Glucocorticoid Cotreatment Induces Apoptosis Resistance toward Cancer Therapy in Carcinomas

Ingrid Herr, Esat Ucur, Kerstin Herzer, Stella Okouoyo, Rüdiger Ridder, Peter H. Krammer, Magnus von Knebel Doeberitz, and Klaus-Michael Debatin

Division of Molecular Oncology/Pediatrics [I. H., E. U., S. O., K.-M. D.] and Tumor Immunology Program [K. H., P. H. K.], German Cancer Research Center, 69120 Heidelberg, Germany; University Children’s Hospital, Ulm, Germany [I. H., E. U., S. O., K.-M. D.]; Division of Molecular Pathology, Department of Pathology, University of Heidelberg, 69120 Heidelberg, Germany [R. R., M. v. K. D.]

ABSTRACT

Chemotherapy and radiation therapy for cancer often have severe side effects that limit their efficacy. Glucocorticoids (GCs) are frequently used as cotreatment because they may have potent proapoptotic properties and reduce nausea, hyperemesis, and acute toxicity on normal tissue. In contrast to the proapoptotic effect of GCs in lymphoid cells, resistance toward cancer therapy-mediated apoptosis was induced in solid tumors of human cervix and lung carcinomas. Filter hybridization, expression data, as well as functional assays identified multiple core apoptosis molecules, which are regulated by GCs in a pro- or antiapoptotic manner. Both antiapoptotic genes such as FLIP and members of the Bcl-2 and IAP family as well as proapoptotic elements of the death receptor and mitochondrial apoptosis pathways were down-regulated in carcinomas resulting in a decreased activity of caspase-8, caspase-9, and caspase-3. In contrast, death receptor and mitochondrial apoptosis signaling as well as caspase activity was enhanced by dexamethasone in lymphoid cells. To restore apoptosis sensitivity in dexamethasone-treated carcinomas, caspase-8 and caspase-9 were transfected. This resensitized tumor cells in vitro and xenografts in vivo to cisplatin induced cell death. These data therefore raise concern about the widespread combined use of GCs with antineoplastic drugs or agents in the clinical management of cancer patients.

INTRODUCTION

GCs such as DEX are of great value in the treatment of inflammatory disorders such as rheumatoid arthritis, asthma, and dermatitis, autoimmune diseases such as Crohn’s disease (1), tissue edema, and GCs also possess antipyretic activity, act as antiinfectives during chemotherapy, and are used in the therapeutic induction of apoptotic cell death in malignant lymphoid cells (2, 3). Before, during, and after chemotherapy of solid tumors, GCs are given at various doses to reduce acute toxicity, particularly hyperemesis, and to protect normal tissue of cancer patients against the long-term effects of genotoxic drugs (4).

The effects of GCs are exerted through the GR, a ligand-induced transcription factor, which belongs to the nuclear receptor superfamily (5). GR controls transcription by binding to GR response elements in regulatory sequences of target genes or by modulating the activity of other transcription factors such as AP-1, NF-AT, and NF-xB (6).

Although hundreds of genes are regulated by GR and despite its clinical importance, the molecular mechanism underlying GC-induced apoptosis is poorly understood.

Chemotherapeutic agents and y-irradiation induce apoptosis signaling, which converges in the activation of initiator caspses (e.g., procaspase-8, procaspase-9), resulting in the proteolytic activation of effector caspses (e.g., caspase-3) that cleave specific substrates. Cross-linking of death receptors by the cognate death ligands or release of apoptogenic factors from mitochondria elicits the activation of initiator caspses. Death signaling may be antagonized by antiapoptotic modulator proteins such as FLIP, members of the IAP family, or proteins of the Bcl-2 family. The death receptor and the mitochondrial death pathway may be linked by the Bcl-2 family member BID, which undergoes caspase-8-mediated cleavage, thereby triggering mitochondrial dysfunction and cytochrome c release (7–9).

To better understand the role of GR-induced apoptosis, we examined the effect of DEX on cancer therapy-mediated death in human lung and cervical carcinoma cells. Surprisingly, and in contrast to lymphoid cells, DEX had a strong antiapoptotic effect in the used carcinoma cells and prevented cancer therapy-induced tumor reduction and apoptosis. This was because of inhibition of key molecules of the death receptor and the mitochondrial apoptosis pathway resulting in a blockade of caspase activity. Direct transfer of caspases restored apoptosis sensitivity of DEX-treated carcinomas in vitro and in vivo. These findings suggest that proapoptotic effects of chemotherapy or radiotherapy regimens in patients with solid tumors might be strongly antagonized by the significant antiapoptotic effects of DEX on solid cancer cells. They therefore suggest a careful considering of GC combination therapy in the treatment of cancer patients.

MATERIALS AND METHODS

Cell Culture. P693 (established directly as a xenograft from a human epidermoid lung carcinoma), P5, and HeLa cell lines were grown at 37°C in DMEM. DMEM and RPMI were obtained from Life Technologies, Inc. (Karlsruhe, Germany) and were supplemented with 10% fetal bovine serum (Life Technologies, Inc.).

Nude Mice and Xenografts. P693 cells (10⁶ cells in 200 μl) were injected s.c. into the dorsal surface of 6–10-week-old NMRI (nu/nu) female mice. Therapy was started 1 week after transplantation when the tumor sizes reached a volume of ~500 mm³. The mice were given 0.28 mg/liter DEX in the drinking water, and the amount of water consumed was monitored. Cisplatin was injected i.p. or i.t. The tumor volumes were measured with calipers and calculated using the formula for the volume of an hemiellipsoid: \[ \pi \times (\text{width}^2 \times \text{length}) / 6 \]. Mice were humanely euthanized at tumor sizes > 3000 mm³.

Stimulation of Cells. Cisplatin (Sigma, Deisenhofen, Germany) was dissolved in DMSO at a concentration of 10 μg/μl. A 25 mm stock of DEX (Sigma) or a 50 mM stock of RU486 (Mifepristone; Sigma) was prepared in ethanol. αAPO-1 antibody was prepared as previously described (10), and human recombinant TRAIL, CD95-L, and TNF-α proteins were obtained from Alexis (Grünberg, Germany). Stock solutions were dissolved in PBS. Z-VAD-fmk was from Calbiochem (Bad Soden, Germany), and a stock solution was prepared in DMSO. Final concentrations of the solvents in medium were ≤0.1%. Cells were γ-irradiated in their flasks using a cesium γ-ray source.
**Measurement of Apoptosis.** Early apoptotic changes were identified by staining of cells with FITC-conjugated annexin V and propidium iodide (BD Biosciences, Heidelberg, Germany) and analyzed by flow cytometry (FACS-can, BD Biosciences) as described previously (11).

**Detection of Cytokeratin Cleavage by Immunohistochemistry.** Cells were grown on chamber slides, fixed in 100% methanol, and permeabilized in 1% Triton X-100. Nonspecific binding was reduced by incubation in 10% Roti-Immunoblock (Rotth, Karlsruhe, Germany). Cleavage of the caspase substrate cytokeratin was examined by monoclonal antibody M30 (Roche, Mannheim, Germany). Bound antibodies were detected by fluorescein-conjugated antimouse IgG (Molecular Probes Europe, Leiden, the Netherlands). Cells were mounted in Kaiser’s glycerol gelatin (Merck, Darmstadt, Germany) and examined by fluorescence microscopy. Controls were stained with mouse IgG1 clone DAK-G01 (Dako, Glostrup, Denmark) directed toward Aspergillus niger glycerol oxidase.

**Western Blot Analysis.** Proteins were harvested and Western blot analysis was performed as described previously (12). Mouse monoclonal antibodies were used for detection of CD95-L, TRAIL, FADD (BD Biosciences, Heidelberg, Germany), and -actin (ICN, Eschwege, Germany). Rabbit polyclonal antibodies were used for detection of GR (Alexis, Grünberg, Germany). Bound antibodies were detected by antimouse/horseradish peroxidase conjugates (Santa Cruz Biotechnology) and enhanced chemiluminescence system.

**RT-PCR.** Total RNA was harvested, converted to cDNA, and PCR was performed as described previously (13). Primer sequences are available upon request.

**Fluorogenic Assays for Detection of Caspase Activity.** Activity of caspase-8, caspase-9, and caspase-3 was detected using fluorescent assay kits according to the instructions of the manufacturer (Clontech, Heidelberg, Germany). In brief, cell lysates were incubated with specific substrates, and the resulting fluorescence according to caspase activity was analyzed in a Victor fluorometer (Wallac, Freiburg, Germany).

**Determination of Mitochondrial Membrane Permeability.** Altered mitochondrial membrane permeability was identified by staining of cells with 10 µg/ml JC-1 (Molecular Probes, Leiden, the Netherlands) according to the instructions of the manufacturer (14). Mitochondrial depolarization was detected by FACS analysis and measuring the percentage of green-fluorescent cells.

**cDNA Constructs in the Mammalian pcDNA3 Expression Vector.** The cDNA for caspase-9-inserted XbaI in pBlueScript SK (+/-) was kindly provided by Dr. Donald W. Nicholson. The caspase-9 cDNA was subcloned into the pcDNA3 vector using the restriction sites NotI and EcoRI. The pcDNA3 expression vector for caspase-9 is described, and a pcDNA3-FADD-DN construct was kindly provided by Dr. Vishva Dixit. pcDNA3-GFP was from Clontech.

**Stable Transfectants.** P5 cells were washed in PBS, resuspended at 5 × 106 cells/200 µl PBS, and transfected with 50 µg of a pcDNA3-FADD-DN expression construct or empty pcDNA3 vector by electroporation (975 µF, 220 V). After transfection, the cells were resuspended in 12 ml of fresh medium. Twenty-four h later, cells were washed in PBS, and selection medium containing 1 mg/ml G418 was added (Calbiochem). Resistant cells were selected over a period of 6 weeks.

**Liposomal-mediated Transfer of Caspase-8 and Caspase-9.** For in vitro transfections, P693 cells were grown on chamber slides, and recombinant human caspase-8 or caspase-9 proteins (Alexis/BioVision, Gruenberg, Germany) were added at a concentration of 2.5 units/5 × 105 cells/200 µl medium using Fugene liposomes (Roche) according to the instructions of the manufacturer. pcDNA3-caspase-8, pcDNA3-caspase-9, or pcDNA3-GFP were added at a concentration of 2.5 µg/5 × 105 cells/200 µl medium using Fugene liposomes. For in vivo transfections, P693 cells were grown as xenografts in nude mice. A mixture of recombinant caspase-8 and caspase-9 proteins (10 units each) and pcDNA3-caspase-8 and caspase-9 vectors (10 µg each) was i.t. injected using Fugene liposomes as vehicle.

**RESULTS**

To additionally elucidate the effect of DEX on cancer therapy-mediated death, we used human lung and cervical carcinoma cells. Patient-derived P693 lung carcinoma xenografts were grown in nude mice. At a tumor size of 500 mm3, cisplatin was injected i.p. weekly over a period of 5 weeks in the absence or presence of DEX, which was added to the drinking water. Weekly measurement of the tumor volume revealed a profound reduction of tumor growth in cisplatin-treated mice (Fig. 1A). DEX prevented the growth-inhibiting effect of cisplatin because the tumors grew faster in combination treatment. Tumor growth of untreated control mice or of mice receiving DEX only increased continuously. Our in vivo results are confirmed by growth curves in vitro in which DEX cotreatment strongly reversed the growth-inhibiting effect of cisplatin in P5 cervix carcinoma cells as observed by counting living cells by trypan blue exclusion during a period of 5 days after treatment (Fig. 1B).

To investigate whether DEX might protect tumors by interfering with cisplatin-induced apoptosis, we examined the cervix carcinoma cell lines P5 and HeLa in vitro. Cells were treated with cisplatin or γ-irradiated in the presence or absence of DEX using therapeutic doses. DEX was preincubated 48 h before cisplatin stimulation. Apoptosis was detectable 24 h after treatment and increased during the next 48 and 72 h (Fig. 2A). Combined DEX treatment strongly reduced the levels of basal or induced apoptosis. Similar results were found for P693 cells in vitro (data not shown). In contrast, DEX alone or in combination with cisplatin had a proapoptotic effect in the human leukemic T cell line CEM (Fig. 2B, compare also Refs. 2, 3). The apoptosis-inhibitory effect of DEX in carcinoma cells might be attributable to a defective GR. Therefore, expression of the GR was analyzed in P5, P693, and HeLa cells by immunoblotting. DEX led to a strong induction of GR within 24 h, which was additionally up-regulated during the next 96 h in all three cells lines (Fig. 2C and data not shown).

Fig. 1. DEX inhibits therapy-induced carcinoma regression in vivo. A, patient-derived human epidermoid lung cancer cells (P693) were injected i.c. into nude mice. At a tumor volume of 500 mm3, cisplatin (CIS, 5 mg/kg/week) was either weekly i.p. injected alone or in combination with DEX (DEX/CIS), which was added to the drinking water (0.28 mg/liter) for the duration of the experiment. The daily rate of DEX consumption was ~30 ng/g body weight. As controls, tumor growth of xenografts from mice that received neither cisplatin nor DEX (CO) or from mice that received DEX only (DEX) was determined. The tumor volume in each animal was measured at weekly intervals for a period of 5 weeks and is shown as cartoon on the right. Data are presented as the mean ± SD of six to eight animals. B, P5 cervix carcinoma cells were treated with cisplatin (2 µg/ml) in the absence or presence of DEX (1 µM) as indicated. Two, 3, 4, and 5 days later, living cells were counted by trypan blue exclusion to determine proliferation.
In addition with RU486 (10 μM, H9262) above, was assessed by annexin staining and flow cytometry. Cells, which were treated with cisplatin and DEX as described previously (17–20), were grown in the absence (CO) or presence (F) of DEX (1 μM), which was preincubated for 48 h. Control cells (CO) treated with DEX alone were examined 96 h after incubation. The percentage of apoptosis was measured by annexin staining and FACS analysis. B, apoptosis of CEM cells, which were treated with cisplatin and DEX as described above, was assessed by annexin staining and flow cytometry. C, protein extracts from P5 cells grown in the absence (CO) or presence of DEX for 24, 48, 72, and 96 h were fractionated by SDS-PAGE, and the GR receptor was visualized with a specific rabbit polyclonal antibody. α-Actin immunoblotting served as loading control. The molecular weights in kDa are indicated on the right. D, apoptosis of P5 cells treated as described above and in addition with RU486 (10 μM) was measured by annexin staining and flow cytometry. SDs are <10%.

Not shown). This finding correlates with the described positive autoregulation of GR (15). Also, RU486, which antagonizes the action of GR (16), restored the apoptosis sensitivity of the above described DEX cotreated cells, whereas RU486 alone did not influence basal or cisplatin-induced apoptosis significantly (Fig. 2D).

To determine whether DEX may inhibit apoptosis in carcinoma cells by up-regulating antiapoptotic proteins expression of Bcl-2, FLIP and members of the IAP-family were analyzed by Western blotting. Compared with the levels in control or cisplatin-treated P5 cells, no up-regulation of any of these proteins by DEX alone or in combination with cisplatin was found (Fig. 3A). Quite the opposite, DEX totally repressed basal and induced protein levels.

To evaluate the involvement of other genes, mRNA expression profiles were performed using nylon membranes containing PCR products of ~800 cDNAs. These selected genes are important for signaling, apoptosis, and regulation of the immune system. The gene list is available on request. DEX alone or in combination with cisplatin significantly altered the expression of genes encoding core apoptosis molecules (data not shown). These microarray data were confirmed by RT-PCR. DEX down-regulated basal and cisplatin-induced expression of CD95-L, TRAIL, FADD, caspase-9, caspase-8, and BID in P5 cells (Fig. 3B), whereas down-regulation of these genes in CEM cells was not observed (data not shown). In lymphoid cells, proapoptotic genes, e.g., CD95, caspase-3 and caspase-4, are up-regulated and antiapoptotic molecules, e.g., Bcl-2 and Bcl-xL, are down-regulated by DEX mediating enhanced apoptosis as described previously (17–20).

DEX-induced down-regulation of CD95-L and TRAIL RNA levels was confirmed by Western blot analysis of the respective proteins (Fig. 4A). To test whether these death ligands might contribute to apoptosis in carcinomas, P5 cells were stimulated with active recombinant CD95-L or TRAIL proteins. Twenty-four h later, apoptosis was found to be enhanced by both death ligands, whereas DEX cotreatment strongly repressed apoptosis (Fig. 4B). These data suggest that an additional blocking effect of DEX downstream of death ligands diminishes apoptosis. To examine whether the DEX-mediated block in death receptor signaling may be the predominant factor in DEX-induced resistance, this situation was mimicked by stably transfecting a FADD-DN construct in P5 cells. Western blot analysis demonstrated a strong overexpression of FADD-DN that resulted in a total resistance toward CD95-induced apoptosis (Fig. 4C and data not shown). Thus, a total inhibition of the death receptor pathway was ensured. However, FADD-DN only retarded drug-induced apoptosis at early time points of apoptosis at 24 h but did not imitate the long-lasting inhibitory effect of DEX at 48 h.

Inhibition of several pro- and antiapoptotic molecules (Bcl-2, FLIP, X-IAP, cIAP-1, CD95-L, TRAIL, FADD, caspase-9, caspase-8, and BID) by DEX in carcinoma cells implicates that the antiapoptotic effect may be attributable to a broad spectrum inhibition of protein synthesis. CHX is another well-known global inhibitor of de novo protein synthesis (21). However, unlike DEX, CHX cotreatment of HeLa and P5 cells did not significantly inhibit apoptosis induced by recombinant death ligands, cisplatin, or γ-irradiation in absence or presence of DEX (Fig. 4D and data not shown). Therefore, the apoptosis-inhibitory effect of DEX in carcinomas may be not attributable to a general inhibition of protein synthesis.

DEX may influence the mitochondrial death pathway in a cell-type-specific manner. Changes in ΔΨm were monitored in P5 and CEM cells by using the potentiometric, fluorescent dye JC-1. The drop of red to green fluorescent cells indicating loss of ΔΨm was analyzed by...
flow cytometry. In P5 cells, cisplatin induced a continuous decrease of \( [\text{H9004}] \) within 72 h, which was prevented at 48 and 72 h by DEX. However, DEX alone or in combination with cisplatin induced an early decrease of \( [\text{H9004}] \) at 12 and 24 h, which dropped back to basal levels during the next 72 h (Fig. 5). In contrast, treatment of CEM cells with DEX, cisplatin, or both together led to a permanent increase of \( [\text{H9004}] \). Thus, the initial decrease of \( [\text{H9004}] \) in DEX-cotreated P5 cells is prevented at later time points by an as yet unknown mechanism.

The DEX-mediated antiapoptotic effect in carcinoma cells might involve inhibition of caspases. To measure activity of caspase-8, caspase-9, and caspase-3 in P5 and CEM cells, we performed fluorometric assays using specific substrates for these caspases. Whereas cisplatin strongly increased the activity of all three caspases in each cell line, the presence of DEX completely blocked substrate turnover in P5 but superinduced it in CEM cells (Fig. 6). RT-PCR was performed using specific primers for CD95-L, TRAIL, FADD, caspase-9, caspase-8, and BID. The sizes of the cDNA products in bp are indicated in the left. GAPDH expression is a marker for equal conditions.

Irrespective of the underlying mechanism, inhibition of both death receptor and mitochondrial apoptosis signaling may be the main reason of DEX-induced therapy resistance in carcinoma cells. Because activation of caspases is a prerequisite in chemotherapy- and radiation therapy-induced apoptosis transfer of the respective initiator caspase-8 and caspase-9 might restore apoptosis sensitivity. P693 cells were transiently transfected with pcDNA3 expression vectors encoding genes for these caspases using liposomes. Forty-eight h later, cleavage of cytokeratin reflecting caspase activity and apoptosis

Fig. 3. DEX interferes with key apoptotic molecules. A, P5 cells were treated with cisplatin (CIS, 2 \( \mu \text{g/ml} \)) in the absence (+DEX) or presence (−DEX) of DEX (1 \( \mu \text{m} \)). Six or 24 h later, proteins were extracted, and expression of FLIP, Bcl-2, X-IAP, and cIAP-1 was detected by Western blot analysis. B, total RNA was harvested from P5 cells that were either untreated, stimulated with cisplatin for 24 h (CIS), preincubated with DEX for 48 h, and then coincubated with cisplatin for additional 24 h (CIS/DEX) or incubated with DEX alone for 72 h (DEX). RT-PCR was performed using specific primers for CD95-L, TRAIL, FADD, caspase-9, caspase-8, and BID. The sizes of the cDNA products in bp are indicated on the left. GAPDH expression is a marker for equal conditions.

Fig. 4. DEX inhibits death receptor signaling. A, P5 cells were treated with cisplatin (CIS, 2 \( \mu \text{g/ml} \)) in the absence or presence of DEX (1 \( \mu \text{m} \)), which was preincubated for 48 h as indicated. Forty-eight h later, proteins were extracted, and expression of CD95-L and TRAIL was examined by Western blot analysis. B, P5 cells were left untreated or were stimulated with recombinant CD95-L or TRAIL proteins (100 ng/ml each) in the absence or presence of preincubated DEX. Twenty-four or 48 h later, the percentage of specific apoptosis was determined by annexin staining and flow cytometry. The percentage of specific apoptosis was calculated as follows: \( 100 \times \left( \frac{\text{experimental apoptosis} \% - \text{spontaneous apoptosis in the control} \%}{\text{spontaneous apoptosis in the control} \%} \right) \). D, HeLa and P5 cells were treated with recombinant CD95-L, TRAIL, or TNF-\( \alpha \) proteins (100 ng/ml each) in the absence or presence of CHX (200 ng/ml) or DEX (1 \( \mu \text{m} \)) as indicated. Twenty-four h later, apoptosis was measured by staining with annexin/propidium iodide using flow cytometry.
was detected by staining with M30 cytodeath antibody and fluorescence microscopy. Transfer of each caspase alone or both together resulted in strong and comparable amounts of cytokeratin cleavage in the absence of DEX, whereas cells transfected with empty vector alone did not show any cleavage (Fig. 7A). However, in the presence of DEX, the fluorescence of caspase-overexpressing cells was somehow weaker, suggesting that DEX also interferes with vector-mediated expression and activity of caspases. To overcome this limiting effect, we transfected recombinant active caspase proteins alone or in combination with the pcDNA3 expression vectors. By this way, both untreated or DEX-treated cells exhibited the same extent of fluorescence. Repression of caspase activity in transfected cells by Z-VAD-fmk resulted in a total absence of fluorescence, which confirmed specific induction of apoptosis by caspases. Correspondingly, induction of caspase activity in untreated cells by the CD95-agonistic αAPO-1 antibody led to a strong fluorescence, thus confirming specificity of the transfection assays. Staining was not observed in transfected control cells, which were incubated with the fluorescein-coupled secondary antibody only. Transfection efficiency was ~70% as tested by a pcDNA3-GFP expression construct and flow cytometry.

To assess whether the transfer of caspase-8 and caspase-9 would sensitize DEX-treated carcinoma cells to cytotoxic therapy in vivo, P693 cells were injected s.c. into nude mice. At a tumor volume of 500 mm³, a mixture of liposomes, caspase-8 and caspase-9 proteins, caspase-8 and caspase-9 pcDNA3 expression vectors, and cisplatin was injected i.t. in the presence or absence of DEX, which was added to the drinking water. Injections were repeated 2 and 12 days after the initial treatment. As already shown, cisplatin strongly inhibited tumor growth, which was neutralized by DEX, whereas tumor growth of untreated control mice increased continuously (Fig. 7B, compare Fig. 1A). Also, the tumor volume of other control mice receiving liposomes together with empty pcDNA3 vector increased continuously. Transfection of caspases alone had a strong tumor reducing effect similar to the one found for cisplatin. Most importantly, the presence of caspases and cisplatin together reduced tumor growth below the levels of single treatment, even in the presence of DEX. Together, transfer of caspase-8 and caspase-9 may be a powerful gene therapeutic approach to overcome DEX-induced apoptosis resistance of solid tumors.

**DISCUSSION**

We report here that DEX confers apoptosis resistance of human cervical and lung carcinoma cells toward apoptosis induced by cisplatin, γ-irradiation, or ligation of death receptors (CD95, TRAIL) in vitro and in xenografted carcinomas by disruption of critical cell death pathways. This effect is not observed in lymphoid cells where GCs are proapoptotic. Because of these proapoptotic properties in lymphoid tissues, GCs are frequently used as cotreatment during cancer therapy and also to reduce nausea, hyperemesis, and acute toxicity on normal tissue. Our finding of apoptosis prevention by GCs in solid tumor cells is not restricted to the cervical and lung carcinoma cells examined in this article but seems to be a more common phenomenon. GCs have been demonstrated to inhibit apoptosis in many normal and carcino-

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Fig. 5. DEX prevents cisplatin-induced loss of Δψm. P5 or CEM cells were left either untreated (CO) or stimulated with cisplatin (CIS, 2 μg/ml) for 12, 24, 48, and 72 h in the absence or presence of preincubated DEX. Cells were stained with JC-1 to indicate mitochondrial depolarization by a drop from red to green fluorescence. The percentage of green-fluorescent cells was determined by flow cytometry.

Fig. 6. DEX blocks expression and activity of caspases. P5 and CEM cells were treated with cisplatin (2 μg/ml) in the absence or presence of preincubated DEX as indicated. Twenty-four h (□) and 48 h (■) after treatment, cells were lysed, and activity of caspase-8, caspase-6/9, and caspase-3 was determined by specific fluorescent assays and analyzed in a victor fluorometer.
Our results suggest a DEX-mediated blockade in expression of key apoptosis molecules in carcinomas but not in lymphoid cells. By using microarray assays, Western blot analysis, and RT-PCR, we identified several genes of the death receptor and mitochondrial apoptosis pathways to be influenced by DEX. DEX antagonizes basal and cisplatin-induced expression of key elements of the cell death receptor pathway such as CD95-L, TRAIL, FADD, and caspase-8 in carcinomas. However, down-regulation of these proapoptotic genes was not observed in lymphoid cells. This observation is confirmed by diverse authors describing up-regulation of proapoptotic genes, e.g., CD95, caspase-3, and caspase-4 as well as down-regulation of antiapoptotic molecules, e.g., Bcl-2 and Bcl-xL after DEX-treatment of lymphoid cells (17–20). This cell-type-specific pro- and antiapoptotic effects of DEX might be the reason for inhibition of apoptosis in carcinoma cells but activation of apoptosis in lymphoid cells. However, the upstream mechanism for this cell-type-specific controversy is unknown as yet.

In functional assays, we observed a DEX-mediated total repression of cisplatin-induced depolarization of the mitochondrial membrane potential together with strong inhibition of expression and activity of caspase-9 in carcinomas but rather up-regulation of these events in lymphoid cells. Also, the expression of BID, which links the death receptor pathway to mitochondria, was strongly down-regulated in carcinomas only. Thus, DEX treatment inhibited in parallel to the death receptor pathway key elements of the mitochondrial death signaling system as the most prominent pathway in chemotherapy-induced apoptosis (7, 8, 26). Interestingly, although DEX prevented cisplatin-induced loss of Δψm in carcinomas at later time points, we observed a direct induction of Δψm at earlier time points (24 h) similar to lymphoid cells. This suggests that the activity of a putative repressor molecule is induced by DEX in carcinoma cells that might prevent
apoptosis induction. Although no such inhibitory molecule was identified as yet, our data are in line with recent reports describing that GC-induced apoptosis in lymphoid cells involves direct mitochondrial damage (2, 3). Furthermore, the markedly reduced GC-mediated apoptosis of thymocytes in caspase-9-deficient mice (27) underscores our finding of DEX-mediated apoptosis induction by caspase-9 activation in lymphoid cells.

DEX-mediated suppression of apoptosis in carcinoma cells is not attributable to a mutation of GR but rather involves transcriptional activity of the functional receptor. We conclude this from Western blot data showing DEX-mediated up-regulation of GR expression and from experiments using the GR inhibitor RU486, which partially rescues apoptosis sensitivity of DEX-treated carcinoma cells. Correspondingly, Mikosz et al. (42) suggest GR-mediated protection of breast cancer cells from serum deprivation-induced apoptosis. Therefore, mutations in the GR may be excluded as a reason for DEX-mediated apoptosis inhibition in certain cell types. More likely, an activated GR may transcriptionally affect gene expression in a cell-type-specific manner as we observed in carcinomas and lymphoid cells. The GR might negatively regulate promoter regions of proapoptotic genes such as CD95 and CD95-L (29–32), whereas promoter regions of other core apoptosis molecules, e.g., caspases, are not characterized until now.

Alternatively, reduction in expression and release of numerous cytokines upon GC treatment may well suffice to induce apoptosis in dependent cells such as observed in leukemic T cells (33), whereas cytokine-independent cells such as peripheral T lymphocytes (34, 35) or carcinoma cells survive. Nevertheless, the question still remains which upstream factor renders DEX-treated solid tumors resistant to therapy-induced apoptosis. Our data suggest a more complex effect of GR on apoptosis inhibition in carcinoma cells because DEX prevented not only expression of proapoptotic genes but simultaneously reduced expression of antiapoptotic genes such as FLIP, Bcl-2, X-IAP, and IAP-1 in carcinoma cells. Therefore, one might speculate that the GR might regulate expression and release of numerous antiapoptotic factors, e.g., caspases, are not characterized until now.

Alternatively, reduction in expression and release of numerous cytokines upon GC treatment may well suffice to induce apoptosis in dependent cells such as observed in leukemic T cells (33), whereas cytokine-independent cells such as peripheral T lymphocytes (34, 35) or carcinoma cells survive. Nevertheless, the question still remains which upstream factor renders DEX-treated solid tumors resistant to therapy-induced apoptosis. Our data suggest a more complex effect of GR on apoptosis inhibition in carcinoma cells because DEX prevented not only expression of proapoptotic genes but simultaneously reduced expression of antiapoptotic genes such as FLIP, Bcl-2, X-IAP, and IAP-1 in carcinoma cells. Therefore, one might speculate that the inhibition of apoptosis by DEX in carcinoma cells is attributable to a broad spectrum inhibition of protein synthesis. However, because the well-known global protein synthesis inhibitor CHX did not prevent therapy-induced cell death in the carcinoma cells used in this study, this assumption may be negligible.

Nevertheless, repression of the death receptor and mitochondrial pathway in carcinoma but not in lymphoid cells results in inhibition of caspase-8 and caspase-9, leading to a decreased activity of the effector caspase-3. In line with these data, decrease of caspase-3 cleavage and apoptosis after exposure of primary human and rat hepatocytes to DEX has been found (22). Therefore, failure to activate caspases appears to be the main reason for DEX-induced apoptosis resistance in carcinoma cells. This suggestion is strongly supported by the results.
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