Abstract. Cisplatin is a DNA-damaging chemotherapeutic drug that may have a role in the adjuvant chemotherapy of several solid tumors, such as malignant glioblastoma, and the status of p53 tumor suppressor protein is a critical determinant of cisplatin chemosensitivity. In the present study, we showed the relationship of p53 status and chemosensitivity of cisplatin between two human malignant glioblastoma cell lines, A172 and T98G, harboring wild-type and mutant-type p53, respectively. Cisplatin was found to be more cytotoxic to A172 than T98G cells in a time- and concentration-dependent manner. Cisplatin-induced cytotoxicity manifested as apoptosis, characterized by genomic DNA fragmentation, nuclear condensation and an increase in sub-G1 population. Cisplatin induced the accumulation of p53 and p21 proteins in A172 cells, but not in T98G cells. The introduction of the adenovirus-mediated wild-type p53 gene into T98G cells resulted in the decrease of viability as well as the increase in sub-G1 population with p53 accumulation, activation of caspase-3 protease and release of cytochrome c from the mitochondria. These data strongly suggest that the expression of p53 is essential for the cytotoxic effect of cisplatin in human malignant glioma cells, A172 and T98G, and the introduction of apoptotic signal molecules, such as p53, will be beneficial to achieve chemosensitivity in malignant glioma.

Introduction

Gliomas are the common primary brain tumor of the adult human central nervous system. Despite innumerable efforts to develop more effective clinical treatment strategies, the median survival time for patients with WHO grade 4 glioma, glioblastoma multiforme, remains at approximately 1 year (1-4). An important factor contributing to this prognosis is the relative chemoresistance of many gliomas to chemotherapy in general (5-7). Nonetheless, chemotherapy is the most widely used modality in the management of gliomas (8). The chemoresistance of tumor cells to anti-cancer drugs is frequently associated with defects in the signaling pathways leading to apoptosis. The product of the p53 tumor suppressor gene has been shown to be responsible for mediating apoptosis after DNA damage in several cell types (9), and the loss of wild-type p53 can render cells resistant to chemotherapeutic agents or radiation (10,11). Actually, the most common genetic alteration in human cancer, including brain cancer, involves the p53 tumor suppressor gene, and mutation or inactivation of p53 is observed in approximately 50% of human cancers. The p53 tumor suppressor gene is the central integrator of the cellular response to DNA damage, oncogenic transformation, and growth factor withdrawal (12-14). p53 plays a central role in the regulation of DNA-damage-induced apoptosis by up-regulating the transcription of genes encoding Bax, Noxa and p53AIPI, which participate in the intrinsic apoptotic pathway (15-17). The release of cytochrome c from mitochondria is a central event in the death receptor-independent intrinsic pathway of apoptosis (18,19). Cytochrome c together with dATP and Apaf-1 facilitates the activation of caspase-9 protease, which further activates procaspase-3 protease (20,21). Caspase-3 protease cleaves their substrates, including PARP, to lead to apoptotic cell death. It has also been reported that the activation of caspase-3 protease in drug-induced apoptosis modulates the activity of caspase-8 protease in a CD95/Fas receptor-independent manner (22-25). Several studies established the involvement of caspase activation in p53-mediated cell death (26). Recently, the requirement of Apaf-1 or caspase-9 protease for the p53-dependent apoptosis of oncogene-transformed murine embryonic fibroblasts has conclusively been demonstrated (27). In addition to involvement in the regulation of apoptosis, p53 also plays a role in DNA-damage repair processes by transcriptional regulation of several downstream mediators that are involved in cell-cycle regulation. p21/WAF1/CIP1 is
of DNA repair. A known DNA repair system, the nuclear single-strand break repair (SSBR) system, was used in these experiments. SSBR is a system that can repair single-strand breaks in DNA, which is important in maintaining genetic stability during cellular proliferation. The SSBR system was evaluated by measuring the percentage of cells that exhibited a 53BP1 focus, a marker of DNA repair dysfunction. In the presence of DNA-damaging agents, the percentage of 53BP1 focus-positive cells increased, indicating impaired DNA repair. The degree of DNA repair impairment correlated with the cytotoxicity of cisplatin. This finding suggests that the SSBR system plays a role in the cytotoxicity of cisplatin in glioma cells. Further studies are needed to elucidate the mechanisms underlying the relationship between DNA repair and the cytotoxicity of cisplatin.
obtained above was measured by proteolytic cleavage of 100 μM 7-amino-4-methylcoumarin (AMC)-DEVD motif-specific peptide (Calbiochem) as a fluorogenic substrate for 1 h and AMC as a standard in an assay buffer (100 mM HEPES, 10% sucrose, 0.1% CHAPS, pH 7.5, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 2 mM DTT) using an excitation wavelength of 380 nm and emission wavelength of 460 nm. Similarly, cleavage of fluorogenic substrate, Ac-LEHD-7-amino-4-trifluoromethylcoumarin (AFC) (Calbiochem) by caspase-9 protease was measured by spectrofluorometer (Jasco FR-777, Germany) at 405/505 nm.

Flow cytometry. After an appropriate treatment, A172 and T98G cells (1x10^6 cells/ml) were harvested by centrifugation and washed with PBS. The cells were fixed with ice-cold 80% ethanol for 30 min, washed with PBS and then treated with 0.25 ml of 0.5% Triton X-100 solution containing 1 mg/ml propidium iodide for 30 min in the dark. Samples were run through a FACStar Vantage to count the number of cells (Becton Dickinson, San Jose, CA).

Statistical analysis. Each experiment was performed at least three times and all values are represented as means ± SD of triplicates. Statistical comparisons between groups were performed using one-way ANOVA, followed by the Student’s t-test.

Results

Induction of apoptosis and cytotoxicity by cisplatin in human malignant glioblastoma A172 and T98G cells. Previous studies showed that anti-cancer drugs, such as cisplatin, induced apoptosis and that p53 status was thought to be an important factor in explaining the difference in cisplatin chemosensitivity among human glioblastoma cell lines (37,38). However, the molecular mechanisms of cisplatin-induced apoptosis remain unclear. In the present study, human malignant glioblastoma cells, A172 and T98G, were used to study the mechanism of p53 involvement in apoptosis induced by cisplatin. First, we treated the cells with cisplatin for an indicated time and dose and cell viability was measured by MTT assay. As shown in
Fig. 1A, cisplatin decreased the viability of A172 cells in a time- and dose-dependent manner, whereas there was only a marginal effect on T98G cells. Furthermore, cisplatin-induced cytotoxicity in A172 cells showed characteristics of apoptosis, manifested by genomic DNA fragmentation in agarose gel electrophoresis as well as nuclear condensation by Hoechst staining and activation of caspase-3-like protease (Fig. 1B-D) and caspase-9 (data not shown); however, this was to a lesser degree in T98G cells. These data clearly showed the difference in cisplatin sensitivity in the induction of apoptosis between the two cell lines.

Induction of p53 and p21/WAF1/CIP1 proteins by cisplatin in human malignant glioblastoma A172 and T98G cells. To investigate whether the expression of p53 and the p53-response gene, p21/WAF1/CIP1, would affect the cytotoxicity of cisplatin, we performed Western blot analysis and found that the accumulation of p53 and p21 proteins was increased in A172 cells in a time-dependent manner but not in T98G cells (Fig. 2). These data indicate that cisplatin induced the accumulation of p53, followed by p21/WAF1/CIP1, in A172 cells harboring a functional p53 gene; however, this was not the case in T98G cells harboring a mutant p53 gene.

Expression of p53 and p21/WAF1/CIP1 proteins and apoptotic cell death in p53-infected T98G cells. To study whether the different sensitivity of A172 and T98G cells to cisplatin was associated with mutation of the p53 gene, we performed Western blot analysis and found that the expression of p53 and p21 proteins was increased in A172 cells in a time-dependent manner but not in T98G cells (Fig. 2). These data indicate that cisplatin induced the accumulation of p53, followed by p21/WAF1/CIP1, in A172 cells harboring a functional p53 gene; however, this was not the case in T98G cells harboring a mutant p53 gene.

Activation of caspase-3 and PARP degradation in cisplatin-treated A172, T98G cells and Ad.p53-infected T98G cells. To investigate whether the expression of p53 in A172 and T98G cells affected cisplatin-induced apoptotic signaling pathway, the activation of caspase-3 and PARP cleavage was measured by Western blot analysis. As shown in Fig. 4A, cisplatin significantly activated caspase-3 in a time-dependent manner.
in A172 and Ad.p53-infected T98G cells but not in T98G cells. We next examined whether PARP, an intracellular bio-
substrate of caspase-3 protease, was cleaved after cisplatin treatment and found that cleavage of 119 kDa PARP to 85 kDa
was detected at 24 h after cisplatin treatment and maintained for 48 h in A172 and p53-infected T98G cells (Fig. 4B). On
the other hand, cleavage of PARP in T98G cells was detected at 48 h after cisplatin treatment (Fig. 4B). These data suggest
that cisplatin-induced apoptosis was accomplished through p53-mediated caspase-3 activation.

Discussion

Although the action mechanism of cisplatin in various cancer cells is relatively well understood and known to result in the
induction of apoptosis, its effect on brain tumor cells remains poorly characterized. Accordingly, we attempted to identify
and functionally characterize the p53 status that may be involved in cisplatin-induced cell death (39-41). In order to
more clearly understand the role of p53 in cisplatin-induced apoptosis, we employed two glioma cell lines, A172 and T98G
which have wild-type and mutant-type p53 respectively, and examined the possible association of defect in the signal
pathways with difference of sensitivity to cisplatin treatment. Indeed, the results clearly showed that cisplatin induced p53-
mediated apoptosis in A172 through the activation of caspase-3-like protease; however, it only had a marginal effect on
T98G human glioblastoma cells. The introduction of Ad.p53 into T98G cells increased the apoptosis to cisplatin, thus
revealing that cisplatin-induced chemosensitivity was at least partly mediated by the induction of p53 in glioma cells.

It is well known that p53 has to be accumulated and
activated in response to various DNA-damaging agents in
order to induce apoptotic cell death (42) and that p21 is a
universal and potent cdk inhibitor under the control of p53
product (43). Our results clearly showed that cisplatin increased
the p53 level in A172 cells followed by the induction of p21
expression, whereas cisplatin failed to accumulate and
activate p53 and p21 proteins in T98G cells. To identify the
involvement of p53 in the difference of chemosensitivity
between A172 and T98G cells, we transfected wild-type p53,
Ad.p53, in T98G cells and measured the induction of p53
and p21 and the proportion of apoptotic cell death. Our
results clearly revealed that transduction of Ad.p53 enhanced
the expression of p53 and p21, as well as cisplatin cytotoxicity
in T98G cells, thus strongly suggesting that cisplatin induced
p53-mediated apoptosis of both human malignant glioblastoma
A172 and T98G cells via accumulation and activation of p53.

To clearly understand the role of p53 in cisplatin-induced
apoptosis, we assessed caspase-3 activation, PARP cleavage
and mitochondrial cytochrome c release after cisplatin
treatment and found that cisplatin induced conversion of procaspase-3 to active caspase-3 protease, PARP cleavage and mitochondrial cytochrome c release in A172 and Ad.p53-transfected T98G cells, whereas T98G cells failed to show cleavage of caspase-3 and a lesser extent of PARP cleavage and mitochondrial cytochrome c release. Therefore, accumulation and activation of the p53 protein appear to result in p53-dependent apoptotic cell death by activation of caspase-3 protease via the release of cytochrome c from mitochondria in A172 and Ad.p53-transfected T98G cells. These results suggest that there may be two mechanisms for understanding the cisplatin-induced apoptotic signaling pathway in human glioblastoma cell lines: one is that cisplatin induces p53-dependent apoptotic cell death and the other is a different pathway, leading to cleavage of PARP and the release of cytochrome c from the mitochondria.

In conclusion, we demonstrated that p53 played a critical role in cisplatin-induced apoptosis and accumulation and activation of p53 might trigger p53-dependent expression of p21, activation of caspase-3 protease, cleavage of PARP and cytochrome c release in A172 and Ad.p53-transfected T98G human glioblastoma cells. Therefore, the introduction of wild-type p53 in glioma cells lacking functional p53 might be an effective means of extending cisplatin’s therapeutic significance.

References

11. Lowe SW, Ruley HE, Jacks T and Housman DE: p53-dependent apoptosis and mitochondrial cytochrome c release in human glioblastoma cell lines: one is that cisplatin induces p53-dependent apoptotic cell death and the other is a different pathway, leading to cleavage of PARP and the release of cytochrome c from the mitochondria.


