Nitrosourea-induced Sister Chromatid Exchanges and Correlation to Cell Survival in 9L Rat Brain Tumor Cells

Philip J. Tofilon, Mary E. Williams, and Dennis F. Deen

ABSTRACT

The ability of various nitrosoureas to induce sister chromatid exchanges (SCEs) in 9L rat brain tumor cells was investigated. Treatment of cells for 1 hr with the alkylating and cross-linking agents 1,3-bis(2-chloroethyl)-1-nitrosourea or chlorozotocin produced concentration-dependent increases in SCEs; elevations above controls were detected at concentrations of 1 mM or more. Above 0.25 mM, the alkylating agent ethylnitrosourea produced a dose-dependent increase in SCEs. Treatment with the carbamoylating agent 1,3-bis(trans-4-hydroxycyclohexyl)-1-nitrosourea did not induce SCEs. The maximum drug concentration at which SCEs are readily scored kills approximately 50% of cells. When accurate cell survival data in this dose range were obtained, a direct correlation between nitrosourea-induced cell kill, measured by a colony-forming efficiency assay, and SCE induction was found. Thus, analysis of the levels of SCE production may provide information about the efficacy of antineoplastic drugs.

INTRODUCTION

The SCE assay is a sensitive, rapid method for measuring the effects of agents that damage DNA (14). Mutagens and carcinogens induce SCEs in many cell lines at concentrations far below cytotoxic and clastogenic levels (13). Correlations between mutagenesis, neoplastic cell transformation, and the induction of SCEs have been found in several but not in all cell lines tested (2, 9). Because of its sensitivity and ease of performance, the SCE assay is commonly used to assess the mutagenic and/or carcinogenic potential of chemicals introduced into the environment.

The in vitro sensitivity of cells to antineoplastic drugs is often determined from survival curves that are based on CFE assays. The assay reflects the combined effects of many factors that lead to cell death, and the assay is usually used to evaluate cell kill caused by doses of drug that are high enough to kill 50% or more of cells. Thus, the method is relatively insensitive as a measure of drug action.

The cellular site of mutagens, carcinogens, and many cancer chemotherapeutic agents is thought to be nuclear DNA. It is well established that many compounds that damage DNA induce SCEs (14). Therefore, measurement of induced SCEs in cells treated with antineoplastic agents may provide a more sensitive indicator of drug action than the CFE assay. In addition, rather than measuring effects at the cellular level, the SCE assay measures effects at the chromosomal level, which may aid in the study of the mechanisms of drug-induced cell kill.

Nitrosourea compounds are an important class of antineoplastic drugs. In combination protocols with radiation therapy, BCNU is one of the most effective agents for the treatment of malignant gliomas (7). However, in the clinical setting, these protocols do not cure patients harboring tumors. In an attempt to more clearly define the mechanism of nitrosourea action and perhaps to provide a basis for a more effective clinical therapy of brain tumors, we have been investigating the effects of various nitrosourea analogues on monolayer 9L rat brain tumor cells.

In the studies reported here, we used the SCE assay to evaluate the effects of treating 9L rat brain tumor cells in vitro with nitrosoureas that have different chemical properties (11, 12): BCNU has alkylating, carbamoylating, and cross-linking activity; CHLZ has alkylating and cross-linking activity but no carbamoylating activity; ENU has alkylating and carbamoylating activity but no cross-linking activity; and BHCNU has carbamoylating activity but no alkylating activity. We found that nitrosoureas with alkylating activity induce SCEs in 9L cells and that there is a strong correlation between the levels of the cell kill and the SCEs induced by these drugs.

MATERIALS AND METHODS

Cell Culture. 9L rat brain tumor cells (1 to 2 x 10⁶ cells) were seeded into 75-cm² tissue culture flasks and grown in 15 ml of Eagle's minimum essential medium supplemented with nonessential amino acids, 10% newborn calf serum, and gentamicin (50 μg/ml). Before treatment, cells were incubated for approximately 24 hr at 37°C in a humidified 5% CO₂:95% air atmosphere to establish early log phase growth.

Drug Treatment. Stock solutions of BCNU, BHCNU, and ENU were prepared in absolute ethanol; CHLZ was dissolved in 0.2 ml of dimethyl sulfoxide and diluted with 9.8 ml of absolute ethanol. Solutions were prepared immediately before use. The volume of added vehicle was always less than 1% of the final treatment volume and had no effect on SCE induction or cell survival. Exponentially growing 9L cells were treated for 1 hr with various concentrations of each drug.

SCE Assay. After treatment, cells were rinsed, and 25 ml of fresh medium containing 10 or 20 μM BrdUrd were added. Cells were allowed to replicate for 2 cell cycles. Four hr before harvesting, cultures were treated with colcemid (0.04 μg/ml). Mitotic cells were shaken from the flask and treated with 0.05 M KCl. Cells were fixed and washed with freshly prepared methanol:acetic acid (3:1). Sister chromatids were differentially stained using the method of Perry and Wolff (8).

CFE Assay. This assay has been described (3). Each experiment consisted of 8 plates per drug concentration.

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3 The abbreviations used are: SCE, sister chromatid exchange, CFE, colony-forming efficiency; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CHLZ, chlorozotocin; ENU, ethylnitrosourea; BHCNU, 1,3-bis(trans-4-hydroxycyclohexyl)-1-nitrosourea; BrdUrd, bromodeoxyuridine.

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RESULTS

BrdUrd alone can induce SCEs above control levels (5); however, a 30-hr incubation with 5, 10, 15, 20, and 30 μM BrdUrd produced approximately 13 SCEs/metaphase in control 9L cells. Also, the dose-response curve for BCNU (Chart 1A) was not affected by varying the concentration of BrdUrd.

Treatment of 9L cells with BCNU, CHLZ, and ENU produced concentration-dependent increases in SCEs (Chart 1). At the lowest concentration used (1 μM), BCNU and CHLZ produced approximately a 2-fold increase in SCEs; however, a concentration of 100 μM ENU was needed to produce a 2-fold increase in SCEs. Concentrations of BHCNU up to 300 μM did not induce SCE formation above control levels, even though the same concentration of BHCNU killed approximately 90% of cells (data not shown).

Because of the insensitivity of the CFE assay at low levels of nitrosourea-induced cell kill, survival curves are usually generated for cell kills of half a log and greater. However, the concentrations of nitrosoureas that produce more than half a log cell kill induce far too many SCEs to count. By increasing the number of plates used per concentration and performing 3 to 6 experiments, cell survival curves were generated at concentrations of nitrosoureas that kill less than 50% of cells; these data can be compared directly with the nitrosourea-induced SCEs. Chart 2, A to C, contains plots of SCEs versus cell kill for BCNU, CHLZ, and ENU. Linear regression analysis of these data yielded correlation coefficients of 0.93, 0.94, and 0.93, respectively. These results show a direct correlation between SCEs and cell kill for BCNU, CHLZ, and ENU.

DISCUSSION

The nitrosourea analogues used in this study have different alkylating, cross-linking, and carbamoylating activities (11, 12). SCEs were induced in 9L cells only by the alkylating agents BCNU, CHLZ, and ENU. On a molar basis, BCNU and CHLZ induce SCEs and kill cells at considerably lower concentrations than does ENU. The much higher dose of ENU required to produce SCEs and kill cells suggests that cross-linking is important for both induction of SCEs and cell kill. However, the relation between cross-linking and SCE formation has not been clearly defined, and it is not possible to draw any general conclusion about the role of cross-linking on SCE formation from the data reported here.

The characteristics of the CFE assay make it difficult to accurately and reproducibly determine cell kills of less than half a log, and small changes in cytotoxicity that are the result of the use of combinations of drugs, combinations of drugs with radiation, or various fractionated protocols may go undetected. Even though these changes are slight, they may significantly affect the outcome of clinical treatment (4). Therefore, it is important to be able to detect any alteration in drug-induced cytotoxicity that might affect therapeutic effectiveness.

While BHCNU did not induce SCEs at concentrations that kill approximately 90% of cells, we have found a direct corre-

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**Chart 1.** Effects of nitrosoureas on SCEs in 9L cells. Cells were treated for 1 hr and then allowed to replicate in the presence of either 10 μM (——) or 20 μM (……) BrdUrd for 30 hr. Points, mean of 2 to 3 experiments obtained by scoring 20 metaphases per concentration point per experiment; bars, S.E. A, BCNU; B, CHLZ; C, ENU.

**Chart 2.** Correlation between SCEs and percentage of cell kill. Each point represents the mean number of SCEs and percentage of cell kill produced by the same concentration of nitrosourea. Horizontal bars represent the standard error of 3 to 6 cell survival experiments; vertical bars represent the standard error of 2 to 3 SCE experiments. Each line was drawn according to a linear regression analysis. A, BCNU; B, CHLZ; C, ENU.
lation between SCE induction and cell kill for the nitrosoureas with alkylating activity. At the concentrations used, data from the cell survival assay were difficult to obtain, whereas a definite linear response in SCEs was consistently observed. For the nitrosoureas that induce SCEs, the SCE assay appears to have the sensitivity to detect slight changes in drug action that may not be detected by the CFE assay. To our knowledge, this is the first report of a direct correlation between SCE induction and cell kill and suggests that the SCE assay may predict tumor cell sensitivity to cancer chemotherapeutic agents. Among antineoplastic drugs that induce SCEs are busulphan (10), vincristine (10), 5-fluorouracil (10), cis-platinum (1), cyclophosphamide (15), 1-β-D-arabinofuranosylcytosine (6), and hydroxyurea (6). Therefore, when there is a correlation between drug-induced SCEs and cell kill, the SCE assay may be a useful complementary method to the CFE assay for the determination of the in vitro sensitivity of cells to antineoplastic drugs.

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