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Overexpression of Acid Sphingomyelinase Sensitizes Glioma Cells to Chemotherapy

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ABSTRACT

Ceramide has been shown by many studies to induce apoptosis. Therefore, upregulation of ceramide is discussed as a novel approach for tumor treatment. However, it is unknown whether overexpression of acid sphingomyelinase releasing ceramide from sphingomyelin sensitizes cells to chemotherapy and, thus, serves as a potential target to amplify chemotherapy. Here, the authors demonstrate that transfection of human or murine glioma cells with acid sphingomyelinase results in a marked sensitization of glioma cells to gemcitabine and doxorubicin, respectively. Transfected cells responded to chemotherapy with an increased activation of acid sphingomyelinase, elevated ceramide levels, and approximately fourfold higher rates of cell death than control transfected cells. Neutralization of reactive oxygen species prevented these events. The data indicate a significant sensitization of glioma cells to chemotherapy treatment by expression of acid sphingomyelinase and further suggest an activation of acid sphingomyelinase by gemcitabine or doxorubicin, respectively, via reactive oxygen species. *Antioxid. Redox Signal.* 9, 1449–1456.

INTRODUCTION

MALIGNANT GLIOMAS ARE AMONG the most common brain tumors and have a very poor prognosis. Approximately half of the patients with glioma die within 12 months after diagnosis. Gliomas grow very invasively and penetrate the brain diffusely, which very often excludes a surgical treatment of glioma. Therefore, gliomas are commonly treated by chemotherapy in combination with radiotherapy, however with limited success. It is therefore central to define molecules that determine the sensitivity of glioma to chemotherapy as an initial step to develop novel strategies for the sensitization of tumor cells to chemotherapeutic treatment. A study by Riboni *et al*. demonstrated that cellular ceramide levels decreased during the progress from astrocytoma grade I via astrocytoma grade II and III to highly malignant glioma grade IV (19). Acid sphingomyelinase hydrolyzes sphingomyelin to ceramide in the outer leaflet of the cell membrane, which finally results in the formation of ceramide-enriched membrane domains (7, 8). These distinct membrane domains are formed by biophysical properties of ceramide (*i.e.*, the tendency of ceramide molecules to associate with each other to form ceramide-enriched microdomains that spontaneously fuse to large ceramide-enriched membrane macrodomains) (7, 10, 16). We and others previously demonstrated that ceramide-enriched membrane domains serve to cluster activated death receptors, for instance, CD95 or DR5 (3, 5, 7–9), and are central for the induction of apoptosis via these receptors after stimulation with the respective ligand (5, 7). Acid sphingomyelinase is also activated by stress stimuli such as irradiation, UV-light, and some chemotherapeutic drugs, in particular cisplatin, gemcitabine and doxorubicin (11, 12, 14, 15, 17, 20, 21). It was demonstrated that cells

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or mice deficient for acid sphingomyelinase resist the induction of apoptosis by these stimuli, indicating that the acid sphingomyelinase/ceramide system is critically involved in the regulation of the cellular sensitivity to these stress stimuli (12, 15, 17, 20, 21). These studies suggest that a lack of acid sphingomyelinase in tumor cells renders the cells resistant to treatment.

Several experiments indicate an activation of acid sphingomyelinase by reactive oxygen species (5, 13, 18). Thus, it was demonstrated that ligation of DR5 by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) triggers the release of reactive oxygen species (ROS) that mediate a direct or indirect activation of acid sphingomyelinase (5). Furthermore, Cu^{2+} induces a release of oxygen intermediates that stimulate acid sphingomyelinase, events prevented by pretreatment with radical scavengers (13). Finally, a study by Qui *et al.* demonstrated that oxidation of the acid sphingomyelinase at cysteine 629 results in activation of the enzyme *in vitro* (18).

Here, we investigated whether overexpression of acid sphingomyelinase in glioma cells sensitizes the tumor cells to chemotherapy, especially treatment with gemcitabine and doxorubicin. In particular, we studied whether chemotherapy activates acid sphingomyelinase via the release of ROS. Our data demonstrate that overexpression of acid sphingomyelinase in human and murine glioma cells increases the levels of ceramide after chemotherapy and greatly sensitizes the cells to chemotherapeutic treatment. Doxorubicin and gemcitabine activated acid sphingomyelinase via reactive oxygen species, and pretreatment of the tumor cells with antioxidants prevented both acid sphingomyelinase activation/ceramide release and chemotherapy-induced cell death.

METHODS

Cells

The murine and human glioma cell lines Gl-261 and U-373, respectively, were cultured in MEM medium supplemented with 10% FCS, 10 mM HEPES, 2 m*M* L-glutamine, 1 m*M* sodium pyruvate, 100 μM nonessential amino acids, 100 units/ml penicillin, 100 μg/ml streptomycin (all purchased from GIBCO/BRL-Life Technologies, Karlsruhe, Germany). Cells were stably transfected with an expression vector for acid sphingomyelinase (pEF-ASM) (6), inducing a strong expression of acid sphingomyelinase. Control cells were transfected with the empty vector (pEF) (6). Cells were treated with gemcitabine or doxorubicin for the indicated time, if indicated the cells were pretreated with 1 m*M* Tiron for 10 min prior to addition of the chemotherapeutic drugs.

Cell death

Cells were stimulated for 48 h with 0.03, 0.15, or 0.3 μM gemcitabine or doxorubicin, respectively. The culture supernatants were collected, adherent cells were trypsinized, the two fractions were combined and washed three times in HEPES/Saline (132 mm NaCl, 20 mm HEPES (pH 7.4), 5 mm KCl, 1 mm $CaCl_2$, 0.7 mm $MgCl_2$, 0.8 mm $MgSO_4$). Cells were then stained with FITC-Annexin V (Roche, Mannheim, Germany) for 15 min at 22°C and analyzed by flow cytometry.

Alternatively, cells were stained with Trypan Blue and apoptosis was determined 36 or 48 h, respectively, after incubation with the indicated drug by assessing typical changes of apoptotic cells, such as chromatin fragmentation, condensation, or membrane blebbing. The data given in the manuscript display the results obtained with Trypan Blue staining and, thus, show global cell death. However, all data were confirmed by FITC-Annexin V staining.

Acid sphingomyelinase activity

To determine the activity of acid sphingomyelinase, cells were treated with 3 μM gemcitabine or 3 μM doxorubicin for the indicated time in cell culture medium. Higher doses of the drugs were used for measuring activity of acid sphingomyelinase and ceramide levels than for the induction of cell death to obtain a synchronized cell response in these assays. The medium was removed, the cells were lysed in 250 m*M* sodium acetate (pH 5.0), 1.3 m*M* EDTA, and 1% NP-40 for 10 min on ice, the samples were diluted to 0.1% NP-40, 250 m*M* sodium acetate (pH 5.0) and 1.3 m*M* EDTA and the enzyme reaction was initiated by addition of substrate [^{14}C]sphingomyelin. Prior to addition to cell lysates, the substrate [^{14}C]sphingomyelin was dried, resuspended in 250 m*M* sodium acetate (pH 5.0), 1.3 mM EDTA and 0.1% NP-40, micelles were formed by a 10 min bath sonication and an aliquot was added to the cell lysates. The samples were incubated for 30 min at 37°C, the reaction was stopped by addition of 800 μl $CHCl_3$:CH_3OH (vol/vol, 2:1) and 250 μl H_2O, phases were separated by 5 min centrifugation at 14,000 rpm and the release of [^{14}C]phosphorylcholine into the aqueous phase by acid sphingomyelinase activity was determined by liquid scintillation counting of an aliquot of the upper phase.

Ceramide levels

Cells were treated with gemcitabine or doxorubicin as above. The stimulation was terminated by extraction in $CHCl_3$:CH_3OH:1N HCl (100:100:1), phases were separated, and the lower phase was collected. The samples were dried and 20 μl of a detergent solution consisting of 7.5% (wt/vol) *n*-octylglucopyranoside, 5 m*M* cardiolipin in 1 m*M* DETAPAC (diethylenetriamine pentaacetic acid) were added to the samples. To promote the formation of micelles, the samples were sonicated for 10 min in a bath sonicator and 50 μl assay buffer consisting of 0.1 *M* imidazole/HCl (pH 6.6), 0.1 *M* NaCl, 25 m*M* $MgCl_2$, and 2 m*M* EGTA, 2.8 m*M* DTT, 5 μM ATP and 10 μCi [^{32}P]γATP and 10 μl diluted DAG kinase (dilution buffer: 0.01 *M* imidazole/HCl, 1 m*M* DETAPAC (pH 6.6) were added. The kinase reaction was performed for 30 min at 22°C and terminated by extraction in 1 ml $CHCl_3$:CH_3OH:1N HCl (vol/vol/vol, 100:100:1). The samples were supplemented with 170 μl buffered saline solution (135 m*M* NaCl, 1.5 m*M* $CaCl_2$, 0.5 m*M* $MgCl_2$, 5.6 m*M* glucose, 10 m*M* HEPES, pH 7.2) and 30 μl of a 100 m*M* EDTA solution, vortexed, phases were separated and the lower phase collected. Samples were then dried, resuspended in 20 μl $CHCl_3$:CH_3OH (vol/vol, 1:1) and separated on a Silica G60 TLC plate with $CHCl_3$:CH_3OH:CH_3COOH (65:15:5). The plates were dried, exposed, ceramide spots removed from the plates, and incor-

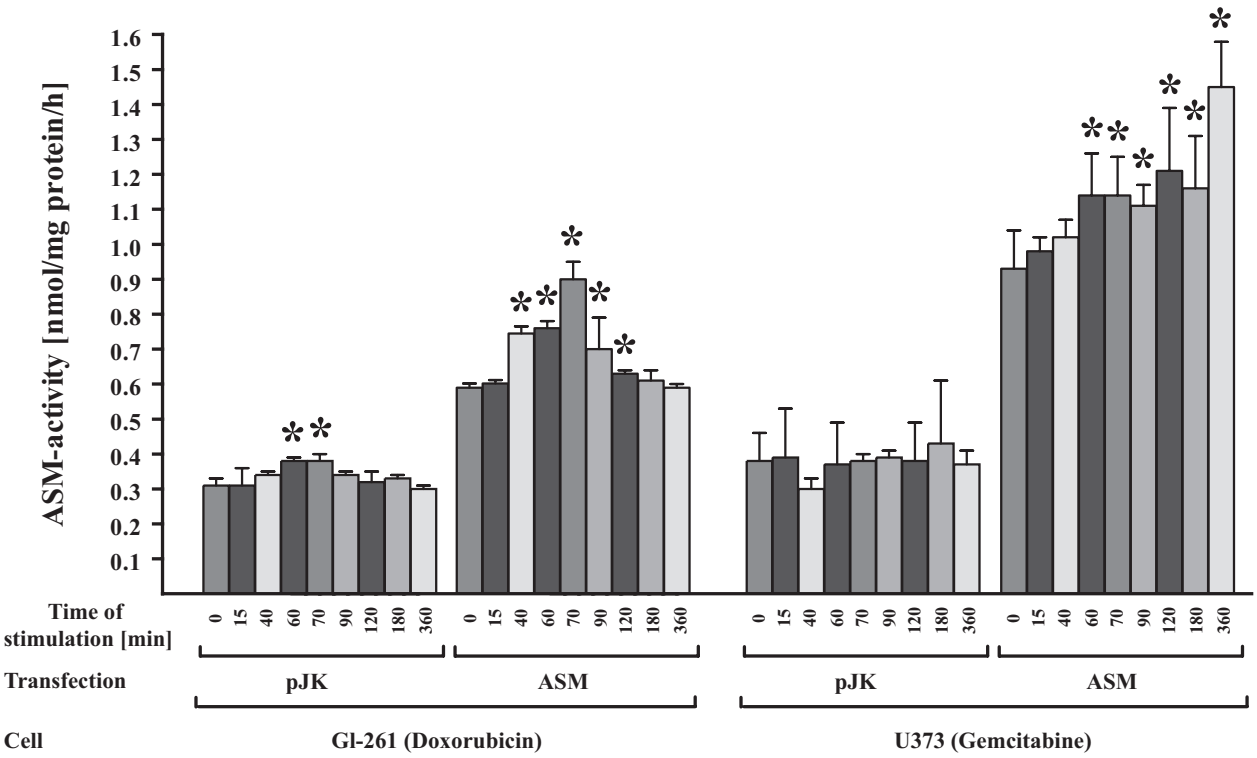

FIG. 1. Doxorubicin and gemcitabine activate the acid sphingomyelinase in glioma cells. Doxorubicin and gemcitabine induce an activation of acid sphingomyelinase in murine Gl-261 and human U373 cells. Cells were each treated with 3 μM doxorubicin or gemcitabine, respectively, lysed, and the activity of acid sphingomyelinase was determined in the lysates. The results indicate a two- to threefold overexpression of acid sphingomyelinase in pEF-ASM transfected cells. Stimulation of the cells results in an activation of the enzyme in acid sphingomyelinase-transfected cells, while control transfected glioma cells only display minor changes of acid sphingomyelinase activity. Displayed are the mean ± SD of each three independent experiments. Significant differences to controls are indicated by asterisks ($p \leq 0.05$, *t*-test).

poration of [^{32}P] into ceramide was quantified by liquid scintillation counting. Ceramide spots were identified by comigration with a C_{16}-ceramide standard. Comparison with a standard curve using C_{16}-ceramide was used to determine the cellular concentration of ceramide. All ceramide values presented in the manuscript give the total cellular concentration of ceramide.

RESULTS

Several studies indicated that expression of acid sphingomyelinase is required for the induction of cell death by cisplatin, doxorubicin, or irradiation (12, 15, 17, 21). However, at present, it is unknown whether overexpression of acid sphingomyelinase is able to sensitize tumor cells to treatment with chemotherapeutic drugs.

Thus, we transfected human and murine glioma cells with an expression vector of the acid sphingomyelinase or with the control vector. Experiments determining the activity of the acid sphingomyelinase confirm a two- to threefold overexpression of the acid sphingomyelinase in Gl-261 and U373 glioma cells, respectively (Fig. 1).

Stimulation of Gl-261 cells with doxorubicin or U373 cells with gemcitabine resulted in an activation of the acid sphingomyelinase within 40–60 min (Fig. 1). Whereas doxorubicin induced a transient increase of acid sphingomyelinase activity in control and ASM-transfected Gl-261 cells, the effect of gemcitabine was sustained and lasted even longer than 6 h. The drugs stimulated acid sphingomyelinase in both control and acid sphingomyelinase-transfected cells; however, the stimulation was more pronounced in cells overexpressing acid sphingomyelinase (Fig. 1). The activation of acid sphingomyelinase in control or acid sphingomyelinase-transfected glioma cells correlated with increased cellular levels of ceramide that was also observed after 40–60 min and was transient after stimulation with doxorubicin, while long lasting after gemcitabine (Fig. 2).

Next, we determined the biological significance of overexpression of acid sphingomyelinase for induction of cell death in glioma cells by chemotherapy. Control and acid sphingomyelinase overexpressing Gl-261 or U373 glioma cells were stimulated for 48 h with doxorubicin or gemcitabine, and cell death was measured by flow cytometry after FITC-Annexin V or by Trypan Blue staining. The results show a marked sensitization of glioma cells overexpressing acid sphingomyelinase to chemotherapy (Fig. 3). While 8–15% of control transfected Gl-261 or U373 glioma cells, respectively, died after chemotherapy with doxorubicin or gemcitabine, this percentage increased to 31–33% in the acid sphingomyelinase overexpressing cells.

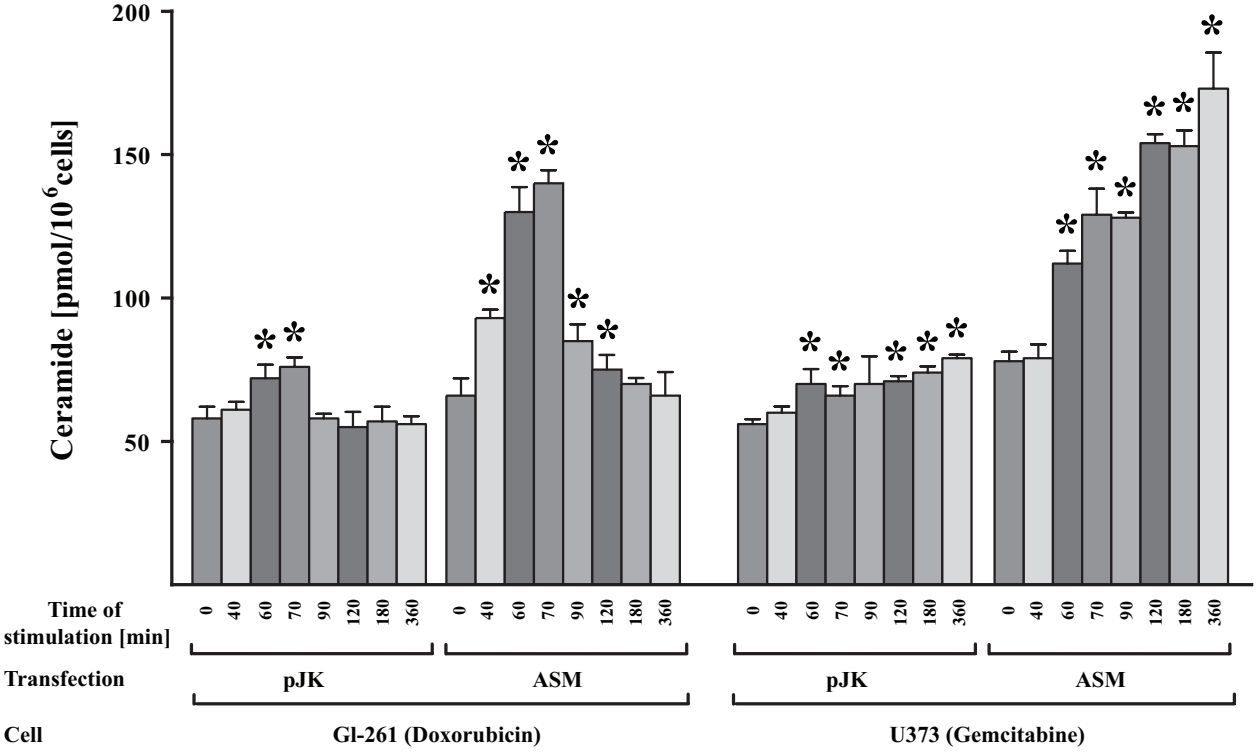

FIG. 2. Doxorubicin and gemcitabine increase cellular levels of ceramide in glioma cells. Gl-261 cells were stimulated with doxorubicin (3 μM), U373 cells with gemcitabine (3 μM), and ceramide levels were determined. The drugs induced an increase of cellular ceramide levels that was more pronounced in cells overexpressing acid sphingomyelinase than in control transfected cells. Ceramide was determined by the DAG-kinase assay. Shown are the mean ± SD of each three independent experiments. Significant differences to controls are indicated by asterisks ($p \leq 0.05$, *t*-test).

These results indicate that overexpression of acid sphingomyelinase greatly sensitizes glioma cells to chemotherapy and suggest acid sphingomyelinase as a key molecule controlling sensitivity of glioma cells to chemotherapy treatment. To address some of the mechanisms that mediate activation of acid sphingomyelinase by doxorubicin and gemcitabine, we determined whether neutralization of reactive oxygen species affects the stimulation of acid sphingomyelinase by the drugs and, thus, the induction of cell death.

The results show that treatment of control or acid sphin-

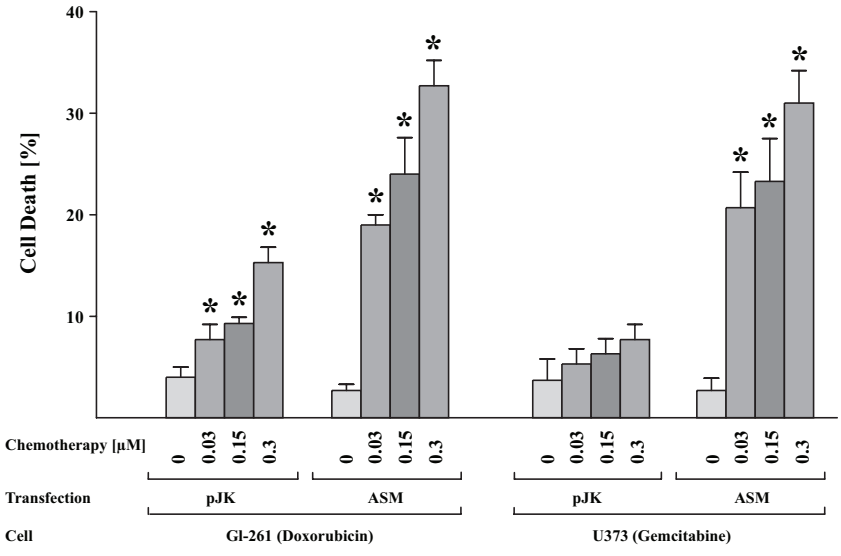

FIG. 3. Overexpression of acid sphingomyelinase sensitizes glioma cells to chemotherapy. Treatment of acid sphingomyelinase or control transfected glioma cells with the indicated concentrations of doxorubicin (Gl-261 cells) or gemcitabine (U373 cells) triggers cell death, which is enhanced in cells overexpressing acid sphingomyelinase. This indicates that overexpression of acid sphingomyelinase sensitizes glioma cells to chemotherapy-induced cell death. Cell death was determined by Trypan Blue staining 36 h after initiation of treatment and confirmed by FACS-analysis after FITC-Annexin V staining (not shown). Displayed is the percent increase of death compared to control of three independent experiments (mean ± SD). Significant differences to controls are indicated by asterisks ($p \leq 0.05$, *t*-test).

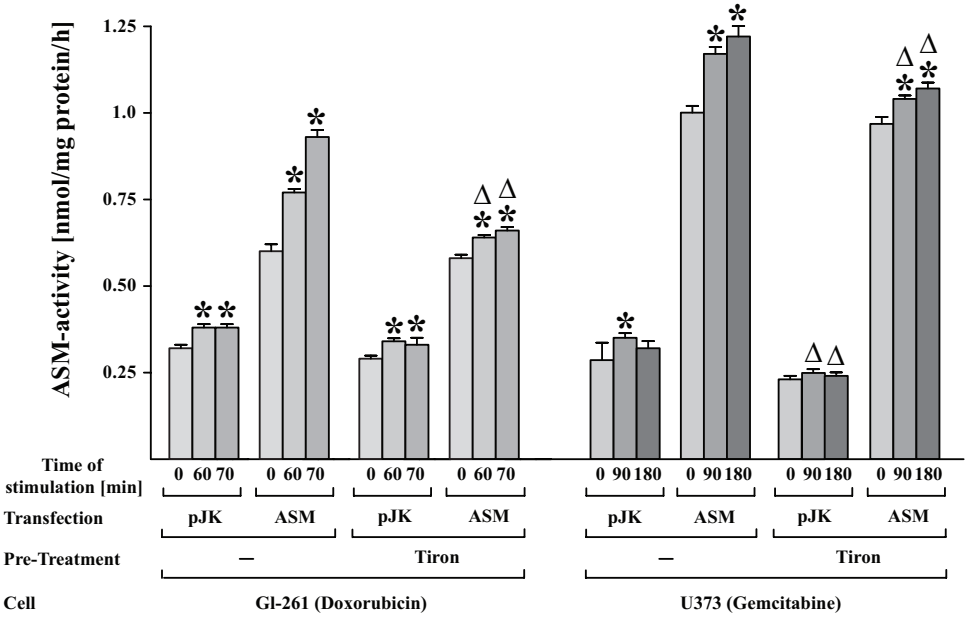

FIG. 4. Neutralization of oxygen radicals prevents activation of acid sphingomyelinase in glioma cells by chemotherapy. Pretreatment of acid sphingomyelinase or control transfected glioma cells with 1 m*M* Tiron blocks activation of acid sphingomyelinase by doxorubicin or gemcitabine. Activity of acid sphingomyelinase was determined as above by measuring the consumption of [^{14}C]sphingomyelin upon addition of cell extracts. Shown are the mean ± SD of three independent experiments. Significant differences to controls are indicated by asterisks ($p \leq 0.05$, *t*-test). Significant differences between Tiron-treated and untreated samples are indicated by a delta ($p \leq 0.05$, *t*-test).

gomyelinase-transfected Gl-261 or U373 cells with the oxygen radical scavenger Tiron significantly blocked the activation of acid sphingomyelinase after treatment with doxorubicin or gemcitabine (Figs. 4 and 5).

Likewise, the neutralization of oxygen radicals prevented an increase of cellular ceramide levels in control and acid sphingomyelinase-transfected glioma cells after treatment (Figs. 6 and 7).

The significance of oxygen radicals for the biological effect of doxorubicin and gemcitabine was evidenced in experiments that revealed an inhibition of cell death in cells expressing endogenous levels of acid sphingomyelinase or overexpressing the enzyme after treatment with doxorubicin or gemcitabine, respectively (Fig. 8).

DISCUSSION

The present data demonstrate that overexpression of acid sphingomyelinase greatly sensitizes human murine glioma cells to the chemotherapeutic drugs gemcitabine and doxorubicin. The drugs trigger a rapid activation of acid sphingomyelinase and increase the cellular concentration of ceramide to induce cell death. Studies employing the radical scavenger Tiron indicate that the drugs gemcitabine and doxorubicin activate acid sphingomyelinase and trigger an increase of cellular ceramide levels via oxygen radicals, which seem to be essential for the induction of death in these glioma cells.

Several previous studies demonstrated a critical role of acid sphingomyelinase expression for the induction of apoptosis by death receptors such as CD95 or DR5 (7, 5, 9). Cells lacking acid sphingomyelinase were resistant to the induction of cell death (3, 5, 7, 9). Furthermore, irradiation was shown to trigger an activation of acid sphingomyelinase and a release of ceramide mediating cell death *in vitro* and *in vivo* (17, 21). Studies employing the cytostatic drugs cisplatin and doxorubicin also demonstrated that downregulation or genetic deficiency of acid sphingomyelinase prevented death induced by these stimuli (12, 15). However, if acid sphingomyelinase shall serve as a therapeutical target in cancer therapy, it is required to define whether an upmodulation of the acid sphingomyelinase/ceramide pathway increases sensitivity of tumor cells to treatment. Here, we demonstrate that even a moderate overexpression of acid sphingomyelinase is sufficient to induce a marked sensitization of the glioma cells to treatment. At present, reagents that induce expression of acid sphingomyelinase need to be defined, but our studies suggest that a search for those reagents might be a novel approach to sensitize tumors, for instance, to chemotherapy.

At present, it is unknown how acid sphingomyelinase is activated. Several studies indicated a role of oxygen radicals in the activation of the acid sphingomyelinase by DR5 or treatment with Cu^{2+} (5, 13). Scavengers of reactive oxygen intermediates inhibited the stimulation of acid sphingomyelinase by these stimuli. Studies by Qui *et al.* also suggested a mechanism how reactive oxygen species directly act on acid sphingomyelinase (18). These investigators demonstrated that oxidation of the cysteine residue 629 in acid sphingomyelinase results in activation and dimerization of the enzyme, at least *in*

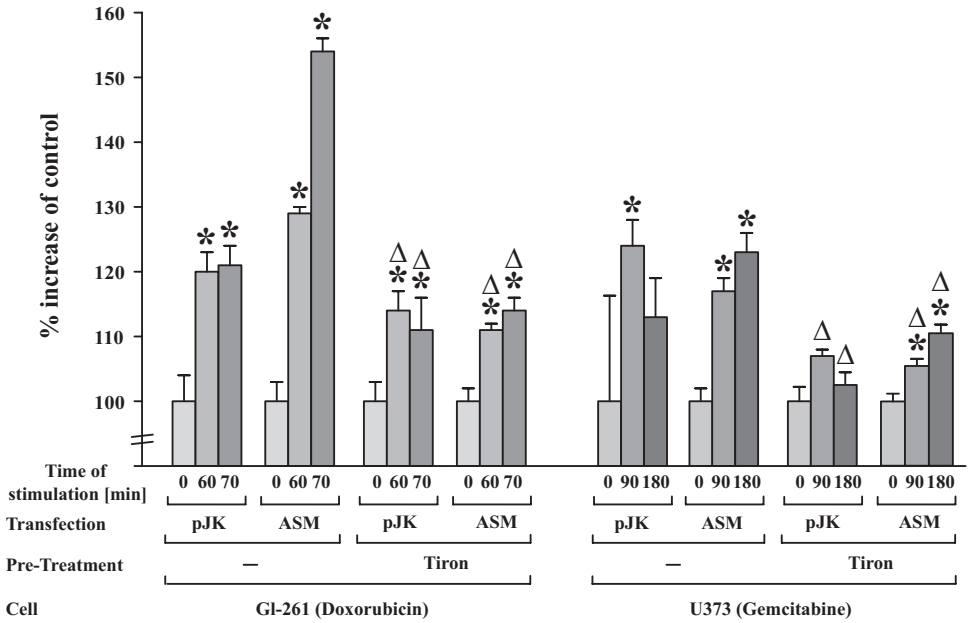

FIG. 5. Chemotherapy activates acid sphingomyelinase via oxygen radicals. Shown are the percent changes of acid sphingomyelinase activities compared to control after treatment with doxorubicin or gemcitabine as described in Fig. 4. Significant differences to controls are indicated by asterisks ($p \leq 0.05$, *t*-test), between Tiron-treated and untreated samples by a delta ($p \leq 0.05$, *t*-test).

vitro using purified enzyme. However, other studies failed to show a dimerization of acid sphingomyelinase in cells treated with Cu^{2+} (18). Thus, at present, it remains to be defined whether oxygen radicals trigger a dimerization of acid sphingomyelinase *in vivo* (*i.e.*, in whole cells). A recent study demonstrated that phosphorylation of acid spingomyelinase at serine 508 is mediated by protein kinase Cδ, activates the enzyme, and leads to translocation of the enzyme onto the extracellular leaflet of the cell membrane (24). Thus, it might be possible that reactive oxygen species stimulate PKC isoforms to phosphorylate and activate acid sphingomyelinase.

Our data indicate that a moderate overexpression of acid sphingomyelinase is sufficient to greatly increase cell death after treatment with chemotherapeutic drugs. This discrepancy might be explained by the presence of acid sphingomyelinase in two pools, a lysosomal pool that serves the constitutive membrane turnover, and a signaling pool in secretory vesicles. These vesicles seem to be mobilized upon stimulation and fuse with

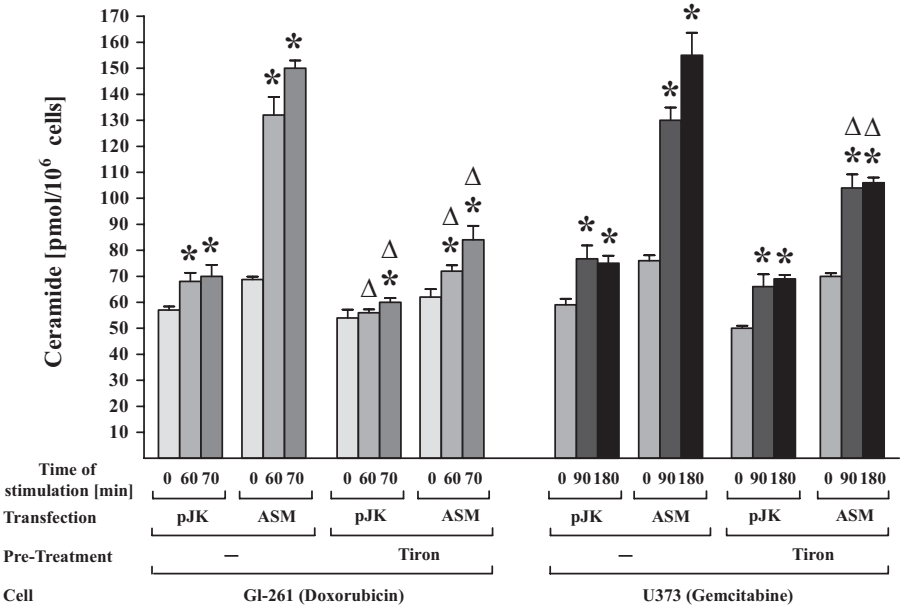

FIG. 6. Neutralization of oxygen radicals prevents the increase of cellular ceramide levels in glioma cells after chemotherapy. Tiron (1 m*M*) significantly reduces the increase of cellular ceramide levels from acid sphingomyelinase or control transfected glioma cells treated with each 3 μM doxorubicin or gemcitabine, respectively. Ceramide levels were determined by the DAG kinase assay. The panel shows the mean ± SD of three independent experiments and gives the absolute cellular ceramide levels. Significant differences to controls are indicated by asterisks ($p \leq 0.05$, *t*-test). Significant differences between Tiron-treated and untreated samples are indicated by a delta ($p \leq 0.05$, *t*-test).

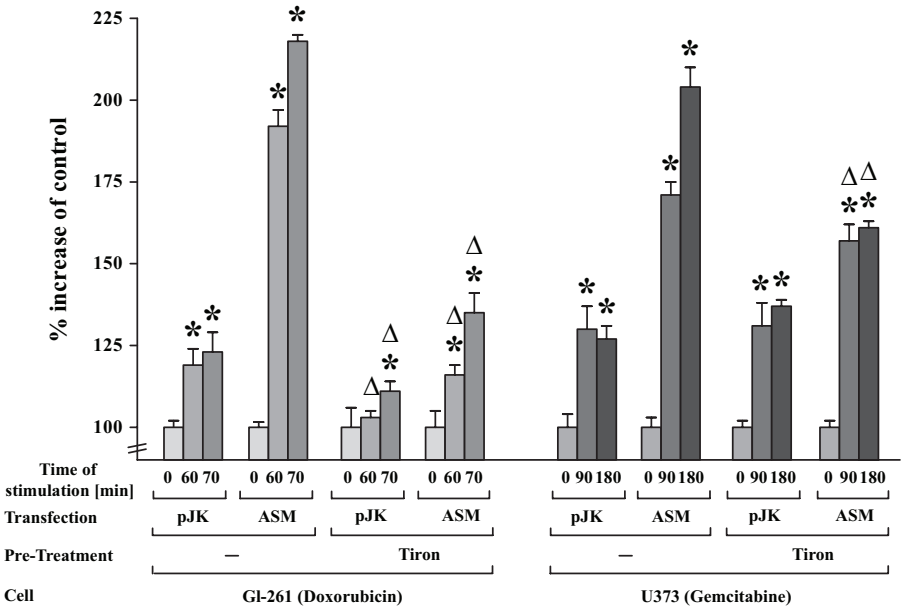

FIG. 7. Oxygen radicals trigger cellular ceramide in glioma cells after chemotherapy. The panel shows the percent increase of cellular ceramide concentrations compared to control from the values displayed in Fig. 6. Asterisks indicate significant differences to controls, deltas significant differences between Tiron-treated and untreated samples ($p \leq 0.05$, *t*-test).

the cell membrane, resulting in exposure of acid sphingomyelinase on the cell surface and released ceramide after cellular stimulation. Cellular transfection with acid sphingomyelinase may result in a particular increase of acid sphingomyelinase in this signaling pool caused by saturation of the pathway transporting the acid sphingomyelinase into the lysosomal pool (22, 23).

Ceramide was shown to form distinct, large membrane domains, also named ceramide-enriched membrane platforms, upon stimulation via death receptors, but also after treatment with UV-A light (11, 14, 20). Although not proven, it is very likely that doxorubicin and gemcitabine also induce the formation of ceramide-enriched membrane platforms that mediate cell death. However, preliminary studies indicated inconsistent formation of ceramide-enriched membrane platforms in the plasma membrane after chemotherapy, which might be due to the slow kinetics of ceramide-release after chemotherapy. Further, it might be possible that chemotherapeutic drugs primarily induce the formation of ceramide-enriched membrane domains in intracellular organelles such as mitochondria or the endoplasmatic reticulum. Ceramide-enriched membrane platforms serve to cluster receptors and signalling molecules, facilitating the transmission of the specific signal via the cognate receptor, for example, the induction of apoptosis (5, 7, 9). It might be speculated that ceramide-enriched membrane platforms also trigger ligand-independent clustering and subsequent activation of death receptors, finally resulting in transactivation of intracellular enzymes associating with these receptors and cell death. In addition, treatment-induced formation of ceramide-enriched membrane platforms may induce conformational changes and

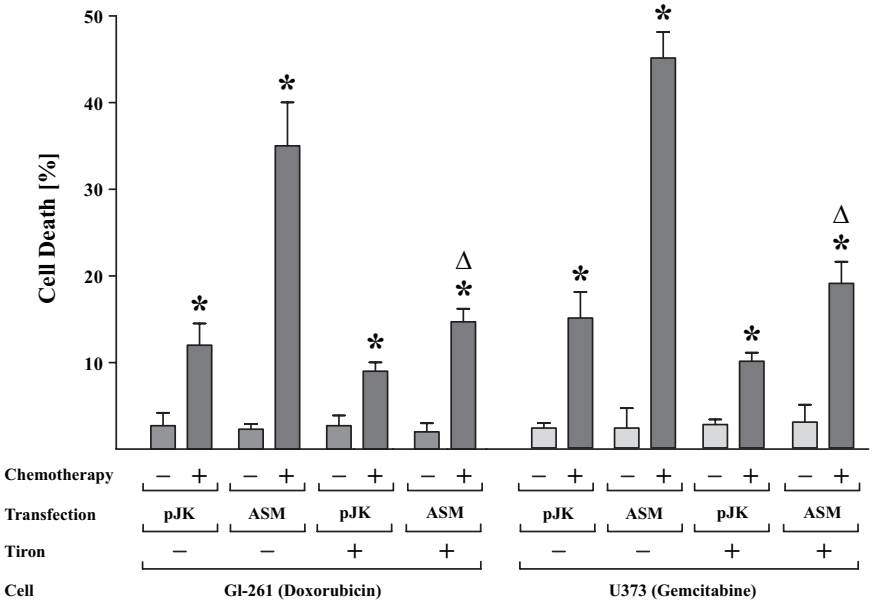

FIG. 8. Neutralization of oxygen radicals prevents acid sphingomyelinase-mediated death of glioma cells triggered by chemotherapy. Pretreatment of acid sphingomyelinase or control transfected glioma cells with 1 m*M* Tiron prevents cell death induced by exposure to doxorubicin or gemcitabine. Cell death was determined by Trypan Blue staining 36 h after addition of the chemotherapeutic drugs. Cells were treated with each 0.3 μM doxorubicin (Gl-261) and gemcitabine (U373). The figure shows the mean ± SD of three independent experiments. Significant differences to controls are indicated by asterisks ($p \leq 0.05$, *t*-test), significant differences between Tiron-treated and untreated samples are indicated by a delta ($p \leq 0.05$, *t*-test).

activation of pro-apoptotic Bcl2-like proteins and, thus, cell death (1). Finally, it might be also possible that chemotherapeutics trigger the formation of ceramide and ceramide domains in mitochondria, which might be involved in recruitment and/or integration of pro-apoptotic proteins into mitochondrial membranes, finally resulting in the induction of apoptosis (2, 4).

In summary, our data indicate that overexpression of acid sphingomyelinase in glioma cells greatly sensitizes the cells to treatment with doxorubicin and gemcitabine. Thus, acid sphingomyelinase might be an interesting target for the development of drugs that sensitize tumors to irradiation or chemotherapy.

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ABBREVIATIONS

ASM, acid sphingomyelinase; CD95, cluster of differentiation 95; DAG, diacylglycerol; DR5, death receptor 5; FITC, fluorescein-isothiocyanate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PKC, protein kinase C; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; UV-light, ultraviolet light.

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