapeutic treatment of lymphomas (25), to study the repair capacity of XP cells. In whole cell experiments analyzed by alkaline sucrose gradient centrifugation, we have examined the ability of untransformed XP cells to repair the DNA lesions produced by BLM treatment. BLM produces single- and double-stranded breaks (9, 17, 18) as well as alkaline-sensitive (apurinic) sites (10, 23) in isolated DNA. Studies in bacteria have shown that the repair of BLM damage appears to involve normal excision repair pathway functions. However, not all the "early" functions of this pathway are needed. For example, the uvrA gene function of Escherichia coli is not required (24). It has not been shown that the incision at the site of BLM damage is enzymatic. An efficient repair system for BLM damage exists in human cells, involving incision of treated DNA, repair of damaged sites, and ligation of the sites into parental DNA (12, 13).

In the results reported here, we observe that incision of DNA after BLM treatment seems deficient in some XP cell lines and that stable low-molecular-weight DNA present in some XP complementation groups disappears after BLM treatment. If the BLM treatment is carried out in the presence of inhibitors of DNA synthesis, HU and ara-C, a normal dose response to the drug is observed. Our interpretation of these data is that a deficit in the normal DNA repair response to BLM damage exists in XP cells and that a repair function not observed in the response of normal cells is observed in XP cells.

MATERIALS AND METHODS

Cell Cultures. Fibroblast strains used in these experiments are catalogued in Table 1. Cells were grown in DMEM in 10% CO2 in the absence of antibiotics. The growth medium was supplemented with 10% fetal calf serum obtained from Grand Island Biological Co., except in the case of XP cell strains which were grown in medium supplemented with 20% fetal calf serum. All cell culture was carried out in Falcon plastic flasks and plates. Cell strains were in passages 4 to 10 and were tested monthly for the presence of Mycoplasma by the fluorescent DNA stain technique developed by DeGuidice and Hopps (5). From 48 to 72 hr prior to experimental treatment, confluent monolayers were treated with trypsin, and 60-mm culture plates were seeded with 1.5 x 106 cells. Plates were approaching confluence or were confluent at the time of the experiments. All incubations for DNA labeling, drug treatment, and repair were carried out in the dark.

DNA Labeling. DNA of the cells was labeled with [3H]dThd (25 μCi/ml; 15 Ci/mmol) in DMEM for 1 hr. [3H]dThd was obtained from Schwarz-Mann. After the 1-hr labeling period, the radioactive medium was removed, and the cells were washed with warm DMEM. A 1-hr incubation in 10-5 cold dThd followed the labeling period to chase the label into high-molecular-weight DNA.

BLM Treatment. Bleomycin (Blenoxane) was a gift from Bristol Laboratories. Stock solutions of BLM in PBS were brought to a final concentration of 5 mg/ml (10 units/ml). The drug treatment period was 30 min when dose effects of 5 to 500 μg/ml were examined. When the effect of BLM with time was examined, the dose was fixed at 50 μg/ml, and the incubation time varied from 0 to 30 min. In incision experiments,
ice-cold stopping buffer (PBS-0.02 M sodium azide-0.01 M sodium pyrophosphate) was added at the end of the drug treatment period. In repair experiments, BLM was removed at the end of the incubation period, the cells were washed with warm DMEM, and incubation in fresh medium followed for the time period indicated. Cells were harvested by scraping with a Teflon policeman, and pellets were collected with a Beckman microfuge. Cell pellets were resuspended in 200 µl of lysing buffer (0.5 M NaCl-1% Sarkosyl-4 mM EDTA-0.01 M Na2HPO4-10 mM H2O-0.5 M NaOH) and lysed overnight at 4°C.

Alkaline Sucrose Gradient Analysis. Five to 20% alkaline sucrose gradients (0.3 M NaOH-0.7 M NaCl-0.1 mM EDTA-50 mM Tris-HCl, pH 8.0) were poured on a 70% sucrose cushion. Cell lysates were layered on the gradients and incubated overnight. The gradients were centrifuged in an SW50.1 rotor for 9 hr at 26,000 rpm at 20°C. Fractions were collected from the bottom of the gradients by pump and precipitated with 10% trichloroacetic acid at 4°C. Precipitates were collected on Whatman GF/C filters, washed with 0.01 M HCl, and dried. Radioactivity was counted in a liquid scintillation counter.

Inhibition of Repair of BLM-treated DNA. HU was prepared in PBS to a final concentration of 1 mM. A 10 mM stock solution of ara-C was prepared in PBS. Both HU and ara-C were obtained from Sigma Chemical Co. In experiments using the inhibitors, cells were seeded, cultured, and prelabeled as described. HU and ara-C were added, and cells were incubated for 30 min without BLM (0 control) or added with BLM for the 30-min incubation. Final concentrations in the media were 0.1 mM ara-C and 10 mM HU. Cells were harvested, and gradient analysis was conducted as described.

RESULTS

Abnormal Incision in XP Cells. During BLM treatment, both incision and reformation of damaged DNA should occur. Incised DNA molecules not yet repaired at the time of analysis would appear as low-molecular-weight DNA on alkaline sucrose gradients. The decrease in molecular weight of DNA could be due to enzymatic incision at damaged sites (by the drug itself or by repair enzymes) or hydrolysis at alkaline-sensitive sites produced by BLM. As we have reported (12, 13), a fall in molecular weight of DNA, directly related to the dose of BLM, was observed in fibroblasts isolated from patients diagnosed as having CS, BS, Fanconi’s anemia, and ataxia telangiectasia, as well as in normal cell strains. Chart 1 illustrates a typical dose-dependent fall in molecular weight of DNA in BLM-treated normal cells (C. C.). In experiments reported here, we used BLM concentrations in a range causing DNA damage in vitro and in cells (18, 24). We extended the dose range to completely characterize the cellular response.

Chart 2A presents a summary of cell lines showing a normal response to BLM. Prelabeled, untreated cells contain very little low-molecular-weight DNA (<16S) under our experimental conditions (<5% of total cpm of [3H]dThd) (13). We used 16S as a definition of low-molecular-weight DNA because this size range has been used by Anderson and DePamphilis (1) as a convenient definition of low-molecular-weight DNA. The amount of DNA <16S increases with increasing BLM dose. Untreated cells from patients with CS contain more DNA <16S than do the other cells. CS cells, however, do respond to BLM in the expected dose-dependent manner observed in the other strains shown in Chart 2A.

XP cells produced abnormal results. (a) They contained 3 to 4 times as much DNA <16S in the absence of drug treatment as did other cells examined (Chart 2, B and C) (14). (b) When low doses of BLM were added to these cells, the amount of DNA...
Chart 2. Incision of BLM-treated DNA by human fibroblasts. Strains utilized are listed in Table 1. Cells were treated as described in "Materials and Methods." The radioactive dThd in the top half of the gradients (≤16S) was summed and shown as a percentage of total cpm (tcpm). The method of data analysis of Anderson and DePamphilis (1) utilized for maturation of Okazaki fragments is used. AT, ataxia telangiectasia; Var., variant strain; Hetero., heterozygote strain(s).

≤16S decreased in some strains instead of increasing in the manner observed in other cells. This is an apparent lack of incision. Chart 2C shows that the XP-C and XP-B heterozygote strains appeared to be unresponsive to low doses of the drug. There was a difference of less than 5% of total cpm of [3H]dThd in "small" DNA at doses of BLM from 0 to 50 µg BLM/ml in both lines. Even the high dose, 500 µg/ml, did not produce an increase in low-molecular-weight DNA in strains from XP-A and XP-C patients. This indicates that BLM was not acting on DNA after lysis of the cells; in that case, we would expect all cells to show the same results at a given BLM dose.

The variant strain contained less low-molecular-weight DNA in the absence of BLM than any other XP cell strain examined. However, this strain responded to BLM at low doses (5 and 50 µg/ml) in the same manner as the other XP strains in Chart 2B; i.e., there was a disappearance of low-molecular-weight DNA in these gradients. At the high dose (500 µg), the variant showed a fall in DNA molecular weight comparable to the responses to the high dose seen in normal cells.

Chart 3. Dose response of human fibroblasts to BLM. cpm were summed for DNA ≤16S, and the ratio of this small DNA after BLM treatment to small DNA before BLM was determined for each strain.

Chart 4. Effect of time on BLM treatment in human fibroblasts. cpm were summed for DNA ≤16S, and the ratio of material ≤16S after BLM treatment to ≤16S DNA before BLM treatment was determined for each strain. Incub., incubation; hetero, heterozygote strain.

Normalizing the data by determining the ratio of low-molecular-weight DNA in BLM-treated cells to that in untreated cells, with increasing dose of BLM, allows direct comparisons (Chart 3). This ratio was obtained by dividing the summed percentage of the total cpm of [3H]dThd in DNA ≤16S in BLM-treated cells by the summed percentage of cpm in DNA ≤16S in untreated cells of the same strain. There is an apparent lack of incision in several groups. Data from all cell strains are not shown; if incision ratios for XP-B (heterozygote) and XP-D were shown, they would fall in the area shown by XP-C and XP-A (under a ratio of 2 even at 500 µg/ml). Group XP-E is slightly higher (2.8). The variant has a ratio of under one until the high dose, where it rises to the
normal ratio (around 7). Thus, the XP cell strains examined for Complementation Groups A to E show essentially the same apparent lack of incision after BLM treatment. The XP variant shows a fall in molecular weight of DNA only at the highest dose of BLM. Only the BS strain and the XP-G cells, the ratios of which went much higher, fell above the distribution shown for normal cells and CS.

A puzzling aspect of the apparent defect in the incision process on BLM-damaged DNA in XP cells is that BLM itself can cause incisions on isolated DNA (10, 23). Cells were collected and resuspended in buffer not containing BLM prior to lysis, so BLM should not be causing cleavage of liberated DNA after lysis. Since incisions at the high dose are not observed (in XP-A), the data appear to show that the incisions observed are enzymatic and that XP-A cells are defective in the incision step.

Further examination of this apparent lack of incision at BLM-damaged sites in XP cells was carried out by looking at the response of cells to a fixed dose of the drug (50 µg/ml) with increasing time of incubation. Chart 4 shows the ratios of the amount of low-molecular-weight DNA in cells treated with BLM for 10, 20, or 30 min to the amount of low-molecular-weight DNA before drug treatment (0 time). Again, there is an apparent lack of incised sites in the DNA of BLM-treated XP-A cells. XP-A, XP-D, and XP-E cells have ratios under one at all time points, due to decreased low-molecular-weight DNA in the presence of BLM. The XP-B heterozygote strain shows a ratio of around one at all time points, which would appear to indicate a defect in incision of BLM-treated DNA. In contrast, the normal cells (C. C.) show an increasing ratio of small DNA with time of drug incubation, as was observed with increasing dose (Chart 3).

The results of our incision studies might be summarized as showing an apparent lack of incision after BLM treatment in some cell lines and a paradoxical decrease in the amount of low-molecular-weight DNA present in the cells with low doses of BLM. In evaluating these responses, it should be noted that most of the XP lines contain increased amounts of low-molecular-weight DNA with no treatment (14).

Reformation of BLM-damaged DNA. As has been discussed, BLM did not produce the expected fall in molecular weight of the DNA in some of the XP cell strains examined in these studies, even at the highest doses. However, as Chart 2B illustrates, high-dose (500 µg/ml) treatment of XP-D, XP-E, and the variant produced a response which falls within the normal range.

Therefore, we studied the ability of these XP cells to convert the low-molecular-weight DNA (produced by high-dose BLM treatment) to high molecular weight. These experiments included removal of BLM (500 µg/ml) after 30 min of exposure and continued incubation in the absence of the drug. Chart 5 shows alkaline sucrose gradient analysis of an ataxia telangiectasia strain (GM1588) and XP-E (GM2415). Chart 5, A and B (GM1588), shows the return of damaged DNA (A) to high molec-
Chart 7. Effect of inhibitors of DNA synthesis on incision of BLM-treated DNA. Radioactivity was summed and presented as described in Chart 2. Experimental conditions are described in "Materials and Methods." These experiments were performed with normal strain M. K. (control), XP-A strain GM2994, XP-D strain GM434, and XP-E strain GM2415.

The amount of DNA ≤16S in BLM-treated normal cells was almost the same in the presence or absence of the 2 inhibitors. Only at the high dose did inhibition by HU and ara-C result in more low-molecular-weight DNA, which showed a normal response to BLM at high dose, possess the repair enzymes required to repair the damaged DNA molecules but perhaps lack optimal levels, since the extent of repair is not as great as is seen in cells which respond normally.

**Incidences in the Presence of HU and ara-C.** If the disappearance of low-molecular-weight DNA seen in untreated XP cells (Chart 2) following BLM exposure was the result of a repair process activated by BLM, HU and ara-C which are inhibitors of DNA synthesis should affect the change in amount of DNA ≤16S observed in XP cells. A review of the use of these inhibitors in DNA repair studies was published by Downes et al. (6). The BLM treatment was performed in the presence of HU and ara-C, as described by Collins et al. (4) and Dunn and Regan (7). The results of our experiments are shown in Chart 7, which shows results obtained in normal cells. The amount of DNA ≤16S in BLM-treated normal cells was almost the same in the presence or absence of the 2 inhibitors. Only at the high dose did inhibition by HU and ara-C result in more low-molecular-weight DNA,
probably due to inhibition of repair synthesis. However, the results obtained with XP-A, XP-D, and XP-E cells showed a marked effect in BLM-treated cells in the presence of HU and ara-C. In cells from all 3 XP complementation groups, the disappearance of DNA ≤16S was blocked, and the normal dose response to the drug was obtained; i.e., there was an increase in DNA ≤16S. If HU was used alone (Chart 8), the same overall results were obtained; i.e., treatment of normal fibroblasts with BLM after pretreatment with HU produced the same response as did treatment with BLM in the absence of HU (data not shown). However, if XP-E cells (GM2415) were pretreated with HU, the decrease in DNA ≤16S seen after low doses of BLM was not observed. Thus, an apparent normal incision response to BLM can be obtained in XP cells if gap-filling processes are inhibited. The results obtained with HU alone can rule out artificial production of low-molecular-weight DNA by ara-C as a mechanism for the reversal in the response of XP cells to BLM. The mechanism of disappearance of DNA ≤16S in BLM-treated XP cells must involve repair and reformation into high-molecular-weight DNA.

**DISCUSSION**

The following conclusions may be drawn from these data and those reported earlier (12, 13). (a) An efficient DNA repair pathway for BLM damage exists in both normal human fibroblasts and fibroblasts taken from patients with some of the putative DNA repair diseases. Our data show that, in these cells, incision of BLM-treated DNA occurs, and the gap-filling processes required to return the damaged DNA to high molecular weight are present. (b) This repair process is altered in XP cells, such that there seems to be unresponsiveness to BLM. Since BLM is capable of stoichiometric fragmentation at high doses, one would expect to overcome the system, however unresponsive, and cause fragmentation of DNA. This was not observed, notably in XP-A cells. (c) The disappearance of the low-molecular-weight DNA after BLM treatment observed in XP cells is the result of a gap-filling process, as evidenced by Charts 7 and 8. (d) The paradoxical disappearance of the low-molecular-weight DNA observed in XP cells suggests induction of repair. The loss of the normal response to BLM damage (due to the XP repair defect) may allow one to observe a second DNA repair pathway not detected in normal cells. This repair pathway would be inhibited with the use of inhibitors of DNA synthesis, HU and ara-C, allowing accumulation of DNA ≤16S. The presence of low-molecular-weight DNA in XP cells, coupled with the addition of a DNA-damaging agent (BLM), may cause induction of this second repair process.

A clear picture of the repair pathways in mammalian cells in general remains to be developed. However, our results indicate that, even under unchallenged conditions, the DNA of XP cells carries single-strand breaks or alkaline-sensitive sites greater in number than in other cells examined, by at least a factor of 2. Perhaps a threshold is crossed when even more damage is produced by BLM, and this activates a repair system, such as the SOS repair systems observed in bacteria (2, 28, 27). Observations which could be considered as evidence for SOS-like repair systems in mammalian cells have been reported (11, 22), and enhanced mutagenesis (UV induced) and induced viral reactivation (20, 21) have been observed in XP cells.

Our results suggest that a repair system is activated in the presence of BLM in XP cells but is not observed in the normal cellular response to BLM. Whether or not this is an SOS-like inducible repair process remains to be elucidated.

**REFERENCES**

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