Attenuation of WAF1/Cip1 Expression by an Antisense Adenovirus Expression Vector Sensitizes Glioblastoma Cells to Apoptosis Induced by Chemotherapeutic 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 renders glioma cells resistant to chemotherapy agents. In this study, we investigated whether down-regulation of WAF1/Cip1 would sensitize gliomas to chemotherapy. An adenoviral vector expressing antisense WAF1/Cip1 was constructed and used to infect D54 glioma cells, which express a high level of endogenous WAF1/Cip1. After D54 cells were infected with antisense WAF1/Cip1 adenovirus, Western blotting revealed a significant decrease in the WAF1/Cip1 protein level. Down-regulation of WAF1/Cip1 alone resulted in the death of many cells. In situ end-labeling assay by flow cytometry revealed that many cells died of apoptosis. Our results show that the attenuation of WAF1/Cip1 expression initiated glioma cell death and sensitized glioma cells to apoptosis induced by 1,3-bis(2-chloroethyl)-1-nitrosourea and cisplatin. Thus, blocking WAF1/Cip1 production may serve as a useful chemosensitization regimen for treating glioma.

INTRODUCTION

Resistance to chemotherapy is a major barrier to the eradication of human glioma and a major reason for the poor survival of glioma patients. It is important, therefore, to understand the mechanisms of chemoresistance in gliomas and to develop new therapeutic strategies to overcome this resistance. Several mechanisms have been shown to contribute to the chemoresistance of brain tumors. In one of these, many drugs fail to reach brain tissues because of the blood-brain barrier. However, progress has already been made toward overcoming this obstacle: agents, such as bradykinin, have been developed that facilitate the crossing of the blood-brain barrier. The second mechanism involves repair of DNA damage caused by DNA-alkylating agents, such as BCNU. The nitrosourea forms an alkyl substitution at the O6 position of guanine and then cross-links with DNA. The enzyme alkylguanine DNA alkyltransferase can remove the alkyl group and restore DNA to its undamaged state, thus conferring resistance. Elevated levels of this enzyme are seen in some gliomas and are associated with clinical resistance to BCNU.

Recently, a new molecule, WAF1/Cip1, has been shown to contribute to cancer chemoresistance. On its face, this seems counterintuitive because: (a) WAF1/Cip1 is a protein known to be involved in cell cycle control (WAF1/Cip1 inhibits cyclin-dependent kinases) and DNA replication (WAF1/Cip1 binds to proliferating cell nuclear antigen; Refs. 21–26); and (b) WAF1/Cip1 is transcriptionally activated by the tumor suppressor p53. Nevertheless, the link was established in a study in which the expression of WAF1/Cip1 in samples of tumors from patients with acute myelogenous leukemia was correlated with the patients’ clinical outcome. Seventeen % of the patients studied had elevated WAF1/Cip1 expression in their leukemias, and those patients were more resistant to chemotherapy than were patients with low levels of WAF1/Cip1. Also, overexpression of WAF1/Cip1 is found in the majority of gliomas, whereas it is expressed at extremely low levels in normal glial cells. It has also been shown that WAF1/Cip1 induction in glioma cells confers resistance to the DNA alkylating agents BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; MOI, multiplicity of infection.

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3 The abbreviations used are: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; MOI, multiplicity of infection.
agents BCNU and cisplatin and increases the cells’ DNA repair ability (20). Furthermore, Waldman et al. (18) established a colon cancer cell line that lacked the WAF1/Cip1 gene and showed that these WAF1/Cip1-defective cells were more sensitive to apoptosis induced by doxorubicin (Adriamycin) than were parental cells (18). Similarly, McDonald et al. (19) demonstrated that these WAF1/Cip1-defective cells were less efficient in repairing DNA damage induced by UV radiation or cisplatin than were parental cells. Loss of WAF1/Cip1 also resulted in increased cell killing by ionizing radiation (27). Other studies have shown that WAF1/Cip1 actually protects certain cancer cells from apoptosis (28–30).

These previous studies demonstrate that overexpression of WAF1/Cip1 is a common phenotype of gliomas and may account for glioma chemoresistance. On the basis of these findings, WAF1/Cip1 emerges as a target for therapeutic intervention. Therefore, in this study, we tested the hypothesis that down-regulation of WAF1/Cip1 expression can sensitize glioma cells to chemotherapeutic-induced cell death. We constructed adenoviral vectors with either antisense or sense WAF1/Cip1 cDNA and used these vectors to infect D54 glioma cells, which express a high level of endogenous WAF1/Cip1. Our results show that adenovirus-mediated transfer of antisense WAF1/Cip1 decreases the expression of endogenous WAF1/Cip1 and greatly enhances the sensitivity of D54 cells to apoptosis induced by BCNU or cisplatin.

MATERIALS AND METHODS

Cells and Chemotherapeutic Agents. D54 glioblastoma cells were purchased from the American Type Culture Collection (Manassas, VA). LN-Z308 glioblastoma cells were generously provided by Erwin Van Meir (University Hospital, Lausanne, Switzerland). These cells were maintained in DMEM/Ham’s F-12 medium supplemented with 10% fetal calf serum at 37°C in an incubator containing 5% CO2. BCNU and cisplatin were obtained from Bristol-Myers Squibb Co. (Princeton, NJ).

Trypan Blue Exclusion Assay. Cell viability was measured using the trypan blue exclusion assay. Harvested cells were mixed with an equal volume of trypan blue dye. Dead cells absorbed the blue dye and appeared blue under a light microscope. For quantitation of cell viability, 250–300 cells were counted, and viability was calculated as the percentage of live (bright) cells in the total cell population.

Construction of Sense and Antisense WAF1/Cip1 Adenoviral Vectors. To generate replication-deficient recombinant viruses carrying either sense or antisense WAF1/Cip1 cDNA, we isolated a 2.1-kb EcoRI fragment from pCEP-WAF1-S (20) and ligated it with pAdE1 CMV/PA, as described previously (31, 32). After restriction enzyme mapping, one vector containing WAF1/Cip1 cDNA in the sense orientation (pW8) and one vector containing WAF1/Cip1 cDNA in the antisense orientation (pW5) were cotransfected with pJM17 into 293 cells to generate recombinant viruses. To produce a large number of viral stocks, the recombinant viruses were harvested from the 293 cells after 36 h of infection and were subjected to two cycles of cesium chloride gradient ultracentrifugation. After dialysis overnight, the stocks of viruses were aliquoted and stored at −80°C until use. The average titers of viral stocks were determined using the plaque assay in triplicate (31, 32).

Infection of Glioma Cells with WAF1/Cip1 Viral Vectors. Glioma cells were seeded 24 h prior to infection and allowed to grow to 50%–70% confluence. Viruses were incubated with cells at a MOI of 65 overnight. The cells were then rinsed once with medium and cultured in medium with or without chemotherapeutic agents for the duration of the experiments. DL317 is a control adenovirus vector that has no WAF1/Cip1 insert.

Western Blotting. Proteins were extracted and analyzed by SDS-PAGE, as described previously (16). After transfer to Immobilon membranes (Millipore, Bedford, MA), the proteins were incubated overnight with antibodies against WAF1/Cip1 (generously provided by Wade Harper, Baylor College of Medicine, Houston, TX) and actin (Amersham, Arlington Heights, IL). The levels of protein were analyzed using the enhanced chemiluminescence system (Amersham) according to the manufacturer’s instructions.

Flow Cytometry Assay. The in situ end-labeling assay was used to measure apoptosis. Briefly, 1 × 10⁶ cells were washed twice in PBS and fixed with methanol and lyzing solution containing paraformaldehyde (Becton Dickinson, San Jose, CA) for 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 dehydration in PBS, cells were resuspended for 1 h at 37°C in 40 µl of a solution containing 0.2 M potassium cacodylate, 2.5 mM Tris-HCl (pH 6.6), 2.5 mM CoCl₂, 0.25 mg/ml bovine serum albumin, 7 units of terminal deoxynucleotidyl transferase, and 0.5 mM of biotin-dUTP (all reagents were purchased from Boehringer Mannheim, Indianapolis, IN). After incubation, cells were rinsed twice in cold PBS and resuspended in 100 µl of a solution containing 4× concentrated saline-sodium citrate buffer, 2.5
mg/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 twice in PBS. As a control, cells from each sample that lack the terminal deoxyribonucleotidyl 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 (Becton Dickinson). The data from 1 × 10^4 cells were collected, stored, and analyzed using Lysys II software (Becton Dickinson).

Electron Microscopy. Cells were scraped from culture dishes and collected by centrifugation. The cell pellet was fixed with a fixative containing 3% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.3) for 1 h at room temperature. The samples were treated with 1% tannic acid in cacodylate buffer for 30 min, rinsed two times in 0.1 M cacodylate-buffered osmium tetroxide for 1 h. The samples were rinsed in distilled water three times for 5 min each, and fixed in cacodylate-buffered uranium acetate for 1 h. The samples were washed again in distilled water three times for 5 min each, dehydrated in increasing concentrations of ethanol, infiltrated, and embedded in Spurr’s low-viscosity medium. The blocks were polymerized for 2 days at 60°C. Thin sections were cut in an LKB Nova Ultramicrotome (Leica, Deerfield, IL) and stained with uranyl acetate and lead citrate in an LKB Ultrastainer. Sections were examined in a JEOL 1200-EX transmission electron microscope at an accelerating voltage of 100 kV.

RESULTS

Down-Regulation of WAF1/Cip1 Expression by Antisense Adenovirus Vector. To modulate the expression of WAF1/Cip1 in human glioma cells, we infected D54 glioma cells with either sense or antisense WAF1/Cip1 adenovirus vectors with a MOI of 50. The infection rate at this MOI was >80% (data not shown). Two days after infection, the cells were collected, and total proteins were isolated for analysis of endogenous WAF1/Cip1 protein level by Western blotting. As shown in Fig. 1, infection with antisense WAF1/Cip1 adenovirus markedly reduced the endogenous level of WAF1/Cip1 in D54 cells. In contrast, infection with sense WAF1/Cip1 adenovirus significantly elevated the endogenous level of WAF1/Cip1. Because WAF1/Cip1 has a moderate growth-inhibitory effect on glioma cells, we also analyzed the cell cycle profile in infected cells. Cells infected with sense WAF1/Cip1 adenovirus had a slightly increased proportion of cells in G1 (74%), and cells infected with antisense WAF1/Cip1 adenovirus had a decreased propor-
Chemosensitization of Glioma by Antisense WAF1/Cip1

One day after infection, the virus-infected cells were treated with one of two chemotherapeutic agents, BCNU or cisplatin. Cell conditions were monitored by light microscopy daily for 3 consecutive days. Interestingly, in the absence of BCNU or cisplatin, antisense WAF1/Cip1 adenovirus-infected D54 cells became unhealthy by day 3, and some of the cells rounded up and detached from plates (data not shown). However, few detached cells were stained positive by trypan blue exclusion assay, and in situ end-labeling assay with DNA degradation (Fig. 2). To gain insight into the state of the infected cells, we analyzed their nuclear structures using electron microscopy. As shown in Fig. 3, many cells infected with antisense WAF1/Cip1 adenovirus had fragmented nuclei, an early indication of apoptosis (Fig. 2). To gain insight into the state of the infected cells, we analyzed their nuclear structures using electron microscopy. As shown in Fig. 3, many cells infected with antisense WAF1/Cip1 adenovirus had fragmented nuclei, an early indication of apoptosis. In contrast, sense WAF1/Cip1 virus-infected cells had a lower incidence of nuclear fragmentation. Therefore, reduction of the WAF1/Cip1 protein level had a deleterious but not fatal effect on D54 cells. The fatal effect was seen after treatment with BCNU or cisplatin. As shown in Fig. 4, D54 cells infected with control virus DL317 alone, sense WAF1/Cip1 virus, or antisense WAF1/Cip1 virus for 24 h and then treated with 55 μg/ml BCNU or 10 μM cisplatin for 3 days. Photographs were taken under an inverse light microscope.

Reduction of WAF1/Cip1 Initiates Apoptosis and Sensitizes D54 Glioma Cells to BCNU and Cisplatin. One day after infection, the virus-infected cells were treated with one of two chemotherapeutic agents, BCNU or cisplatin. Cell conditions were monitored by light microscopy daily for 3 consecutive days. Interestingly, in the absence of BCNU or cisplatin, antisense WAF1/Cip1 adenovirus-infected D54 cells became unhealthy by day 3, and some of the cells rounded up and detached from plates (data not shown). However, few detached cells were stained positive by trypan blue exclusion assay, and in situ end-labeling assay detected no marked DNA degradation (Fig. 2). To gain insight into the state of the infected cells, we analyzed their nuclear structures using electron microscopy. As shown in Fig. 3, many cells infected with antisense WAF1/Cip1 adenovirus had fragmented nuclei, an early indication of apoptosis. In contrast, sense WAF1/Cip1 virus-infected cells had a lower incidence of nuclear fragmentation. Therefore, reduction of the WAF1/Cip1 protein level had a deleterious but not fatal effect on D54 cells. The fatal effect was seen after treatment with BCNU or cisplatin. As shown in Fig. 4, D54 cells infected with control virus DL317 alone, sense WAF1/Cip1 virus, or antisense WAF1/Cip1 virus for 24 h and then treated with 55 μg/ml BCNU or 10 μM cisplatin for 3 days. Photographs were taken under an inverse light microscope.

To further determine whether the cell death was apoptotic, the treated cells were subjected to in situ end-labeling assay with flow cytometry to measure DNA degradation. Consistent with the results of the trypan blue exclusion assay, antisense-WAF1/Cip1 adenovirus-infected D54 cells treated with BCNU or cisplatin had marked DNA degradation (Fig. 2 and Table 2). These results indicated that attenuation of WAF1/Cip1 expression sensitized D54 cells to apoptosis induced by these chemotherapeutic agents. One intriguing observation was that more sense WAF1/Cip1-infected cells were apoptotic than DL317 infected cells after BCNU treatment (Table 2). This may have been due to toxic effects from the extremely high levels of WAF1/Cip1 protein in sense WAF1/Cip1-infected cells (Fig. 1).

### DISCUSSION

Our previous studies demonstrated that WAF1/Cip1 is overexpressed in glioma (17) and that overexpression of WAF1/Cip1 increases glioma’s resistance to the chemotherapeutic agents BCNU and cisplatin (20). These studies revealed a novel mechanism of drug resistance in gliomas and identified a potential new target, WAF1/Cip1, for therapeutic intervention. A straightforward hypothesis was that down-regulation of WAF1/Cip1 would sensitize glioma cells to chemotherapeutic agents. In this study, we tested this hypothesis by using an antisense approach to down-regulate the endogenous WAF1/Cip1 levels in glioma. We first constructed adenoviruses expressing antisense WAF1/Cip1. When we used these viruses to infect D54 cells, which express high levels of endogenous WAF1/Cip1, WAF1/Cip1 was down-regulated, and the infected cells were readily killed by BCNU or cisplatin. Therefore, this in vitro cell

### Table 1 Decrease of WAF1/Cip1 expression sensitizes D54 cells to cell death

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Viable cells (%) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>55 μg/ml BCNU</td>
<td></td>
</tr>
<tr>
<td>Sense WAF1/Cip1</td>
<td>81 ± 5</td>
</tr>
<tr>
<td>Antisense WAF1/Cip1</td>
<td>24 ± 5</td>
</tr>
<tr>
<td>DL317</td>
<td>77 ± 2</td>
</tr>
<tr>
<td>10 μM cisplatin</td>
<td></td>
</tr>
<tr>
<td>Sense WAF1/Cip1</td>
<td>97 ± 1</td>
</tr>
<tr>
<td>Antisense WAF1/Cip1</td>
<td>55 ± 3</td>
</tr>
<tr>
<td>DL317</td>
<td>94 ± 2</td>
</tr>
</tbody>
</table>

* Results represent mean ± SD from three experiments.

### Table 2 Decrease of WAF1/Cip1 expression sensitizes D54 cells to apoptosis

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Apoptotic cells (%) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>55 μg/ml BCNU</td>
<td></td>
</tr>
<tr>
<td>Sense WAF1/Cip1</td>
<td>26 ± 5</td>
</tr>
<tr>
<td>Antisense WAF1/Cip1</td>
<td>77 ± 3</td>
</tr>
<tr>
<td>DL317</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>10 μM cisplatin</td>
<td></td>
</tr>
<tr>
<td>Sense WAF1/Cip1</td>
<td>16 ± 8</td>
</tr>
<tr>
<td>Antisense WAF1/Cip1</td>
<td>70 ± 4</td>
</tr>
<tr>
<td>DL317</td>
<td>18 ± 2</td>
</tr>
</tbody>
</table>

* Results represent mean ± SD from three experiments.
culture study provided evidence that down-regulation of WAF1/Cip1 may be a useful approach to improving the efficacy for chemotherapy for glioma.

Our results show that WAF1/Cip1, indeed, protects cells from the initiation of apoptosis. Reduction of WAF1/Cip1 expression in D54 cells resulted in nuclear breakdown, although marked DNA degradation did not occur and the cells were still alive according to trypan blue exclusion assay. This “primed” state, which perhaps represents an early stage of apoptosis, renders D54 cells vulnerable to further insult inflicted by exposure to DNA-damaging agents.

WAF1/Cip1 is a negative regulator of cell cycle, and we showed that antisense WAF1/Cip1 infection led to less cells in G1 phase and sense WAF1/Cip1 infection led to more cells in G1 phase. One explanation is that increased cell proliferation in antisense WAF1/Cip1-infected cells renders sensitivity to chemotherapy agents because many chemotherapeutic agents preferentially kill cycling cells. However, we excluded this mechanism because BCNU is a cycle-independent cytotoxic agent. Indeed, when apoptosis was analyzed simultaneously with cell cycle distribution in BCNU-treated, antisense WAF1/Cip1-infected cells, apoptosis occurred in cells at all stages of cell cycle (data not shown).

Sensitization of glioma cells to chemotherapy by attenuation of WAF1/Cip1 is an important finding because gliomas are notoriously resistant to chemotherapy, and patients have very short survival as a result. That the attenuation of WAF1/Cip1 alone led glioma cells to a preapoptotic state is not only pleasing but also leads to several inferences. First, nuclear integrity appears to be the front-line defense against cell death. Weakening of this defense can be easily envisioned to result in increased susceptibility of the nucleus to damaging agents, both endogenous and exogenous. Cells at this stage are not physiologically dead, although they may lose their ability to reproduce if the damage is not repaired. Second, WAF1/Cip1 appears to be closely involved in this defense system because down-regulation of WAF1/Cip1 resulted in nuclear fragmentation. One possibility is that WAF1/Cip1 regulates cyclin-dependent kinases/cyclin B kinases that phosphorylate proteins such as nuclear lamins involved in nuclear structure.

Alternatively, D54 cells may have defects in nuclear integrity, and overexpression of WAF1/Cip1 may serve to protect cells from the nuclear alterations, allowing the defective cells to survive and become cancerous. Although highly speculative, this hypothesis can be tested in the future. Notwithstanding the mechanism, the adenovirus-mediated antisense approach is very attractive because it may target tumor cells without affecting normal brain cells, which express extremely low levels of WAF1/Cip1. Future in vivo experiments will shed light on the feasibility of this approach for glioma treatment.

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