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ORIGINAL ARTICLE

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Epidermal growth factor enhances cisplatin-induced apoptosis by a caspase 3 independent pathway

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Abstract *Purpose*: Activation of the epidermal growth factor (EGF) receptor has previously been shown to increase the sensitivity of cancer cells to DNA-damaging agents, including cisplatin, UV-B, and γ-radiation. We now investigated the mechanisms by which EGF enhances the sensitivity of human ovarian cancer cells to cisplatin. *Results*: The effect of EGF on cisplatin sensitivity could not be entirely explained by alterations in the cellular detoxification of cisplatin by glutathione or DNA repair of transcribed genes, as assessed by a plasmid reactivation assay. Furthermore, EGF did not affect the levels of several proteins that regulate apoptotic pathways, including bcl2, bclX_L, bax and p53. Cisplatin treatment resulted in activation of caspase 3 and subsequent cleavage of specific substrates containing the DEVD (Asp-Glu-Val-Asp) amino acid sequence, including PARP (poly(ADP-ribose) polymerase). The EGF-mediated increase in cisplatin-induced apoptosis, however, did not result in increased caspase 3 activity. Moreover, apoptosis induced by cisplatin alone was completely inhibited by the caspase 3 inhibitor DEVD-CHO, whereas cell death induced by combined treatment with cisplatin and EGF was not prevented by inhibition of caspase 3. *Conclusion*: Our results suggest that, although cisplatin alone induces apoptosis by a caspase 3 dependent pathway, EGF enhances cisplatin-induced cell death by activating an apoptotic pathway that is independent of caspase 3.

Key words Apoptosis · Caspase · Chemotherapy · Cisplatin · EGF

Abbreviations *AMC*: 7-amino-4-methylcoumarin · *CHAPS*: 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate · *DEVD*: amino acid one-letter code for Asp-Glu-Val-Asp · *DEVD-AMC*: DEVD conjugated to AMC · *DEVD-CHO*: DEVD-aldehyde · *DTT*: dithiothreitol · *EGF*: epidermal growth factor · *EGTA*: ethyleneglycol-bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetate · *HEPES*: *N*-[2-hydroxyethyl]piperazine-*N*′- [2-ethanesulfonic acid] · *PARP*: poly(ADP-ribose) polymerase · *PMSF*: phenylmethylsulfonyl fluoride · *YVAD*: Tyr-Val-Ala-Asp · *YVAD-AMC*: YVAD conjugated to AMC

Introduction

Upon binding of EGF to its cognate receptor, multiple signaling pathways are triggered, and a multitude of cellular responses can be induced (reviewed by Boonstra et al. [3]). Previous work from several groups including our laboratory has shown that exposure to EGF enhances the toxicity of cytotoxic agents, such as cisplatin, 5-fluorouracil, taxol, and γ- and UV-radiation [1, 2, 7, 10, 15, 16]. Even though the degree of EGF-induced sensitization to cisplatin was only in the range of two- to fourfold, this is potentially significant, since resistance in patients failing cisplatin therapy can be partially overcome by a twofold increase of the cisplatin dose [13, 14].

Most agents used in the therapy of cancer induce a dose-dependent cellular injury response, ranging from growth arrest and subsequent recovery after mild cellular injury, to apoptotic or even necrotic cell death after stronger insults [23]. Apoptotic cell death has been shown

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to involve activation of caspases, cysteinyl proteases that cleave peptides after aspartate residues (reviewed by Fraser and Evan [12] and Nicholson and Thornberry [19]).

The aim of the present study was to determine whether EGF increased cisplatin-induced cell death by inducing changes in the cellular pharmacology of cisplatin or by modifying the cellular injury response at a level beyond the specific drug–target interaction. We report that EGF enhanced cisplatin-induced apoptosis without affecting the cellular pharmacology of cisplatin, and that although apoptosis induced by cisplatin alone was caspase 3 dependent, apoptosis induced by combined treatment with cisplatin and EGF was largely independent of caspase 3 activity, suggesting that EGF activated additional apoptotic pathways.

Materials and methods

Cell lines and chemicals

The human ovarian carcinoma cell line 2008 was grown as previously described [7]. The 2008 cells express 1.6×10^5 EGF-binding sites/cell ($K_D = 2.4$ nM EGF) [7]. Cisplatin and recombinant human EGF were obtained from Sigma (St. Louis, Mo.). The fluorogenic caspase substrates DEVD-AMC (Asp-Glu-Val-Asp conjugated to AMC, 7-amino-4-methylcoumarin) and YVAD-AMC (Tyr-Val-Ala-Asp conjugated to AMC, 7-amino-4-methylcoumarin) and the caspase 3 inhibitor DEVD-CHO (Asp-Glu-Val-Asp-aldedyde) were purchased from Oncogene Research/Calbiochem (Cambridge, Mass.). Unconjugated AMC was obtained from Molecular Probes (Eugene, Ore.).

Quantification of apoptotic cells

Cells were treated with EGF for 1 h followed by a second hour with EGF combined to cisplatin. Forty-eight hours after treatment, floating and attached cells were harvested by trypsinization. After centrifugation, cells were resuspended in PBS containing 4 μg/ml acridine orange and 4 μg/ml ethidium bromide and assessed for apoptotic morphology by supravital fluorescence microscopy [17]. This method is based on the ability of both dyes to stain nuclear DNA and on the differential cell membrane permeability of each dye. Cells displaying typical condensed and fragmented nuclear morphology were scored as apoptotic. Cells with apoptotic nuclei, but still capable of excluding ethidium bromide from their cytoplasm were classified as early apoptotic, whereas cells with apoptotic nuclei and disturbed plasma membrane permeability, and thus stained by ethidium bromide, were scored as late apoptotic. Cells excluding ethidium bromide and showing normal nuclear morphology were counted as normal, cells permeable for ethidium bromide and lacking apoptotic nuclei were counted as necrotic cells. Two hundred cells per independent experiment were analyzed and scored as viable, apoptotic, or necrotic.

Clonogenic survival assay

Colony-forming assays were performed by seeding 300 cells per 60 mm tissue-culture plastic dish. The cells were allowed to attach overnight. On the next day, cells were exposed to cisplatin for 1 h in the presence or absence of EGF. EGF-treated cells were exposed to 10 nM EGF for 1 h and then to both EGF and cisplatin during the second hour. The media was changed and cells were returned to normal culture conditions. After 10 days, cells were fixed with methanol and stained with 0.01% crystal violet. Cell clusters comprising more than 50 cells were scored as a colony.

Plasmid reactivation assay

The repair of an actively transcribed reporter gene was measured as the luciferase activity generated from a transiently transfected cisplatin-damaged plasmid, as described in detail before [5]. Luciferase expression from a constitutive CMV (human cytomegalovirus) promoter was repressed by pretreating the plasmid DNA with cisplatin, resulting in purified platinated plasmid containing 1.5 ± 1.4 pg Pt/μg DNA (equivalent to 9.3 adducts/plasmid or 3.2 adducts per luciferase-coding region and promoter). Cells were transfected with lipofectin (Life Technologies; Gaithersburg, Md.) in serum-free medium. At given time points, the cells of triplicate wells were lysed in luciferase buffer 25 mM glycylglycine pH 7.8, 15 mM $MgSO_4$, 4 mM ethyleneglycol-bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetate (EGTA), 1% Triton X-100, and 1 mM dithiothreitol (DTT). Aliquots of the lysates were assayed for luciferase activity [4]. The luciferase activities of the platinated vector were normalized to the unplatinated control.

Determination of intracellular glutathione

The levels of free intracellular glutathione were measured on a flow cytometer as the enzymatically generated fluorescent monochlorobimane–glutathione derivative [20]. Cells were stained with 25 μM monochlorobimane at room temperature until steady-state levels of the fluorescent monochlorobimane derivative were reached. Cellular fluorescence was measured on a flow cytometer with excitation and emission settings of 395 and 490 nm, respectively. To avoid reequilibration of intracellular glutathione levels, EGF pressure was maintained throughout the whole experiment.

Immunoblotting

For the detection of poly(ADP-ribose) polymerase (PARP) cleavage, cells were collected by scraping, including floating cells, washed once with ice-cold phosphate-buffered saline, and lysed in SDS-PAGE (sodium dodecylsulfate-polyacrylamide gel electrophoresis) sample buffer containing 6 M urea. Lysates were sonicated and aliquots equivalent to 100,000 cells were separated on 8% SDS-PAGE. The monoclonal C-2–10 anti-PARP antibody was from Dr. G. Poirier (University of Laval, Canada). For the detection of all other proteins, cells were lysed in 0.15 M NaCl, 5 mM EDTA, 1% Triton X100, 5 mM DTT, 100 μM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml pepstatin A, 20 μg/ml leupeptin, 10 μg/ml aprotinin, 10 mM TRIS pH 7.4 for 10 min on ice and centrifuged at 15,000×*g* for 20 min at 4 °C. Equal amounts of protein were separated on SDS-PAGE and immunoblotted. The proteins were revealed with the chemiluminescence reagent ECL (Amersham; Arlington Heights, Ill.). The following antibodies were used: anti-bcl2 (100), anti-p53 (DO-1) and anti-bax (N-20 and P-19) from Santa Cruz Biotechnology (Santa Cruz, Calif.), anti-bclX_L (Ab-1) from Oncogene Research Products (Cambridge, Mass.), anti-caspase 3 precursor (19) from Transduction Laboratories (Lexington, Ky.), anti-caspase 1 precursor (06-503) from Upstate Biotechnology (Lake Placid, N.Y.), anti-lamin A was a generous gift of Dr. B. Burke (University of Calgary, Canada). Where indicated, Jurkat human leukemic T cells where used as positive control for apoptosis.

Caspase activity assay

The enzymatic activity of caspase 3 was assessed by fluorometric determination of free fluorescent AMC generated by proteolytic cleavage from the nonfluorescent conjugated substrate DEVD-AMC. The amount of free fluorescent AMC generated is proportional to the concentration of activated caspase 3 in the lysate. Similarly, caspase 1 activity was assessed with the specific substrate YVAD-AMC. In brief, cells were collected by scraping, washed once with ice-cold phosphate-buffered saline, and lysed in protease

buffer (10 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES) pH 7.4, 0.1% 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate (CHAPS), 2 mM EDTA, 5 mM DTT, 1 mM PMSF, 10 μg/ml pepstatin A, 20 μg/ml leupeptin, 10 μg/ml aprotinin). The lysates were cleared by centrifugation at 15,000×*g* for 10 min and caspase activity was measured in 50-μl aliquots adjusted to 1 μg/ml protein in 96-well microtiter plates. The reactions were started by addition of an equal volume of 50 μM DEVD-AMC or 20 μM YVAD-AMC substrate in protease buffer. The generation of fluorescent AMC was monitored at room temperature in 5-min intervals over 30–60 min at an excitation wavelength of 380 nm and an emission wavelength of 460 nm. From the slope of the linear increase in AMC fluorescence, the protease activity was calculated as the amount of free AMC generated per unit time (fmol AMC min^{-1} μg $protein^{-1}$).

Statistical analysis

Statistical significance was determined by Student's *t*-test and expressed as *P* values. Generally, three or more independent experiments performed on different days were used for statistical analysis.

Results

Effect of EGF on cisplatin-induced cell death

To determine the effect of EGF on cisplatin-induced cell-death, fixed concentrations of EGF and cisplatin were chosen so that sensitization by EGF was easily detected, according to full dose–response curves [7]. Treatment of the human ovarian carcinoma cell line 2008 with 5 μM cisplatin for 1 h induced cell death with the morphological characteristics of apoptosis, including cell shrinkage, membrane blebbing, as well as nuclear condensation and fragmentation. Apoptotic morphology was detectable 24–72 h after treatment, with a maximum after 48 h. Concurrent treatment with 10 nM EGF for 2 h and 5 μM cisplatin during the second hour induced a 1.9 ± 0.3-fold (mean ± SD, $n = 3$) increase in the fraction of cells with apoptotic morphology (Fig. 1A), as measured by supravital staining with acridine orange and ethidium bromide 48 h after treatment ($n = 3$; $P = 0.003$ by *t*-test). The distribution between early and late apoptotic cells, still excluding ethidium bromide or having lost membrane integrity, respectively, was not affected by EGF. Similarly, the number of necrotic cells remained unaffected.

Effect of EGF on cisplatin sensitivity

Cells were exposed to cisplatin and EGF as described in the preceding section. The fraction of surviving cells was determined by a clonogenic survival assay. Combined treatment with 10 nM EGF and 5 μM cisplatin reduced the fraction of colony-forming units by 2.3 ± 0.4-fold (mean ± SD, $n = 3$; $P = 0.034$ by *t*-test) relative to treatment with 5 μM cisplatin alone (Fig. 1B). Similar results were obtained by flow-cytometric analysis of annexin-V-labeled cells (data not shown).

Effect of EGF on reactivation of reporter plasmid damaged by cisplatin

We have previously reported that EGF had only a small effect on the formation of DNA intrastrand guanine–guanine platinum adducts in 2008 [6, 8]. After a 24-h repair period, EGF induced a 10% decrease in the rate of adduct removal [6, 8]. To better understand the biological relevance of this finding, we assessed the effect of EGF on the reactivation of a platinated plasmid, a system more representative for repair of transcribed genes. Treatment of the 2008 cells with 10 nM EGF either prior to or during transfection of the plasmids did not significantly affect the efficiency of reactivation of platinated pKEX-2-XR-Luc plasmids (Fig. 2). As determined in three separate experiments, each performed with triplicate samples, the ratio of the reactivation efficiency in the absence of EGF to the efficiency in

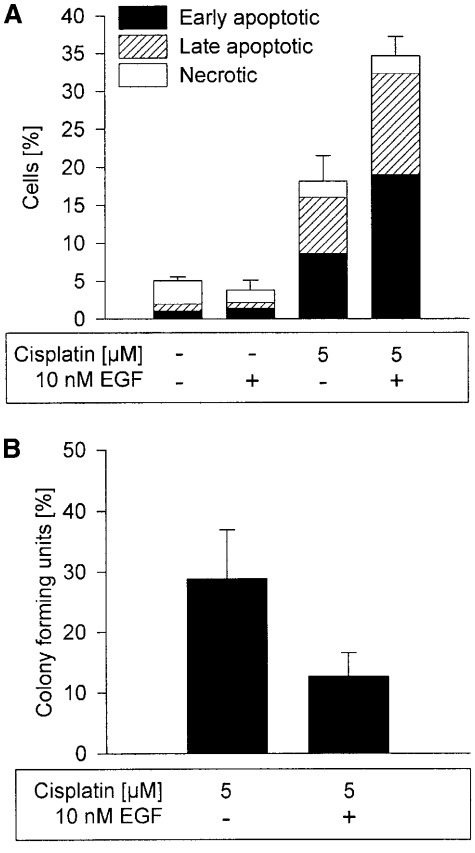


Fig. 1A, B Effect of EGF on cisplatin-induced cell death. **A** Apoptotic morphology: 2008 cells were exposed to EGF (10 nM) alone for 1 h and then to both EGF (10 nM) and cisplatin (5 μM) during the second hour. Combined treatment with EGF plus cisplatin increased the fraction of cells with apoptotic morphology by 1.9 ± 0.3-fold compared to treatment with cisplatin alone, as measured by supravital staining 48 h after treatment (mean ± SD, $n = 3$, $P = 0.003$ by *t*-test). **B** Effect of EGF and cisplatin on colony formation in 2008 cells: Colony formation was 2.3 ± 0.4-fold higher in cells treated with cisplatin alone compared to treatment with the combination of EGF and cisplatin (mean ± SD, $n = 3$, $P = 0.034$ by *t*-test)

the presence of EGF was 0.94 ± 0.06 (mean $\pm$ SD), not significantly different from 1 ($n = 3$; $P = 0.21$ by t-test).

Effect of EGF on cellular glutathione levels

Cisplatin can be detoxified by intracellular glutathione [9]. Therefore, intracellular glutathione concentrations were assessed by flow cytometry by measurement of the fluorescent glutathione–monochlorobimane product. In the 2008 cells, treatment with 10 nM EGF for 2 h increased the generation of glutathione–monochlorobimane fluorescence to $134 \pm 18\%$ (mean $\pm$ SD, $n = 3$) of the untreated controls. The EGF-induced increase in glutathione levels was statistically significant ($P < 0.05$ by two-sided t-test). This effect is opposite to that expected to be associated with enhanced sensitivity to cisplatin.

Effect of EGF and cisplatin on proteins that control apoptosis

The 2008 cells were exposed to 10 nM EGF for 1 h and then to both EGF and cisplatin during the second hour. A cisplatin concentration of 5 μM corresponded to 17% and 36% cells with an apoptotic morphology, scored as described above, in the absence and presence of EGF, respectively. A cisplatin concentration of 10 μM corresponded to 44% and 56% apoptotic cells in the absence and presence of EGF, respectively. The effect of EGF and cisplatin on the protein levels of bcl2, bclX_L, bax, and p53 was assessed by immunoblotting. Treatment with cisplatin alone and treatment with cisplatin combined with EGF had no significant effect on bcl2, bax, or bclX_L proteins (Fig. 3). Moreover, cisplatin-induced accumulation of p53 protein was not affected by concurrent treatment with EGF.

Effect of EGF and cisplatin on PARP cleavage

To qualitatively understand the mechanism of cisplatin-induced apoptosis, we assessed the cleavage of PARP by immunoblotting. PARP is an endogenous and physiological substrate of caspase 3 and was thus used as a marker for caspase 3 activation. An accurate quantification of caspase activity based on a biochemical protease assay is described below. Treatment of the 2008 cells with 10 μM cisplatin resulted in cleavage of full-length 116-kD PARP to the characteristic 85-kD ΔPARP fragment (Fig. 4). Pretreatment of the 2008 cells with 10 nM EGF for 1 h followed by concurrent treatment with 10 μM cisplatin and 10 nM EGF for a second hour also resulted in cleavage of PARP, although the intensity of the 85-kD ΔPARP band appeared slightly reduced compared to that after treatment with cisplatin alone. The treatment resulted in 44 and 56% apoptotic cells for cisplatin alone and its combination with EGF, respectively, as described above.

Effect of EGF and cisplatin on caspase 1 and caspase 3 precursors and lamin A

Similarly, the activation of further caspases by treatment with cisplatin and EGF was qualitatively monitored by measuring the levels of the precursors of caspase 3 and caspase 1 and the levels of endogenous lamin A, a caspase 6 specific substrate. Treatment with cisplatin resulted in activation of caspase 3, as determined by a reduction in caspase 3 precursor levels; however, EGF had no effect on cisplatin-induced changes in caspase 3 precursor levels (Fig. 5). Jurkat cells treated with 1 μM staurosporine (STA) for 4 h served as a positive control.

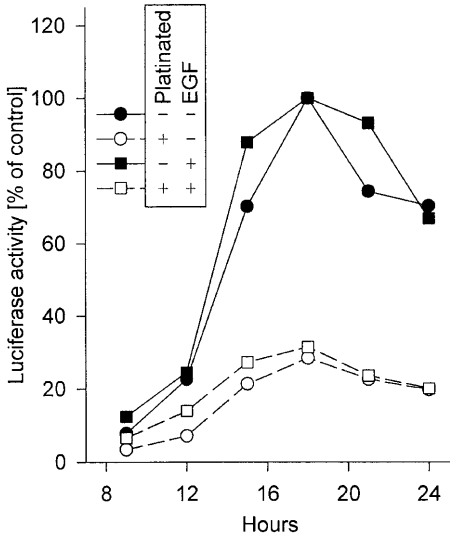


Fig. 2 Effect of EGF on the cellular pharmacology of cisplatin. Reactivation of a reporter plasmid damaged by cisplatin: luciferase activity was determined following transfection of pKEX-2-XR-Luc in 2008 cells. Treatment with EGF (*squares*) did not affect the generation of luciferase activity compared to control cells (*circles*), after transfection of unplatinated (*closed symbols*) or platinated (*open symbols*) pKEX-2-XR-Luc. Luciferase activity generated from the platinated vector was normalized to the activity of the unplatinated vector for each treatment. Averages of triplicate samples from one representative experiment of three are shown

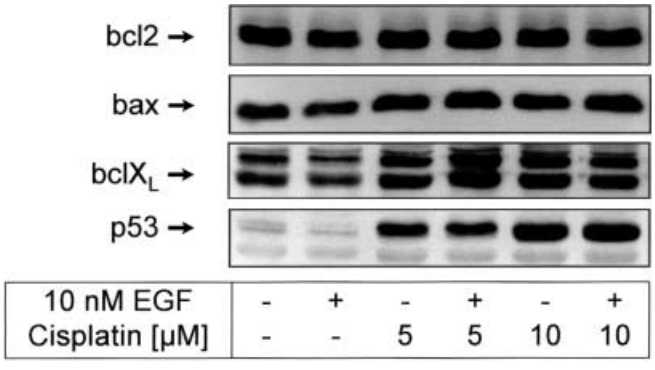


Fig. 3 Effect of EGF and cisplatin on apoptosis-related proteins. Lysates of 2008 cells were prepared 48 h after treatment with EGF and cisplatin. Immunoblots with specific antibodies revealed no change in the levels of bcl2, bax, and bclX_L. EGF had no effect on cisplatin-induced accumulation of p53

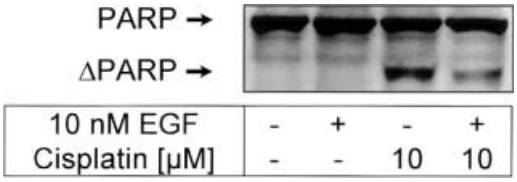


Fig. 4 Effect of EGF and cisplatin on PARP cleavage. Treatment of 2008 cells with 10 μM cisplatin for 1 h resulted in cleavage of PARP to a characteristic 85-kD ΔPARP fragment detected 48 h after treatment. Treatment with EGF and cisplatin resulted in a similar cleavage of PARP

Compared to Jurkat cells, the 2008 cells had lower baseline levels of the caspase 3 precursor and the cisplatin-induced decrease in the caspase 3 precursor level was less marked. The levels of the caspase 1 precursor and lamin A were not affected by treatment with cisplatin or combined treatment with EGF and cisplatin, indicating that caspase 1 and caspase 6 were not activated.

Effect of EGF and cisplatin on DEVD and YVAD cleavage activity

Since the cleavage of endogenous caspase substrates detected by immunoblot, as shown in Figs. 4 and 5, is not an accurate quantitative measure of caspase activity, a biochemical protease assay was utilized. Caspase-specific fluorogenic substrates were chosen to quantitatively assess changes in protease activity induced by treatment with cisplatin and EGF. The DEVD-AMC substrate contains the amino acid sequence of the PARP-cleavage site and is preferentially cleaved by caspases like caspase 3, whereas the YVAD-AMC substrate contains the pro-interleukin-1 cleavage site that is preferentially cleaved by caspases like caspase 1. Treatment with 5 and 10 μM cisplatin alone for 1 h induced a time- and concentration-dependent increase in DEVD-cleavage activity (Fig. 6A). Combined treatment with EGF and cisplatin resulted in less DEVD-cleavage activity relative to treatment with cisplatin alone. Repeated measurements of DEVD-cleavage activity 48 h after treatment showed that EGF reduced DEVD cleavage induced by 5 μM and 10 μM cisplatin by $30.1 \pm 13.4\%$ ($P = 0.071$) and $26.3 \pm 7.1\%$ ($P = 0.002$), respectively (mean ± SD, $n = 3$, t-test). Neither treatment with cisplatin alone nor combined treatment with cisplatin and EGF induced YVAD-cleavage activity like that of caspase 1 (Fig. 6B).

Fig. 5 Effect of cisplatin and EGF on levels of caspase precursors and lamin A. Treatment of 2008 cells with cisplatin reduced caspase 3 precursor levels at 48 h after treatment. EGF had no effect on cisplatin-induced changes in caspase 3 precursor levels. Staurosporine-treated Jurkat cells were used as a positive control. The levels of caspase 1 precursor and lamin A, a caspase 6 specific substrate, were not affected by treatment with EGF and cisplatin

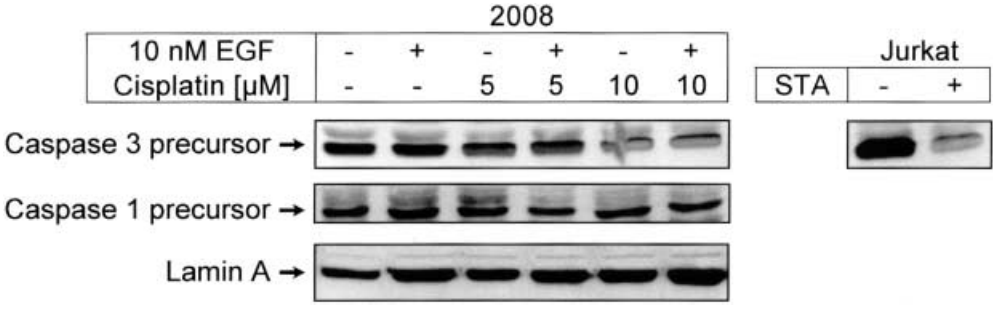
Effect of caspase 3 inhibition on apoptosis induced by cisplatin and EGF

The requirement for caspase 3 activity in apoptosis induced by cisplatin and EGF was determined by use of the specific caspase 3 inhibitor DEVD-CHO. The appearance of cells with apoptotic morphology in cisplatin-induced apoptosis was completely inhibited when the 2008 cells where treated with 100 μM DEVD-CHO concurrently to cisplatin treatment and over 48 h after cisplatin was washed off (Fig. 7; $n = 4$, $P = 0.0002$ by t-test). Apoptosis induced by combined treatment with cisplatin and EGF, however, was only minimally inhibited by exposure to DEVD-CHO. Similar results were obtained in clonogenic assays (data not shown).

Discussion

A short exposure to EGF enhances the cytotoxicity of chemotherapeutic agents in cell lines that overexpress the EGF receptor [1, 2, 7, 10, 15, 16]. The mechanism of this effect is not well understood, although for one experimental system an effect on DNA repair has been suggested [10]. Interestingly, both EGF and an inhibitory anti-EGF-receptor antibody can modulate the extent of drug-induced cell death (reviewed by Mendelsohn and Fan [18]) and the reason for this apparent ambivalence is still elusive. A possible explanation could be the different treatment schedules: a short pretreatment with EGF compared to treatment over several days with the inhibitory antibody.

We now show that the effect of EGF on chemosensitivity is mediated by events that are downstream from the induction of DNA damage by cisplatin. Activation of the EGF receptor has no effect on the extent of cisplatin–DNA adduct formation or on the uptake of



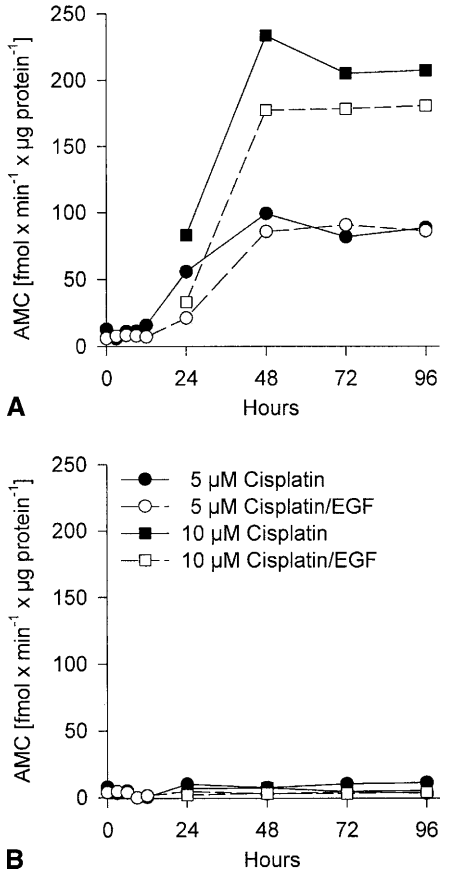


Fig. 6A, B Caspase activities induced by treatment with EGF and cisplatin. **A** Effect of EGF and cisplatin on DEVD cleavage activity: Treatment with 5 µM (*closed circles*) and 10 µM cisplatin (*closed squares*) for 1 h resulted in a time- and dose-dependent increase in DEVD-cleavage activity. *Open symbols* represent values after pretreatment with 10 nM EGF for 1 h and then concurrently with 5 µM (*circles*) or 10 µM (*squares*) cisplatin for a second hour. One representative experiment of three is shown. **B** Effect of EGF and cisplatin on YVAD cleavage activity: Same treatment and representation as in **A**. One representative experiment of three is shown

radioactively labeled cisplatin [6]. EGF did not affect the generation of a functional reporter protein from a transiently transfected plasmid that had been platinated before introduction into the cells. EGF produced a small increase in cellular glutathione levels, an effect opposite to that expected to be associated with enhanced sensitivity to cisplatin. Taken together, these findings indicate that EGF sensitizes cells by mechanisms that are independent of the cellular pharmacology of cisplatin. This is consistent with the observation that EGF did not affect cisplatin-induced accumulation of p53, a protein known to be an essential part of the DNA-damage response, and it is also consistent with the observation that EGF enhances the cytotoxicity of a large number of agents with different mechanisms of action, including γ-radiation and UV-B radiation.

Apoptosis induced by combined treatment with EGF and cisplatin was morphologically indistinguishable from apoptosis induced by cisplatin alone and the kinetics of morphologic changes induced by cisplatin were not changed by EGF. Neither treatment affected the levels and migration pattern of bcl2, bax, or $bclX_L$, proteins known to affect drug-induced apoptosis. It is remarkable that bcl2 and bax protein levels were not significantly affected, although both can be transcriptionally regulated by p53, and p53 levels were increased by cisplatin treatment. It is likely that cisplatin treatment may induce the accumulation of p53 protein without induction of its transcriptional activity. In 2008 cells, cisplatin-induced apoptosis was associated with cleavage of PARP; this suggests that cisplatin activates caspase 3, the caspase with the highest avidity to cleave PARP [22]. Furthermore, treatment with cisplatin resulted in a decrease in caspase 3 precursor levels, confirming cisplatin-induced activation of caspase 3. We found no evidence for caspase 1 activation in response to treatment with cisplatin alone or combined treatment with cisplatin and EGF, either at the level of the precursor protein and of the proteolytic activity. Moreover, the specific caspase 1 inhibitor YVAD-CHO did not affect apoptosis induced by either treatment (data not shown). Similar findings have been reported for etoposide-induced apoptosis [11]. Caspase 6 exhibits partially overlapping cleavage specificity with caspase 3, and caspase 6 is thought to preferentially activate other caspases [19]. However, caspase 6 activation in cisplatin-treated 2008 cells is unlikely, since treatment with cisplatin and EGF did not result in

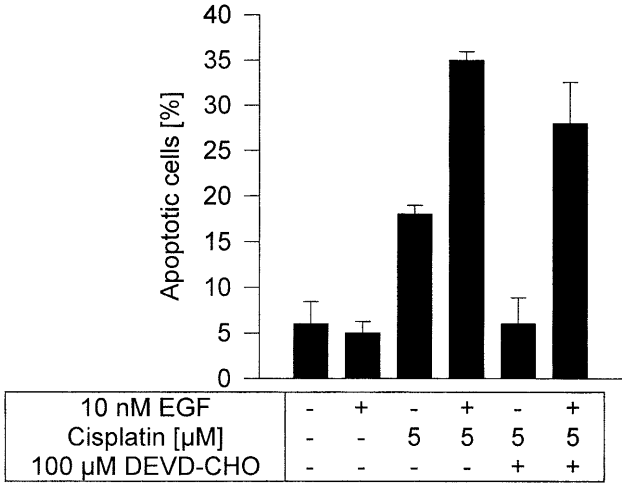


Fig. 7 Effect of caspase 3 inhibition on apoptosis induced by cisplatin and EGF. The appearance of apoptotic cells, as determined by supravital staining with acridine orange and ethidium bromide, was determined 48 h after treatment with 5 µM cisplatin for 1 h, or combined treatment with cisplatin and EGF. The addition of 100 µM of the specific caspase 3 inhibitor DEVD-CHO concurrently to the cisplatin treatment and throughout the 48 h after cisplatin and EGF were washed off completely inhibited apoptosis induced by cisplatin alone ($P = 0.0002$, $n = 4$, *t*-test). In contrast, DEVD-CHO had no significant effect on apoptosis induced by combined treatment with cisplatin and EGF. Averages ± SD from four independent experiments are shown. All samples were corrected for equal content of DEVD-CHO solvent

degradation of lamin A, the preferred endogenous substrate for caspase 6 [21]. Even though EGF caused a twofold increase in cisplatin-induced apoptotic cell death, treatment with EGF did not enhance cisplatin-induced DEVD-cleavage activity, PARP proteolysis, or caspase 3 precursor degradation. Moreover, the specific caspase-3 inhibitor DEVD-CHO completely inhibited cell death induced by cisplatin alone, but failed to inhibit cell death induced by treatment with cisplatin and EGF.

Thus, we conclude that although cisplatin-induced apoptosis requires DEVD-cleavage activity, combined treatment with cisplatin and EGF triggers a separate apoptotic pathway that is largely independent of DEVD cleavage. This suggests that specific signaling agents can directly modulate cell death, not by changing the cellular pharmacology of cisplatin, but by activating additional apoptotic pathways. The ability of EGF to increase cell death induced by agents acting by different mechanisms may offer the opportunity to modulate general apoptotic pathways triggered by chemotherapeutic agents and indicate new strategies to improve existing cancer chemotherapy by modulators of apoptosis.

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