Differential Inhibition of the Rejoining of X-ray-induced DNA Strand Breaks in Normal and Transformed Human Fibroblasts Treated with 1,3-Bis(2-chloroethyl)-1-nitrosourea in Vitro

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ABSTRACT

The effects of 1,3-bis(2-chloroethyl)-1-nitrosourea on the rejoining of X-ray-induced DNA strand breaks were examined in normal human fibroblasts (WI-38) and a simian virus 40-transformed derivative (VA-13) with the use of alkaline sucrose sedimentation. 1,3-Bis(2-chloroethyl)-1-nitrosourea was capable of partially inhibiting repair of X-ray-produced DNA strand breaks in both cell types when the drug was added to the culture medium immediately after X-irradiation. However, when 1,3-bis(2-chloroethyl)-1-nitrosourea exposure preceded X-ray by 1 hr, DNA repair was inhibited to a much greater extent than it was when 1,3-bis(2-chloroethyl)-1-nitrosourea followed X-ray. The inhibition of DNA repair by 1,3-bis(2-chloroethyl)-1-nitrosourea appeared to be complete in the transformed VA-13 cells, while only partial inhibition of repair was observed in the normal WI-38 cells.

INTRODUCTION

Although the chloroethylnitrosoureas have shown important antitumor activity against a variety of animal neoplasms (12), their activities against human tumors have been less than expected based on animal data (2). In general, the chloroethylnitrosoureas decompose under physiological conditions to yield an alkylating moiety capable of interacting with nucleic acids and proteins (16) and a carbamoylating moiety, which interacts primarily with proteins (3, 13). The alkylating activity of various chloroethylnitrosoureas is capable of cross-linking DNA in vitro (10). BCNU3 is capable of cross-linking the DNA of cultured L1210 cells (8) and the DNA of normal and SV40-transformed human cells grown in vitro (7). In addition to DNA cross-linking, BCNU produces DNA strand breaks or alkali-labile sites in the DNA of cultured human cells (7) and L1210 cells (8).

Since chlorozotocin lacks carbamoylating activity (11), yet is cytotoxic and has antitumor activity, the alkylating activity of the chloroethylnitrosoureas may be responsible for their antitumor effects. However, interesting observations have been reported that are attributable to the carbamoylating activity of nitrosoureas. Among these are reports that show that BCNU is capable of inhibiting DNA polymerase II from rat liver cells (1) and human leukemia cells (4) in an in vitro DNA synthesis assay. Recently, Kann et al. (9) have reported that BCNU and 1 of its breakdown products, chloroethylisocyanate, inhibited the rejoining of X-ray-induced DNA strand breaks when BCNU was added to L1210 cultures immediately following X-irradiation. This inhibition was attributed to the interaction of these drugs with enzymes involved in DNA ligation.

In a recent report (7), we showed that SV40-transformed human fibroblasts (VA-13) were more sensitive to BCNU treatment than were their normal counterparts (WI-38). VA-13 cultures declined in cell number following BCNU exposure with little regrowth, whereas WI-38 cultures were relatively unaffected by BCNU exposure. In that study it was also noted that BCNU produced DNA strand breaks that were gradually repaired following drug removal. However, if the medium containing BCNU and/or its breakdown products was not removed from cultures, no repair of the DNA breaks was observed. Since Kann et al. (9) had previously shown that BCNU added to L1210 cells immediately following X-irradiation partially inhibited DNA strand rejoining, we wondered if BCNU pretreatment would be more effective at inhibiting X-ray repair. We also wanted to determine whether the transformed VA-13 cells would respond similarly to the normal WI-38 cells when exposed to BCNU plus X-ray.

In this report we present evidence that BCNU produces a modest inhibition of X-ray repair when added to WI-38 and VA-13 cultures following X-irradiation. However, if BCNU treatment precedes X-irradiation, the inhibition of X-ray repair is more complete. Furthermore, the inhibition of X-ray repair appears to be greater in the transformed VA-13 cells than it is in the normal WI-38 cells.

MATERIALS AND METHODS

Cell Culture. Normal human embryonic lung fibroblasts (WI-38; passages 16 to 32) and SV40-transformed derivatives (VA-13) were serially subcultured once a week at a seeding density of 1.5 to 2.0 × 10⁶ cells in 75-sq cm plastic flasks (Falcon Plastics Co., Oxnard, Calif.). The cells were grown in Eagle's basal medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% fetal calf serum (Flow Laboratories, Rockville, Md.), 1 mEq L-glutamine, and 50 μg of gentamicin (Schering Corp., Kenilworth, N. J.) per ml. The medium contained NaHCO₃, 1.0 g/liter, and the pH of the medium was controlled by filling the flasks with 5% CO₂/95% air before closing. For experiments, 25-sq cm plastic flasks (Falcon Plastics Co.) were seeded with 2.5 × 10⁶ WI-38 or VA-13 cells. The cultures were incubated for 24 hr prior to BCNU exposure. Stock

1 Presented in part at the 68th Annual Meeting of the American Association for Cancer Research (6).
2 To whom requests for reprints should be addressed.
3 The abbreviations used are: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea (NSC 409962); MeCCNU, 1-(2-chloroethyl)-3-(4-methyl-cyclohexyl)-1-nitrosourea (NSC 54441).

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cultures used in these experiments were found to be free of *Mycoplasma* contamination as determined by Flow Laboratories.

**Radioactive Labeling of DNA.** WI-38 and VA-13 cultures in 25-sq cm flasks were labeled with either [2-¹⁴C]thymidine, 0.02 μCi/ml (51.3 mCi/mmol; New England Nuclear, Boston, Mass.) or [methyl-³H]thymidine, 0.05 μCi/ml (50.8 Ci/mmol; New England Nuclear) for 3 to 4 days. The radioactive medium was replaced after 36 to 48 hr of incubation and replaced again with fresh nonradioactive medium several hr before BCNU treatment. The final medium change was used to avoid labeled low-molecular-weight DNA in the succrose gradients.

**BCNU Treatment of Cell Cultures.** 1,3-Bis(2-chloroethyl)-1-nitrosourea (NSC 409962) was obtained from the Drug Development Branch, National Cancer Institute, NIH, Bethesda, Md. BCNU stock solutions were prepared in 70% ethanol 5 to 10 min before treatment of cell cultures. Ten or 20 μl of stock solutions were added to flasks for treatment. Control flasks receiving 10 or 20 μl of 70% ethanol showed no effect on growth or DNA integrity when compared to untreated controls. The same lot of serum was used for all experiments, and fresh medium was applied 30 min before drug addition for consistent pH and serum conditions.

**X-irradiation of Cell Cultures.** Cell cultures were maintained at 37° prior to irradiation. The T-25 flasks were not chilled to avoid cold shock of cultures treated with drug and X-ray. Unless otherwise noted, the flasks were irradiated at room temperature at a rate of 600 R/min. X-ray was delivered by 2 vertically opposed Westinghouse Quadra-conex X-ray tubes or 2 vertically opposed Phillips RT-250 X-ray tubes. Both units operated at 200 kV, 15 ma, and were equipped with 0.25-mm copper and 0.55-mm aluminum filters. Following X-irradiation, flasks were either transferred to a portable 37° constant temperature box or their medium was replaced with ice-cold medium and the flasks were maintained on ice. For cell harvest these flasks were warmed to 37° for 3.0 min during trypsinization, as described below. In some control experiments flasks were chilled on ice before X-irradiation, irradiated in ice, and maintained on ice until harvest. These cells were harvested by treating the cultures with ice-cold trypsin for 15 min at 0°.

**Preparation of Cell Lysates.** In preparation for alkaline sedimentation, the cultures were rinsed with 10 ml of Hanks' balanced saline solution containing 0.02% disodium EDTA (pH 7.2), treated with 1.0 ml of 0.05% trypsin/0.02% disodium EDTA at 37° for 3 min, and suspended by vigorous pipetting in 4 ml of cold Eagle's basal medium containing serum. The cell number was determined using a Coulter counter, Model B (Coulter Electronics, Hialeah, Fla.), and the suspension was maintained in ice until lysis. A volume of medium containing 10⁶ cells was centrifuged at 1000 x g for 10 min at 0°; the medium was decanted, and the cell pellet was resuspended in 0.4 ml of ice-cold phosphate-buffered saline (0.15 M NaCl/0.014 M KH₂PO₄/0.086 M K₂HPO₄). Cell lysates were prepared by adding the following components (5, 7): 0.1 ml of ¹⁴C-labeled cells; 0.1 ml of ⁵³H-labeled cells; 50 μl of a solution containing 10 mg of proteinase-K (E. M. Laboratories, Elsford, N. Y.) per ml, 2% sodium dodecyl sulfate, 5 mM CaCl₂, 1 mM disodium EDTA, and 0.01 M Tris (pH 8.0); and 0.2 ml of 2% Sarkosyl (Geigy-Ciba, Ardsley, N. Y.). The tubes were gently rocked to mix the components. The lysate was incubated at 50° for 1 hr and gently layered onto a 4 to 20% linear sucrose gradient with a wide bore 1-ml pipet.

**Alkaline Sucrose Sedimentation.** Alkaline sucrose gradients were prepared from stock solutions containing: (a) 4% sucrose, 0.9 M NaCl, 0.05 M Na₂-EDTA, and 0.2 M NaOH and (b) 20% sucrose, 0.9 M NaCl, 0.05 M Na₂-EDTA, and 0.3 M NaOH. The gradients in cellulose nitrate tubes were centrifuged in a Beckman SW-40 Ti rotor at 9500 rpm, 20°, for 16.5 hr, ω²t = 6.177 x 10⁶ rad²/sec. The gradients were fractionated from the top by displacement with a solution of 60% sucrose/1 M NaCl. Fractions (0.5 ml) were collected in scintillation vials and mixed with 3 ml of distilled water and 10 ml of Aquasol (New England Nuclear) containing 0.7% glacial acetic acid. DNA sedimenting to the bottom of the tube was recovered by cutting off the tube bottom and washing it with 1 ml of 0.1 M NaOH/0.3% Sarkosyl; this sample was plotted as the last fraction. The samples were counted in a Packard Tri-Carb 2450B liquid scintillation counter (Packard Instruments, Chicago, Ill.). Weight average (M₊) and number average (Mₙ) molecular weights were computed as described by Dingman and Kakunaga (5). Induced break frequencies were computed as previously described (7).

## RESULTS

**Effects of BCNU or X-ray on the Growth of WI-38 and VA-13.** In a previous study (7), we showed that transformed VA-13 cultures were more sensitive to BCNU treatment than were their normal WI-38 counterparts. Chart 1 shows that, when these cultures are exposed to 50 or 100 μM BCNU (the treatments used in the present study) for 2 hr, there is a reduction in cell number and a delayed recovery in the VA-13 cultures, but less of an effect on WI-38 cultures. When assayed for colony-forming ability (Chart 2, right), VA-13 cells are more sensitive to BCNU than are the WI-38 cells. However, when assayed following X-irradiation (Chart 2, left) both cell types show similar dose-dependent reductions in colony-forming ability. These results demonstrate that BCNU is selectively toxic to VA-13, whereas X-irradiation produces no differential in cell kill.

**Analysis of DNA Strand Rejoining following X-irradiation of WI-38 and VA-13 Cultures.** To determine whether X-ray repair capabilities were similar in WI-38 and VA-13, we analyzed the DNA strand breaks present following 4000 R of X-ray and after 1 hr of incubation at 37° following 4000 R. Chart 3 shows the alkaline sucrose sedimentation patterns of WI-38 and VA-13 cells following 4000 R of X-ray (B and E) and following 4000 R and 1 hr of repair at 37° (C and F). These cells were irradiated at room temperature and also were warmed to 37° during harvest (see “Materials and Methods”) so that some DNA strand rejoining had occurred. X-ray produces similar numbers of DNA strand breaks in both cell types. Following 1 hr of incubation at 37°, DNA strand breaks have been repaired similarly in both cell types.

**Effect of BCNU Treatment on the Repair of X-ray-induced DNA Damage in WI-38 and VA-13.** The effect of BCNU treatment on the ability to repair X-ray-induced DNA
strand breaks was studied with respect to timing of the BCNU exposure relative to X-irradiation. In these experiments cultures were incubated at 37° for 1 hr following 4000 R of X-irradiation to determine the effect of the BCNU treatment on the X-ray repair. Subsequent sedimentation patterns should be compared to Chart 3 (C and F) for the typical 1-hr repair sedimentation patterns.

The effect of 100 μM BCNU on X-ray repair in WI-38 and VA-13 was studied in the following experiments. BCNU produces DNA damage in the form of DNA strand breaks and/or alkali-sensitive lesions (7). Strand breaks resulting from exposure to 100 μM BCNU are shown in Chart 4, A and F (2-hr exposure) and C and H (1-hr exposure). As was first demonstrated by Kann et al. (9), addition of BCNU immediately following X-irradiation produces a modest inhibition of DNA repair. This effect is shown in Chart 4 (E and J) for WI-38 and VA-13, respectively. However, if BCNU is present for the 1-hr preceding X-ray treatment (Chart 4, D and I), the repair of X-ray-induced DNA strand breaks is inhibited more than it is when BCNU is added after irradiation. The most striking effect of BCNU on X-ray repair is seen when BCNU is present for both the hr preceding X-ray and the hr of incubation at 37° following X-ray (Chart 4, B and G). In these experiments the inhibition of X-ray repair is more extensive in the VA-13 cells than it is in the WI-38 cells.

Because 100 μM BCNU is toxic to both cell types, we studied the effect of 50 μM BCNU on X-ray repair. These experiments are shown in Chart 5. DNA strand breaks produced by 50 μM BCNU for 2 hr are shown in Panels A and D. When 50 μM BCNU was added immediately following X-irradiation (C and F), only slight inhibition of X-ray repair was seen. However, similar to the 100 μM BCNU experi-
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Chart 4. Alkaline sucrose sedimentation of DNA from WI-38 cells (•, O) and VA-13 cells (A, Δ) treated with 100 μM BCNU or 100 μM BCNU and 4000 R of X-ray. Symbols are as in Chart 3. Treatment: 100 μM BCNU for 2 hr (A and F); 100 μM BCNU for 1 hr (C and H); 100 μM BCNU for 1 hr, 4000 R of X-ray, 1 hr of incubation at 37° (D and I); 4000 R of X-ray, 100 μM BCNU added immediately after X-ray, 1 hr of incubation at 37° (E and J).

ments, when 50 μM BCNU was present for the hour before and the hour following X-ray the inhibition of X-ray repair was clearly evident (B and E). As was seen with the 100 μM dose, the effect on X-ray repair was greater in the VA-13 cells than it was in the WI-38 cells.

DNA strand break frequencies for all experiments described above are shown in Chart 6. This chart schematically indicates the drug and X-ray protocols and the resulting DNA break frequencies. BCNU treatment, X-ray, drug-free post-X-ray incubation at 37°, and cell harvest are indicated. DNA break frequencies were determined from the following relationship (7):

\[
\text{DNA breaks/10}^8 \text{ daltons} = \frac{1}{M_{n,t}} - \frac{1}{M_{n,o}}
\]

where \(M_{n,t}\) and \(M_{n,o}\) are the number average molecular weight of treated \(^{14}\)C-labeled cells and untreated \(^3\)H-labeled cells, respectively.

Examination of the DNA break frequencies in Chart 6 reveals that in several protocols the VA-13 cells have more DNA breaks than can be accounted for by summing the breaks produced by 4000 R of X-ray and the BCNU-induced breaks. This finding was puzzling until we considered the fact that, since irradiation was delivered with the cells at approximately 35–37° and the cells were warmed to 37° for 3 min during trypsinization, some repair could have taken place. If this were the case, then the 4000-R, no repair sedimentation patterns (Chart 3, B and E) underestimated the DNA strand breaks produced by 4000 R. To test this possibility we chilled cells on ice to 0°, X-irradiated them while on ice, and harvested them by treatment for 15 min with ice-cold trypsin. The cells were then analyzed by alkaline sucrose sedimentation as before. Chart 7 shows the sedimentation patterns of WI-38 and VA-13 cells irradiated with 4000 R of X-ray and harvested without warming. The sedimentation patterns of both cell types closely resemble the VA-13 sedimentation profiles in Chart 4 (G and I) and Chart 5 (E). The estimated break frequencies are 1.16 and 1.39 breaks/10^8 daltons for VA-13 and WI-38, respectively. Since little or no DNA repair could have occurred in these cells while on ice, these sedimentation patterns show the complete effect of 4000 R of X-ray. The fact that the VA-13 sedimentation patterns and DNA break frequencies in the BCNU plus X-ray experiments are similar to those shown in Chart 7 suggests that X-ray repair is completely inhibited in VA-13, while it is only partially inhibited in WI-38.
DISCUSSION

In this study we have examined the effect of BCNU on the repair of DNA strand breaks produced by X-irradiation in normal and SV40-transformed human fibroblasts. The experiments presented here confirm and extend the finding of Kann et al. (9) that BCNU added to cell cultures following X-irradiation is capable of partially inhibiting DNA strand rejoining. However, in this study, when BCNU preceded X-irradiation for 1 hr or was present the hour prior to X-ray and the hour following X-ray the inhibition of X-ray repair was much greater than when the drug was present only following irradiation. The inhibition of DNA strand rejoining in the transformed cells was apparently complete, whereas in the normal cells there was only partial inhibition of the repair process.

At this point any description of the mechanism of the inhibition of X-ray repair by BCNU and reasons for the differential effect on the transformed VA-13 cells must be considered speculative. BCNU is capable of inhibiting DNA polymerases from several cell types (1, 4). Since BCNU decomposes rapidly in aqueous solutions to generate alkylating and carbamoylating moieties (16), it is possible that the pre-X-ray BCNU treatment of the cells in this study allowed the generation of a breakdown product responsible for the observed effects. In the post-X-ray BCNU treatments, much of the repair of the DNA breaks could have taken place before the inactivation of the repair system by the drug. If the responsible inhibitor is chloroethylisocyanate, as proposed by Kann et al. (9), this molecule may be generated during the pre-X-ray incubation and subsequently inactivate the DNA ligase involved in X-ray strand rejoining. Whether the observed effect is due to a stable or reversible inhibitor remains to be determined. We have previously observed (7) that the repair of BCNU-induced DNA damage is inhibited by the presence of BCNU and/or its breakdown products in the culture medium. This inhibition was reversed by replacing the drug treatment medium with fresh medium. These observations would be consistent with the presence of a reversible inhibitor or the stimulation of synthesis of new repair enzymes by the fresh medium.

The selective effect of BCNU on VA-13 cells could be attributed to differential uptake of BCNU by these cells. However, the high lipid solubility of BCNU and its ability to cross the blood-brain barrier suggest that the drug should be easily taken up by cells. Furthermore, in our previous study (7), we observed comparable levels of DNA cross-linking in WI-38 and VA-13 cells exposed to BCNU for 1 hr, and in prolonged exposures to BCNU we observed similar levels of DNA strand breaks in both cell types. These observations imply, at least, that comparable amounts of drug reached the DNA of both cell types. Another explanation for the selective action of BCNU on the VA-13 cells is that these cells are metabolically dead and therefore are incapable of repairing the X-ray damage. Since the 4000 R of X-ray plus BCNU treatment is highly toxic (30 μM BCNU plus 400 R of X-ray yields less than 1% survival in cloning assays of both cell types), this suggestion is possible. However, in the 50 μM BCNU survival experiments (Charts 1 and 2), some VA-13 cells do survive, and both cells can repair the damage caused by 4000 R of X-ray (Chart 3) even though these cells are destined to die. Examination of cultures with a phase microscope at the time of harvest did not show any signs of gross cell death following the BCNU plus X-ray protocol. Therefore, although the cells treated with BCNU plus 4000 R of X-ray eventually will die, the repair systems should be able to repair the DNA damage in the time periods that were studied. In fact, WI-38 cells treated with 50 μM BCNU and 4000 R (Chart 5B and Chart 6) do show substantial repair of the X-ray damage.

BCNU and MeCCNU have been used in combination with radiotherapy for the clinical treatment of malignant glioma in man (14, 15). In these trials BCNU plus radiotherapy was found to give median survivals superior to those given by...
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The single regimens of BCNU, MeCCNU, or radiotherapy. BCNU plus radiotherapy also gave responses superior to MeCCNU plus radiotherapy. DNA is presumably an important target of radiotherapy, although DNA single strand breaks may not be a major cause of lethality. However, if DNA damage can be prolonged by inhibiting DNA strand rejoining, then DNA damage might become important in cell kill. To determine whether DNA breaks persist following 100 μM BCNU and X-ray, we have followed the inhibition of repair of 4000 R-induced DNA damage for 1, 2, and 3 hr. In both cell types we observed no further increase in molecular weight after the first hr, indicating that the DNA damage does in fact persist.

In a recent report Wheeler et al. (17) have shown that BCNU treatment of rat 9L brain tumor cells prior to X-irradiation produced greater cell kills than did BCNU treatment that followed X-ray. Cell kill was maximum when BCNU treatment preceded X-irradiation by 5 to 15 hr. We do not know if that schedule of drug and X-ray would also inhibit X-ray repair as observed in WI-38 and VA-13 in this report.

At this time it is not known whether the inhibition of DNA strand rejoining by BCNU is responsible for increased cellular lethality. We are currently comparing the survival of cells treated with combinations of X-ray and drugs that inhibit DNA repair with the survival of cells treated with X-ray and drugs that do not inhibit repair. However, regardless of the outcome of those studies, based on the present work and that of Wheeler et al. (17), we feel that scheduling of drug treatment relative to radiotherapy should be considered when these treatments are used in combination therapy.

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