Mitochondrial Amplification of Death Signals Determines Thymidine Kinase/Ganciclovir-triggered Activation of Apoptosis

Christian Beltinger, Simone Fulda, Thomas Kammertoens, Wolfgang Uckert, and Klaus-Michael Debatin

University Children’s Hospital, 89075 Ulm (C. B., S. F., K.-M. D.), and Max-Delbrueck Center for Molecular Medicine, 13092 Berlin (T. K., W. U.), Germany

ABSTRACT

Previous clinical experience shows that the efficacy of suicide gene transfer in tumor therapy is limited, resulting from inefficient gene transfer or, alternatively, from intrinsic resistance of the tumor in vivo. Herpes simplex virus thymidine kinase/ganciclovir (TK/GCV), a paradigmatic suicide gene therapy system, has been described to exert its cytotoxic effect, at least in part, by inducing apoptosis in target cells. Here, we report that mitochondria amplify TK/GCV-induced apoptosis by regulating p53 accumulation and the effector phase of apoptosis. Treatment with TK/GCV led to mitochondrial perturbations including loss of the mitochondrial membrane potential and release of cytochrome c from mitochondria into the cytosol, inducing caspase activation and nuclear fragmentation. Inhibition of TK/GCV-induced mitochondrial perturbations by Bcl-2 overexpression or by the mitochondrion-specific inhibitor bongkrekic acid; strongly inhibited TK/GCV-induced activation of caspases and apoptosis. TK/GCV-induced mitochondrial perturbations depended on caspase activity possibly initiated by death receptor signaling. Perturbation of mitochondrial function mediated accumulation of wild-type p53 protein, since Bcl-2 overexpression, bongkrekic acid, or inhibition of mitochondrial protein synthesis with chloramphenicol strongly reduced TK/GCV-induced accumulation of wild-type p53 protein. These findings suggest that TK/GCV therapy may be less efficient in tumors in which the mitochondrial amplification of TK/GCV-induced apoptosis is blocked, e.g., by Bcl-2 overexpression. Given the low efficacy of currently used gene therapy systems, our data on molecular mechanisms that regulate sensitivity or resistance toward TK/GCV-induced cytotoxicity might have important implications to improve the clinical application of suicide gene therapy.

INTRODUCTION

After an initial wave of enthusiasm, suicide gene therapy systems such as the herpes simplex TK/GCV system did not meet the expectations once they entered the clinical stage. Low target specificity and low transfection efficacy of target cells have been discussed to contribute once they entered the clinical stage. Low target specificity and low transfection efficacy of target cells have been discussed to contribute once they entered the clinical stage.

In the present report, we investigated the role of mitochondria in suicide gene therapy and found that mitochondria amplify TK/GCV-induced apoptosis by regulating both the initiation and the effector phase of apoptosis.

MATERIALS AND METHODS

Drugs. GCV (Hoffmann La Roche, Grenzach, Germany) was provided as pure substance and dissolved in water and stored at −80°C. Cell Culture. SHEP neuroblastoma cells transfected with control vector (pLXSN) or TK (pHyTK) or double transfected with Bcl-2 and TK (pHyTK/Bcl-2) were cultured in RPMI 1640 (Life Technologies, Inc., Eggenstein, Germany) supplemented with 10% heat-inactivated FCS (Conco, Wiesbaden, Germany), 100 U/ml penicillin (Life Technologies, Inc.), 100 μg/ml streptomycin (Life Technologies, Inc.), 2 mM L-glutamine (Biochrom), 0.5 mg/ml G418 (Life Technologies, Inc.), or 0.5 mg/ml hygromycin (Life Technologies, Inc.) as previously described (1, 11, 15).

Determination of Apoptosis. Quantification of DNA fragmentation was performed by FACS analysis of propidium iodide-stained nuclei as previously described (1, 11, 15).

Inhibition of Drug-induced Apoptosis or Loss of ΔΨm by zVAD.fmk or BA. The broad spectrum tripeptide inhibitor of caspases zVAD.fmk (Enzyme Systems Products, Livermore, CA) was used at a concentration of 60 μM, and the mitochondrion-specific inhibitor BA was used at a concentration of 30 μM (kindly provided by Dr. Duine, University of Delft, Delft, the Netherlands).

Assessment of Mitochondrial Potential. The cationic lipophilic fluorescence probe DiOC6(3) (460 nm/510 nm; Molecular Probes, Eugene, OR) was used to measure the ΔΨm. Cells were incubated for 12 min at 37°C in the presence of the fluorescein, washed in PBS/1% FCS, and immediately analyzed by flow cytometry (FACSscan). ΔΨm was recorded in fluorescence 1. The percentage of cells with low mitochondrial potential was calculated in comparison with untreated control cells.
Western Blot Analysis. Cells were lysed for 30 min at 4°C in PBS with 0.5% Triton X (Serva, Heidelberg, Germany) and 1 mM PMSF (Sigma, Deisenhofen, Germany) followed by high speed centrifugation. Membrane proteins were eluted in buffer containing 0.1 M glycine, pH 3.0, and 1.5 mM Tris, pH 8.8. Protein concentration was assayed by bicinchoninic acid (Pierce, Rockford, IL). Protein, 40 μg/lane, was separated by 12% SDS-PAGE and electroblotted onto nitrocellulose membranes (Amersham Pharmacia, Freiburg, Germany). Equal protein loading was controlled by Ponceau red electroblotted onto nitrocellulose membranes (Amersham Pharmacia, Freiburg, Germany). Equal protein loading was controlled by Ponceau red staining of membranes. After blocking for 1 h in PBS supplemented with 2% BSA (Sigma) and 0.1% Tween 20 (Sigma), immunodetection of caspase-8, caspase-3, PARP, and cytochrome c protein was conducted with the use of mouse anti-caspase-8 monoclonal antibody C15 (1:10 dilution of hybridoma supernatant (17)), mouse anti-caspase-3 monoclonal antibody (1:1,000; Transduction Laboratories, Lexington, KY), rabbit anti-PARP polyclonal antibody (1:10,000; Boehringer Mannheim, Mannheim, Germany), or mouse anti-cytochrome c monoclonal antibody (1:5,000; Pharmingen, San Diego, CA) and goat anti-mouse IgG or goat anti-rabbit IgG (1:5,000; Santa Cruz Biotechnology, Santa Cruz, CA). ECL (Amersham) was used for detection.

Preparation of Mitochondria, Cytosolic Extracts, and Nuclei. For isolation of mitochondria, cells (3 × 10⁶/sample) were washed twice with ice-cold PBS and resuspended with 5 volumes of buffer A [50 mM Tris, 1 mM EGTA, 5 mM 2-mercaptoethanol, 0.2% BSA, 10 mM KH₂PO₄ (pH 7.6), 0.4 M sucrose] and allowed to swell on ice for 20 min. Cells were homogenized with 30 strokes of a Teflon homogenizer and centrifuged at 10,000 × g for 10 min at 4°C. The resulting pellets were resuspended in buffer B [10 mM KH₂PO₄ (pH 7.2), 0.3 mM mannitol, 0.1% BSA]. Mitochondria were separated by sucrose gradient [lower layer, 1.6 M sucrose, 10 mM KH₂PO₄ (pH 7.5), 0.1% BSA; upper layer, 1.2 M sucrose, 10 mM KH₂PO₄ (pH 7.5), 0.1% BSA]. Interphase contaminants mitochondria were washed with buffer B at 18,000 × g for 10 min at 4°C, and the resulting mitochondrial pellets were resuspended in buffer B. For preparation of cytosolic extracts, cells (1 × 10⁶/sample) were washed twice with ice-cold PBS, resuspended with 1 volume of buffer A, and allowed to swell on ice for 20 min. Cells were homogenized with 30 strokes of a Dounce homogenizer and centrifuged at 15,000 × g for 15 min at 4°C. The protein concentration of mitochondria or cytosolic extracts was determined using bicinchoninic acid (Pierce, Rockford, IL). For isolation of nuclei, cells were washed twice in ice-cold PBS, resuspended in 10 volumes of buffer C (10 mM PIPES, pH 7.4, 10 mM KCl, 2 mM MgCl₂, 1 mM DTT [Merck, Darmstadt, Germany], 1 mM PMSF (Sigma), 10 mM cytochalasin B (Sigma)], allowed to swell on ice for 20 min, and homogenized in a Teflon homogenizer. Homogenates were layered over 30% sucrose in buffer C and centrifuged at 800 × g for 10 min. The resulting nuclear pellets were resuspended in buffer C and washed three times. Nuclei were stored at −80°C in aliquots of 10⁶ nuclei/ml.

Cell-Free System of Apoptosis. For determination of nuclear fragmentation, nuclei (10⁷/μl) were incubated with mitochondria (1 μg/μl) in buffer D (10 mM Hepes (pH 7.4), 50 mM NaCl, 2 mM MgCl₂, 5 mM EGTA, 1 mM DTT, 2 mM ATP (Sigma), 10 mM phosphocreatine (Sigma), 50 μg/ml creatine kinase (Sigma), 10 μM cytochalasin B) for 2 h at 37°C. Nuclei were stained with propidium iodide (10 μg/μl) and analyzed by flow cytometry. For determination of mitochondrial cytochrome c content, mitochondria were lysed in lysis buffer, and proteins were separated by 15% SDS-PAGE. Western blot analysis was performed as described above.

Inhibition of Mitochondrial Protein Synthesis. Mitochondrial protein synthesis was inhibited by treating SHEP pHyTK11 cells with 50 μg/ml chloramphenicol (Sigma-Aldrich, Steinheim, Germany) for 3 weeks in medium containing 110 μg/ml sodium pyruvate (Biochrom), 50 μg/ml uridine (Sigma), and 5 mg/ml glucose (Sigma).

TK/GCV-induced Apoptosis during Inhibition of Mitochondrial Protein Synthesis. SHEP pHyTK11 cells (2 × 10⁶) in which mitochondrial protein synthesis had been inhibited as described above were incubated with GCV in increasing concentrations for 72 h in the presence of sodium pyruvate, uridine, and glucose. Control SHEP pHyTK11 cells were also incubated in medium containing the same concentrations of pyruvate, uridine, and glucose starting 3 weeks prior and continued during the experiment. Specific apoptosis was measured by enumerating propidium iodide stained hypodiploid nuclei by flow cytometry.

p53 Induction by TK/GCV. SHEP pHyTK11 cells (5 × 10⁶) were treated with 10 μM GCV for increasing times, with or without 50 μg/ml chloramphenicol (Sigma-Aldrich). When the effect of chloramphenicol was examined, both the chloramphenicol-treated cells and the untreated controls were adapted to medium containing pyruvate, uridine, and glucose starting 3 weeks prior and continued during the experiment. Western blotting was performed as described above with mouse anti-p53 monoclonal antibody (1:1000; Transduction Laboratories). Mouse anti-α-tubulin monoclonal antibody (1:3000; Calbiochem, Bad Soden, Germany) was used to control for equal gel loading.

Detection of Mitochondrial DNA Damage. DNA was extracted using the QIAamp Blood Kit (Qiagen, Hilden, Germany) according to the instruction of the manufacturer. To detect mitochondrial DNA damage, the entire coding region of the mitochondrial DNA was amplified by long range PCR with the use of primers 5′-CCCAGTTTATGTAGCCTCTCTCTCA-3′ (nucleotides 571–598 of the Cambridge human mitochondrial DNA sequence) and 5′-TTGATTGCTGTACTTGCTTGTAAGCATG-3′ (nucleotides 16,220–16,193). Long range PCR was performed with the Expand Long Template PCR System (Boehringer Mannheim) according to the manufacturer’s instructions with the following modifications. Denaturing temperature was 92°C, annealing temperature was 65°C, extension time was 14 min during the first 10 cycles and 14 min plus 20 s for each consecutive cycle during the subsequent 12 cycles. The final extension time was 14 min. The 15.6-kb PCR products were resolved on a 0.7% agarose gel and visualized by ethidium bromide. As a positive control, mitochondrial DNA damage was induced in 1 × 10⁶ SHEP pHyTK11 cells by treatment with H₂O₂ (Mallinckrodt-Baker, Deventer, the Netherlands) in concentrations ranging from 6.25 to 200 μM at 37°C for 1 h. To detect TK/GCV-induced DNA damage, 1 × 10⁶ SHEP pHyTK11 cells were treated with 10 μM GCV with increasing duration before DNA extraction.

RESULTS

To investigate whether mitochondria are involved in TK/GCV-triggered apoptosis, we studied apoptosis after GCV treatment in TK- and Bcl-2-transfected cells or with BA, a specific inhibitor of mitochondrial permeability transition pores. Induction of apoptosis in response to treatment with GCV was strongly inhibited in SHEP neuroblastoma cells transfected with both TK and Bcl-2 compared with cells expressing TK, but not Bcl-2 (Fig. 1A). Similarly, TK/GCV-triggered apoptosis was blocked in TK-expressing cells in the presence of BA (Fig. 1A), indicating that mitochondria were involved in mediating TK/GCV gene therapy-induced apoptosis.

To study the effect of mitochondrial activation on the cleavage of caspases, we monitored cleavage of initiator (caspase-8) and effector (caspase-3) caspases by Western blot analysis in TK- and Bcl-2-transfected cells. After incubation with GCV, cleavage of the effector caspase-3 and processing of the caspase substrate PARP were inhibited in cells overexpressing Bcl-2 and TK compared with cells expressing TK, but not Bcl-2 (Fig. 1A). Similarly, TK/GCV-triggered apoptosis was blocked in TK-expressing cells in the presence of BA (Fig. 1A), indicating that mitochondria were involved in mediating TK/GCV gene therapy-induced apoptosis.

To further define alterations in mitochondrial function after treatment with TK/GCV, we assessed the mitochondrial transmembrane potential ΔΨₘ using the potential-sensitive fluorochrome DiOC₆(3). GCV treatment was associated with a time-dependent loss of the ΔΨₘ in TK-transfected cells with a maximum at 96 h of GCV treatment, whereas no significant alteration of the ΔΨₘ was found in mock transfected control cells (Fig. 2A). In addition, loss of the ΔΨₘ upon TK/GCV gene therapy was strongly reduced in TK and Bcl-2 double-transfected cells or in the presence of BA (Fig. 2B). To dissect mitochondrial-driven effects of TK/GCV therapy from its effect on other subcellular systems, we used a cell-free system. Mitochondria were isolated from TK/GCV-treated cells and incubated with cytosolic extracts prepared from untreated cells. In this experimental setting, isolated mitochondria induced cleavage of caspase-8,
caspase-3, and PARP in cytosolic extracts (Fig. 3A). In addition, mitochondria isolated from TK/GCV-treated cells triggered nuclear fragmentation when incubated with nuclei isolated from untreated cells (Fig. 3B). However, cleavage of caspases and nuclear fragmentation was blocked when mitochondria isolated from TK/GCV-treated cells transfected with Bcl-2 were used (Fig. 3, A and B). These findings indicate that mitochondria of TK/GCV-treated cells were able to trigger full activation of apoptosis effectors programs.

On induction of apoptosis, mitochondria can release apoptogenic factors such as cytochrome c into the cytosol which may directly activate downstream effector caspases such as caspase-3 (18, 19). Therefore, we next investigated cytochrome c release after TK/GCV gene therapy. Treatment of TK-transfected neuroblastoma cells resulted in a time-dependent release of cytochrome c from mitochondria into the cytosol which was inhibited in TK and Bcl-2 double-transfected cells (Fig. 4). This suggests that mitochondria contribute to TK/GCV-triggered apoptosis by releasing cytochrome c into the cytosol before the breakdown of the mitochondrial membrane potential. Bcl-2 interfered with TK/GCV-triggered apoptosis by inhibiting cytochrome c release. TK/GCV-induced release of cytochrome c from mitochondria into the cytosol was inhibited in cells incubated with zVAD.fmk (Fig. 4), indicating that mitochondrial dysfunctions in TK/GCV gene therapy required activation of upstream caspases.

Because GCV has been reported to cause chain termination and single-strand breaks on incorporation into nuclear DNA after conversion to active metabolites by TK, we asked whether TK/GCV gene therapy would have a direct damaging effect on mitochondrial DNA (3). However, with a PCR-based method, no damage to mitochondrial DNA was detected during treatment of cells with TK/GCV, whereas mitochondrial DNA damage was detectable after incubation with H$_2$O$_2$ known to induce DNA damage (Fig. 5). This suggests that TK/GCV therapy did not involve obvious damage to mitochondrial DNA replication detectable by PCR.

To test whether mitochondrial DNA would play any role in TK/GCV-induced apoptosis, we studied the effect of proteins encoded by the mitochondrial genome on TK/GCV-induced apoptosis. To inhibit mitochondrial protein synthesis, we used chloramphenicol which specifically blocks synthesis of mitochondrial encoded proteins (20). Treatment of TK-transfected neuroblastoma cells with chloramphenicol resulted in inhibition of mitochondrial metabolic function as measured by the dependence on uridine (data not shown). Pyruvate was added to the medium because cells rendered respiratory deficient by chloramphenicol depend on exogenous pyruvate. Interestingly, pyruvate alone markedly decreased TK/GCV-induced apoptosis. However, treatment of TK-transfected neuroblastoma cells with chloramphenicol did not further inhibit TK/GCV-induced apoptosis, indicating that proteins encoded by the mitochondrial genome and involved in mitochondrial redox function did not contribute to TK/GCV-induced apoptosis (Fig. 6).

Because damage to nuclear DNA may involve accumulation of wild-type p53 protein and because release of mitochondrial factors may contribute to nuclear DNA damage, we then studied whether interfering with mitochondrial functions would have any influence on p53 accumulation. Accumulation of wild-type p53 protein was reduced in TK- and Bcl-2-transfected cells or in TK-transfected cells in the presence of BA (Fig. 7). In addition, accumulation of wild-type p53 protein after GCV treatment was markedly diminished in cells in which mitochondrial protein synthesis was blocked by chloramphenicol (Fig. 7). This suggests that mitochondria might be involved in mediating accumulation of p53 protein after GCV treatment in the absence of detectable damage to mitochondrial DNA replication.

**DISCUSSION**

Thus far, clinical results with suicide gene therapy systems such as the HSV-TK/GCV system did not fulfill the initial expectations for an...
Treatment with TK/GCV triggered mitochondrial perturbations, e.g., loss of the mitochondrial transmembrane potential and cytochrome c release, leading to activation of apoptosis effector programs. Inhibition of TK/GCV-induced mitochondrial perturbations by Bcl-2 overexpression or by the mitochondrion-specific inhibitor BA strongly reduced TK/GCV-induced apoptosis. Mitochondria from TK/GCV-treated cells fully initiated activation of apoptosis effector programs leading to DNA fragmentation and cell death by releasing apoptogenic factors such as cytochrome c. Cytochrome c has been described to form a complex together with Apaf-1 and procaspase-9, resulting in activation of procaspase-9 and leading to cleavage of downstream caspases such as caspase-3 and cleavage of CAD (caspase-activated Dnase) or DNA fragmentation factor, resulting in nuclear fragmentation (21, 22). TK/Bcl-2 double-transfected cells that were blocked at the mitochondrial level did not release cytochrome c from mitochondria and did not activate caspases after treatment with GCV.

In addition to regulation of the effector phase of apoptosis, mitochondria were also involved in mediating accumulation of wild-type p53 protein, because accumulation of wild-type p53 after treatment with TK/GCV protein was blocked by overexpression of effective new therapeutic approach in the treatment of malignancies, especially for patients with malignant brain tumors. Therefore, in addition to improving target specificity and gene transfer efficacy, a better understanding of the underlying mechanisms that regulate sensitivity or resistance to TK/GCV-induced cytotoxicity may be critical to improve its efficacy. The cytotoxic effect of TK/GCV therapy is, at least in part, mediated by induction of apoptosis (1, 2). Here, we report that mitochondria are centrally involved in the regulation of TK/GCV-induced apoptosis by amplifying TK/GCV-induced activation of apoptosis pathways.

Fig. 2. TK/GCV-induced apoptosis involves loss of mitochondrial membrane potential. A, kinetics of TK/GCV-induced loss of mitochondrial membrane potential. SHEP neuroblastoma cells transfected with pHyTK (+TK) or mock transfectants (-TK) were treated with 10 μM GCV for indicated times. ΔΨm was determined by staining cells with the potential-sensitive fluorochrome DiOC6(3). B, inhibition of TK/GCV-induced loss of mitochondrial membrane potential by Bcl-2 overexpression or by mitochondrion-specific inhibitor BA. SHEP neuroblastoma cells transfected with pHyTK (+TK), mock transfectants (-TK) or pHyTK/Bcl-2 double transfectants (+TK/Bcl-2) were treated with 10 μM GCV for 72 h in the presence or absence of 50 μM BA. ΔΨm was determined by staining cells with the potential-sensitive fluorochrome DiOC6(3).

Fig. 3. TK/GCV-induced mitochondrial alterations are involved in activation of caspases and nuclear fragmentation. A, TK/GCV-induced mitochondrial alterations mediate activation of caspases. SHEP neuroblastoma cells transfected with pHyTK (+TK), mock transfectants (-TK), or pHyTK/Bcl-2 double transfectants (+TK/Bcl-2) were treated with 10 μM GCV for 24 h. Mitochondria were isolated and incubated with cytotoxic extracts from untreated cells for 6 h. Proteins (40 μg/lane) were separated by 12% SDS-PAGE. Immunodetection of caspases-3 and -8 and PARP protein was performed by mouse anti-caspase-3 monoclonal antibody, mouse anti-caspase-8 monoclonal antibody, rabbit anti-PARP polyclonal antibody, and ECL. B, TK/GCV-induced mitochondrial alterations mediate nuclear fragmentation. SHEP neuroblastoma cells transfected with pHyTK (+TK), mock transfectants (-TK), or pHyTK/Bcl-2 double transfectants (+TK/Bcl-2) were treated with 10 μM GCV for 24 h. Mitochondria were isolated and incubated with nuclei from untreated cells. Nuclear apoptosis was determined by FACs analysis of propidium iodide-stained DNA content.

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Bcl-2, by blockade of the mitochondrial megapore using BA, or by inhibition of synthesis of mitochondrial encoded proteins. At present, the underlying mechanism(s) regulating mitochondria-mediated p53 accumulation are not known and may involve production of reactive oxygen species, release of soluble factors from mitochondria or TK/GCV-induced damage to mitochondrial DNA, although we could not detect obvious damage to mitochondrial DNA replication. To investigate whether mitochondrial encoded respiratory chain proteins contribute to TK/GCV-induced p53 accumulation and apoptosis we blocked mitochondrial protein synthesis by the specific inhibitor chloramphenicol. Pyruvate was added to the medium since cells rendered respiratory deficient by chloramphenicol become auxotrophic for pyruvate. Interestingly, pyruvate alone markedly decreased TK/GCV-induced apoptosis, in line with a cytoprotective effect described for pyruvate (23, 24). Since pyruvate levels are increased in hypoxic areas of solid tumors this may decrease TK/GCV-induced apoptosis in vivo. Chloramphenicol treatment ablated TK/GCV-induced p53 accumulation while not decreasing TK/GCV-induced apoptosis. This may suggest that TK/GCV-mediated apoptosis is independent of respiratory function and of p53. p53 independence during respiratory deficiency would contrast with our previous finding that p53 is associated with TK/GCV-induced death receptor aggregation during normal cellular respiration (1). Alternatively, the cytoprotective effect of pyruvate may have masked any decrease of apoptosis caused by chloramphenicol. Chloramphenicol treatment markedly decreased tumor cell proliferation (data not shown). This has been explained by decreased G1-S transition linked to purine auxotrophy caused by respiratory deficiency (25). Because the efficacy of TK/GCV depends on replicating DNA, one would expect an inhibition of TK/GCV-induced apoptosis in the presence of chloramphenicol. As mentioned, this was not observed, suggesting that purine auxotrophy induced by chloramphenicol led to sufficient incorporation of the purine analogue GCV triphosphate despite decreased DNA replication. Similar to cells experiencing chloramphenicol-induced respiratory deficiency, cells in hypoxic tumor areas slow down at G1 (26). Our finding that tumor cells are not completely resistant to TK/GCV despite decreased proliferation and increased pyruvate under conditions of respiratory deficiency supports the use of TK/GCV for targeting hypoxic tumors (27).

The molecular mechanisms leading to perturbations of mitochondria in TK/GCV therapy are not understood. Although TK/GCV has been reported to have a direct damaging effect on nuclear DNA by inhibiting DNA polymerase resulting in termination of DNA synthesis, TK/GCV therapy did not lead to damage of mitochondrial DNA replication detectable by PCR. Moreover, TK/GCV therapy probably did not directly induce perturbation of mitochondrial function. Caspases or caspase-dependent signaling events operating upstream of mitochondria are required for triggering mitochondrial permeability transition because TK/GCV-triggered mitochondrial alterations such as loss of mitochondrial membrane potential or release of cytochrome c depended on caspase activity. In addition, activation of the initiator caspase-8 was not blocked by Bcl-2 overexpression indicating that cleavage of caspases occurred, at least to some extent, independent and upstream of mitochondria in TK/GCV-treated cells. Bid, a recently identified BH3 domain-containing molecule, or CAF (caspase...
activated factor) may link caspase-8 cleavage at the activated CD95 receptor to mitochondrial pathways (28, 29).

Thus, mitochondria play a central role as amplifier of TK/GCV-induced apoptosis by their effect on the effector phase of apoptosis and on p53 accumulation. Mitochondria trigger apoptosis effector systems such as caspases through the release of cytochrome c into the cytosol. Because p53 can mediate up-regulation of CD95 resulting in the formation of the CD95 death-inducing signaling complex and activation of the caspase cascade (1) that may in turn lead to mitochondrial perturbations, mitochondria might be part of a positive, albeit redundant amplification loop in TK/GCV-induced apoptosis, at least in some cell types.

The fact that TK/GCV therapy-induced cell death is strongly reduced in cells overexpressing Bcl-2 might have important clinical implications given the fact that a large proportion of brain tumors, a potential target for TK/GCV therapy, express high levels of Bcl-2 (30). By demonstrating that mitochondria are amplifiers of TK/GCV-induced apoptosis, our findings contribute to the molecular understanding of TK/GCV gene therapy, a paradigmatic gene therapy system. Furthermore, because TK/GCV gene therapy is a model of drug-induced cytotoxicity apart from its clinical application, our findings provide insights into molecular mechanisms of cytotoxicity by showing that mitochondria are involved in the regulation of cell death and accumulation of p53 protein.

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