Strand Breaks in DNA from Normal and Transformed Human Cells Treated with 1,3-Bis(2-chloroethyl)-1-nitrosourea

Leonard C. Erickson, Matthews O. Bradley, and Kurt W. Kohn

Laboratory of Molecular Pharmacology, Division of Cancer Treatment, National Cancer Institute, NIH, Bethesda, Maryland 20014

SUMMARY

The DNA of human cells treated with 1,3-bis(2-chloroethyl)-1-nitrosourea was examined for the appearance of single-strand breaks or alkali-labile sites. Normal human embryo fibroblasts (WI-38) were compared with an SV40-transformed derivative line (VA-13) which was found to have an increased sensitivity to the cytotoxic actions of the drug. Drug treatment induced single-strand breaks and/or alkali-labile sites in both cell types as measured by two methods: alkaline sedimentation (conducted at pH 13) and alkaline elution (conducted at pH 12.1). The apparent single-strand break frequencies (which may include alkali-labile sites) were much greater when measured by alkaline sedimentation than when measured by alkaline elution. When measured by alkaline sedimentation, the apparent break frequencies were greater in WI-38 cells than in VA-13 cells, whereas by alkaline elution the reverse was true. The results may reflect differences between the two cell types in the relative frequencies of drug-induced alkali-labile sites as opposed to actual single-strand breaks.

The DNA breaks and/or alkali-labile sites appear to be repaired upon replacement of drug treatment medium with fresh medium. If the medium was not replaced, there was no repair, even after times much longer than the reported half-times for 1,3-bis(2-chloroethyl)-1-nitrosourea decomposition. This might be due to an inhibitory effect of a relatively stable drug product or to a stimulatory effect of fresh medium. The apparent rates of repair were similar in the two cell types studied.

INTRODUCTION

In a recent review, Schabel (18) concluded that nitrosoureas related to BCNU and CCNU are among the most active anticancer drugs against rodent tumors, both in the magnitude of the cell kill in vivo and in the breadth of the spectrum of effectiveness against different tumors. Although the nitrosoureas are clinically active against a variety of human neoplasms (2), their effectiveness against human disease has not been as outstanding as might have been hoped on the basis of the animal data.

One approach to an understanding of this problem is to determine the molecular basis for the cytotoxic action that produces the antitumor effect. Unfortunately, there is presently no direct way to identify with certainty the critical molecular lesions. It is, however, possible to obtain correlative evidence between molecular effects and cytotoxic effects in various cell types, which could lead to a probable identification of the critical lesions. In the current work, we studied DNA single-strand breaks and/or alkali-labile sites in BCNU-treated normal human embryo fibroblasts (WI-38) and in an SV40-transformed derivative (VA-13) that exhibited increased sensitivity to BCNU.

Nitrosoureas react with biological macromolecules by 2 mechanisms: alkylation, which affects both proteins and nucleic acids; and carbamoylation, which affects proteins but not nucleic acids (3, 22). Since certain nitrosoureas, e.g., chlorozotocin, specifically lack the carbamoylation effect and yet retain antitumor potency (10), it is probably the alkylation activity that is primarily responsible for the antitumor action. Our working hypothesis is that the crucial activity stems from alkylation of DNA and that this activity may be reflected by effects on DNA macromolecular structure.

Simple alkylnitrosoureas, such as methyl- or ethylnitrosourea, alkylate DNA at multiple sites (16, 20). Alkylations at guanine-N-7 and adenine-N-3 lead to depurination (15), and the resulting apurinic sites are susceptible to phosphodiester cleavage by specific repair enzymes, as well as to cleavage by alkali (21). Alkali lability can also result from alkylation of DNA phosphate groups (19). Thus alkylation by simple nitrosoureas produces DNA lesions that can lead to enzymatically produced single-strand breaks and to alkali-labile lesions that can be converted to single-strand breaks by alkali. The sites of DNA alkylation by chloroethyl nitrosoureas, such as BCNU and CCNU, however, have not been fully determined. The production of single-strand breaks or alkali-labile sites by BCNU and CCNU have been reported previously in abstracts (6, 11).

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 1.5 to 2.0 x 10^6 cells in 75-sq cm plastic flasks (Falcon Plastics, 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, 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, Oxnard, Calif).
in cellulose nitrate tubes, were centrifuged in a Beckman SW40 Ti rotor at 9500 rpm, 20°, for 16.5 hr, $\omega^2 t = 6.177 \times 10^{10}$ s$^2$/sec. The gradients were fractionated from the top by displacement with a solution of 60% sucrose/1 M NaCl. Five-tenths-ml fractions were collected in scintillation vials and mixed with 2.5 ml of 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 450B liquid scintillation counter (Packard Instruments, Chicago, Ill.). Weight-average molecular weight was computed as described by Dingman and Kakunaga (4).

**Alkaline Elution.** This method of estimating DNA single-strand breakage is based on the relationship between DNA single-strand length and the rate of elution of these strands from cell lysates at pH 12.1 (14). Cells were labeled with $[^{14}C]$thymidine, treated with BCNU, and suspended by trypsinization as described above. The cells were then collected on a membrane filter and subjected to lysis and alkaline elution as previously described (7, 9, 14). Included in each assay as internal reference were $[^{3}H]$thymidine-labeled L1210 cells irradiated with 150 rads. Relative elution rate ($B$) was calculated from the formula:

$$B = \frac{\log R(0.8) - \log R(0.5)}{\log(0.8) - \log(0.5)}$$

where $R(0.8)$ and $R(0.5)$ are the fractions of the $[^{14}C]$DNA that remained on the filter when 0.8 or 0.5 of the reference $[^{3}H]$DNA remained on the filter. The relative elution rate produced by 150 R of X-ray is 1.0 by definition. X-ray was delivered to cells at ice temperatures by 2 vertically opposed Phillips RT-250 X-ray tubes operating at 200 kV and 15 ma, with 0.25-mm copper and 0.55-mm aluminum filters.

**RESULTS**

**Differential Effects of BCNU on WI-38 and VA-13 Cell Cultures**

BCNU produced a differential effect on growth of WI-38 and VA-13 cell cultures that was clearly evident at 50 $\mu$M (Chart 1). In these experiments the treatment medium (containing BCNU and its decomposition products) was replaced with fresh drug-free medium either 1 or 24 hr after drug addition. Treated WI-38 cells continued to proliferate, although at a reduced rate, and eventually reached saturation densities similar to those of control cultures. No gross cell death or floating cell debris was evident on examination of the cultures with a phase microscope. In contrast to WI-38 cells, treated VA-13 cells exhibited gross cell death, which was evident by reduction in the number of attached cells and by the appearance of cell debris in the medium. Treatments with BCNU concentrations of 10 $\mu$M or less had no effect on either cell type, whereas treatments with 100 $\mu$M completely inhibited the proliferation of both cell types.

The possibility was considered that the cell loss in the
L. C. Erickson et al.

Both cell types. Drug effects were sensitively detected by sedimentation of DNA from mixtures of treated cells labeled with [14C]thymidine and untreated cells labeled with [3H]thymidine. Chart 2 shows the effects of treatment of WI-38 or VA-13 cells with 100 μM BCNU for 1 hr on DNA sedimentation in triplicate experiments. The treatment produced small but quite reproducible reductions in sedimentation, and this effect appeared to be greater in the WI-38 cells than in the VA-13 cells (Table 1). From the sedimentation changes, we estimated frequency of drug-induced DNA strand breaks (see Table 1); the observed break frequencies may include both strand breaks that exist as such in the cells and breaks that are generated from alkali-labile DNA lesions by the high pH of the gradients.

The time course of the formation and disappearance of apparent strand breaks during and after a 1-hr exposure to 100 μM BCNU is shown in Chart 3. The frequency of drug-induced DNA breaks was again consistently greater in WI-38 cells than in VA-13 cells. In both cell types, however, most of the apparent DNA breakage disappeared within 24 hr after removal of drug. Part of this recovery in apparent DNA strand length might be related to the delayed formation of DNA interstrand cross-links (7, 13).

VA-13 cultures might be related to an alteration in surface attachments of these cells. The experiment was therefore repeated with a subline of VA-13 cells (VA-13B) selected for its ability to form clones from single attached cells. This subline is thus more capable of proliferation from isolated viable cells. After BCNU treatment, these cultures declined less in cell number, but the prolonged delay in recovery of proliferation was similar to that seen in the original VA-13 line. It therefore seems unlikely that the differential effect of BCNU on WI-38 and VA-13 cells is merely due to differences in surface attachment.

**Brief Exposures to BCNU**

**Alkaline Sedimentation Studies.** We determined DNA single-strand-length distributions by an alkaline sedimentation procedure described by Dingman and Kakunaga (4), in which cell lysates are proteolytically digested prior to layering onto alkaline sucrose gradients. Although the DNA may be sheared to some extent during handling in this procedure, mixing of experimental and control cells before lysis should assure that any shearing will be equivalent in both cell types. Drug effects were sensitively detected by sedimentation of DNA from mixtures of treated cells labeled with [14C]thymidine and untreated cells labeled with [3H]thymidine. Chart 2 shows the effects of treatment of WI-38 or VA-13 cells with 100 μM BCNU for 1 hr on DNA sedimentation in triplicate experiments. The treatment produced small but quite reproducible reductions in sedimentation, and this effect appeared to be greater in the WI-38 cells than in the VA-13 cells (Table 1). From the sedimentation changes, we estimated frequency of drug-induced DNA strand breaks (see Table 1); the observed break frequencies may include both strand breaks that exist as such in the cells and breaks that are generated from alkali-labile DNA lesions by the high pH of the gradients.

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BCNU-induced DNA Strand Breaks in Human Cells

Table 1
Apparent frequency of DNA single-strand breaks due to treatment of cells with BCNU: alkaline sedimentation method

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Control (H)</th>
<th>Treated (C)</th>
<th>Induced break frequency (× 10⁻¹⁵ dalton⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WI-38</td>
<td>2.72</td>
<td>2.30</td>
<td>13.5</td>
</tr>
<tr>
<td></td>
<td>2.71</td>
<td>2.38</td>
<td>10.5</td>
</tr>
<tr>
<td></td>
<td>2.74</td>
<td>2.32</td>
<td>16.6</td>
</tr>
<tr>
<td>VA-13</td>
<td>2.36</td>
<td>2.15</td>
<td>7.9</td>
</tr>
<tr>
<td></td>
<td>2.27</td>
<td>2.15</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>2.20</td>
<td>2.07</td>
<td>5.7</td>
</tr>
</tbody>
</table>

*Induced break frequency = \( \frac{1}{M_\alpha M_\beta} \)

where \( M_\alpha \) and \( M_\beta \) are number-average molecular weights of ¹⁴C-labeled treated cells and ³H-labeled control cells, respectively, sedimented in the same tube. (Number-average molecular weights estimated as one-half of weight-average molecular weights.)

Alkaline Elution Studies. DNA strand break frequency can also be estimated from the kinetics of the alkaline elution of DNA from cell lysates (14). Cells from WI-38 and VA-13 cultures treated with 50 or 100 µM BCNU were analyzed by alkaline elution. In order to check the calibration of the method, experiments with cells exposed to low doses of X-ray were also included. A set of elution kinetic curves following BCNU treatment of cultures is illustrated in Chart 4, and the results from a number of independent experiments, expressed as induced break frequencies, are summarized in Table 2. The BCNU treatments produced dose-dependent increases in elution rates that were greater in VA-13 cells than in WI-38 cells (Chart 4). The elution rates due to 150 R of X-ray, on the other hand, were similar in the 2 cell types.

Although alkaline sedimentation and alkaline elution both showed the production of DNA single-strand breaks by BCNU, the apparent break frequencies estimated by alkaline sedimentation were 2 to 7 times as great as those estimated by alkaline elution (Tables 1 and 2). By alkaline sedimentation more breaks appeared to be induced in WI-38 cells than in VA-13 cells, whereas by alkaline elution the reverse was the case. Several possibilities were considered in an attempt to explain these differences. First, the 2 methods differed in that the cell lysate was subjected to proteolytic digestion in the alkaline sedimentation analyses but not in the alkaline elution analyses. A proteolytic digestion step can, however, be included in the alkaline elution procedure (7, 8); although it was not feasible to carry out the digestion at 50°, as was done in the sedimentation procedure, an incubation at room temperature was effective in reducing the amount of protein retained on the filter (7). The addition of a proteolytic digestion step in alkaline elution assays of BCNU-treated cells did not significantly alter the estimated induced break frequencies (Table 2). Proteolytic treatment is therefore probably not responsible for the difference in the break frequency estimates between the 2 methods.

A 2nd possibility that we have examined is that DNA breaks may be partially hidden by the simultaneous production of DNA cross-links (8). Cross-linking of DNA by BCNU has been demonstrated in isolated DNA (13) and in cultured L1210 cells (7). DNA cross-linking can be estimated in alkaline elution from the reduction in the effect of a standard X-ray on elution kinetics (7, 8). The effects of BCNU and X-ray on elution rates are shown in Table 3. The upper 2 lines show the increase in elution rate caused by BCNU alone; the increase is greater in VA-13 than in WI-38 cells and is interpreted as being due to a greater induction of single-strand breaks in the former cell type. Lines 3 and 4 in Table 3 show the increase in elution rate caused by X-ray alone. Several possibilities were considered in an attempt to explain these differences. First, the 2 methods differed in that the cell lysate was subjected to proteolytic digestion in the alkaline sedimentation analyses but not in the alkaline elution analyses. A proteolytic digestion step can, however, be included in the alkaline elution procedure (7, 8); although it was not feasible to carry out the digestion at 50°, as was done in the sedimentation procedure, an incubation at room temperature was effective in reducing the amount of protein retained on the filter (7). The addition of a proteolytic digestion step in alkaline elution assays of BCNU-treated cells did not significantly alter the estimated induced break frequencies (Table 2).
Effects of Prolonged Exposure to BCNU

Treatment with 50 μM BCNU for 1 hr produced little DNA strand breakage detectable by alkaline sedimentation in either cell type. Continued incubation in the same medium, however, resulted in a progressive accumulation of DNA single-strand breaks over a 24-hr period (Charts 5 and 7). If the medium was then replaced with a fresh drug-free medium, DNA sedimentation returned to near normal over the next 48 hr (Charts 6 and 7). If the medium was not replaced, the DNA did not recover (Chart 7). The recovery of the DNA sedimentation patterns was similar in both cell types. These observations pertain to [14C]DNA that was synthesized prior to addition of drug.

The possibility could be entertained that 14C label may be released from dying cells and reincorporated into DNA in surviving cells, thus giving the illusion of DNA repair. The following experiment addressed this question. The 24-hr BCNU treatment protocol was repeated with cells in which the DNA had been prelabeled with [14C]thymidine, and the medium was assayed for released radioactivity over a 120-hr period. No radioactivity was detected in the WI-38 culture medium, indicating that prelabeled DNA was not degraded. DNA strands shortened by BCNU treatment in WI-38 cells therefore must be able to recover in length upon posttreatment incubation. In VA-13 cultures, however, large amounts

3 show that the increase in elution rate due to X-ray alone is similar in the 2 cell types. The bottom 2 lines in the table show that pretreatment with BCNU reduces the elution rate produced by X-ray alone. This effect of BCNU is attributed to the formation of cross-links involving DNA (7). The magnitude of the cross-linking effect was similar in the 2 cell types. Therefore, cross-linking cannot fully explain the differences in break frequencies estimated by alkaline sedimentation and alkaline elution.

A 3rd possible explanation for the difference in break frequency estimates is that the ratio of alkali-labile sites to DNA strand breaks may differ between the 2 cell types. The pH of the alkaline gradients is approximately 1 pH unit higher than the alkaline elution solution (pH 13 versus 12.1) and may hydrolyze all alkaline-sensitive lesions. In the alkaline elution assay some or most of these sites may remain uncleaved. Thus the elution experiments may measure primarily DNA strand breaks existing in cells, whereas the sedimentation experiments may measure both actual breaks and alkali-labile sites.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>WI-38 (x 10^{-10} dalton^{-1})</th>
<th>VA-13 (x 10^{-10} dalton^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 μM BCNU, 1 hr</td>
<td>1.08 ± 0.12 (6)</td>
<td>1.43 ± 0.20 (6)</td>
</tr>
<tr>
<td>100 μM BCNU, 1 hr</td>
<td>1.93 ± 0.37 (4)</td>
<td>3.03 ± 0.34 (4)</td>
</tr>
<tr>
<td>50 μM BCNU, 1 hr (proteinase-K included in assay)</td>
<td>1.17 ± 0.29 (3)</td>
<td>1.32 ± 0.14 (3)</td>
</tr>
<tr>
<td>150 R X-ray</td>
<td>4.36 ± 0.37 (4)</td>
<td>4.69 ± 0.17 (4)</td>
</tr>
</tbody>
</table>

\* Mean ± S.D.

\* Numbers in parentheses, number of independent experiments.
BCNU-induced DNA Strand Breaks in Human Cells

The simplest explanation for this difference would be that alkali-labile sites are much more frequent in treated WI-38 cells and DNA strand breaks are more frequent in VA-13.

Although chloroethylnitrosoureas are known to alkylate DNA (3), the sites of alkylation and the subsequent chemical events have not been fully defined. It nevertheless seems likely that the single-strand breaks and/or alkali-labile sites that we have observed arise either by direct action of the drug or from the repair of BCNU-induced DNA alkylation.

Both alkali-labile sites and single-strand breaks occur in the course of repair of DNA alkylation damage. Alkylation at guanine-N-7 and adenine-N-3 leads by depunination to base-free sites that are convertible in alkali to single-strand breaks (21). Apurinic sites can be converted to single-strand breaks intracellularly by a specific endonuclease (5, 21). These breaks may then be repaired by the DNA ligase system. The sequence of intracellular events thus is as follows:

Alkylated purine $\rightarrow$ apurinic site $\rightarrow$ single-strand break $\rightarrow$ rejoined DNA

If we assume that alkaline sedimentation measures the sum of apurinic sites and single-strand breaks while alkaline elution measures mainly single-strand breaks, our findings would suggest that in WI-38 cells compared with VA-13 cells the frequency of apurinic sites is higher while the frequency of single-strand breaks is lower. This would suggest that there may be important differences between the 2 cell types in the relative rates of the individual steps of the above reaction sequence. Such differences between cell types could bear on the observed difference in cytotoxicity. An additional factor that may enter into the final interpretation, however, is the possible role of DNA phosphate alkylations, which also may confer alkali lability (19).

After addition of BCNU to either cell type, we found a steady increase in apparent DNA break frequencies for 24 hr (Chart 7). Since BCNU decomposes in physiological media with a half-time of approximately 1 hr (17), it might be supposed that the prolonged accumulation of DNA breaks is a secondary effect occurring after the disappear-

of soluble and acid-precipitable radioactivity were released into the medium, so that reincorporation of released label in these cells is not excluded.

DISCUSSION

We have compared the effects of BCNU treatment of normal human embryo cells (WI-38) and an SV40-transformed derivative of these cells (VA-13). The WI-38 cells were less sensitive to BCNU than were VA-13 cells when assayed either by change in cell number (Chart 1) or by loss of ability to proliferate into clones (M. O. Bradley, unpublished observations). In the current work we have compared the 2 cell types with regard to the appearance of DNA single-strand breaks as measured by 2 alkaline assay methods. Both cell types when treated with BCNU for 1 hr exhibited single-strand breaks by both methods. The BCNU-induced break frequencies estimated by sedimentation, however, were much greater than the estimates by alkaline elution (Tables 1 and 2). This difference cannot be attributed to calibration errors, since both methods were calibrated in terms of the efficiencies for single-strand break production by X-ray under similar experimental conditions. A possible explanation for the break frequency difference is that most of the breaks observed by sedimentation were derived from alkali-labile sites due to the higher pH of the gradients (pH 13) and that a large fraction of these sites were not converted to breaks at the lower pH of the elution method (pH 12.1). Whereas alkaline sedimentation disclosed more breaks in WI-38 than in VA-13 cells (Table 1), alkaline elution indicated the opposite (Table 2).
ance of active drug species. Contrary to this supposition, however, was the finding that replacement of the medium, containing drug and its decomposition products, with fresh drug-free medium was followed by a gradual decline of apparent strand breaks, whereas the continued presence of drug medium prevented this recovery. We do not yet know whether a stable inhibitor is formed in treatment medium or whether recovery is stimulated by fresh medium; the findings are consistent with either or both of these possibilities. DNA repair processes have been reported to be inhibited by BCNU breakdown products related to chloroethylisocyanate (12). Although isocyanates themselves would be too unstable to account for the present findings, a reaction of an isocyanate with another substrate might generate a stable inhibitor.

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REFERENCES

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