Overexpressed WAF1/Cip1 Renders Glioblastoma Cells Resistant to Chemotherapy Agents 1,3-Bis(2-chloroethyl)-1-nitrosourea and Cisplatin

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ABSTRACT

Previous studies have shown that the negative cell cycle regulator WAF1/Cip1 is often overexpressed in human gliomas and that WAF1/Cip1 overexpression may be a factor in cancer chemoresistance. We established a doxycycline-inducible WAF1/Cip1 expression system in two glioblastoma cell lines and examined the role of WAF1/Cip1 in their response to the chemotherapy agents 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and cis-diaminedichloroplatinum (cisplatin), in an isoegenic background. Our results showed that the induction of WAF1/Cip1 expression rendered glioma cells resistant to cell death induced by BCNU and cisplatin. Using an in vivo host-cell reactivation DNA repair assay, we demonstrated that WAF1/Cip1 enhances the repair of BCNU-induced DNA damage. We conclude that WAF1/Cip1 allows repair of BCNU- and cisplatin-damaged DNA and protects glioma cells from chemotherapy agent-induced apoptosis. Thus, blocking WAF1/Cip1 production or function may serve as a useful chemosensitization regimen for glioma.

INTRODUCTION

Gliomas are the most common primary tumor in the central nervous system, and glioblastoma multiforme is the most advanced stage (1). Chemotherapy agents used to treat brain tumors include the alkylating agent BCNU (carmustine) and cisplatin (2, 3). Patient response to chemotherapy, however, is rather poor, and median survival of glioblastoma patients is less than 1 year (4). The seriousness of this tumor type is evidenced by its refractory response to current therapy regimens and by the psychosocial and financial costs it inflicts on patients, their families, and society. It is important, therefore, to understand the mechanisms for chemoresistance of gliomas and to develop strategies to improve therapeutic response in glioblastoma patients.

Normal cell proliferation is regulated by essential proteins at checkpoints in the cell cycle. These proteins either stimulate or inhibit cell cycle progression (5, 6). The most studied of the positive regulators are CDKs that associate with cyclins and PCNA, forming a kinase complex (7).

WAF1/Cip1 (also known as p21, Sdi1, Plc1, and CAP20) is a target of p53 transactivation (8) and an inhibitor of a variety of CDKs (9–11). Overexpression of WAF1/Cip1 from a transfected expression vector inhibits the growth of various tumor cells to different degrees in clonogenic assays (8, 12, 13). The WAF1/Cip1 protein also interacts with PCNA directly and inhibits DNA replication independent of cyclin-CDK activity (14). Interaction of WAF1/Cip1 with PCNA may also affect the DNA repair process, because PCNA is an important factor in DNA repair (15).

To understand the role of WAF1/Cip1 in human cancers, we previously analyzed the expression of WAF1/Cip1 in samples of tumors from patients with AML and from patients with glioblastoma. Samples from 17% of the AML patients studied had elevated WAF1/Cip1 expression, and those patients were four times more resistant to chemotherapy than patients with low levels of WAF1/Cip1 (16). Similarly, WAF1/Cip1 was found to be overexpressed in the majority of gliomas, whereas it was expressed at extremely low levels in normal glial cells (17). We hypothesized that elevated WAF1/Cip1 is an important factor in the chemoresistance of gliomas and that WAF1/Cip1 repairs DNA damage caused by chemotherapy agents. Several recent publications support this hypothesis. Using a homologous recombination technique, Waldman et al. (18) established a colon cancer cell line that has the WAF1/Cip1 gene deleted. The WAF1/Cip1-defective cells were more sensitive to apoptosis induced by the chemotherapy agent doxorubicin (Adriamycin) than were parental cells (18). McDonald et al. (19) further demonstrated that these WAF1/Cip1-defective cells were less efficient in repairing DNA damaged by UV radiation or cisplatin than were parental cells. Along the same lines and in contrast to original speculation (20), WAF1/Cip1 did not promote cell death (21) and actually protected certain cancer cells from apoptosis (22, 23).

In this study, we established a stable doxycycline-inducible WAF1/Cip1 expression system in glioblastoma cell lines LN-Z308 and U251. (Doxycycline is a derivative of tetracycline.) This doxycycline turn-on-inducible system (24) allows a target gene to express in cells when doxycycline is added to the cell culture. Using this inducible isogeneic on-inducible system (24) allows a target gene to express in cells when doxycycline is added to the cell culture. Using this inducible isogeneic system, we showed that WAF1/Cip1-induced cells were significantly more resistant than were parental cells to BCNU- and cisplatin-caused cell death. The WAF1/Cip1-induced cells also were better able to repair BCNU-generated DNA damage. Therefore, we identified a novel mechanism of chemoresistance in gliomas and a new target for therapeutic chemosensitization regimens.

MATERIALS AND METHODS

Cells and Reagents. U251 glioblastoma cells were purchased from the American Type Culture Collection (Rockville, MD). LN-Z308 glioblastoma cells were generously provided by Dr. Erwin Van Meir (University Hospital, Lausanne, Switzerland). A mutant p53 is present in U251 cells (25), and no p53 is expressed in LN-Z308 cells (26). Transfection experiments were done using the Superfect method (Qiagen, Inc., Chatsworth, CA), which routinely achieves a 30–40% transfection efficiency.

BCNU, cisplatin, and doxorubicin (Adriamycin) were obtained from The University of Texas M. D. Anderson Cancer Center Physician's Referral Service and by NIH Grants CA67987 and CA55164. To whom requests for reprints should be addressed, at Department of Neuro-Oncology, Box 316, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: (713) 792-3778; Fax: (713) 745-1183; E-mail: 12507@utmdacc.mdadm.utoh.edu.

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2 To whom requests for reprints should be addressed, at Department of Neuro-Oncology, Box 316, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: (713) 792-3778; Fax: (713) 745-1183; E-mail: 12507@utmdacc.mdadm.utoh.edu.

3 The abbreviations used are: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; cisplatin, cis-diaminedichloroplatinum; CDK, cyclin-dependent kinase; PCNA, proliferating cell nuclear antigen; AML, acute myelogenous leukemia; AGAT, O*-alkylguanine-DNA alkyltransferase; tTA, tetracycline transcriptional activator.

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at 20°C for 1-2 days. After rehydration in PBS, cells were resuspended for chemiluminescence system (Amersham), according to the manufacturer’s instructions.

2.5 μg/ml streptavidin-FITC (Becton Dickinson), and 0.05% Triton X-100: cells were washed twice in PBS and fixed using X-100: cells were incubated in this solution for 30 min at room temperature in a cacodylate buffer containing 0.2 M potassium cacodylate, 2.5 mm Tris-HCl (pH 6.6), 2.5 mm CaCl2, 0.25 mg/ml BSA, 7 units of terminal deoxynucleotidyl transferase, and 0.5 nmol biotin-dUTP (all reagents were purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN). After incubation, cells were rinsed twice in cold PBS and resuspended in 100 μl of a solution containing 4X concentrated saline-sodium citrate buffer, 2.5 μg/ml streptavidin-FITC (Becton Dickinson), and 0.05% Triton X-100; cells were incubated in this solution for 30 min at room temperature in the dark and then rinsed in PBS twice. As a control, duplicates from each sample that lack the terminal deoxynucleotidyl transferase enzyme in the dark and then rinsed in PBS twice.

Establishment of WAFI/Cipl Doxycycline-inducible System in Glioma Cells. The tTA vector, containing the full-length mutant tetracycline repressor (terR) and the acidic transcriptional activation domain of the herpes simplex viral protein VP16, and the tetO vector were generously provided by Dr. H. Bujard (University of California, Berkeley, CA; Ref. 24). First, we transfected glioma cells with tTA vector and selected for cells that stably transfected (i.e., that expressed the fusion of mutant terR and transcriptional factor VP16). The 1.1-kb EcoRI fragment of WAFI/Cipl cDNA, including the open reading frame, was then cloned into the tetO vector to generate tetO-WAFI. tetO-WAFI was cotransfected with hygromycin-expression vector into the glioblastoma cells stably transfected with tTA vector. Stable clones were selected in hygromycin for 1 month and further expanded. Western Blotting. Proteins were extracted and analyzed on a SDS-polyacrylamide gel, as described previously (17). After transfer to Immobilon membranes (Millipore, Bedford, MA), the proteins were incubated overnight with antibodies against WAFI/Cipl (generously provided by Wade Harper, Baylor College of Medicine, Houston, TX) and actin (Amersham Corp., Arlington Heights, IL). The levels of protein were analyzed using the enhanced chemiluminescence system (Amersham), according to the manufacturer’s instructions.

Flow Cytometry Assays. An in situ end-labeling assay was used to measure apoptosis. Briefly, 1 × 10^6 cells were washed twice in PBS and fixed using paraformaldehyde (Becton Dickinson, San Jose, CA) at 15 min at 4°C. The cells were washed twice in PBS and resuspended in 5 ml of 70% cold (−20°C) ethanol. The samples were stored at −20°C for 1-2 days. After rehydration in PBS, cells were resuspended for 1 h at 37°C in 40 μl of a cacodylate buffer containing 0.2 M potassium cacodylate, 2.5 mm Tris-HCl (pH 6.6), 2.5 mm CaCl2, 0.25 mg/ml BSA, 7 units of terminal deoxynucleotidyl transferase, and 0.5 nmol biotin-dUTP (all reagents were purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN). After incubation, cells were rinsed twice in cold PBS and resuspended in 100 μl of a solution containing 4X concentrated saline-sodium citrate buffer, 2.5 μg/ml streptavidin-FITC (Becton Dickinson), and 0.05% Triton X-100; cells were incubated in this solution for 30 min at room temperature in the dark and then rinsed in PBS twice. As a control, duplicates from each sample that lack the terminal deoxynucleotidyl transferase enzyme in the incubation buffer were treated according to the same procedure. Cells were then resuspended in 500 μl of PBS and analyzed on a FACScan flow cytometer. The data from 1 × 10^6 cells were collected, stored, and analyzed using Lyssys II software.

DNA Damage and Host Cell Reactivation Assay. Firefly luciferase reporter PGL2-Luc was incubated with 0.2-2 μM BCNU in a cacodylate buffer (pH 7.4) at 37°C for 2-24 h, as described previously (27). Tissue culture cells were transfected with damaged or undamaged PGL2-Luc. As an internal control, Renilla luciferase vector PRL-CMV was included in the transfection. Seventy-two h after transfection, luciferase activity was analyzed with a luminometer, normalized against a Renilla luciferase internal standard, and indicated relative to the luciferase activity obtained for the undamaged PGL2-Luc plasmid. Plasmids and substrates were purchased from Promega (Madison, WI).

**RESULTS**

Establishment of Doxycycline-inducible WAFI/Cipl Expression System in Glioma Cell Lines. To establish the doxycycline turn-on system, LN-Z308 and U251 glioblastoma cells were cotransfected with tTA vector and CMV.neo selection vector. These two glioblastoma cell lines were used, because they express relatively low levels of endogenous WAFI/Cipl (13). After selection with G418, two stable cell lines, tTA-LN-Z308 and tTA-U251, were established. The luciferase-tetO reporter gene construct was transiently transfected into the established cells and tested for inducibility in both cell lines. A 25-fold induction was observed in the transient system (data not shown), demonstrating the feasibility of using the inducible system in these glioma cells. We cloned WAFI/Cipl cDNA into tetO vector. After transfection of tetO-WAFI/Cipl and hygromycin selection vectors into tTA-LN-Z308 and tTA-U251 cells and after 4 weeks of selection in the presence of hygromycin, a series of clones was expanded and tested for WAFI/Cipl inducibility by doxycycline. Several inducible clones demonstrated a 5-10-fold induction of WAFI/Cipl protein levels after treatment with doxycycline for 2 days (Fig. 1). One clone from each cell line (LN-Z308-ind and U251-ind) was used for additional experiments.

First, cell cycle profiles of the clones before and after WAFI/Cipl induction were analyzed by flow cytometry. Insignificant change in growth profile was observed (data not shown), consistent with our previous finding that WAFI/Cipl only moderately inhibited glioma cell growth in clonogenic assays (13).

**Doxycycline Induction of WAFI/Cipl Renders Glioma Cells Resistant to BCNU and Cisplatin.** Three chemotherapy agents, BCNU, cisplatin, and doxorubicin, were used to test the effect of
WAF1/Cip1 confers chemoresistance

**Table 1** WAF1/Cip1 induction protects LN-Z308 cells from cell death

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Viable cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-Dox, -BCNU</td>
<td>97 ± 1</td>
</tr>
<tr>
<td>+Dox, -BCNU</td>
<td>97 ± 1</td>
</tr>
<tr>
<td>-Dox, 40 µg/ml BCNU</td>
<td>14 ± 5</td>
</tr>
<tr>
<td>+Dox, 40 µg/ml BCNU</td>
<td>57 ± 6</td>
</tr>
<tr>
<td>-Dox, -cisplatin</td>
<td>98 ± 1</td>
</tr>
<tr>
<td>+Dox, -cisplatin</td>
<td>97 ± 1</td>
</tr>
<tr>
<td>-Dox, 10 µg cisplatin</td>
<td>42 ± 2</td>
</tr>
<tr>
<td>+Dox, 10 µg cisplatin</td>
<td>76 ± 2</td>
</tr>
</tbody>
</table>

*a* Results were derived from three experiments. Medium was not changed during the 5-day experiments. All cells (attached and detached) were used for the trypan blue assay. A total of 250 live (clear) and dead (blue) cells were counted, and viability was calculated as the percentage of live cells. Dox, doxycycline; -, absence; +, presence.

**Table 2** WAF1/Cip1 induction protects U251 cells from BCNU-induced cell death

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Viable cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-Dox, -BCNU</td>
<td>94 ± 0</td>
</tr>
<tr>
<td>+Dox, -BCNU</td>
<td>94 ± 1</td>
</tr>
<tr>
<td>-Dox, 3.5 µg/ml BCNU</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>+Dox, 3.5 µg/ml BCNU</td>
<td>80 ± 3</td>
</tr>
<tr>
<td>-Dox, 10 µg/ml BCNU</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>+Dox, 10 µg/ml BCNU</td>
<td>60 ± 2</td>
</tr>
<tr>
<td>-Dox, -cisplatin</td>
<td>78 ± 2</td>
</tr>
<tr>
<td>+Dox, -cisplatin</td>
<td>84 ± 3</td>
</tr>
<tr>
<td>-Dox, 1.5 µM cisplatin</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>+Dox, 1.5 µM cisplatin</td>
<td>74 ± 2</td>
</tr>
</tbody>
</table>

*a* Results were derived from three experiments. Medium was not changed during the 7-day experiment. All cells (attached and detached) were used for the trypan blue assay. A total of 250 live (clear) and dead (blue) cells were counted, and viability was calculated as the percentage of live cells. Dox, doxycycline; -, absence; +, presence.

WAF1/Cip1 in glioma cells. The cells were cultured either with or without doxycycline for 24 h, and then different concentrations of chemotherapy agents were added to the cultures. Cell death was monitored by microscopy daily for 7 days. Cell death was indicated by cell round-up and detachment. When differences between WAF1/Cip1-induced and -uninduced cells were observed, cell viabilities were quantitated by trypan blue exclusion assay and in situ end-labeling assay with flow cytometry. For LN-Z308-ind cells, a significant difference in cell death was observed in WAF1/Cip1-induced and -uninduced cells when 40 µg/ml BCNU was used (Fig. 2). The WAF1/Cip1-uninduced cells were rounded up, detached from the culture plate, and aggregated, whereas many of the WAF1/Cip1-induced cells remained healthy and attached to the dish. Similar morphological differences were observed in induced and uninduced cells after treatment with 10 µM cisplatin (data not shown). After collecting both floating and attached cells, we performed trypan blue exclusion assays to measure cell viability. Consistent with the morphological observations, most WAF1/Cip1-uninduced LN-Z308 cells died, whereas WAF1/Cip1-induced cells showed high viability levels (Table 1). For U251-ind cells, when 40 µg/mL BCNU was used, most cells died, and the cell death rates of the WAF1/Cip1-induced and -uninduced cells were not significantly different. However, when the concentration of BCNU was decreased to 3.5–10 µg/mL, cell killing was very different in cells with and without WAF1/Cip1 induction. The doxycycline-treated WAF1/Cip1-induced cells had significantly higher levels of viability than the WAF1/Cip1-uninduced clone (Table 2). This result showed that WAF1/Cip1 conferred resistance to BCNU at different doses in different cell lines. WAF1/Cip1-induced U251 cells also were resistant to 1.5 µM cisplatin-induced cell death, as judged by morphological features (Fig. 2) and quantitated by trypan blue exclusion assay (Table 2). WAF1/Cip1 induction did not cause any differences in cell death in response to doxorubicin (0.2 to 2 µg/mL) in either cell line (data not shown). Cell survival was also examined after 2–3 weeks for long-term effect. Virtually all of the WAF1/Cip1-uninduced, drug-treated U251 and LN-Z308 cells were detached from the culture plate and died, whereas many of the WAF1/Cip1-induced, drug-treated cells were still present on the plates; however, those cells became more elongated and flat and failed to form distinct colonies (data not shown).

Because the trypan blue assay analyzes total cell death, we used the in situ end-labeling assay to examine cellular apoptosis in WAF1/Cip1-induced and -uninduced cells after chemical treatment. In this assay, apoptotic cells are demonstrated by DNA degradation. As summarized in Table 3 and shown in Fig. 3, WAF1/Cip1-induced LN-Z308 cells and U251 cells had fewer DNA-damaged cells than WAF1/Cip1-uninduced cells after treatment with BCNU and cisplatin. These results indicate that WAF1/Cip1 confers resistance to these chemotherapy agents either by protecting cells from DNA damage or by aiding in the repair of damaged DNA.

**Repair of BCNU-damaged DNA in WAF1/Cip1-induced Cells.** Because DNA is an alkylating agent that causes DNA breaking and cross-linking (27, 28), we hypothesized that WAF1/Cip1 protects cells against BCNU-induced death by facilitating the repair of BCNU-induced DNA damage. We tested this hypothesis in host-cell reactivation assays. First, we established in an in vitro system that luciferase reporter DNA plasmid (pG3) can be damaged by BCNU treatment. Plasmid DNA was incubated with BCNU overnight at 37°C in a 20 mM sodium cacodylate buffer (pH 7.4). After precipitation, DNA was...
analyzed on an agarose gel. As seen in Fig. 4, BCNU treatment caused a dose-dependent conversion of normal supercoiled DNA (form I) to nicked forms of DNA (forms II and III). A concentration of 2 mM BCNU caused such severe DNA damage that supercoiled DNA (form I) disappeared. DNA damage induced by cisplatin was also examined using this in vitro assay, but we failed to detect DNA damage as seen with BCNU treatment.

The luciferase reporter plasmids, which were treated with 0.2, 0.75, and 2 mM BCNU, were transfected into the WAF1/CIP1-inducible U251 clone after the cells were incubated either with or without doxycycline for 24 h. The undamaged Renilla luciferase plasmid was cotransfected as an internal control for transfection efficiency. The transfected cells were incubated for an additional 48 h. The firefly and Renilla luciferase activities were then determined on two substrates using a luminometer, and the ratios of firefly luciferase (from the BCNU-treated plasmid):Renilla luciferase (from the control, untreated plasmid) were calculated (Table 4). It is evident that luciferase activity derived from the BCNU-treated plasmid decreased as the BCNU concentration used to treat the plasmids increased, which was consistent with the extent of DNA damage (Fig. 4). The firefly:Renilla luciferase ratios of transfected WAF1/CIP1-induced and -uninduced cells were compared to determine whether WAF1/CIP1 contributes to the repair of damaged firefly luciferase reporter plasmid. DNA damage from 2 mM BCNU was so severe that no DNA repair in WAF1/CIP1-induced cells could be observed (ratios of WAF1/CIP1-induced cells are equivalent to ratios from control cells). In contrast, 2-fold DNA repair was detected in WAF1/CIP1-induced U251 cells when 0.2 or 0.75 mM BCNU-treated firefly luciferase plasmids were transfected (i.e., twice as much firefly luciferase activity was detected when damaged DNA was transfected into WAF1/CIP1-induced cells as in control cells).

**DISCUSSION**

Primary anaplastic glial neoplasms are notoriously refractory to therapy. Although surgery, radiotherapy, and chemotherapy can prolong survival, these treatments usually have a poor therapeutic index and high toxicity. Multiple mechanisms may account for the refractory nature of gliomas. In the case of resistance to alkylating agents, the nitrosourea can first form an alkyl substitution at the O\(^{\alpha}\) position of guanine and then cross-link DNA. AGAT can repair O\(^{\alpha}\)-alkylguanine adducts and thereby confer resistance to tumor cells treated with alkylating agents (29, 30).

Here, we demonstrated a novel mechanism of drug resistance in gliomas. The central player of this mechanism is WAF1/CIP1, the previously known functions of which include inhibition of CDKs and DNA replication. We demonstrated that WAF1/CIP1 can also repair DNA damage and confer to glioma cells chemoresistance to the alkylating agents BCNU and cisplatin.

This study stemmed from two previous studies. Our clinical correlation study of WAF1/CIP1 in human AML first revealed that WAF1/CIP1 positively contributes to the chemoresistance of these cancer cells (16). We then showed that WAF1/CIP1 overexpression constitutes a phenotype of human gliomas (17), which are notorious for their resistance to chemotherapy. Accumulating evidence supports this thesis. In a recent study, WAF1/CIP1 was deleted from a colon cancer cell line and WAF1/CIP1-negative cells were found to be more sensitive to doxorubicin-induced apoptosis (18). Along the same lines, WAF1/CIP1 was found to protect cells from apoptosis during cell differentiation (22, 23, 31, 32).

In this study, we formally tested our hypothesis that WAF1/CIP1 contributes to chemoresistance in glioma cells. To obtain a definitive answer, we established a doxycycline-inducible WAF1/CIP1 expression system to test our hypothesis in an isogenic background. Using inducible clones of two glioma cell lines, we found that WAF1/CIP1-induced cells are more resistant to BCNU- and cisplatin-induced cell death than are WAF1/CIP1-uninduced cells. Our host-cell reactivation assays showed that WAF1/CIP1 confers this resistance at least in part by enhancing DNA repair ability. Therefore, these results provided direct evidence that WAF1/CIP1 contributes to glioma chemoresistance, which explains our initial observation of elevated WAF1/CIP1 expression in tumor cells from patients with primary gliomas. The AGAT is known to provide a mechanism for gliomas to resist BCNU (29, 30). It is possible that WAF1/CIP1 affects AGAT expression and by this means confers chemoresistance. It would be interesting to investigate AGAT activity after WAF1/CIP1 induction in glioma cells. However, alteration of AGAT expression may not be the mechanism of WAF1/CIP1 conferred chemoresistance, because WAF1/CIP1 also renders cells resistant to cisplatin, whereas AGAT does not (33). Nevertheless, considering the finding that WAF1/CIP1 is involved in nuclear factor \(\kappa\)B-dependent gene expression through p300 (34), it is conceivable that WAF1/CIP1 is involved in the expression of chemoresistance genes. Because the p53 gene is defective in both U251 and LN-Z308 cells, the p53 is not involved in this process.

Our study showed that the effect of WAF1/CIP1 on the response of cells to chemotherapy agents is not universal. WAF1/CIP1 induction did not affect the response of the cells to doxorubicin. Failure of WAF1/CIP1 to affect doxorubicin-induced cell death was also reported in a recent study with a colorectal carcinoma cell line (35). In contrast, WAF1/CIP1 was observed to protect a different colon cancer cell line from doxorubicin (18). This difference may reflect that doxorubicin works on different pathways in different types of cancer cells and that WAF1/CIP1 does not cause a general resistance to cell-killing agents. Although doxorubicin is not used for glioma treatment, investigation of the effect of this agent on gliomas may provide additional clues to the mechanism of WAF1/CIP1-associated chemoresistance.

**Table 4 Repair of BCNU-damaged DNA in WAF1/CIP1-induced U251 cells**

<table>
<thead>
<tr>
<th>BCNU (mM)</th>
<th>Control cells</th>
<th>WAF1/CIP1 induced</th>
<th>Doxorubicin treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>50 ± 10</td>
<td>50 ± 10</td>
<td>50 ± 10</td>
</tr>
<tr>
<td>0.75</td>
<td>25 ± 1</td>
<td>25 ± 1</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>2.0</td>
<td>13 ± 1</td>
<td>13 ± 1</td>
<td>13 ± 1</td>
</tr>
</tbody>
</table>

\*Results were derived from three experiments. The numbers shown are the ratios of firefly luciferase activity (from the BCNU-treated plasmid: Renilla luciferase activity (from the control undamaged plasmid). Higher number indicates the presence of more functional firefly luciferase plasmid in cells. Two-fold higher values were seen in WAF1/CIP1-induced cells than in cells without WAF1/CIP1 induction when 0.2 and 0.75 mM BCNU-treated plasmids were transfected.
One mechanism that our results can exclude is a cell cycle-dependent event, for the following reasons: (a) BCNU is a cycle-independent cytotoxic agent, yet WAFI/Cipl induction had a profound protective effect; (b) WAFI/Cipl-induction did not provide protection against doxorubicin-induced cell killing, which occurs predominantly in replicating cells; and (c) WAFI/Cipl induction had little effect on cell cycle profiles of the glioma clones during the course of our experiments.

Our DNA repair studies demonstrated that WAFI/Cipl provides chemoresistance at least partially through playing an active role in DNA repair. This conclusion is supported by a finding by McDonald et al. (19), who found that WAFI/Cipl-deleted colon cancer cells had less DNA repair ability. These investigators further showed that the role of WAFI/Cipl in DNA repair is dependent on its interaction with PCNA: deletion of the PCNA-interaction domain from WAFI/Cipl abolished its contribution to DNA repair. Thus, finding agents that disrupt the interaction of WAFI/Cipl and PCNA as a means of sensitizing glioma cells to chemotherapy agents may be an attractive strategy. Another, perhaps more direct, strategy is to down-regulate WAFI/Cipl expression by delivery of antisense WAFI/Cipl, which is currently under study in our laboratory.

We should point out that our findings and those of McDonald et al., which were derived from in vivo experiments, appear to be contrary to conclusions concerning the role of WAFI/Cipl in DNA replication reached by other groups. Using in vitro purified WAFI/Cipl protein in a reconstitution assay, Li et al. (36) and Shrivij et al. (37) reported that WAFI/Cipl did not inhibit DNA excision repair. Conversely, in a similar in vitro study by Pan et al. (38), WAFI/Cipl was found to inhibit DNA excision repair. One reason that different results were obtained from those in vitro experiments is that added recombinant WAFI/Cipl may be unstable to form specific complexes. In our recent preliminary experiments, we isolated nuclear proteins from WAFI/Cipl-induced and -uninduced cells and performed in vitro DNA repair assay. We failed to detect a marked difference in DNA repair (data not shown), consistent with the notion that the putative WAFI/Cipl DNA repair complexes are not stable. Nevertheless, results from in vivo assays appear to support a positive role for WAFI/Cipl in DNA repair.

It would be interesting to identify two groups of glioma patients who express low and high levels of WAFI/Cipl and investigate whether the low-WAFI/Cipl expressors are relatively more responsive to therapy. Initial results suggest that this pattern may hold for a subpopulation of human glioblastoma cells. From a therapeutic point of view, WAFI/Cipl, which is located downstream from p53, may be a better target for intervention.

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