Interleukin-1β-converting Enzyme Mediates Cisplatin-induced Apoptosis in Malignant Glioma Cells

Seiji Kondo, Barbara P. Barna, Tatsuo Morimura, Juji Takeuchi, Junying Yuan, Aytacl Akbasak, and Gene H. Barnett

ABSTRACT

Increasing the susceptibility of tumor cells to apoptotic cell death following chemotherapy is of importance to the outcome of cancer treatment. Although the tumor suppressor gene p53 is required for efficient induction of apoptosis by chemotherapeutic agents, it is not the only apoptosis mediator gene. The molecular mechanisms mediating apoptosis following chemotherapy via p53-dependent or p53-independent pathways remain unclear. We show here that cis-diaminedichloroplatinum (cisplatin) induces the expression of interleukin-1β-converting enzyme (ICE), a mammalian homologue of the Caenorhabditis elegans cell death gene ced-3, in murine and human malignant glioma cells during apoptosis regardless of their p53 status. Furthermore, overexpression of the murine ICE gene induces apoptosis in these tumor cells. The apoptosis induced by cisplatin treatment or murine ICE overexpression can be suppressed by the tetrapeptide ICE inhibitor Ac-YVAD-CMK or the apoptosis inhibitors bcl-2 or bcl-2-related bcl-Xi gene. These findings suggest that ICE may mediate apoptosis induced by chemotherapy, and its induction could represent a novel approach for the effective treatment of malignant glioma.

INTRODUCTION

Malignant gliomas are the most common malignant brain tumors and are considered incurable (1). The infiltrative growth pattern of these tumors precludes curative neurosurgery, and tumor cells fail to respond to irradiation, chemotherapy, or immunotherapy (2). Malignancy has been recognized to result not only from enhanced cell proliferation but also from decreased physiological programmed cell death (apoptosis; 3, 4). Apoptosis is a genetically encoded cell death program defined by characteristic morphological and biochemical changes (5, 6) and is a pathway that may be disrupted in tumor cells, conferring a survival advantage. Apoptosis also can be induced in certain cell types by ionizing radiation and many of the DNA-damaging drugs used in cancer treatment (7–12). However, the precise molecular mechanisms regulating apoptosis by DNA damage are unknown. For successful cancer treatment, it may be important to improve our understanding of how apoptosis provoked by DNA damage is regulated.

The tumor-suppressor gene p53 is a nuclear phosphoprotein, which is involved in the regulation of fundamental biological processes in cell proliferation and differentiation (13, 14). Recent reports demonstrate a crucial role for p53 in the induction of some forms of apoptosis (15–17). The p53-inducible gene WAF1/CIP1 has been shown to trigger G1 arrest or apoptosis via a p53-dependent pathway involving inhibition of cyclin-dependent kinases (18). In tumor cells, a loss of wild-type p53 function may occur and may prevent the activation of this p53-WAF1/CIP1 pathway. This failure to induce transcriptionally active p53 may play a role in the unregulated growth of tumors and also in the failure to respond to chemotherapeutic agents, which trigger p53-regulated cell arrest or apoptosis normally (18). In contrast, other studies have demonstrated, however, that not all forms of apoptosis require p53 (7, 16, 19, 20). The molecular mechanisms by which DNA damage can induce apoptosis by not only p53-dependent but also p53-independent pathways are unknown.

Recently, the ICE3 gene, a mammalian homologue of the Caenorhabditis elegans cell death gene ced-3 (21), has been identified as inducer of apoptosis in Rat-1 fibroblasts and chicken ganglion neurons (22, 23). We hypothesized that ICE might play a role in the apoptosis of malignant glioma cells induced by the chemotherapeutic agent cisplatin. Here we report that cisplatin increased the mRNA expression of ICE and induced apoptosis in malignant glioma cells regardless of their p53 status. We also demonstrate that the mICE gene caused malignant glioma cells to undergo apoptosis in a transient transfection assay. This apoptosis, induced by cisplatin treatment and mICE overexpression, was suppressed by the tetrapeptide ICE inhibitor Ac-YVAD-CMK or the bcl-2 and bcl-2-related bcl-Xi genes, which have been shown to inhibit apoptosis previously (24–29).

MATERIALS AND METHODS

Tumor Cells. Malignant glioma GL26 (30), human glioblastoma U87-MG (12), and GB-1 cells (11) were used in this study. Tumor cells were cultured in DMEM (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated FCS (GIBCO), 4 mM glutamine, 50 units/ml penicillin, and 50 g/ml streptomycin.

Chemotherapeutic Reagent. Cisplatin was a generous gift from Nippon Kayaku Co. (Tokyo, Japan). It was obtained in powder form, from which 0.4 mg/ml stock solution was prepared from normal saline as described previously (11, 12).

Cell Viability Assay. The cytotoxic effect of cisplatin on tumor cells was quantified using a modified MTT (Chemicon, Temecula, CA) colorimetric assay (29). Tumor cells were seeded at 104 cells/well (0.1 ml) in 96-well, flat-bottom plates (Corning Glass Co., Corning, NY) and incubated overnight at 37°C. Then, either 5.0 or 10.0 g/ml cisplatin were added to wells. After incubation for 1–4 days, the MTT assay was performed. The statistical significance of the findings was assessed using the paired Student’s t test.

Apopotic Features of Tumor Cells Treated with Cisplatin. To determine whether tumor cells treated with cisplatin display an apoptotic morphology, tumor cells were stained with Hoechst 33258 (8@ml) as described previously (31). Five hundred cells were counted and scored for the incidence of apoptotic chromatin changes under UV fluorescence microscopy. Furthermore, a DNA fragmentation assay was performed using methods previously described (10). The cells were lysed in 1.0 ml buffer consisting of 10 mM Tris-HCl, 10 mM EDTA, and 0.2% Triton X-100 (pH 7.5). After 10 min on ice, the costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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3 The abbreviations used are: ICE, interleukin-1β-converting enzyme; cisplatin, cis-diaminedichloroplatinum; mICE, murine ICE gene; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyldiaminotetrazolium bromide; Hoechst 33258, the DNA-binding fluorochrome bis(benzimidaz)-thiobilirubindihydrochloride; Ab, antibody; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; X-Gal, X-galactosidase.
the lysate was centrifuged (13,000 × g) for 10 min at 4°C in an Eppendorf microtube. Then, the supernatant (containing RNA and fragmented DNA but not intact chromatin) was extracted first with phenol and then with phenol-chloroform:isoamyl alcohol (24:1). The aqueous phase was made to 300 mM NaCl, and nucleic acids were precipitated with 2 volume ethanol and then dissolved in 20 μl TE buffer (10 mM Tris-HCl and 1 mM EDTA (pH 7.5)). Following digestion of RNA with RNase A (0.6 mg/ml at 37°C for 30 min), the sample was electrophoresed in a 2% agarose gel with TE buffer. DNA was then visualized with ethidium bromide staining.

**Immunoblotting Assay.** The soluble protein for immunoblotting was harvested from tumor cells lysed in 500 μl freshly prepared extraction buffer (10 mM Tris-HCl (pH 7), 140 mM sodium chloride, 3 mM magnesium chloride, 0.5% Nonidet P-40, 2 mM phenylmethylsulfonfyl fluoride, 1% aprotinin, and 5 mM DTT) for 20 min on ice as described previously (31). Equal amounts of protein estimated by the Bio-Rad (Richmond, CA) Protein Assay were separated by electrophoresis on a 10 or 12% polyacrylamide gel in SDS and subjected thereafter to electrophoretic transfer to nitrocellulose. Filters were subjected to immunoblotting using the Enhanced Chemiluminescence (Amersham, Arlington Heights, IL) detection system according to the manufacturer's instructions. The specific Abs used were anti-p53 (Ab1 and Ab2; Oncogene Science, Uniondale, NY), PAbl12 (Boehringer Mannheim, Indianapolis, IN), and anti-bcl-2 (Biochemicals, Tokyo, Japan). Equivalent sample loading of the intact protein was confirmed by blotting using the anti-Actin (Oncogene Science) Ab.

**Northern Blot Analysis.** Total RNA was prepared by CsCl gradient ultracentrifugation of guanidine isothiocyanate-lysed malignant glioma cells as described (32). Twenty μg denatured total RNA were electrophoresed through a 1.0% agarose gel containing 6% formaldehyde and were then transferred to a Magna NT nylon membrane, UV cross-linked, and baked. The following [32P]dCTP-labeled cDNA probes were used for hybridization: human p53 (Oncogene Science), WAF1 (kindly supplied by Dr. B. Vogelstein; Ref. 33), mICE (22), bcl-Xi (26), and human GAPDH (Oncogene Science). The filters were washed, with the final wash in 0.1 × saline-sodium phosphate-EDTA and 0.1% SDS at 55°C. Autoradiography and quantification of band intensities were performed using a Phosphor Imager (Molecular Dynamics, Inc., Sunnyvale, CA). The relative transcript accumulation for a given band was calculated from the ratio of counts in the band to counts in the GAPDH band of that same lane.

**ICE Transfection.** To determine whether overexpression of ICE induces apoptosis in malignant glioma cells, the ile-acZ fusion gene (pShactlOZ containing the intact mICE CDNA fused to the Escherichia coli lacZ gene) was used (22). The day before ICE transfection, tumor cells were seeded at 10³ cells/ml in each of the six-well dishes. For each well, each 5 μg pICE-lacZ and control gene (parbgal) constructs were transfected into tumor cells by lipofectamine method as described above and incubated for 24 h. To detect the expression of the chimeric gene in transfected tumor cells, cells were fixed with 1% formaldehyde and 0.2% glutaraldehyde for 5 min, rinsed three times with PBS, and stained in X-Gal buffer [0.4 mg/ml 5-bromo-4-chloro-3-indoxyl β-galactoside, 4 mM KFe(CN)₆, 4 mM K₄Fe(CN)₅·3H₂O, and 2 mM MgCl₂ in 0.1 M sodium phosphate buffer (pH 7.5)] at 37°C for 3 h. Three tumor cells were also stained with Hoechst 33258 as described above to detect the apoptotic morphology.

**ICE Inhibition Assay.** We determined whether a specific tetrapeptide ICE inhibitor, Ac-YVAD-CMK (BACHEM, Inc., Torrance, CA) affects the apoptosis in malignant glioma cells induced by cisplatin. This inhibitor was added (10 μM) to aliquots 1 day prior to adding cisplatin. Then, tumor cells were stained with Hoechst 33258 as described above to detect the apoptotic morphology.

**bcl-2/bcl-Xi Transfection.** Murine glioma GL26 cells were infected by adding the filtered supernatant of the iZ2 fibroblast cells secreting the MPZenNeo(bcl-2) or MPZenNeo virus with incubation for 24 h (24, 27–29). Infected GL26 cells were selected in G418. The production of the bcl-2 gene in tumor cells was assessed by immunoblotting as described above. In addition, human glioma cells were transfected with the pSPFF-Neo plasmid containing bcl-Xi (26) by lipofectamine-mediated gene transfer (GIBCO). The cells were incubated for 5 h in OPTI-MEM medium (GIBCO) containing 5 μg DNA and 10 μl lipofectamine; then, an equal volume of culture medium containing 20% FCS was added without removing the transfection mixture and allowed to continue for incubate for 48 h. Transfectants were selected in G418. bcl-Xi expression was confirmed by Northern blot analysis as described above.

**RESULTS**

**Induction of Apoptosis in Malignant Glioma Cells by Cisplatin.** Exponentially growing GL26, U87-MG, and GB-1 cells were treated with cisplatin. Using a modified MTT assay, as shown in Fig. 1A, cisplatin inhibited the viability of tumor cells in a time-dependent manner. When assayed 2 days after adding cisplatin, about 55% of the GL26 cells displayed a typical apoptotic morphology, including nuclear condensation and fragmentation (Fig. 1, B and C). There was a time-dependent increase in the proportion of all tumor cells undergoing apoptosis (Fig. 1B). Furthermore, the treatment of cisplatin induced DNA fragmentation corresponding clearly to the nucleosome ladders characteristic of apoptosis in GL26 cells after 2 days (Fig. 1D). These data suggested that the process of cisplatin-induced apoptosis in all glioma cell lines used in this study was activated by 2 days after the initiation of treatment.

**Expression of p53 Protein during Apoptosis.** To determine whether cisplatin affected the p53 protein level in tumor cells during apoptosis, three types of anti-p53 protein Abs reacting with different epitopes were used. Untreated GL26 and GB-1 cells expressed the p53 protein (with Ab1, PAb122, and/or Ab2), but cisplatin treatment reduced p53 expression to undetectable levels within 2 days (Fig. 2A). Untreated U87-MG cells expressed no p53 (with Ab1, Ab2, and PAb122), but the expression of p53 was remarkably increased by 2 days after cisplatin treatment.

**Expression of p53, WAF1/CIP1, ICE, and bcl-Xi mRNA during Apoptosis.** To determine whether cisplatin affected the expression of the apoptosis-related genes p53, WAF1/CIP1, ICE, and bcl-Xi during apoptosis, we isolated total cellular RNA from tumor cells prior to and 4, 24, and 48 h after treatment. Northern blot analysis showed that GL26 cells expressed low and relatively constant levels of p53 during apoptosis (Fig. 2, B and C). Only the U87-MG cell line treated with cisplatin showed higher p53 and WAF1/CIP1 mRNA levels than untreated cells. In contrast, treated GB-1 cells showed a remarkable decrease in p53 mRNA compared with untreated cells. The expression of WAF1/CIP1 was undetectable in GL26 and GB-1 cells either before or after treatment. Interestingly, all of the glioma cell lines tested in this study showed a higher level of ICE mRNA after cisplatin treatment than did untreated cells (Fig. 2B). Band intensities were used to quantitate ICE mRNA relative to GAPDH (Fig. 2D). The ICE transcript accumulated significantly during apoptosis in GL26, U87-MG, and GB-1 cells (P < 0.01, 0.005, and 0.01, respectively). These results suggested that the accumulation of ICE expression may play an important role in apoptosis of malignant glioma cells induced by cisplatin.

**Overexpression of mICE Induces Apoptosis in Malignant Glioma Cells.** To examine the function of ICE, we transfected the mICE-lacZ chimeric gene into glioma cells and examined gene expression 24 h later using X-Gal reactivity. In addition, we determined whether ICE overexpression induced apoptosis in tumor cells by DNA staining (Fig. 3). X-Gal-reactive GL26 cells transfected with the mICE-lacZ construct showed apoptotic features such as condensed and fragmented nuclei. In contrast, GL26 cells transfected with the control lacZ (pactoM10Z) construct retained normal nuclear architecture and viability. Transfection of the mICE-lacZ chimeric gene into U87-MG and GB-1 cells also induced apoptosis in both cell lines (Table 1). Thus, our results suggested that overexpression of mICE induced apoptosis in malignant glioma cells.

**Effect of ICE Inhibitor Ac-YVAD-CMK, bcl-2, or bcl-Xi on Apoptosis in Malignant Glioma Cells.** From the results of the Northern blot and ICE transfection assay, we determined whether a specific inhibitor of ICE, Ac-YVAD-CMK, prevents cisplatin-induced apoptosis in malignant glioma cells. In mammals, bcl-2 pre-
ICE MEDIATES CISPLATIN-INDUCED APOPTOSIS IN GLIOMA CELLS

Fig. 1. Apoptotic features of malignant glioma cells induced by cisplatin. A, cytotoxic effect of cisplatin on tumor cells. Tumor cells were seeded at a density of 10^4 cells/well (0.1 ml) in 96-well, flat-bottom plates and incubated at 37°C. GL26, U87-MG, and GB-1 cells were treated with cisplatin (5, 5, and 10 μg/ml, respectively). Viability was determined by a modified MTT assay. Values represent the mean ± SD of results of three independent experiments. B, effect of cisplatin on the percentage of tumor cells exhibiting morphological changes of apoptosis. A total of 500 cells was counted for each data point. Values represent the mean ± SD of results of three independent experiments. C, morphological changes associated with apoptosis in GL26 cells treated with 5 μg/ml cisplatin for 2 days. The cells were then fixed and stained with Hoechst 33258 (×200). D, induction of DNA fragmentation by cisplatin. GL26 cells were treated without (Lane 2) or with 5 μg/ml cisplatin for 1 day (Lane 3) or 2 days (Lane 4). Fragmented DNA was isolated and electrophoresed in a 2.0% agarose gel containing 0.5 μg/ml ethidium bromide. Molecular weight standards of multiples of a 123-bp DNA ladder (GIBCO BRL, Tokyo, Japan) are shown in Lane 1.

vents certain cells from undergoing apoptosis (24–29). Therefore, we also determined whether the apoptosis of malignant glioma cells induced by cisplatin treatment and mICE overexpression could be blocked by bcl-2 and bcl-XL. The expression of bcl-XL was undetectable in any of the parental tumor cells during apoptosis, although bcl-XL-transfected cells showed high levels of expression (data not shown). As shown in Table 1, the administration of Ac-YVAD-CMK inhibited the apoptosis in malignant glioma cells by cisplatin partially. Moreover, overexpression of bcl-2 in GL26 cells resulted in 90% high viability despite 2 days of cisplatin treatment. In addition, U87-MG and GB-1 cells overexpressing bcl-XL also acquired resistance to cisplatin. When the mICE-lacZ construct was transfected into tumor cells that overexpressed bcl-2 or bcl-XL, 75 to 90% of X-Gal-reactive cells retained normal morphology (Table 1). These results indicated
ICE MEDIATES CISPLATIN-INDUCED APOPTOSIS IN GLIOMA CELLS

Fig. 2. Expression of apoptosis-related genes in malignant glioma cells treated with cisplatin. A, expression of the p53 protein in tumor cells. Immunoblotting using anti-p53 protein monoclonal antibodies (Ab1, Ab2, and PAb122) was performed with the equal amounts of proteins. The anti-Actin Ab was used for protein-loading equivalence. Data shown are representative of two independent experiments. B, expression of p53, WAF1/CIP1, ICE, and bcl-XL mRNA in tumor cells treated with cisplatin. Aliquots of 20 μg RNA from each sample were subjected to Northern blotting. The blot was reacted with each probe and rehybridized with a GAPDH-specific probe to control the amount of RNA loaded. Data shown are representative of three independent experiments. C and D, analysis of band intensities on the Northern blot (B). Relative transcript accumulation is expressed as the ratio of counts in p53 (C) and ICE (D) bands to counts to the GAPDH band. Means ± SD were calculated from triplicates.
ICE MEDIATES CISPLATIN-INDUCED APOPTOSIS IN GLIOMA CELLS

Fig. 3. Induction of apoptosis in malignant glioma cells by overexpression of mICE. GL26 cells were transiently transfected with mICE-lacZ and the control pact@3gal' vector and, 24 h later, fixed and stained with Hoechst 33258 following X-Gal staining (×400). The top and bottom panels are from the same fields and at the same magnification, respectively.

that Ac-YVAD-CMK, bcl-2, or bcl-X_L prevents cisplatin or mICE transfection-induced apoptosis in malignant glioma cells.

DISCUSSION

The results with U87-MG cells showing that cisplatin induced the expression of p53 and WAF1/CIP1 during apoptosis are consistent with several recent investigations indicating that DNA-damaging agents induce the accumulation of p53 and WAF1/CIP1 (9, 18, 34). On the other hand, the decrease in p53 protein in GL26 and GB-1 cells during apoptosis may have been due to nonspecific degradation of cells during apoptosis. Taken together, we suggest that cisplatin induced apoptosis in GL26 and GB-1 glioma cells via a p53-independent pathway and in U87-MG glioma cells via a p53-dependent pathway. Intriguingly, we also observed that cisplatin increased the expression of the ICE gene in all three glioma cell lines during apoptosis. Furthermore, overexpression of mICE alone induced apoptosis in all three glioma lines. ICE or the ICE-like, protease-specific inhibitor Ac-YVAD-CMK prevented the apoptosis in malignant glioma cells by cisplatin. Therefore, we suggest that ICE mediates cisplatin-induced apoptosis in glioma cells regardless of p53 status.

ICE is an unusual cytoplasmic cysteine protease that was first isolated from cells of monocytic origin (35, 36). It cleaves at the Asp-116—Ala-117 bond in the inactive intracellular precursor for interleukin-1β to generate the biologically active form of the cytokine. Two groups have cloned cDNA for human ICE and found that it is not related to previously known proteases (37, 38). The recent discovery that the product of ced-3 is similar in sequence to ICE suggests the intriguing possibility that there may be a family of cytoplasmic proteases involved in both the generation of cytokines and the mediation of apoptosis (21). Overexpression of mICE has been shown to induce apoptosis in rat fibroblasts (22) and chicken ganglionic neurons (23). Another member of the ICE family (ICE and ced-3), Nedd-2/Ich-1, also induces apoptosis when transfected into rodent fibroblasts (39, 40). More recent studies suggest that all ICE family members are Asp-specific proteases (41, 42), and that cells may contain a common substrate, which, when cleaved by an appropriate Asp-specific protease, can cause apoptosis (43). The identity of such a substrate, however, is presently unknown. ICE may cause cell death directly by cleaving proteins essential for cell viability proteolytically, and thus the activation of ICE-like proteases may represent a final common pathway of apoptosis (22). However, there is evidence that ICE may be not the only protease in the normal apoptotic pathway, because thymocytes and macrophages from the ICE-deficient mice undergo apoptosis normally (43). There may be two or more proteases that function redundantly in the induction of apoptosis such that the elimination of ICE alone has no effect (43).

The first inhibitor of apoptosis to be identified (24) was the cytoplasmic membrane protein encoded by bcl-2, the gene translocated frequently to an immunoglobulin locus in human follicular lymphoma (44). Overexpression of bcl-2 in normal and tumor cells is shown to prevent apoptosis induced by DNA damage (19, 25, 28, 29). In addition, bcl-2 is a homologue of the C. elegans gene ced-9 (45), which inhibits the activity of ced-3 similar to ICE (21, 22). Recent demonstrations showing that ICE-induced apoptosis can be suppressed by bcl-2 (22, 23) are consistent with our present study. Strasser et al. (19) suggest that Bcl-2 may block apoptosis by inhibiting the activity of the Ced-3/ICE homologue, either by preventing cleavage of the proenzyme, directly interfering with proteolytic activity, or sequestering a relevant target protein. In this study, bcl-2 prevents apoptosis induced by cisplatin treatment and overexpression of mICE. bcl-X_L, a structural and functional homologue of bcl-2 (26),

Table 1  Effect of cisplatin and mICE overexpression on apoptosis in malignant glioma cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GL26</th>
<th>GL26/bcl-2</th>
<th>U87-MG</th>
<th>U87-MG/mICE</th>
<th>GB-1</th>
<th>GB-1/bcl-X_L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cisplatin</td>
<td>65 ± 1.5 (550)</td>
<td>12 ± 0.3 (490)</td>
<td>45 ± 1.1 (540)</td>
<td>7.2 ± 0.2 (530)</td>
<td>37 ± 1.1 (520)</td>
<td>5.2 ± 0.3 (500)</td>
</tr>
<tr>
<td>Cisplatin/Ac-YVAD-CMK</td>
<td>32 ± 2.5 (250)</td>
<td>ND*</td>
<td>28 ± 3.3 (250)</td>
<td>ND</td>
<td>18 ± 2.3 (250)</td>
<td>ND</td>
</tr>
<tr>
<td>Ac-YVAD-CMK</td>
<td>2.2 ± 0.2 (230)</td>
<td>1.9 ± 0.5 (250)</td>
<td>3.5 ± 0.3 (220)</td>
<td>2.5 ± 0.2 (260)</td>
<td>1.5 ± 0.5 (230)</td>
<td>2.1 ± 0.4 (210)</td>
</tr>
</tbody>
</table>

* The numbers in parentheses represent the total numbers of cells counted.
* ND, not determined.
also confers resistance to both cisplatin and mICE expression in tumor cells. Taken together, we hypothesize that the activation of ICE or also confers resistance to both cisplatin and mICE expression in tumor cells. We also thank Dr. James P. McAllister for helpful suggestions and Robert Connelly, Isabel Lewis, Rami Kaakaji, Barbara Jacobs, and Talat Haqqi for technical assistance.

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