Sensitization of Glioma Cells to Fas-Dependent Apoptosis by Chemotherapy-Induced Oxidative Stress

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Abstract

A prominent feature of glioblastoma is its resistance to death from Fas pathway activation. In this study, we explored the modulation of Fas-induced glioblastoma death with chemotherapeutic agents. Camptothecin significantly increased the glioblastoma cell death response to Fas receptor activation regardless of p53 status. Sublethal concentrations of camptothecin reduced the IC50 of agonistic anti-Fas antibody (CH-11) 10-fold, from 500 to 50 ng/mL, in human U87 glioblastoma cells (p53 wild-type). Cell viability in response to camptothecin, CH-11 alone, and the combination of camptothecin + CH-11 was found to be 84%, 85%, and 47% (P < 0.001), respectively. A similar pattern of relative cytotoxicity was found in U373 cells (p53 mutant). We further examined the pathways and mechanisms involved in this apparent synergistic cytotoxic response. Cell death was found to be predominantly apoptotic involving both extrinsic and intrinsic pathways as evidenced by annexin V staining, cleavage of caspases (3, 8, and 9), increased caspase activities, Smac release, and cytoprotection by caspase inhibitors. Expression of Fas-associated death domain, and not Fas, Fas ligand, or caspase proteins, increased following cell treatment with camptothecin + CH-11. Camptothecin treatment enhanced c-jun-NH2-kinase activation in response to CH-11, but inhibition of c-jun-NH2-kinase did not prevent cell death induced by the combination treatment. Reactive oxygen species, especially H2O2, were elevated following camptothecin treatment; and H2O2 enhanced cell death induced by CH-11. The antioxidants glutathione and N-acetyl-cysteine prevented cell death induced by camptothecin + CH-11. These findings show that camptothecin synergizes with Fas activation to induce glioblastoma apoptosis via a mechanism involving reactive oxygen species and oxidative stress pathways.

Introduction

Glioblastoma is the most common and aggressive primary human brain tumor. Despite considerable progress in modern tumor therapy, the prognosis for patients with glioblastoma has improved only marginally (1). A prominent feature of glioblastoma is its relative resistance to cytotoxic chemotherapy and ionizing radiation. Cellular studies have shown that this resistance could be related to alterations of certain genes involved in the apoptotic response, such as p53, Bcl-2, etc. (2, 3). Eliciting mechanisms by which glioblastoma resists cytotoxic death and developing new strategies to enhance glioblastoma apoptosis are expected to have a major impact on therapeutic outcomes. One promising experimental therapeutic strategy employs the death receptor pathway members Fas and Fas ligand (4). Fas (otherwise known as APO-1, CD95, or TNFRSF6) is a tumor necrosis/nerve growth factor receptor family member (for reviews, see refs. 5, 6). Fas activation, by either ligand or agonistic anti-Fas antibodies, signals caspase-dependent apoptotic cell death in sensitive cells. Activated transmembrane Fas receptors trimerez and recruit the cytoplasmatic adapter protein Fas-associated death domain (7, 8). Recruited Fas-associated death domain interacts with procaspases 8 and 10 through its death effector domain to form a death-inducing signaling complex (8–10). Complexes of Fas-death-inducing signaling complex stimulate caspase 8/10 activation that induces apoptotic cell death by activating caspase 3 either directly or indirectly via the mitochondrial apoptotic pathway. Caspase 8 can also activate the intrinsic/mitochondrial apoptotic pathway by cleaving the BH-3 only protein Bid (10–13). Thus, death receptors are able to commit cells to apoptosis by converging on caspase 3 via multiple mitochondrial-independent and -dependent pathways (8, 9).

Targeting death receptors to trigger apoptosis in tumor cells is an attractive concept for cancer therapy because the cytotoxic effect of death receptor activation is relatively selective to cancer cells compared with normal cells; and death receptor-mediated cell death can be independent of p53 and other antiapoptotic mutations common in cancer (14–16). Fas death receptors have been identified in most glioblastoma cells and expression levels correspond to the grade of tumor malignancy (17–20). However, because the majority of glioblastoma cells are resistant to apoptosis induced by Fas ligand, harnessing the death-inducing pathway in gliomas will require sensitization by other means (21–23). In this study, we explored the possibility of modulating the susceptibility of glioblastoma cells to death receptor activation with chemotherapeutic agents and further investigated the mechanisms involved in this Fas-dependent cell death cascade. We found that there is synergistic cell apoptosis with combining certain chemotherapeutic agents and Fas activation. Production of reactive oxygen species (ROS) in response to the chemotherapeutic agents tested was found to mediate this enhanced cell death response.

Materials and Methods

Reagents. All reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated. Drugs were made in stock and diluted in cell culture medium. Camptothecin was dissolved in 0.1 N NaOH. Adriamycin was dissolved in water. Anti-Fas antibody (clone CH-11) was purchased from Upstate (Charlottesville, VA). Caspase-3, -8, -9 inhibitors were dissolved in DMSO. Glutathione (GSH) and N-acetyl-cysteine stock were dissolved in water.
blotting were according to the recommendations of the manufacturers. All the secondary antibodies conjugated to horseradish peroxidase were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) and were used in Western blotting at 1:1,000 dilution. D-JNK1 was purchased from Alexis (San Diego, CA). CEP-11004 was kindly provided by Cephalon, Inc. (West Chester, PA).

**Cell culture.** U87 and U373 glioblastoma cell lines were originally purchased from American Type Culture Collection (Rockville, MD). U87 cells were grown in Eagle's MEM (Cellgro, Washington, DC) supplemented with 10% fetal bovine serum (FBS; Cellgro), 0.15% sodium bicarbonate (Gibco, Rockville, MD), 1 mmol/L sodium pyruvate (Gibco), 0.1 mol/L nonessential amino acids (Gibco), and 500 μg/ml penicillin-streptomycin (Gibco). U373 cells were grown in Dulbecco's modified essential medium (Gibco) supplemented with 10% FBS, 20 mmol/L HEPES (Gibco), and penicillin-streptomycin. All cells were grown at 37°C in a humidified incubator with 5% CO₂.

**Cell viability assay.** Cell viability was measured using the substrate 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), which is reduced by cells with functional mitochondria and considered a biochemical index of cellular viability. Cells were plated at 5,000 cells/well in 24-well tissue culture plates and cultured for 48 to 72 hours before treatment with different drugs. After 24 hours of treatment, MTT was added to each well at a final concentration of 150 μg/ml, and the cells were incubated for 1 to 2 hours at 37°C. The medium was then removed, and the formazan reaction product was dissolved with DMEM and spectrophotometrically quantified at 570 nm using a Spectra MAX 340pc plate reader (Molecular Devices, Sunnyvale, CA). The results are expressed as a percentage of absorbance measured in control cultures after subtracting the background absorbance from all values.

**Annexin V and propidium iodide staining.** Apoptosis was quantified using the annexin V-FITC apoptosis kit (BD Biosciences, San Diego, CA) according to the instructions of the manufacturer. Briefly, U87 cells were trypsinized (Gibco), pelleted by centrifugation, and resuspended in annexin V binding buffer (150 mmol/L NaCl, 18 mmol/L CaCl₂, 10 mmol/L HEPES, 5 mmol/L KCl, 1 mmol/L MgCl₂). FITC-conjugated annexin V (1 μg/ml) and propidium iodide (50 μg/ml) were added to cells and incubated for 30 minutes at room temperature in the dark. Analyses were done on a FACScan (Becton Dickinson, Mountain View, CA). The data were analyzed with CellQuest software (Becton Dickinson).

**Caspase activity assays.** The activities of caspases 3 and 8 were measured using the colorimetric assay kit (Sigma) according to the instructions of the manufacturer. Briefly, cells grown in 10 cm diameter culture dishes were treated with drugs for 24 hours and harvested by centrifugation. The cell pellets were lysed with 100 μL lysis buffer and the lysates were diluted with 50 μL of 2% assay buffer and incubated with 200 μmol/L p-nitroanilide–conjugated colorimetric substrate of either caspase 3 or caspase 8. After incubation for 1 to 2 hours at 37°C, released p-nitroanilide was detected using a Spectra Max 340pc plate reader (Molecular Devices). Protein concentration was determined using the Coomassie Protein Assay Reagent. Thirty micrograms of protein were run on 8% SDS-PAGE gels. Membranes were rinsed, and antibody binding was detected with the enhanced chemiluminescence system (Amersham, Little Chalfont, United Kingdom).

**Western blot analysis.** Cells grown in 10 cm diameter tissue culture dishes were lysed with radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% NP40, 0.25% Na-deoxycholate] containing 1× protease and phosphatase inhibitor cocktail (Calbiochem, San Diego, CA). After sonication for 15 seconds, the suspensions were centrifuged at 3,000 × g for 10 minutes. Protein concentrations were determined using the Coomassie Protein Assay Reagent. Thirty micrograms of protein were subjected to 4% to 20% SDS-PAGE electrophoresis and was transferred to nitrocellular membrane for 1 hour. The membrane was then blocked in 5% nonfat dry milk (Carnation, Nestle Food Co., Glendale, CA) in TBS Tween 20 (TBST) for 1 hour, and then incubated overnight at 4°C in 5% nonfat dry milk or 5% bovine serum albumin containing the primary antibody. Membranes were subsequently rinsed in TBST, and then incubated for 1 hour at room temperature with secondary antibody conjugated with horseradish peroxidase. After incubation, membranes were rinsed, and antibody binding was detected with the enhanced chemiluminescence system (Amersham, Little Chalfont, United Kingdom).

**Statistical analysis.** Statistical analysis consisted of one-way ANOVA followed by the Turkey multiple comparison test using InStat software (GraphPad, San Diego, CA). All experiments reported here represent at least three independent replications. Data are represented as mean ± SE.

**Results**

Synergistic glioma cell death induced by death receptor activation and chemotherapeutic drugs. Human U87 (p53 wild-type) and U373 (p53 mutant) glioblastoma cells express moderate levels of the death receptor Fas (24), but they are not sensitive to cell death induced by death receptor activation (24, 25). We examined the influence of chemotherapeutic reagents on glioma cell sensitivity to Fas-activating agonistic anti-Fas antibody (CH-11). In U87 glioblastoma cells, viability as measured by MTT assay was reduced only at high CH-11 concentrations (Fig. 1). Similarly, cytotoxic reagents such as the topoisomerase inhibitor camptothecin or Adriamycin as single agents minimally affected U87 cell viability in the investigated concentration ranges (Fig. 1). However, cell death was prominent in response to the simultaneous application of CH-11 with either camptothecin or Adriamycin for 24 hours (Fig. 1). U87 cell viability as determined by MTT in response to camptothecin alone (50 μmol/L), CH-11 alone (50 μg/mL), and the combination of camptothecin + CH-11 was found to be 84%, 85%, and 47%, respectively (Fig. 1B). Camptothecin reduced the median inhibitory concentration (IC₅₀) of CH-11 10-fold in U87 cells, from 500 to 50 ng/mL (Fig. 1B). A similar pattern of relative cytotoxicity was found in human U373 glioblastoma cells. U373 cells were not sensitive to CH-11 alone, but coapplication of CH-11 with camptothecin or Adriamycin induced significant cell death (Fig. 1D). We tested other glioblastoma cell lines for their sensitivity to camptothecin and CH-11. A172 (p53 wild-type) and SNB19 (p53 mutant) cells are sensitive to camptothecin alone. The combination of camptothecin with CH-11 (50 μg/mL) induced synergistic cell death in both cell lines (Fig. 1E). Thus, the cytotoxic chemotherapeutic agents examined significantly increased the susceptibility of human glioblastoma cells to death pathway activation regardless of their p53 status.

**Death pathways involved in cell death potentiation by combining CH-11 with cytotoxic reagents.** We used the U87 cell line to study the death pathways activated by combining Fas...
activation with either Adriamycin or camptothecin. Under phase-contrast microscopy, the cell death induced by CH-11 + camptothecin or CH-11 + Adriamycin showed features typical of apoptosis (i.e., cell shrinkage, membrane blebbing, and apoptotic bodies). Apoptosis was quantified by FITC-conjugated annexin V and flow cytometric analysis. In U87 cells, there were ~4% annexin V–positive cells following 24 hours of treatment with camptothecin (100 μmol/L), 9.5% following CH-11 (50 ng/mL) alone, and 32% in response to camptothecin + CH-11 (Fig. 2A and B). Western immunoblotting was used to examine the activation of various caspases under the death-promoting conditions. Caspases 3, 8, and 9 were found to be activated following treatment with CH-11 alone and caspase activation was amplified by combining CH-11 with camptothecin or Adriamycin (Fig. 3A). Caspase-3 and -8 activities were quantified by colorimetric enzyme assay. Compared with control, caspase-3 activity was elevated 17-fold and 22-fold with the combination of CH-11 + Adriamycin or CH-11 + camptothecin, respectively. Caspase-8 activity was elevated 2-fold and 2.6-fold with CH-11 + Adriamycin and CH-11 + camptothecin, respectively (Fig. 3B and C). Furthermore, cell death induced by the combination of camptothecin + CH-11 was inhibited by pharmacologic inhibitors of caspase 3 (Z-DEVD-FMK), caspase 8 (Z-IETD-FMK), and caspase 9 (Z-LEHD-FMK; Fig. 3D). Together, these findings implicate a caspase-dependent cell death pathway that is predominantly apoptotic involving both the extrinsic and intrinsic pathways. To further document activation of the intrinsic apoptotic pathway, we separated U87 mitochondria from cytosol and detected released cytosolic Smac in response to each drug alone.
and cotreatment. Densitometry of Western blots (normalization to actin) revealed that Smac release in response to camptothecin + CH-11 was 32% higher when compared with camptothecin alone and 15% higher when compared with CH-11 alone (Fig. 3A).

Modulation of death receptor pathway by camptothecin. We investigated the mechanisms involved in the cooperative and potentially synergistic cytotoxic response from combining camptothecin with Fas activation. First, we examined effects on expression levels of the principal components of the death receptor pathway, such as Fas, Fas ligand, and Fas-associated death domain. Flow cytometry with anti-Fas and anti–Fas ligand antibody showed no change in the cell surface pool of these proteins following treatment with camptothecin + CH-11 compared with treatment with CH-11 alone (Fig. 4A and B). This was further confirmed by Western blot analysis of whole cell lysates (data not shown). However, Fas-associated death domain expression was moderately increased following treatment with camptothecin alone as well as in combination with CH-11 (Fig. 4C). We also examined the components in the intrinsic apoptosis pathway. Western immunoblotting showed that in response to camptothecin + CH-11, caspases 3 and 8 were cleaved with the reduction of procaspase level (Fig. 3A). However, the total protein levels of procaspases 3 and 8 were not changed by camptothecin alone as different concentrations of camptothecin did not increase or decrease the total procaspase proteins in Western blot (data not shown).
expression levels of other proapoptotic or antiapoptotic proteins, such as Bax, Bcl-2, and Bcl-xL, were not altered in response to camptothecin, CH-11 alone, or both (Fig. 4C).

**Stress-activated protein kinase/c-jun-NH2-kinase, AKT, and mitogen-activated protein kinase pathways do not mediate glioma cell death induced by camptothecin and Fas activation.** We examined the involvement of upstream signaling pathways in the cell death response to Fas activation and camptothecin. Others have suggested that stress-activated protein kinase/c-jun-NH2-kinase (SAPK/JNK) mediates the death response of prostate or ovarian carcinoma cells to the combination of chemotherapeutic drugs and Fas activation (26, 27). We asked if CH-11, camptothecin, or their combination induced JNK phosphorylation (i.e., activation) in U87 glioma cells. JNK1 phosphorylation was gradually increased by either CH-11 or camptothecin treatment alone in a time-dependent manner, with the maximal increase (i.e., 2.5-fold) at 6 hours (Fig. 5A). In response to combination of camptothecin + CH-11, JNK phosphorylation increased as early as 1 hour, with the maximal increase (2.5-fold) at 6 hours (Fig. 5A). Thus, JNK activation was augmented and developed earlier in response to camptothecin + CH-11 when compared with either treatment alone. To test the role of JNK in the cell death response to camptothecin + CH-11, we asked if pretreating U87 cells with the JNK inhibitor D-JNKI1 (1 μmol/L) or an inhibitor of the upstream kinase MKK4/7 (CEP-11004, 10 μmol/L; ref. 28) had a protective effect. Neither inhibitor prevented cell death caused by camptothecin + CH-11 based on MTT assay (Fig. 5B). As a control, we further examined whether CEP-11004 blocked JNK activation. Preincubation with CEP-11004 (10 μmol/L) for 30 minutes inhibited camptothecin + CH-11–induced JNK phosphorylation by ~80% (Fig. 5C). Furthermore, both D-JNKI1 and CEP11004 could reverse U87 cell death induced by JNK activation in response to anisomycin (data not shown). These findings indicate that the enhanced activation of the JNK pathway is not involved in the cell death pathway caused by the combination of camptothecin + CH-11. We also examined components of other survival pathways. Activation of mitogen-activated protein kinase (MAPK), AKT, and nuclear factor κB (NF-κB) was slightly increased with the combined treatment (data not shown). Thus, down-regulation of survival kinase activities such as AKT and MAPK is not involved in the enhanced cell death from combination camptothecin and Fas activation.

**Reactive oxygen species trigger the enhanced cell killing effect from combining camptothecin with Fas pathway activation.** ROS have been found to play a role in the death response to death receptor activation in certain cell types (29–31). We investigated the generation of O2•− and H2O2 in glioma cells by FACS analysis of dihydroethidium and DCFDA oxidation, respectively. There was no significant change in dihydroethidium oxidation in response to either agent alone or their combination (data not shown). In contrast, DCFDA oxidation increased 2-fold within 30 minutes and 2.5-fold after 24 hours of camptothecin treatment. CH-11 had no effect on DCFDA oxidation. In response to camptothecin + CH-11, DCFDA oxidation increased 2-fold within 30 minutes and 5-fold at 24 hours (Fig. 6A). To determine if enhanced oxidative stress mediates the synergistic killing from combining camptothecin with CH-11, cells were preincubated with antioxidants GSH and N-acetyl-cysteine before treatment with camptothecin + CH-11. Both GSH and N-acetyl-cysteine prevented cell death caused by CH-11 + camptothecin in a concentration- and time-dependent fashion as quantified by annexin V/FACS.
(Fig. 6B and C). GSH at 10 or 20 mmol/L inhibited cell death by ~80%, whereas 1 mmol/L inhibited cell death by only 16% (Fig. 6D). Pretreating or simultaneously applying the free radical scavenger with CH-11 + camptothecin showed the same extent of protection. In contrast, adding GSH or N-acetyl-cysteine 4 hours after camptothecin + CH-11 failed to protect against cell death (Fig. 6C). To explore further the role of ROS in potentiating the Fas pathway, we examined the effect of H2O2 on CH-11–dependent apoptosis. Exogenous H2O2 was found to potentiate the ability of CH-11 to induce apoptosis in U87 cells (Fig. 6D). Together these findings support the mechanism by which camptothecin and other chemotherapeutic agents potentiate Fas ligand–mediated glioma cell death via ROS generation and oxidative stress.

Discussion
Malignant gliomas of astrocytic lineage, including astrocytoma, anaplastic astrocytoma, and glioblastoma, are the most common human primary brain tumors. Despite intensive research, the most current treatment modalities have only modestly improved patient survival over the last decades. Developing strategies such as targeting death receptors to trigger apoptosis in tumor cells remains an untested and promising alternative for cancer therapy (1). An obstacle to this approach is that despite the fact that high-grade gliomas express death receptors (such as Fas/CD95) in modest to high levels, glioblastoma cells are usually resistant to apoptosis induced by death receptor ligands. Here, we show that treating glioblastoma cells with either of two conventional chemotherapeutic drugs results in their sensitization to apoptosis induced by Fas activation. The finding that the sensitization of cells to death receptor ligand was independent of p53 status in glioblastomas is particularly relevant to therapeutic considerations in the clinical setting where more than 50% of malignant gliomas exhibit p53 mutations or p53 pathway inactivation secondary to MDM2 gain-of-function (2, 3). We further investigated the cell death pathways enhanced by combining DNA-damage reagents with Fas pathway activation. Both intrinsic and extrinsic apoptosis pathways were found to be activated and required for cell death potentiation.

Cytotoxic drugs can directly target mitochondria or can alter various signaling pathways responsible for modulating cell death and survival. We explored the molecular mechanisms responsible for potentiating Fas-dependent glioma cell death. The mechanisms were dissected by examining signaling pathways known to be engaged by both Fas activation and cytotoxic reagents. There are several pathways shared between chemotherapeutic drug–induced cell death and death receptor–induced apoptosis. These include the death receptor cascade itself (32–34), the caspase signaling cascades (35, 36), the JNK/SAPK signaling pathway (26, 27, 37), and the ceramide pathway (23, 25). We carefully examined these possibilities and ruled out unlikely mechanisms. First, it has been shown that cell death induced by chemotherapeutic drugs and death receptor ligands can converge at the level of the death receptor itself. Mechanisms proposed by others include death receptor up-regulation by p53, ceramide synthesis, JNK/SAPK pathway activation, and NFκB activation (32, 33). Others have reported that ionizing radiation, camptothecin 83, or cis-diaminedichloroplatinum (II) sensitizes glioblastoma multiforme cells to Fas-mediated apoptosis by up-regulating Fas receptor (34, 38). Therefore, we evaluated the effect of CH-11 and camptothecin, alone and in combination, on Fas and Fas ligand expression in our system. We found that treating U87 cells with CH-11 induced only a slight increase in Fas expression, which was not changed by adding camptothecin. There was no detectable Fas ligand on U87 cell surfaces and treatment with either drug alone or in combination with CH-11 did not induce Fas ligand expression. Thus, the up-regulation of Fas or Fas ligand cannot explain the potentiation of Fas-induced cell death by camptothecin or Adriamycin in our study.

Camptothecin was found to moderately increase Fas-associated death domain levels and to potentiate the activation of caspases 8, 9, and 3 by CH-11 without altering levels of their prospective procaspases. In contrast, levels of other proapoptotic and antiapoptotic proteins such as Bax, Bax-xl, Bcl-2, and Bcl-xL were not changed by either camptothecin or CH-11 alone or in combination. These findings suggested that camptothecin might potentiate Fas-mediated apoptotic glioma cell death by enhancing...
Fas-associated death domain expression/stabilization, leading to activation of both extrinsic and intrinsic apoptotic cascades (39–41). Whereas such a mechanism might occur in certain contexts, it does not seem to play a role in the cell death response observed in our studies. This conclusion is based on our findings that neither antioxidants, which protected cells against death induced by camptothecin + CH-11, nor H$_2$O$_2$, which sensitized cells to Fas-induced death, altered Fas-associated death domain levels (data not shown).

JNK, a member of the MAPK family, phosphorylates c-Jun and mediates either cell death or survival depending on specific cell contexts and environment stresses, including UV, DNA-damaging drugs, ionizing radiation, and Fas activation (42, 43). Recent data suggest a close connection between Fas activation-induced cell death and the activation of cellular stress responses and the JNK pathway. Anticancer drugs including topoisomerase I inhibitors can activate the JNK pathway (44). In human prostate cancer cells and ovarian carcinoma cells, several reports indicate that JNK activation and Fas activation synergistically kill cells (27, 45). Consistent with this role in other systems, we found that JNK was activated by either camptothecin or CH-11 alone and the activation was potentiated with combined treatment. However, pharmacologic agents that directly inhibit JNK or inhibit MKK4/7, the upstream kinase of JNK (28), failed to prevent cell death. Thus, in our model, the JNK pathway does not mediate the synergistic cell death from combination Fas activation and camptothecin.

ROS, such as hydrogen peroxide, hydroxyl radicals, and superoxide anion, are highly reactive molecules with unpaired electrons that are generated in normal physiologic processes or via external stress such as UV light, ionizing radiation, and DNA-damaging drugs (46). Cellular defenses to ROS include antioxidant scavengers, such as ascorbate, GSH, and thioredoxin, and antioxidant enzymes, such as superoxide dismutase, catalase, glutathione peroxidase, and thioredoxin reductase (46, 47). The regulation of oxidation-reduction (redox) reactions is critical in cell signaling and programmed cell death. An increase in the generation of ROS, or a reduction in their detoxification, such as from depleting nonoxidized GSH, results in the induction of apoptosis through depolarization and permeabilization of the mitochondrial membrane (48). In fact, many enzymatic and chemical antioxidants abrogate the apoptotic response to different stimuli (29–31). The antiapoptotic Bcl-2 family members Bcl-2 and Bcl-xL have been shown to antagonize ROS production in apoptosis and to protect cells from exogenous oxidant–induced apoptosis (49, 50). A defined mechanistic model of how ROS are produced during apoptosis and how they contribute to the cascade of apoptotic events is still lacking. We chose to examine the role of ROS generation in the synergistic cell killing effect of camptothecin + CH-11. In U87 glioblastoma cells, anti-Fas antibody CH-11 did not generate detectable free radicals as evidenced with FACS analysis. In contrast, camptothecin treatment generated ROS within 30 minutes. This early wave of ROS generation was found to trigger the synergistic cell death pathway because the antioxidants GSH and N-acetyl-cysteine rescued cell death when administered no later than 4 hours following camptothecin treatment. This was confirmed further by our ability to potentiate Fas-mediated cell death by substituting camptothecin with exogenous H$_2$O$_2$. The large increase in ROS generation seen after 24 hours of treatment with camptothecin + CH-11 was
likely generated by cells already committed to apoptosis. Thus, the ROS generated by camptothecin in turn amplifies cell death as related to tumor Fas (APO-1/CD95) expression. Cancer Res 1999;59:1308–18.


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