Inactivation of p53 Sensitizes U87MG Glioma Cells to 1,3-bis(2-Chloroethyl)-1-nitrosourea

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ABSTRACT

We examined the effect of p53 inactivation on the response of U87MG glioma cells to 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). These studies were motivated by three observations: (a) some human astrocytomas are sensitive to BCNU and some are resistant; (b) chemosensitive astrocytomas are more likely to be found in young adults whose tumors are more likely to harbor a p53 mutation; and (c) mouse astrocytes lacking the p53 gene are more sensitive to BCNU than wild-type cells. Here, we observed that p53 inactivation by transfection with pCMV-E6 sensitized U87MG cells to BCNU. Compared with control U87MG-neo cells with intact p53 function, the clonogenic survival of U87MG-E6 cells exposed to BCNU was reduced significantly. In U87MG-E6 cells, sensitization to BCNU was associated with failure of p21WAF1 induction, transient cell cycle arrest in S phase, accumulation of polyploid cells, and significant cell death. In contrast, resistance to BCNU in U87MG-neo cells was associated with up-regulation of p53, prolonged induction of p21WAF1 sustained cell cycle arrest in S phase, and enhancement of DNA repair. U87MG cells with disrupted p53 function were less able to repair BCNU-induced DNA damage and survive this chemotherapeutic insult. The question arises of whether p53 dysfunction might be a chemosensitizing genetic alteration in human astrocytic gliomas.

INTRODUCTION

Two distinct molecular pathways may be activated by p53 in response to DNA damage. p53 may trigger programmed cell death, but it may also set into motion mechanisms that facilitate cell survival. Ultimately, the balance between these opposing responses to injury, which may be both cell type and insult specific, determines cell fate (1, 2). In the context of neoplasia and chemotherapy, the influence of p53 function on response to treatment is doubly complex because p53 often is inactivated in human cancers (3). For example, p53 might contribute to sensitivity if tumor cells apoptose following drug treatment (4) or to resistance if therapeutic DNA damage is repaired preferentially (5). Conversely, in a cell harboring a mutation, p53 inactivation might contribute to resistance if tumor cells are unable to apoptose or to sensitivity if checkpoint failure leads to ineffective DNA repair. At present, the influence of p53 status on chemosensitivity is unknown for many types of human cancer. In neuro-oncology, there is circumstantial evidence suggesting that the response of astrocytic gliomas to BCNU may be linked to p53 status. Clinicians have noted that chemosensitive tumors are more likely to be found in young adults whose astrocytomas are also more likely to harbor a p53 mutation (6). In related research, it has been observed that neonatal mouse astrocytes lacking the p53 gene are more sensitive to BCNU than wild-type astrocytes (7), whereas p53 knockout fibroblasts (7) and thymocytes are more resistant to BCNU than wild-type cells, indicating cell type-specific p53 effects. Furthermore, these findings suggest that induction of p53 preferentially activates mechanisms that enhance the survival of cells of astrocytic derivation. In this study, we examined the influence of p53 disruption on the viability of human astrocytic glioma cells exposed to BCNU.

MATERIALS AND METHODS

Cell Lines and Drug Sensitivity Assay. U87MG, a human glioma cell line with functional wild-type p53 (8), was purchased from the American Type Culture Collection (Rockville, MD). U87MG and derivative cell lines were maintained in antibiotic-free MEM (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and 1 mM sodium pyruvate. To generate a glioma cell line lacking p53 function, U87MG was transfectected with pCMV-E6; the human papillomavirus E6 gene encodes a protein that binds to and inactivates p53. To serve as a control, U87MG was also transfected with pCMV-neo, a plasmid lacking the E6 gene. pCMV-E6 and pCMV-neo plasmid DNAs were provided by Dr. K. Cho (University of Michigan, Ann Arbor, MI) (9). pG13-CAT plasmid DNA was provided by Dr. B. Vogelstein (Johns Hopkins Cancer Center, Baltimore, MD) (10). Plasmids were transformed and amplified in competent bacteria DH-5α (Life Technologies), and DNA was isolated using the EndoFree plasmid kit (Qiagen) according to the manufacturer’s instructions. Transfections were performed using a calcium phosphate reagent kit (Life Technologies). After incubation in medium containing 400 μg/ml G418 (Life Technologies), individual U87MG clones were isolated and tested for endogenous wild-type p53 activity (see below).

BCNU (M, 214.06) was purchased from Bristol-Myers Pharmaceuticals. A stock solution was prepared by dissolving 100 mg of BCNU in 30 ml of 10% ethanol, and was stored at −70°C to ensure drug stability (12). Subsequently, BCNU stock solution was added directly to culture medium to achieve the desired concentrations for experimental use.

To ascertain sensitivity to BCNU, clonogenic survival assays were performed. Clonogenic assays, more reliably than others, assess whether tumor cells survive and proliferate following drug treatment (11). U87MG-E6 cells (p53 inactivated) and U87MG-neo cells (functional p53) were plated at low density in 100-mm dishes and exposed to BCNU. Cultures were incubated for 2–3 weeks without a change in medium. Colonies were then fixed, stained with 1% crystal violet in methanol, and counted.

Function and Induction Studies. p53 function in U87MG-E6 and U87MG-neo cells was assessed using a CAT enzyme assay system (Promega). Briefly, cultures were transfected with pG13-CAT, a plasmid containing wild-type p53-responsive elements, washed with PBS, and lysed in 1× Reporter Lysis Buffer. As a control for transfection efficiency, 0.5 μg of pSV-β-galactosidase plasmid was cotransfected and β-galactosidase activity was measured using an enzyme assay system (Promega). CAT activity was determined on lysates with equal protein content, washed with PBS, and incubated in medium containing 400 μg/ml G418 and 1 mM sodium pyruvate. For the induction studies, CAT activity was measured using 14C chloramphenicol and TLC or liquid scintillation counting. CAT activity was quantified using a PhosphorImager (Molecular Dynamics) or measured on a scintillation counter. CAT activity was expressed as the

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3The abbreviations used are: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CAT, chloramphenicol acetyl transferase; MGMT, O6-methylguanine-DNA methyltransferase.

4M. C. Zlatescu and J. G. Cairncross, unpublished data.
percentage of conversion of [14C]chloramphenicol to butyrylated forms and was normalized to control activity, which was set at 100%.

p53, p21WAF1, and MGMT expression was assessed by Western blotting in U87MG, U87MG-E6, and U87MG-neo cells in response to BCNU treatment. Cultures were washed twice with PBS, lysed in preheated SDS buffer (1% SDS-5% glycerol), heated to 100°C for 5 min, and stored at −20°C. Protein concentrations were determined using a DC Protein Assay kit (Bio-Rad). Subsequently, lysates were supplemented with 100 mM DTT, heated to 100°C for 5 min, loaded on precast 8–16% gradient SDS-PAGE gels (Novex), and transferred to nitrocellulose membranes in a semi-dry gel transfer module (Novex). Hybridization to antibodies was performed, and signals were visualized using an enhanced chemiluminescence kit (Amer-Sham). Blots were stained to ensure equal protein loading. Antibodies to p53 and p21WAF1 were obtained from Santa Cruz Biotechnology, and antibodies to MGMT were obtained from PharMingen.

Cell Cycle and Death Analyses. Approximately 1 × 10^6 U87MG-E6 and U87MG-neo cells were seeded in 150-mm Petri dishes. Forty-eight h later, cultures were treated with identical concentrations of BCNU. After incubation, suspensions of single nuclei were prepared and stained with propidium iodide as instructed, using a Cycle TEST PLUS DNA reagent kit (Becton & Dickinson). Cells (1 × 10^6) were analyzed on a Beckman Coulter XL-MCL instrument using the FL3 detector with 620 nm band-pass filter. For each sample, 50,000 events were collected. Analyses of the data were performed on un gated histograms by use of MultiCycle software (Phoenix Flow System). For control samples, a zero-order polynomial fit with background subtraction was used. For complex cell cycle histograms, a synchronized S-phase model with a second-order polynomial and background subtraction was used. In addition to measuring sub-G1 DNA contents by flow cytometry, we evaluated cell death by assessing DNA fragmentation of U87MG-E6 and -neo cells exposed to BCNU using both DNA ladder and TdT-mediated dUTP nick end labeling (Roche) methods according to the manufacturer’s instructions.

DNA Repair Assay. DNA repair capacity in U87MG transfectants exposed to BCNU was assessed using a host cell reactivation assay. In this assay, host cells were transfected with a damaged CAT reporter plasmid and then exposed to an active drug or sham treatment. Repair of the plasmid as reflected by CAT activity was then a measure of repair capacity in the host cell (5, 13). Briefly, 2 μg of reporter plasmid DNA pCAT3 (Promega) was incubated with 2 mM BCNU in 20 μl of culture medium (pH 7.5) at 37°C for 2 h. BCNU-damaged plasmid DNA was diluted into 100 μl of Opti-MEM medium (Life Technologies) and mixed with an equal volume of Opti-MEM containing 20 μg of Lipofectin reagent (Life Technologies). The DNA-lipofectin complex was added to exponentially growing U87MG-E6 or U87MG-neo cells seeded 48 h earlier. Immediately prior to transfection, culture medium was replaced with 5 ml of Opti-MEM. As a control for transfection efficiency, 0.5 μg of pSV-β-galactosidase plasmid was cotransfected. After overnight incubation and the addition of fresh medium, each culture that had been transiently transfected with BCNU-damaged plasmid DNAs was treated with BCNU or sham treated. BCNU solution (5 mg/ml in 10% ethanol) or 10% ethanol (sham) was added to a concentration of 15 μg/ml or 0.03%, respectively. BCNU- and sham-treated cultures were incubated for 48 h without a change in medium followed by CAT and β-galactosidase assays. CAT activity was measured by liquid scintillation counting and normalized to β-galactosidase activity (internal control). CAT activities of transfected U87MG-E6 and U87MG-neo cells exposed to BCNU were normalized to sham-treated U87MG-E6 and -neo cultures, respectively, which were set at 100%.

RESULTS

Clonogenic Survival Assay. Following transfection with pCMV-E6, p53 function was assessed in multiple clonal cell lines. Six cell lines in which p53 had been successfully inactivated by E6 were subjected to further analysis; in all clones tested, inactivation of p53 resulted in sensitization to BCNU. BCNU-treated U87MG-E6 cells had significantly reduced clonogenic survival compared with either U87MG-neo or U87MG parental glioma cells (Fig. 1). In addition, the LD_{50} for U87MG-E6 cells was half that of either U87MG-neo or U87MG parental cells, whose LD_{50}s were similar (Fig. 1). These results parallel our earlier findings with neonatal mouse astrocytes; in those studies, astrocytes lacking the p53 gene were significantly more sensitive to BCNU than wild-type astrocytes (7).

p53 and p21WAF1 Induction Studies. p53 and p21WAF1 expression was evaluated in U87MG-E6 and -neo cells. As shown in Fig. 2, lower levels of p53 protein were detected in the U87MG-E6 cells in which p53 function had been inactivated. U87MG-E6 cells had markedly diminished p53 activity, as assessed by CAT assay, compared with both U87MG-neo and U87MG parental cells (Fig. 2). In contrast, p53 protein was readily detected in U87MG-neo cells, and protein levels in U87MG-neo cells, but not in U87MG-E6 cells, increased significantly in response to BCNU treatment (Fig. 2). Increased p53 protein levels could be detected as early as 12 h following exposure to 30 μg/ml BCNU and increased 4-fold over basal levels by 24 h. In light of the induction of p53 by BCNU in U87MG-neo cells, the expression of p21WAF1, an important downstream target for p53 (14), was examined in U87MG-neo cells. Forty-eight h after exposure to BCNU, levels of p21WAF1 had increased dramatically in U87MG-neo cells (Fig. 2). In contrast, p21WAF1 could neither be detected nor induced by BCNU in U87MG-E6 cells.

Cell Cycle Distribution in U87MG-E6 Cells after BCNU Exposure. Given the absence of p21WAF1 in U87MG-E6 cells and the role of p21WAF1 in cell cycle regulation, we evaluated the cell
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Figure 2. Levels of p53 and p21WAF1 proteins and p53 transcription activity are depicted. A, Western blot analysis of p53 and p21WAF1 levels in U87MG-neo and U87MG-E6 cells (clone 6) sham treated or treated with BCNU. Lanes 1–4, BCNU treated; Lanes 5–8, sham treated. Lane 9, lanes 1–4 are BCNU treated. Lanes 1–2 depict the results 12, 24, 48, 72, and 96 h after BCNU treatment, respectively; Lanes 3 and 4 depict the results 48 and 72 h after such treatment. p53 and p21WAF1 levels increased in U87MG-neo cells only. Forty-eight h after treatment, densitometer analyses indicated that p53 levels had increased 3.5-fold and that p21WAF1 levels had increased 10-fold. B, shown are CAT activities from cell lysates of U87MG-neo cells (Lane 1) and multiple U87MG-E6 clones (Lanes 2–6) transiently transfected with pG13-CAT plasmid containing equal amounts of β-galactosidase activity. U87MG-E6 clones 5 (Lane 2) and 12 (Lane 4) had CAT activities that were 0.06 and 3.5% of that observed in U87MG-neo cells (Lane 1), which was set at 100%. Cm, chloramphenicol; bCm, butyrylated chloramphenicol. The findings depicted in A and B represent the results of at least three independent experiments.

DISCUSSION

In this study, we demonstrate that inactivation of p53 by E6 sensitizes the human astrocytic glioma cell line U87MG to BCNU, the drug of choice for patients with anaplastic astrocytoma and glioblastoma multiforme. Furthermore, sensitization of U87MG-E6 to BCNU is associated with a failure of p21WAF1 induction, transient cell cycle

cycle distributions of U87MG-E6 and U87MG-neo cells in response to BCNU treatment. As shown in Fig. 3, 30 h after exposure there was a dramatic increase in S-phase populations in both U87MG-E6 and U87MG-neo cells compared with sham-treated controls. Ninety-six h after exposure to BCNU, the G2-M phase population had increased substantially in U87MG-E6 cells, whereas U87MG-neo cells with intact p53 function remained arrested in S phase (Fig. 3). Moreover, at 96 h, 20–40% of U87MG-E6 cells had polyploid nuclei with a DNA content of 8n. In addition, there was a substantially larger sub-G1 fraction in U87MG-E6 cells after BCNU exposure, indicating greater cell death in the absence of p53 function (Fig. 3). Although the precise mechanism of enhanced killing of E6 cells remains to be elucidated, exposure to BCNU did not appear to trigger substantial programmed cell death in either U87MG-E6 or -neo cells. Low molecular weight DNA ladders were not detected in either U87MG-E6 or -neo cells exposed to BCNU (data not shown). Moreover, after BCNU treatment, TdT-mediated dUTP nick end labeling was observed in <10% of both U87MG-E6 and -neo cells (data not shown).

Decreased DNA Repair in p53-inactivated U87MG-E6 Cells. A host cell reactivation assay was used to assess DNA repair capability in U87MG-E6 and -neo cells. Prolonged induction of p21WAF1 in U87MG-neo cells by BCNU, and a report of enhanced DNA repair in glioma cells overexpressing p21WAF1 (13), suggested that the relative resistance of U87MG-neo cells to BCNU might be explained by more effective DNA repair in the presence of intact p53 function. Relative to sham-treated cells, CAT activity increased by 50% in BCNU-treated U87MG-neo cells (Fig. 4). In U87MG-E6 cells, however, CAT activity after BCNU exposure decreased slightly compared with sham-treated cells (Fig. 4). Hence, following exposure to BCNU, enhanced DNA repair occurred in U87MG-neo cells with intact p53 function, whereas U87MG-E6 cells with disrupted p53 function were unable to augment DNA repair following such treatment. Although the specific mechanism by which DNA repair is enhanced in U87MG-neo cells is unknown at present, repair enhancement was not explained by up-regulation of MGMT, a suicide DNA repair protein whose activity has been implicated in BCNU resistance (15); in keeping with a previous report (16), neither MGMT mRNA nor protein were detectable in U87MG cells (data not shown).
arrest, accumulation of polyploid glioma cells, and significant cell death. These findings are congruent with those of Waldman et al. (17), who reported that treatment with anticancer drugs in cells with inactivated p21WAF1 could lead to failure of cell cycle arrest, repetitive abnormal mitoses, and the accumulation of polyploid cells, which then apoptose. In contrast to U87MG-E6, resistance to BCNU in U87MG-neo cells with intact p53 function was associated with up-regulation of p53, prolonged induction of p21WAF1, sustained cell cycle arrest, and significant enhancement of DNA repair. These findings and those of Nutt et al. (7) in mouse astrocytes suggest that DNA repair, not programmed cell death, is the principal consequence of p53 induction in both normal and neoplastic astrocytic cells and raise the further possibility that DNA repair in response to injury is a fundamental property of astrocytes, a cell type with barrier and detoxification functions in the mammalian central nervous system (18).

Following exposure to BCNU, both U87MG-E6 and U87MG-neo cells arrested in S phase. Although unusual, S-phase arrest has been reported previously in some types of cells after exposure to specific cytotoxic drugs. Bunch and Eastman (19), for example, observed that Chinese hamster ovary cells treated with cisplatin arrested in S phase. Following abrogation of S-phase arrest, Chinese hamster ovary cells progressed to G2 and, subsequently, to apoptotic cell death. S-phase arrest in U87MG glioma cells occurred in two steps. The initial step, which appeared promptly in both U87MG-E6 and U87MG-neo cells, was p53 independent. The second step, sustained arrest in S phase, occurred only in U87MG-neo cells with functional wild-type p53. Sustained arrest was associated with a 3.5-fold induction of p53 and a 10-fold induction of p21WAF1; moreover, p21WAF1 induction in U87MG-neo cells was prolonged. In U87MG-E6 cells, however, S-phase arrest was transitory and followed by progression to G2-M. Hence, the relative resistance of U87MG-neo cells to BCNU would appear to be explained by the combined influences of sustained cell cycle arrest and DNA repair enhancement. Conversely, the relative chemosensitivity of U87MG-E6 cells would appear to be attributable to the combined effects of cell cycle checkpoint failure and insufficient DNA repair capacity. Conceptually, our findings are in keeping with those of Bunz et al. (20), who observed that p53 and p21WAF1 together are required for sustained cell cycle arrest (in G2) following DNA damage, and also consistent with the observations of Agami and Bernards (21), who noted that distinct initiation and maintenance mechanisms cooperate to induce cell cycle arrest (in G1) in response to injury.

Future studies will determine whether p53 inactivation is similarly chemosensitizing when U87MG glioma cells are grown in vivo and explore whether p53 disruption renders U87MG sensitive to many types of chemotherapeutic agents, to certain classes of cytotoxic drugs only, or to BCNU. It will also be important to ascertain whether sensitization to BCNU following inactivation of p53 is a feature unique to the human glioma, U87MG, or a property characteristic of astrocytic glioma cells, i.e., whether other human astrocytic cell lines with functional wild-type p53 are also sensitized to BCNU when p53 is inactivated by E6 or by dominant-negative p53 mutant proteins. These studies are also necessary because other investigators who have examined the influence of p53 status on chemosensitivity in glioma cells have yet to discern a consistent effect or pattern (22–24). To date, all investigations of p53 and drug response in glioma have occurred in vitro, and such analyses have limitations (11, 25). Observations in model systems, even those that appear relevant, require confirmation in the clinical setting. U87MG glioma cells resemble de novo glioblastoma (i.e., wild-type p53, PTEN mutation, p16 deletion), but molecular characterization of tumors that have responded, or not, to BCNU will be essential to a fuller appreciation of the molecular determinants of drug response in astrocytic neoplasms. Such studies will bring neuro-oncologists a step closer to rational drug selection for their patients.


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