Impairment of Both Apoptotic and Cytoprotective Signalings in Glioma Cells Resistant to the Combined Use of Cisplatin and Tumor Necrosis Factor α

Lian Duan,1 Masaru Aoyagi,1 Masashi Tamaki,1 Yoshikazu Yoshino,1 Takashi Morimoto,1 Hiroaki Wakimoto,1 Yasuhiro Nagasaka,3 Kimiyoshi Hirakawa,1 Kikuo Ohno,1 and Kiyotaka Yamamoto2

1Department of Neurosurgery, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan; 2Department of Cell Biology, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan; and 3Cytometry Group, Beckman Coulter, Tokyo, Japan

ABSTRACT

Purpose: Tumor necrosis factor (TNF)-α elicits two opposing effects, the induction of apoptosis and the transcription of anti-apoptotic genes. We have recently shown that cisplatin sensitizes glioma cells to TNF-induced apoptosis, but only in some cell lines. To understand the mechanism involved in the different susceptibilities, we examined both the activation of caspases and cytoprotective signaling by TNF-α.

Experimental Design: Caspase activation was examined by estimating the cleavage of substrate peptides and by immunoblot to identify the cleavage of procaspases. Peptide inhibitors of caspases were used to reverse the cytotoxicity. The binding of TNF-α to the receptor was analyzed by flow cytometry. Nuclear factor (NF)-κB activation was assayed by the binding of NF-κB to oligonucleotides containing the consensus binding site. Interleukin (IL)-1β, IL-6, IL-8, and manganese superoxide dismutase (MnSOD) were measured by enzyme-linked immunoassays.

Results: T98G and U87MG underwent apoptosis on treatment with cisplatin and TNF-α, but U373MG and A172 were resistant. Caspases 2, 3, and 6–10, but not caspases 1, 4, and 5, were activated in sensitive cells, and none were activated in resistant cells. The binding of TNF-α to the receptor was the same in all four of the cell lines. In the sensitive cells, NF-κB activation and the production of IL-1β, IL-6, IL-8, and MnSOD were significantly elevated by TNF-α. However, in the resistant cells, the production of IL-1β and IL-6 were specifically impaired in response to TNF-α.

Conclusions: Our results indicate that both apoptotic and cytoprotective pathways are impaired in glioma cells that are resistant to treatment with cisplatin and TNF-α.

INTRODUCTION

Resistance of tumor cells to the induction of apoptosis is one of the main reasons for the failure of anticancer treatments. Tumor necrosis factor (TNF)-α was first described in the serum of endotoxin-treated mice, in which it was found to induce tumor necrosis in vivo and to selectively kill transformed and neoplastic cell lines in vitro.1 TNF-α signaling is transduced through its receptors to simultaneously elicit two opposing effects: the induction of apoptosis and the transcription of anti-apoptotic genes, such as through nuclear factor (NF)-κB and activator protein 1 (AP-1). In certain cell types and under certain conditions, TNF-α can induce apoptosis. However, its clinical use has been limited because numerous tumor cells are naturally resistant to TNF-α-induced apoptosis (4–6).

The binding of TNF-α to TNF receptor (TNFR) results in receptor trimerization (7) and the recruitment of a series of intracellular proteins. Initially, the TNFR-associated death domain (8) binds to TNFR, and then recruits TNFR-associated factor 2 (8), Fas-associated death domain (FADD) (9), and receptor interacting protein (10). Fas-associated death domain interacts with and activates apoptotic proteases, thus triggering cell death (3, 11, 12). TNFR-associated factor 2 has been hypothesized to act mainly by mediating transcription factors NF-κB and activator protein 1 (3, 13, 14). NF-κB has been proposed to switch on the transcription of anti-apoptotic genes (15–17). The first biological role of TNF-stimulated gene expression is to protect cells from TNF cytotoxicity; thus, most TNF-treated cells are resistant to TNF cytotoxicity unless treated with protein or RNA synthesis inhibitors, such as cycloheximide or actinomycin D (18–20).

Recent studies have indicated that the combined use of some anticancer chemotherapeutic drugs and TNF-α can synergistically kill some resistant tumor cells in vitro and in vivo (21–23). We have recently shown that cis-diaminedichloroplatinum (CDDP) or actinomycin D can sensitize glioma cells to TNF-α-induced apoptosis (24). However, the effects of sensitization are limited to a few glioma cell lines. T98G and U87MG cells undergo apoptosis by the combined use of CDDP and TNF-α, but U373MG and A172 cells remain resistant. To understand the mechanism involved in the different susceptibilities, we examined glioma cell lines for both the activation of caspases and the cytoprotective signaling by the combined use of CDDP and TNF-α. The results suggest that both cytoprotec-
tive and apoptotic pathways are impaired in glioma cells that are resistant to the combined use of CDDP and TNF-α.

MATERIALS AND METHODS

Materials. CDDP was a generous gift from Bristol-Myers Squibb (Tokyo, Japan). Human natural TNF-α was a generous gift from Hayashibara Biochemical Laboratories (Okayama, Japan). Interleukin (IL)-1β-converting enzyme inhibitor III (Z-Asp-2, 6-dichlorobenzoyloxymethylketone) was purchased from Takara (Kyoto, Japan). Peptide cell-permeable inhibitors of caspases Ac-YVAD-CHO, Z-DEVD-FMK, Ac-LEVD-CHO, Z-VEID-FMK, Z-IETD-FMK, and Z-LEHD-FMK were obtained from Calbiochem (San Diego, CA), and Z-VDVD-FMK, Z-WEHD-FMK, and Z-AEVD-FMK were obtained from MBL (Medical Biological Laboratories, Nagoya, Japan). SP600125, a Jun NH2-terminal kinase (JNK) inhibitor, was obtained from Calbiochem. U0126, an extracellular signal-regulated kinase (ERK) inhibitor, were obtained from Calbiochem. FMK were obtained from Calbiochem (San Diego, CA), and LEVD-CHO, Z-VEID-FMK, Z-IETD-FMK, and Z-LEHD-FMK were obtained from MBL (Medical Biological Laboratories, Nagoya, Japan). SB203580, a specific p38 mitogen-activated protein kinase (MAPK) inhibitor, and PD98059, an extracellular signal-regulated kinase (ERK) inhibitor, were obtained from Calbiochem. SP600125, a Jun NH2-terminal kinase (JNK) inhibitor, was obtained from BIOMOL (Plymouth Meeting, PA).

Cell Culture. Human malignant glioma cell lines, T98G and A172, were obtained from the Japanese Cancer Research Resources Bank. Human malignant glioma cell lines, U373MG and U87MG, were obtained from the American Type Culture Collection. Cells were maintained in MEM (Life Technologies, England). IL-1β-converting enzyme inhibitor III (100 μg/ml) and/or TNF-α (1000 units/ml) were added together with CDDP and TNF-α to cultures in MEM containing 1% fetal bovine serum, and the number of cells were counted after 24-h incubation.

Western Blot Analysis. The cytosolic lysates were mixed with SDS loading buffer containing DTT and then were boiled for 5 min. Equal amounts of protein, 50 μg/lane, were electrophoresed in a 12.5% SDS-polyacrylamide gel and were transferred to Immobilon P membranes (Millipore, Tokyo, Japan). The membranes were incubated with primary mouse or rabbit antibodies against caspases 7, 8, and 9, and then with alkaline phosphatase-conjugated goat antiserum or rabbit antiserum (ICN, Aurora, OH). The immunoreactive bands were visualized with nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate solution (Roche, Mannheim, Germany). Mouse monoclonal antibodies to human caspase 7 and caspase 8 were obtained from MBL, and the rabbit polyclonal antibody to human caspase 9 was obtained from PharMingen (San Diego, CA). The antibodies recognize the proform as well as the cleaved intermediate or active form of each caspase.

Flow Cytometry by Double Staining with FITC-Labeled Annexin V and PI. Annexin V, a calcium-dependent phospholipid-binding protein with a high affinity for phosphatidylserine, was used to detect the early stages of apoptosis (24, 25). Double staining for FITC-annexin V binding and for cellular DNA using propidium iodide (PI) was performed with an ApoDETECT annexin V-FITC kit (Zymed, San Francisco, CA) as described previously (24). Briefly, the cells were incubated with annexin V-FITC for 10 min in the dark, and then PI was added to the cell suspensions. Cell fluorescence was measured on a flow cytometer (EPICS Elite, Coulter) using an argon ion laser (488 nm). The red (610 nm, PI) and green (525 nm, annexin V) fluorescence emissions were separated optically by separate photomultipliers. The quadrant settings were set so that the negative control allowed less than 1% positivity.

Assay of Caspase Activity. To determine the increased enzymatic activity of caspases in apoptotic cells, we used Colorimetric Assay kits for caspases 3, 8, and 9 (R&D systems, Minneapolis, MN) and for caspases 1, 2, 5, 6, and 10 (MBL). The assay is based on spectrophotometric detection of the chromophore p-nitroaniline (pNA) after cleavage from the substrate peptides conjugated to pNA. The substrate peptides used for the colorimetric assays of caspase classes 1, 2, 3, 5, 6, 8, 9, and 10 were YVAD, VDVD, DEVD, WEHD, VIED, IETD, LEHD, and AEVD, respectively. Cells treated with CDDP and/or TNF-α were collected by centrifugation. The cell pellets were lysed, kept on ice, and then centrifuged. The cell lysates, 100 μg of protein, were incubated with the colorimetric substrate in a reaction buffer containing 10 mM DTT at 37°C for 2 h in a 96-well microplate. The pNA released by cleavage of the peptide was quantified spectrophotometrically at 405 nm in a microtiter plate reader (Bio-Rad, Hercules, CA: Model 550).

Cell Cytoxicity. Subconfluent cultures of glioma cells in MEM supplemented with 1% fetal bovine serum were treated with CDDP (10 μg/ml) and/or TNF-α (1000 units/ml). After 24-h incubation, the numbers of cells were counted with a Coulter counter (Industrial D; Coulter Corporation, Luton, England). IL-1β-converting enzyme inhibitor III (100 μg/ml), a pancaspase inhibitor, and the selective inhibitors (20 μM) of caspases were added together with CDDP and TNF-α to cultures in MEM containing 1% fetal bovine serum, and the number of cells were counted after 24-h incubation.

Flow Cytometric Analysis for TNF-α Binding to the Receptor. Subconfluent cells were detached with 0.5 mM EDTA in PBS and were collected by centrifugation. Cells were incubated with biotinylated recombinant TNF-α for 1 h and then with avidin-fluorescein for 30 min at 4°C in the dark. Unreacted avidin-fluorescein was removed, and the cells were subjected to flow cytometric analysis. Cell fluorescence was measured on a flow cytometer (EPICS Elite, Coulter) using an argon ion laser (488 nm). Polyclonal antibody to human TNF-α was used to block TNF binding to the receptor. Cells were incubated with biotinylated recombinant TNF-α and anti-human TNF-α blocking antibody, and then with avidin-fluorescein. The flow cytometric data were used as negative controls.

NF-κB p50 Assay. NF-κB activation was quantified by estimating the binding of NF-κB to an oligonucleotide containing the NF-κB p50 consensus binding site with a trans-AM NF-κB p50 kit (Active Motif, Carlsbad, CA). Antibodies directed against the NF-κB p50 subunit detect the NF-κB complex bound to the oligonucleotide. Quantification of NF-κB activation was obtained by a colorimetric reaction with a secondary
antibody conjugated with horseradish peroxidase. According to the protocol of the manufacturer, the cytosolic cell lysate was obtained. Equal amounts of protein, 10 μg/well, were used for the assay. The results were obtained by measuring the absorbance at 450 nm with a reference wavelength of 595 nm in a microtiter plate reader (Bio-Rad).

**Immunoassay for IL-1β, IL-6, IL-8, and MnSOD.** Subconfluent cultures of glioma cells in MEM supplemented with 1% fetal bovine serum were treated with TNF-α (1000 units/ml). Conditioned media or cell extracts were collected 2, 12, and 24 h after treatment. The concentrations of IL-6 and IL-8 in the culture medium and those of IL-1β and manganese superoxide dismutase (MnSOD) in the cell extracts were measured with ELISA kits for human IL-1β, IL-6, IL-8 (R & D Systems), and MnSOD (Amersham, Tokyo, Japan). For IL-1β, IL-6, and IL-8, the absorbance was measured at 450 nm, and for MnSOD, it was measured at 490 nm in a microtiter plate reader (Bio-Rad).

**RESULTS AND DISCUSSION**

**Different Susceptibilities of Glioma Cells to Apoptosis by the Combined Use of CDDP and TNF-α.** We previously examined the cytotoxic effects of TNF-α with or without CDDP on human malignant glioma cell lines, T98G, U373MG, A172, and U87MG (24). The results showed that all of the glioma cell lines tested are resistant to TNF-α alone. Although T98G and U87MG cells became sensitive to TNF-α when used in combination with CDDP, U373MG and A172 cells remained resistant.

To determine the occurrence of apoptosis in cells treated with CDDP and TNF-α, we stained the cells with both annexin V-FITC and PI and performed flow cytometry. After treatment with CDDP and TNF-α for 24 h, the annexin-positive and PI-negative cell population (apoptotic cell population) clearly increased in T98G and U87MG cells, but not in U373MG cells, by treatment with CDDP and TNF-α for 24 h. ICE inhibitor III almost eliminated the apoptotic cell population induced by CDDP and TNF-α. Results from three representative cell lines, T98G, U87MG, and U373MG, are shown.

**Activation of Multiple Caspases in Glioma Cells by the Combined Use of CDDP and TNF-α.** To estimate the activation of caspases, we examined the cell cytosolic extracts for their ability to cleave substrate peptides conjugated to pNA. The pNA released from caspase 2-, 3-, 6-, 8-, and 9-like substrates began to be detected in T98G and U87MG cells (24.4% and 27.6%, respectively). IL-1β-converting enzyme inhibitor III, a caspase inhibitor with broad specificity, eliminated the apoptotic cell population induced by CDDP and TNF-α (Fig. 1). The data indicate that treatment with CDDP and TNF-α induces apoptosis only in T98G and U87MG cells.

![Fig. 1 Detection of the apoptotic cell population in glioma cells by flow cytometry using annexin V and propidium iodide (PI). Cultured glioma cells were treated with 10 μg/ml cis-diaminedichloroplatinum (CDDP) and 1000 units/ml tumor necrosis factor (TNF)-α with or without 100 μg/ml interleukin (IL)-1β-converting enzyme inhibitor III (ICE-III) in MEM containing 1% fetal bovine serum for 24 h at 37°C. The cells were detached by brief trypsinization and then were stained with annexin V labeled with FITC (ANNEXIN-FITC) and then with PI. Cell fluorescence was measured on a flow cytometer as described in “Materials and Methods.” The annexin-positive- and PI-negative-cell population (apoptotic cell population) clearly increased in T98G and U87MG cells, but not in U373MG cells, by treatment with CDDP and TNF-α for 24 h. ICE inhibitor III almost eliminated the apoptotic cell population induced by CDDP and TNF-α. Results from three representative cell lines, T98G, U87MG, and U373MG, are shown.](cancerres.aacrjournals.org)
2). No release of pNA from caspase 1- or caspase 5-like substrates was detected in T98G and U87MG. Cell extracts from these glioma cells treated with cis-diamminedichloroplatinum (CDDP) and tumor necrosis factor (TNF)-α were collected at 2, 6, 12, and 24 h. Cell lysates were reacted with p-nitroaniline (pNA)-conjugated substrate peptides. The substrate peptides used for the assays of caspase classes 1, 3, 5, 8, 9, and 10 were YVAD, DEVD, WEHD, IETD, LEHD, and AEVD, respectively. The data using VDVAD and VEID, caspase classes 2 and 6, are not shown. The pNA released by the cleavage of the peptide was quantified spectrophotometrically at a wavelength of 405 nm in a microtiter plate reader.

We then used cell-permeable peptides that can block the activation of specific types of caspases. The cell-permeable peptides were added together with TNF-α and CDDP, and their effects on cell cytotoxicity were estimated by counting the cell numbers after 24 h. The results showed that Z-VDVAD-FMK, Z-DEVD-FMK, Z-VEID-FMK, Z-IETD-FMK, Z-LEHD-FMK, and Z-AEVD-FMK, largely specific for caspase 2, 3, 6, 8, 9, and 10, respectively, significantly reversed the cell cytotoxicity induced by CDDP and TNF-α in T98G and U87MG, whereas Ac-YVAD-CHO, Ac-LEVD-CHO, and Z-WEHD-FMK, largely specific for caspase 1, 4, and 5, respectively, had no effects on cell cytotoxicity (Fig. 3).

We further performed Western blotting to examine the activation of caspase 7 and to confirm the cleavage of the proforms of caspase 8 and 9, the initiators of caspases (Fig. 4). The proforms of caspases are converted to the active subunits during activation. The cleaved bands corresponding to the intermediate or active forms of caspases 7, 8, and 9 began to be detected 12 h after treatment, and their signals increased after 24 h in T98G and U87MG cells. In contrast, no cleavage bands were detected in U373MG and A172 cells (Fig. 4). Interestingly, the band of procaspase 8 in U373MG was extremely weak, whereas those of procaspases 7 and 9 were clearly visualized.

The present model of apoptosis suggests that, after apo-
Reversal of cytotoxicity by peptide inhibitors of caspases. Peptide inhibitors (20 μM) of caspases were added together with cis-diaminedichloroplatinum (CDDP) and tumor necrosis factor (TNF)-α to cultures in MEM containing 1% fetal bovine serum, and the numbers of cells were counted after 24-h incubation. The peptide inhibitors of caspase used were Ac-YVAD-CHO, Z-VDVAD-FMK, Z-DEVD-FMK, Ac-LEVD-CHO, Z-WEHD-FMK, Z-VEID-FMK, Z-IETD-FMK, Z-LEHD-FMK, and Z-AEVD-FMK, largely specific for caspases 1, 2, 3, 4, 5, 6, 8, 9, and 10, respectively. Data are expressed as the ratios to the number of cells treated with CDDP+TNF. Data show the means with SD (bars) calculated from three or more separate experiments. *, P < 0.01, and ns, not significant versus CDDP+TNF by unpaired t test.

Fig. 4 Western blotting for caspases 7, 8, and 9. Cell extracts were collected 2, 6, 12, 24 h after treatment with cis-diaminedichloroplatinum (CDDP) and tumor necrosis factor (TNF)-α. Samples were electrophoresed in a 12.5% SDS-polyacrylamide gel, transferred to Immobilon P membranes, and detected with antibodies against caspases 7, 8, and 9. The antibodies can recognize both proforms and intermediate or active forms: caspase 7 as a 20 kD, caspase 8 as a 43 kD, and caspase 9 as a 48 kD active form. kD, Mr in thousands.

238 Caspases and Cytoprotective Signaling in Gliomas

(29) recently reported that adenovirus-mediated gene transfer of caspase 8 plus an NH2-terminal deleted form of inhibitor κBα can induce apoptosis in U373MG cells in the presence of TNF-α. Taken together with the finding that the levels of procaspase-8 are extremely low in U373 MG, the resistance to CDDP and TNF-α in A172 and U373MG may be due to an impairment in the activation process of caspase 8 through interaction with Fas-associated death domain.

Changes in the Binding of TNF-α to the Receptor. We previously examined the levels of TNFR1 mRNA expression and found them to be essentially the same among four cell lines. TNFR2 mRNA expression was detected only in T98G and U87MG cells, which showed synergistic effects by the combined use of CDDP and TNF-α (24). We suspected possible contributions of TNFR2 to the synergistic effects and investigated the effects of monoclonal antibodies that block TNF-α receptors. The results indicated that TNF-α induces apoptosis in T98G and U87MG cells via signaling through TNFR1 only and that there is no contribution of TNFR2 to the enhanced cytotoxicity caused by the combined use of CDDP and TNF-α (24).

In the present study, we further examined TNF-α binding to the receptors in glioma cells. Glioma cells were incubated with biotinylated TNF-α and then were incubated with avidin–fluorescein. The densities of the stained receptors were determined.

apoptotic stimulation, activation of initiator caspases containing a large prodomain, such as caspases 8/10 and 9, leads to the proteolytic cleavage of downstream executioner caspases. Studies on caspase 8 suggest that this caspase is the most proximal caspase to become activated on ligation of the CD95 (Fas/Apo-1) molecule (11, 12). Recent studies, however, have suggested that caspase 8 is activated not always early but late in the context of CD95 signaling in a mitochondrial-dependent fashion, involving cytoplasmic cytochrome-c release and the activation of caspase 9 (26, 27). The present study indicates that apoptosis occurring in T98G and U87MG cells by treatment with CDDP and TNF-α is induced through the activation of multiple caspases, caspase 2, 3, 6, 7, 8, 9, and 10 and that no caspases, even initiators, are activated in resistant cells, U373MG and A172, by CDDP and TNF-α. No activation of the IL-1β-converting-enzyme subfamilies, caspase 1, 4, and 5, was observed in T98G and U87MG by CDDP and TNF-α. The findings of the activated caspases in the present study are quite consistent with those by Slee et al. (28), who showed caspase 9 is indispensable for cytochrome c-initiated triggering of the death program and occupies an apical point in the caspase cascade in mitochondria. This may suggest that apoptosis induced in T98G and U87MG by CDDP and TNF-α also involves the mitochondrial pathway, although we did not confirm the contribution of cytochrome c during the activation of multiple caspases. It is not clear from the present study whether caspase 8 is activated early, late, or in parallel during apoptosis in T98G and U87MG by CDDP and TNF-α. However, Shinoura et al.

Changes in the Binding of TNF-α to the Receptor. We previously examined the levels of TNFR1 mRNA expression and found them to be essentially the same among four cell lines. TNFR2 mRNA expression was detected only in T98G and U87MG cells, which showed synergistic effects by the combined use of CDDP and TNF-α (24). We suspected possible contributions of TNFR2 to the synergistic effects and investigated the effects of monoclonal antibodies that block TNF-α receptors. The results indicated that TNF-α induces apoptosis in T98G and U87MG cells via signaling through TNFR1 only and that there is no contribution of TNFR2 to the enhanced cytotoxicity caused by the combined use of CDDP and TNF-α (24).

In the present study, we further examined TNF-α binding to the receptors in glioma cells. Glioma cells were incubated with biotinylated TNF-α and then were incubated with avidin–fluorescein. The densities of the stained receptors were determined.

Fig. 3 Reversal of cytotoxicity by peptide inhibitors of caspases. Peptide inhibitors (20 μM) of caspases were added together with cis-diaminedichloroplatinum (CDDP) and tumor necrosis factor (TNF)-α to cultures in MEM containing 1% fetal bovine serum, and the numbers of cells were counted after 24-h incubation. The peptide inhibitors of caspase used were Ac-YVAD-CHO, Z-VDVAD-FMK, Z-DEVD-FMK, Ac-LEVD-CHO, Z-WEHD-FMK, Z-VEID-FMK, Z-IETD-FMK, Z-LEHD-FMK, and Z-AEVD-FMK, largely specific for caspases 1, 2, 3, 4, 5, 6, 8, 9, and 10, respectively. Data are expressed as the ratios to the number of cells treated with CDDP+TNF. Data show the means with SD (bars) calculated from three or more separate experiments. *, P < 0.01, and ns, not significant versus CDDP+TNF by unpaired t test.
by flow cytometric analysis (Fig. 5). The fluorescence intensity of the bound TNF-α was the same in T98G, U373MG, and A172, whereas it was smaller in U87MG than in other cell lines. However, the background fluorescence intensity of U87MG, estimated by using polyclonal blocking antibody, was also smaller than that of other cell lines. This may be attributable to the smaller cell size of U87MG. Collectively, the results showed that the receptor densities are essentially the same in all four of the glioma cell lines.

**Activation of NF-κB and Production of IL-1β, IL-6, IL-8, and MnSOD, by TNF-α in Glioma Cells.** To examine whether the resistance to TNF-α-induced apoptosis is related to the activation of NF-κB, we measured NF-κB p50 activation by TNF-α. The basal level of NF-κB p50 was low in both sensitive
and resistant glioma cell lines. The levels of NF-κB activation increased by treatment with TNF-α in four glioma cell lines in a time-dependent manner, although the levels of NF-κB activation varied among the four cell lines (Fig. 6). Furthermore, CDDP significantly attenuated the levels of activation of NF-κB induced by TNF-α in four glioma cell lines. These observations indicate that NF-κB can be activated by treatment with TNF-α in both sensitive and resistant glioma cell lines and that the inhibition of NF-κB activation by CDDP is not related to the different susceptibilities to the combined use of CDDP and TNF-α among glioma cells.

We next examined the production of cytokines downstream of NF-κB, IL-1β, IL-6, and IL-8, as well as that of MnSOD, a mitochondrial reactive oxygen intermediate also under the control of NF-κB, in TNF-α-stimulated glioma cells. The concentrations of IL-6 and IL-8 in the culture medium and those of IL-1β and MnSOD in cell extracts were measured after stimulating the cells with TNF-α. The steady-state levels of IL-1β, IL-6, IL-8, and MnSOD production in four glioma cell lines remained low during 24 h of incubation. Treatment with TNF-α significantly increased the production of IL-1β, IL-6, IL-8, and MnSOD in T98G and U87MG cells. The production of IL-8 and MnSOD significantly increased in U373MG and A172 cells by TNF-α treatment, whereas the production of IL-1β and IL-6 remained low in these cells (Fig. 7). These results indicate that cytoprotective pathways are also impaired in glioma cells that are resistant to the combined use of CDDP and TNF-α.

In consideration of the different responses between IL-6 and IL-8 release to TNF-α in resistant glioma cells, we further examined the effects of MAPK inhibitors on IL-6 and IL-8
secretion from glioma cells in response to TNF-α (Fig. 8). SB203580 (a p38 MAPK inhibitor) significantly inhibited an increase in IL-6 release from T98G and U87MG cells by TNF-α, whereas SP600125 (a JNK inhibitor) and PD98059 (an ERK inhibitor) did not. SB203580 slightly increased the effects of cytotoxicity on T98G and U87MG cells by CDDP and TNF-α, whereas it had no effect on resistant cell lines (data not shown). SB203580 significantly increased an induction in IL-8 production by TNF-α, not only in sensitive cell lines but also in resistant cell lines. The results indicate that p38 MAPK is also activated in A172 and U373MG cells that showed impairment in IL-6 release by TNF-α. The immunoblot study for p38 MAPK also showed that p38 MAPK is activated by TNF-α in resistant cell lines, although weakly in A172 cells (Fig. 9). Because p38 MAPK was activated even in the steady state of T98G and U87MG cells, activation of p38 MAPK by TNF-α was not clear in these cell lines on immunoblot study.

Relation between Apoptotic and Cytoprotective Signalings. Resistance to TNF-α-induced apoptosis has been explained by the concomitant activation of the transcription factor NF-κB, the target genes of which include caspase inhibitors (15–17, 30, 31). In most cells, the activation of NF-κB is crucial for the inducible expression of the proinflammatory cytokines IL-1β, IL-6, IL-8, and TNF-α (32). MnSOD, located in the mitochondria, is also one of the antiapoptotic enzymes activated by NF-κB, and is essential for cellular resistance to the cytotoxicity of TNF (6, 19, 20, 33). In T98G and U87MG glioma cells that have become sensitive to TNF-induced apoptosis by CDDP treatment, NF-κB activation and the levels of IL-1β, IL-6, IL-8, and MnSOD were all significantly elevated by TNF-α. The result is in line with the previous hypothesis for the resistance to TNF-α-induced apoptosis (15–17). However, in A172 and U373MG cells that are still resistant to TNF-induced apoptosis even with CDDP treatment, the production of IL-1β and IL-6 was not stimulated by TNF-α, although NF-κB activation and the levels of IL-6 and MnSOD were significantly elevated by TNF-α. The result is not as we expected but is consistent with that by Poppenborg et al. (34), who showed the reduced transcription rates of IL-1α, IL-1β, and IL-6 in a CDDP-resistant phenotype of T98G glioma cells. The finding that the impairment in response to TNF is limited to the signalings of IL-1β and IL-6, but is not observed in those of NF-κB, IL-8, and MnSOD, suggests that the site of impairment resides downstream of the TNFRs, consistent with the results of our TNFR assays.

The specific impairment of IL-1β and IL-6 production in response to TNF may also indicate the presence of a distinct signaling pathway independent of NF-κB. Several lines of evidence suggest different regulations of IL-6 and IL-8 secretion from cells in response to TNF-α or other stimuli (35–39). In the case of TNF, the main transcriptional activator for IL-6 gene induction is NF-κB (40). However, the activation of NF-κB and its binding to DNA are not sufficient for IL-6 gene activation by TNF. TNFR-associated factor 2 is also an efficient activator of several MAPKs, including JNK, p38, and ERK 1/2, by TNF-α stimulation (13, 14, 30). The most likely function of JNK and p38 is in the induction of TNF-α-induced activator protein 1 activity (41), which contributes to the induction of TNF-α target genes. Furthermore, JNK and p38 have also been linked to the induction of apoptosis, whereas ERK1/2 is linked to cell survival (42). Beyaert et al. (43) and Vanden Berghe et al. (44) indicated the importance of p38 MAPK pathway as a necessary cooperative mechanism for the transcriptional activity of the IL-6 promoter. In the present study, activation of p38 MAPK, but not JNK nor ERK, contributes to IL-6 secretion in response to TNF-α in glioma cells sensitive to the combined treatment of CDDP and TNF-α. However, the results on IL-8 secretion by TNF-α and those of immunoblot study indicate that p38 MAPK is also activated in the resistant cell lines. The findings suggest that the site of impairment in IL-6 release from the resistant cell lines resides downstream of the p38 MAPK phosphorylation.

Fig. 8  Effects of mitogen-activated kinase inhibitors on interleukin (IL)-6 and IL-8 production from glioma cells stimulated by tumor necrosis factor (TNF)-α. Cultured glioma cells were treated with TNF-α (1000 units/ml) with or without mitogen-activated protein kinase (MAPK) inhibitors. SB203580 (1 μM, IC50 = 0.6 μM), SP600125 (1 μM, IC50 = 0.11 μM), and PD98059 (10 μM, IC50 = 2 μM) are used as a p38 MAPK inhibitor, a JNK (JNK) inhibitor, and an extracellular signal-related kinase (ERK) inhibitor, respectively. The concentrations of IL-6 and IL-8 in the culture medium were measured with ELISA kits for IL-6 and IL-8. Staining was detected by measuring the absorbance at 450 nm in a microtiter plate reader. Data show the means with SD (bars) calculated from three or more separate experiments. ns, not significant, and *, P < 0.01 versus TNF-α by unpaired t test.
Caspases and Cytoprotective Signaling in Gliomas

The specific impairment of cytoprotective signaling potentially relates to the failure in the activation of apical caspases in glioma cells resistant to the combined use of CDDP and TNF-α, although the direct causal relation is not known at present. Additional investigations focusing on the mechanisms of the inactivation of caspases and the impairment of the specific cytoprotective response to TNF-α will facilitate the elucidation of the mechanisms of resistance that are essentially important for the treatment of currently incurable malignant gliomas.

ACKNOWLEDGMENTS

We thank Dr. Margaret Dooley Ohto for reviewing the manuscript.

REFERENCES


Impairment of Both Apoptotic and Cytoprotective Signalings in Glioma Cells Resistant to the Combined Use of Cisplatin and Tumor Necrosis Factor α

Lian Duan, Masaru Aoyagi, Masashi Tamaki, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/10/1/234

Cited articles
This article cites 44 articles, 17 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/10/1/234.full#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/10/1/234.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://clincancerres.aacrjournals.org/content/10/1/234.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.