Inhibition of apoptosis facilitates necrosis induced by cisplatin in gastric cancer cells

Lin Jie Zhang[a], Yuan Zhang Hao[a], Chun Song Hu[a], Yan Ye[a], Qi Peng Xie[a], Rick F. Thorne[b], Peter Hersey[c] and Xu Dong Zhang[a,c]

Although cisplatin has been shown to induce both apoptosis and necrosis in cancer cells, the potential interconnections between these modes of cell death induced by the drug remain unknown. We studied this phenomenon in gastric cancer cell lines and identified one cell line (SGC-7901) that underwent apoptosis, and another cell line (BGC-823) that primarily underwent nonapoptotic cell death, in response to cisplatin. Apoptosis in cisplatin-treated SGC-7901 cells seemed to be caspase dependent and was mediated, at least in part, by the BH3-only protein, Noxa. This was evidenced by the rapid upregulation of Noxa and inhibition of apoptosis by small interfering RNA knockdown of Noxa. Nonapoptotic cell death induced by cisplatin in BGC-823 cells was characterized by lack of DNA fragmentation, delayed externalization of phosphatidylserine, caspase independence, plasma membrane disruption, and intracellular vacuole formation, indicative of necrosis. Surprisingly, blockage of apoptosis induction by a general caspase inhibitor or by Noxa small interfering RNA in SGC-7901 failed to protect against cisplatin-induced cell death. Under such conditions, SGC-7901 cells displayed cellular features associated with necrosis. Cisplatin-induced apoptosis, thus, seems to precede necrosis when the apoptotic machinery is operative. When the apoptosis program is defective, necrotic cell death takes place as an alternative pathway leading to cell demise. Induction of different modes of cell death that are interrelated in the same cells by cisplatin has the potential to be exploited in formulating new adjuvant cancer therapies. *Anti-Cancer Drugs* 19:159–166 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2008, **19**:159–166

Keywords: apoptosis, cisplatin, gastric cancer, necrosis, noxa

[a]Department of Immunology, The Anhui Medical University, Hefei, China, [b]Cancer Research Unit, School of Biomedical Sciences, The University of Newcastle and [c]Immunology and Oncology Unit, Calvary Mater Newcastle Hospital, New South Wales, Australia

Correspondence to Dr Xu Dong Zhang, Room 442, David Maddison Clinical Sciences Building, Cnr King & Watt Streets, Newcastle, NSW 2300, Australia
Tel: +61 2 49 236194; fax: +61 2 49 236184;
e-mail: Xu.Zhang@newcastle.edu.au

Received 29 August 2007 **Revised form accepted** 7 October 2007

Introduction

Apoptosis and necrosis are the two major forms of cell death that were originally defined with morphological and biochemical differences [1–4]. Apoptosis is an energy-driven process by which a cell actively destroys itself in response to extracellular or intracellular signals, and is characterized by nuclear condensation and fragmentation, ordered degradation of cellular proteins and organelles, and packaging of the intracellular contents into apoptotic bodies without plasma membrane rupture [1,2]. In contrast, necrosis is often considered as a passive process in which a cell dies as a result of chemical and/or mechanical damage, and is characterized by vacuolation of the cytoplasm, loss of plasma membrane integrity, and release of cellular contents into the extracellular environment [1,3].

Many chemotherapeutic agents used against cancer, including DNA-damaging drugs, mediate their effects by induction of apoptosis, which is commonly executed by the mitochondrial-mediated 'intrinsic' apoptotic pathway [4,5]. This involves release of mitochondrial apoptotic proteins, and activation of the caspase cascade. The Bcl-2 family members play a central role in regulating mitochondrial apoptotic events by preserving or damaging the integrity of the outer mitochondrial membrane [6,7]. Among them, BH-3-only proteins such as Bim, Noxa, and PUMA can act as apoptosis 'sensors', to initiate apoptotic cascade in response to stimuli [6,7].

In addition to inducing apoptosis, a number of chemotherapeutic agents have been shown to induce nonapoptotic forms of cell death [1,8,9]. The significance of nonapoptotic cell death in chemotherapy and the mechanism(s) by which they are induced remain less understood. Given the fact that most cancer cells have defects in the response to induction of apoptosis [4,5], it would be desirable if therapeutic agents can still kill cancer cells resistant to apoptosis through alternative mechanisms.

In the current study, we have examined one gastric cancer line that is sensitive to cisplatin-induced apoptosis and another that is susceptible to cisplatin-induced necrosis. We report that apoptosis induced by cisplatin was caspase dependent and mediated, at least in part, by the BH-3-only

0959-4973

protein of the Bcl-2 family, Noxa. Inhibition of apoptosis by either a general caspase inhibitor or small interfering RNA (siRNA) knockdown of Noxa converted the mode of cell death to necrosis in the apoptosis-sensitive cell line, indicating that cisplatin-induced apoptosis and necrosis are interrelated in gastric cancer cells.

Materials and methods

Cell culture and reagents

Human gastric adenocarcinoma cell lines SGC-7901 and BGC-823 were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (Commonwealth Serum Laboratories, Melbourne, Australia). Cisplatin was supplied by Pharmacia Upjohn (Sydney, New South Wales, Australia). The cell-permeable pancaspase inhibitor Z-Val-Ala-Asp(OMe)-CH2F (z-VAD-fmk) was purchased from Calbiochem (La Jolla, California, USA). The rabbit monoclonal antibody against the active form of caspase-3, the rabbit polyclonal antibodies against poly(ADP ribose) polymerase (PARP), and fluorescein isothiocyanate (FITC)-conjugated annexin V were purchased from Pharmingen (Bioclone, Marrickville, Australia). The rabbit polyclonal antibody against Bim and the monoclonal antibodies against Noxa and PUMA were from Imgenex (San Diego, California, USA).

Cell-viability assays

The cytotoxic effect of cisplatin was determined using MTT assay, as described previously. Briefly, cells were seeded at 5000/well onto flat-bottomed 96-well culture plates and allowed to grow for 24 h, followed by the desired treatment. Cells were then labeled with MTT from the Vybrant MTT Cell Proliferation Assay Kit (Molecular Probes, Eugene, Oregon, USA) according to the manufacturer's instructions.

Apoptosis

Quantitation of apoptotic cells by measurement of sub-G_1 DNA content using the propidium iodide (PI) method or by annexin V staining was carried out as described elsewhere [10]. 4′,6-Diamidino-2-phenylindole (DAPI) staining was performed according to the manufacturer's instructions (Molecular Probes), and was described previously [11].

Propidium iodide-uptake assay

Tumor cells were seeded at 1×10^5 cells/well in 24-well plates and allowed to reach exponential growth for 24 h before treatment. The PI-uptake assay was performed, as described previously [11]. Briefly, adherent cells and nonadherent cells were collected and washed with ice-cold phosphate-buffered saline. Cells were then resuspended in 100 ml of binding buffer at 1×10^6/ml and stained with PI. After incubation at room temperature for 15 min, an additional 400 ml of binding buffer was added, and cells were analyzed by flow cytometry within 1 h. Analysis of nontreated cells was used to create a threshold that defines live cells that exclude PI. Data are expressed as the percentage of dead cells that clearly exceeded this fluorescence-intensity threshold.

Measurement of intracellular ATP content

Intracellular ATP of melanoma cells was measured by a luciferin–luciferase bioluminescence assay using the ATP Determination Kit (Molecular Probes). Cells were washed and collected in ice-cold phosphate-buffered saline. Then, 10 µl of cell lysate was added to 90 µl of working buffer containing 0.5 mmol/l luciferin, 1.25 µg/ml luciferase, 25 mmol/l Tricine buffer (pH 7.8), 5 mmol/l $MgSO_4$, 100 µm EDTA, and 1 mmol/l DTT. Luminescence was then analyzed with a luminometer. A standard curve was generated from known concentrations of ATP supplied with the kit, and was used to calculate the concentration of ATP in each sample.

Western blot analysis

Methods used were as described previously [10,11]. Briefly, a total of 20–30 µg of protein was electrophoresed on 10–15% SDS–PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked, incubated with primary antibodies at the appropriate concentration, and subsequently incubated with horseradish peroxidase-conjugated goat antirabbit or goat antimouse immunoglobulin (1 : 3000 dilution, respectively; Bio-Rad, Regents Park, New South Wales, Australia). Labeled bands were detected by Renaissance Western Blot Chemiluminescence Reagent (New England Nuclear Life Science Products, Boston, Massachusetts, USA) and exposed on Hyper MP autoradiography film (Amersham, Piscataway, New Jersey, USA).

Transmission electron microscopy

Cells were rinsed with serum-free Dulbecco's modified Eagle's medium, and fixed with prewarmed 2.5% glutaraldehyde or 2% formaldehyde in 0.1 mol/l sodium cacodylate buffer for 1 h. Cells were washed, postfixed with 2% osmium tetroxide, dehydrated with ascending grades of ethanol and propylene oxide, and embedded in LX-112 medium (Ladd, Research Industries, Burlington, Vermont, USA). After polymerization, ultrathin (90 nm) sections were cut with a diamond knife, collected on uncoated copper grids, and stained with uranyl acetate (1%) and lead citrate (0.2%). Samples were examined with a JEOL-1010 electron microscope (JEOL, Tokyo, Japan) operated at 80 kV.

Small interfering RNA

Cells were seeded at 4×10^4 cells/well in 24-well plates and allowed to reach approximately 50% confluence on the day of transfection. The siGENOME SMARTpool Noxa (M-004380-01-0010) and the nontargeting siRNA control SiConTRolNon-targeting SiRNA pool (D-001206-

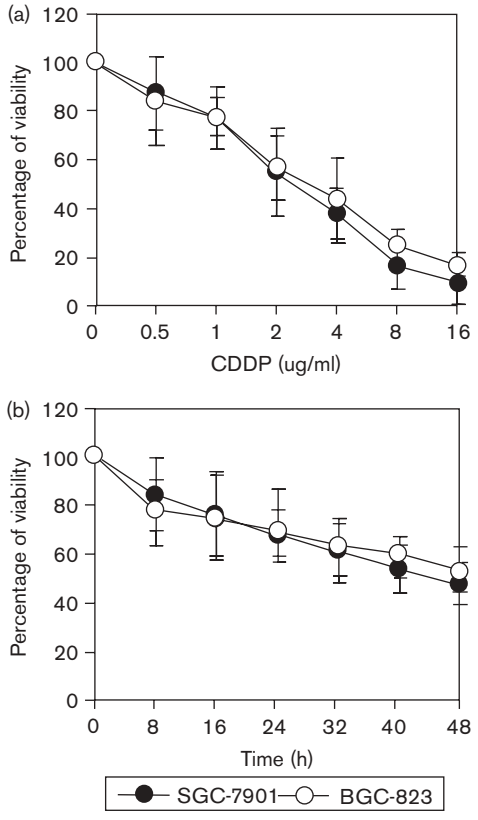
13-20) were obtained as the siGENOME SMARTpool reagents (Dharmacon, Lafayette, Colorado, USA), Cells were transfected with 50–100 nmol/l siRNA in Opti-MEM medium (Invitrogen, Carlsbad, California, USA) using Lipofectamine reagent (Invitrogen), according to the manufacturer's protocol. Efficiency of siRNA was measured by Western blot analysis.

Results

Cisplatin exhibits similar cytotoxic potential in SGC-7901 and BGC-823 cells

We examined the cytotoxic potential of cisplatin in two gastric cancer cell lines, SGC-7901 and BGC-823, by MTT assays after treating the cells with a range of concentrations of the compound for 48 h. As shown in Fig. 1a, cisplatin induced reduction in cell viability in a dose-dependent manner, with an IC_{50} value of approximately 3 μg/ml in both cell lines. Figure 1b shows that cisplatin at 3 μg/ml induced reduction in cell viability in a time-dependent manner in both cell lines with similar kinetics.

Fig. 1



Cisplatin (CDDP) exhibits cytoxic effects against gastric cancer cell lines. (a) SGC-7901 and BGC-823 cells seeded onto flat-bottomed 96-well plates at a fixed density for 24 h hours were treated with cisplatin at indicated concentrations for 48 h, followed by MTT assays. The data shown are the mean ± SE of three individual experiments. (b) Cells seeded as in (a) were treated with cisplatin at 3 μg/ml for the indicated time periods, followed by MTT assays. The data shown are the mean ± SE of three individual experiments.

Cisplatin induces apoptosis in SGC-7901, but nonapoptotic cell death in BGC-823 cells

We studied whether cisplatin-mediated cytotoxicity against gastric cancer cells was due to the induction of apoptosis, by measuring sub-G_1 content of SGC-7901 and BGC-823 cells after exposure to cisplatin at 3 μg/ml for varying time periods. Figure 2a shows that cisplatin induced relatively high levels of apoptosis in SGC-7901 cells, which could be detected as early as 8 h after treatment, with 48% of the cells being apoptotic at 48 h. In contrast, BGC-823 cells seemed to be resistant to cisplatin-induced apoptosis, with less than 20% of the cells being apoptotic at 48 h. The difference in sensitivity to cisplatin-induced apoptosis between SGC-7901 and BGC-823 cells was further confirmed by visualization of DNA fragmentation by DAPI staining (Fig. 2b). Although DNA fragmentation and/or chromatin condensation was frequently observed in SGC-7901 cells, the majority of nuclei of BGC-823 cells did not exhibit the apoptotic characteristics after exposure to cisplatin. Collectively these results suggest that cisplatin induced apoptosis largely in SGC-7901, but nonapoptotic cell death in BGC-823 cells (Fig. 2c).

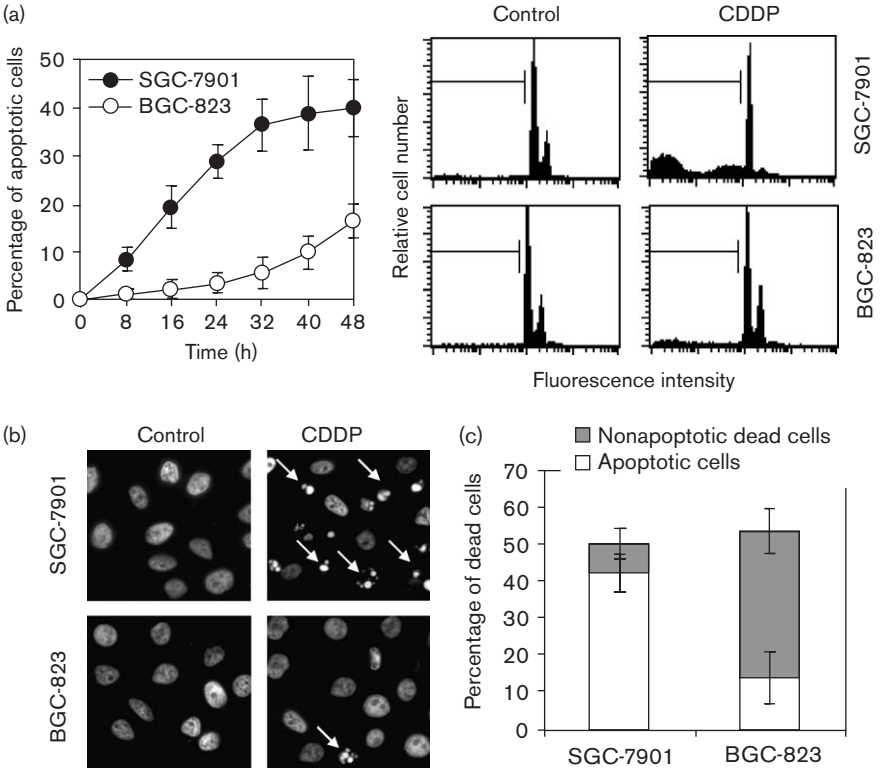
Cisplatin induces necrosis in BGC-823 cells

Exclusion of PI is a test for plasma membrane integrity and indicates primary necrosis when observed in the absence of apoptosis [11,12]. As shown in Fig. 3a, marked uptake of PI was observed in BGC-823 cells as early as 8 h after treatment with cisplatin when minimal PI uptake was seen in SGC-7901 cells. By 48 h, 52% of BGC-823 cells and 38% of SGC-7901 cells seemed to be PI-positive. We analyzed the relationship between the uptake of PI induced by cisplatin and an early apoptotic event, externalization of phosphatidylserine (PS), detected by fluorescein isothiocyanate-conjugated annexin V. Figure 3a also shows that the percentages of PI-positive cells were higher than those of annexin V-positive cells in BGC-823, but lower in SGC-7901 cells at all the time points tested. This indicates that unlike SGC-7901 cells, the loss of plasma membrane integrity visualized by PI exclusion in cisplatin-treated BGC-823 cells preceded the externalization of PS detected with annexin V binding.

Next we examined intracellular ATP levels in SGC-7901 and BGC-823 cells before and after treatment with cisplatin as loss of ATP levels occurs rapidly during necrosis but not during the early stages of apoptosis. Figure 3b shows that the levels of ATP in SGC-7901 cells were relatively stable with 74% of the control levels remaining at 8 h after treatment with cisplatin. In contrast, BGC-823 cells showed a rapid decrease in the levels of ATP with only 43 and 18% of the control levels remaining after 4 and 8 h, respectively.

We also examined the morphologic features of cell death in SGC-7901 and BGC-823 cells induced by

Fig. 2



Cisplatin (CDDP) induces apoptosis in SGC-7901 cells, but not in BGC-823 cells. (a) Left panel: SGC-7901 and BGC-823 cells were treated with cisplatin at 3 μg/ml for the indicated time periods before quantitation of apotosis by measuring the sub-G_1 contents with propidium iodide (PI), using flow cytometry. The data shown are the mean ± SE of three individual experiments. Right panel: representative flow cytometric histograms of measurement of sub-G_1 contents with PI in SGC-7901 and BGC-823 cells without (control) or with cisplatin treatment (3 μg/ml) for 48 h. (b) Representative microphotographs of nuclear morphology. SGC-7901 and BGC-823 cells were treated with cisplatin (3 μg/ml) for 48 h, followed by visualization of DNA by staining with 4′,6-diamidino-2-phenylindole. Images were then recorded using a fluorescent microscope. The data shown are representative of three individual experiments. (c) A summary of studies of cell death relative to apoptosis induced by cisplatin in SGC-7901 and BGC-823 cells. Cells were treated with cisplatin at 3 μg/ml for 48 h, followed by either MTT assays or quantitation of apoptotic cell death by measuring sub-G_1 content with PI in flow cytometry. The data shown are the mean ± SE of three individual experiments.

cisplatin by electron microscopy (Fig. 3c). After treatment with cisplatin for 48 h, BGC-823 cells exhibited morphologic changes characteristic of necrosis, including organelle swelling, intracellular vacuole formation, and plasma membrane disintegration. In contrast, SGC-7901 cells displayed apoptotic morphological changes such as condensed chromatin and no notable disintegration of the cell body. Taken together, these results indicate that cisplatin induced primarily necrotic cell death in BGC-823 cells and apoptosis in SGC-7901 cells.

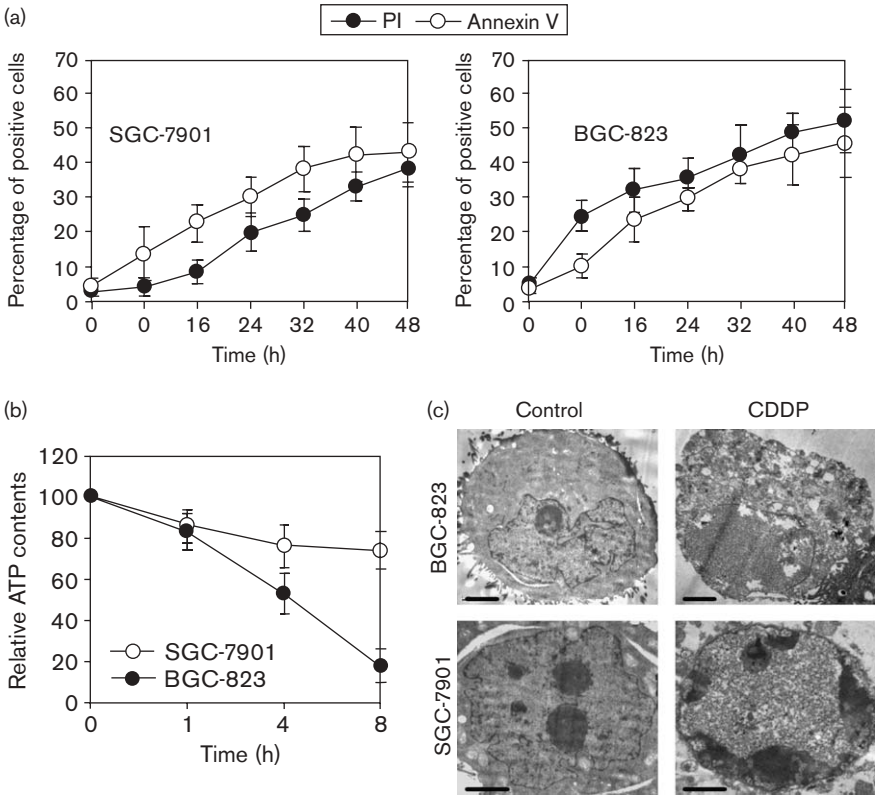
The BH-3-only protein Noxa plays an important role in cisplatin-induced apoptosis of SGC-7901 cells

We examined whether cisplatin-induced apoptosis in SGC-7901 is caspase dependent by treating the cells with the pan-caspase inhibitor, z-VAD-fmk, 1 h before the addition of cisplatin. Figure 4a shows that z-VAD-fmk markedly inhibited apoptosis. The involvement of the caspase cascade in cisplatin-induced apoptosis was also evidenced by the activation of caspase-3 and the cleavage of its substrate PARP in SGC-7901, but not in BGC-823 cells (Fig. 4b and data not shown).

To understand further the mechanism(s) by which cisplatin induces apoptosis, we evaluated expression of the BH3-only proteins of the Bcl-2 family, Noxa and Bim, before and after treatment with cisplatin. As shown in Fig. 5a, Noxa was markedly upregulated in SGC-7901 cells as early as 8 h after treatment. In contrast, Noxa was not induced in BGC-823 cells by cisplatin and remained low throughout the experiment. Little change was found in the overall protein levels of Bim in SGC-7901 cells, but the levels of Bim in BGC-823 cells seemed to be reduced within 24 h presumably owing to general protein degradation accompanying necrosis.

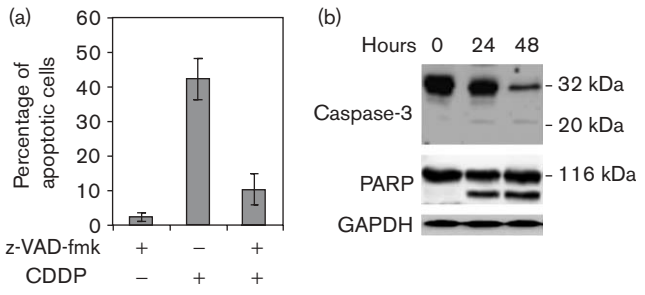
To confirm the role of Noxa in cisplatin-induced apoptosis of gastric cancer cells, we silenced Noxa expression in SGC-7901 cells using siRNA. Figure 5b

Fig. 3



Cisplatin (CDDP) primarily induces necrosis in BGC-823 cells. (a) SGC-7901 (right panel) and BGC-823 (left panel) cells were treated with cisplatin at 3 μg/ml for the indicated time periods, followed by flow cytometric quantitation of both uptake of propidium iodide (PI) and externalization of phosphatidylserine using fluorescein isothiocyanate-conjugated annexin V staining. The data shown are the mean ± SE of three individual experiments. (b) SGC-7901 and BGC-823 cells, with or without cisplatin treatment at 3 μg/ml for the indicated periods, were analyzed for intracellular ATP levels. The data shown are percentages of the control value and are the mean ± SE of three individual experiments. (c) SGC-7901 and BGC-823 cells, with or without cisplatin treatment at 3 μg/ml for 48 h, before analysis of morphological changes by electron microscopy. The data shown are representative of two individual experiments.

Fig. 4



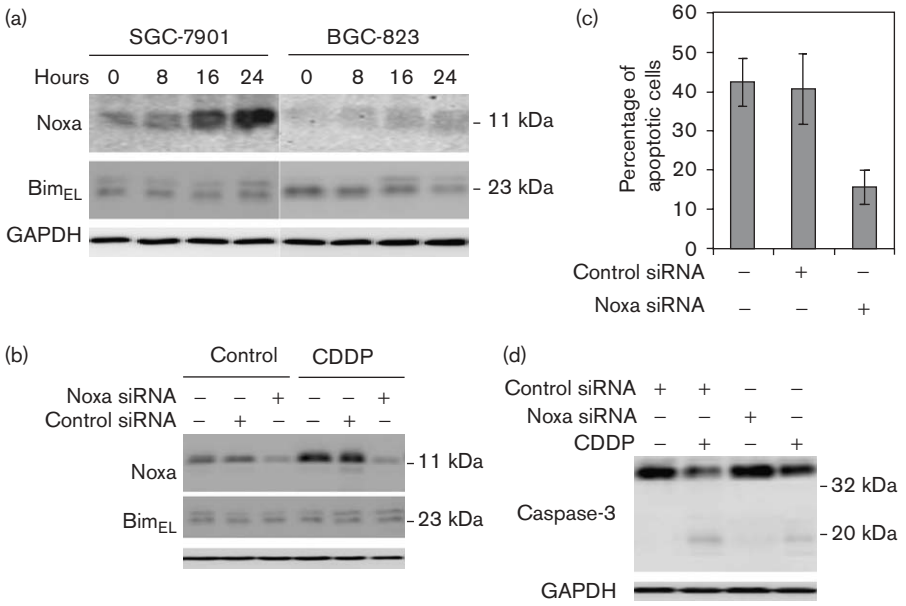
Cisplatin (CDDP)-induced apoptosis of SGC-7901 cells is caspase dependent. (a) SGC-7901 cells treated with z-VAD-fmk at 30 μmol/l for 1 h before the addition of cisplatin (3 μg/ml) for a further 48 h were subjected to flow cytometry quantitation of apoptosis by measuring sub-G_1 contents with propidium iodide. The data shown are the mean ± SE of three individual experiments. (b) Cisplatin induces caspase-3 activation and cleavage of its substrate poly(ADP ribose) polymerase (PARP). Whole-cell lysates from SGC-7901 cells treated with cisplatin (3 μg/ml) for the indicated periods were subjected to Western blot analysis. The data shown are representative of three individual experiments.

shows that siRNA-mediated knockdown of Noxa markedly inhibited its expression before and after exposure to cisplatin, but had no effect on the expression of Bim that was used as a control for the specificity of the siRNA. Inhibition of Noxa by siRNA markedly reduced the level of apoptosis induced by cisplatin, in comparison with cells transfected with control RNA (Fig. 5c). Similarly, siRNA knockdown of Noxa markedly attenuated caspase-3 activation induced by cisplatin (Fig. 5d).

Inhibition of apoptosis facilitates necrosis in gastric cancer cells

Although apoptosis was inhibited, a large proportion of SGC-7901 cells still seemed to be rounded and detached from culture plates after incubation with cisplatin in the presence of the pan-caspase inhibitor z-VAD-fmk (Fig. 6a), and this also occurred following siRNA-mediated knockdown of Noxa (data not shown). In view of this, we compared uptake of PI in SGC-7901 cells treated with cisplatin alone or cisplatin in the presence of z-VAD-fmk or inhibition of Noxa. Figure 6b illustrates that the proportion

Fig. 5



Cisplatin (CDDP)-induced apoptosis of SGC-7901 Cells is mediated at least in part by Noxa. (a) Cisplatin upregulates Noxa in SGC-7901, but not in SGC-823 cells. Whole-cell lysates from SGC-7901 and BGC-823 cells, with or without cisplatin treatment at 3 μg/ml for the indicated time periods, were subjected to Western blot analysis for Noxa, Bim, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control. The data shown are representative of three individual experiments. (b) Knockdown of Noxa by small interfering RNA (siRNA) inhibits cisplatin-induced upregulation of Noxa in SGC-7901 cells. Whole-cell lysates from SGC-7901 cells transfected with the control or Noxa siRNA, with or without cisplatin treatment (3 μg/ml) for 24 h, were subjected to Western blot analysis. Western blot analysis of Bim was used as a control for the specificity of the Noxa siRNA. The data shown are representative of three individual experiments. (c) Knockdown of Noxa inhibited cisplatin-induced apoptosis of SGC-7901 cells. SGC-7901 cells transfected with the control or Noxa siRNA were treated with cisplatin (3 μg/ml) for 48 h, before quantitation of apoptotic cell death by measuring the sub-G_1 content with propidium iodide in flow cytometry. The data shown are the mean ± SE of three individual experiments. (d) Knockdown of Noxa inhibited cisplatin-induced activation of caspase-3 in SGC-7901 cells. Whole-cell lysates from SGC-7901 cells transfected with the control or Noxa siRNA, with or without cisplatin treatment (3 μg/ml) for 48 h, were subjected to Western blot analysis. The data shown are representative of three individual experiments.

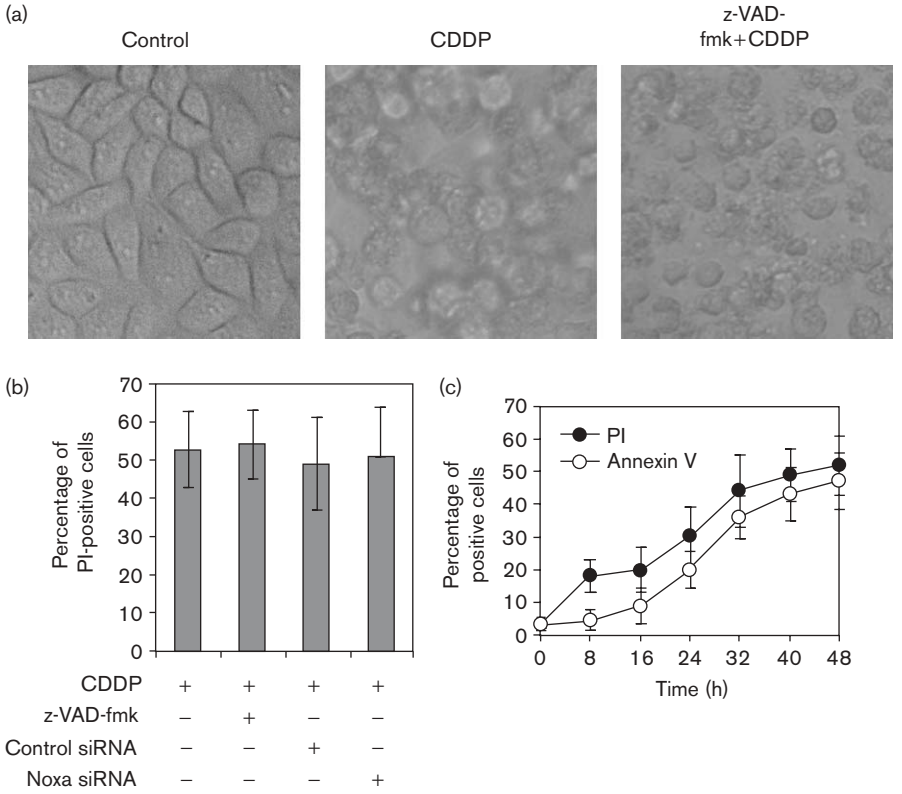
of PI-positive cells induced by cisplatin remained largely unchanged with or without pretreatment with z-VAD-fmk. This result contrasts with the effects of z-VAD-fmk, which strongly inhibits apoptosis under the same conditions (Fig. 4a). Similar effects were observed with the SGC-7901 cells that had been treated with Noxa siRNA (Fig. 6b). Therefore, we undertook kinetic studies to measure the uptake of PI in relation to the externalization of PS in the presence of z-VAD-fmk. We observed that, in contrast to the cells treated with cisplatin alone (Fig. 3a), SGC-7901 cells now displayed higher staining for PI: this was observed before they became positive for annexin V (Fig. 6c). Taken together, these results indicate that inhibition of apoptosis cannot inhibit reduction in the viability of SGC-7901 cells induced by cisplatin, but it can change the mode of cell death from apoptosis to necrosis.

Discussion

We demonstrated in this study that the commonly used chemotherapeutic drug cisplatin kills gastric cancer cells by the induction of dual modes of cell death – apoptosis and necrosis. Induction of apoptosis by cisplatin seemed to be caspase dependent and was mediated, at least in part, by the BH3-only protein of the Bcl-2 family, Noxa. Inhibition of apoptosis, however, did not seem to attenuate the cytotoxicity of cisplatin against gastric cancer cells. Instead, it converted the mode of cell death from apoptosis to necrosis.

As a typical DNA-damaging agent, cisplatin can induce apoptosis by a p53-dependent mechanism(s) [13]. In particular, the transcriptional activity of p53 seems to be critical [14]. The current study shows that the BH-3-only protein of the Bcl-2 family, Noxa, plays a critical role in cisplatin-induced apoptosis of gastric cancer cells. This is because (i) Noxa was rapidly upregulated by cisplatin in the apoptosis-sensitive, but not in the resistant, gastric cancer cell line, and (ii) knockdown of Noxa by siRNA markedly inhibited cisplatin-induced apoptosis. As a BH-3-only protein, Noxa can directly, or indirectly through neutralization of antiapoptotic Bcl-2 family members by binding to proteins such as Mcl-1, Bcl-2, and Bcl-X_L, activate the multidomain proapoptotic proteins Bax and/or Bak [15,16]. This, in turn, leads to

Fig. 6



Inhibition of apoptosis converts the mode of cell death in SGC-7901 cells to necrosis. (a) Representative phase-contrast microscopic photographs of SGC-7901 cells treated with cisplatin (CDDP; 3 μg/ml) for 48 h, with or without pretreatment with z-VAD-fmk at 30 μmol/l 1 h. (b) SGC-7901 cells pretreated with z-VAD-fmk as in (a) or cells transfected with the indicated small interfering RNAs (siRNAs) were then treated with cisplatin (3 μg/ml) for 48 h, followed by the measurement of the uptake of propidium iodide (PI) by flow cytometry. (c) SGC-7901 cells were pretreated with z-VAD-fmk at 30 μmol/l 1 h before the addition of cisplatin (3 μg/ml) for the indicated time periods. Cells were then analyzed by flow cytometry for the uptake of PI and the externalization of phosphatidylserine using fluorescein isothiocyanate-conjugated annexin V. The data shown are the mean ± SE of three individual experiments.

the permeabilization of the mitochondrial outer membrane, release of mitochondrial apoptosis mediators such as cytochrome *c* and Smac/DIABLO, activation of caspases, and eventually apoptosis [6,15]. It remains unknown how Noxa is unregulated by cisplatin in one but not in another gastric cancer cell line. Given the high frequency of p53 mutations in gastric cancer [17], it is conceivable that p53 might be mutated in the cells in which Noxa cannot be transcribed as a p53 target gene in response to DNA damage.

Of significance, the apoptosis-resistant gastric cancer cells were still susceptible to cisplatin-mediated cytotoxicity. Several lines of evidence suggest that the cytotoxic effect of cisplatin on the cells was due to induction of necrosis. These included lack of DNA fragmentation, delayed externalization of PS, plasma membrane perturbations, organelle swelling, and intracellular vacuole formation. Although cisplatin has been reported to induce necrotic cell death when used at relatively high concentrations [18,19], our results indicate that cisplatin predominantly induces necrosis in gastric cancer cells resistant to apoptosis at the same doses that otherwise primarily induce apoptosis. How cisplatin induces necrosis in gastric cancer cells remains unclear. It was previously shown that increased PARP activity can play an important role in cisplatin-induced necrosis in apoptosis-resistant melanoma cells, in that rapid PARP activation and depletion of ATP preceded the features of necrosis [11].

The most important finding of this study is that inhibition of apoptosis cannot inhibit reduction in cell viability induced by cisplatin, but can convert the mode of cell death from apoptosis to necrosis in the same cell line (SGC-7901). This indicates that although apoptosis might be the first line of response of gastric cancer cells to cisplatin-induced killing, necrosis can serve as an alternative way for cisplatin to exert its cytotoxicity against gastric cancer cells when the apoptotic machinery is defective. Although it is well known that DNA damage can initiate apoptosis [13], it has been recently reported that DNA-damaging agents could kill $Bax^{-/-}$, $Bak^{-/-}$, or

$p53^{-/-}$ cells by induction of primary necrosis, indicating that an intact apoptotic pathway is not required for killing of cells with DNA damage [8]. In fact, it has also been suggested that the initiation of apoptosis might actively suppress necrotic cell death because activated caspases might degrade the proteins required for necrosis [20]. Our results support these views, in that cisplatin induced necrosis only in apoptosis-resistant gastric cancer cells or in apoptosis-sensitive cells when apoptosis was inhibited.

Although numerous mechanisms involved in resistance to chemotherapeutic drugs were discovered in recent years, acquired resistance of tumor cells to apoptosis is believed to be a major obstacle for cancer chemotherapy [4,21]. Our finding that cisplatin can induce necrosis in cells that are rendered resistant to apoptosis can have important clinical implications. Gastric cancer cells that acquire resistance to apoptosis *in vivo* might still be able to respond to cisplatin treatment by committing necrotic cell death. This knowledge can be exploited in designing optimal combinations of adjuvant therapies. In addition, our results indicate that the sensitivity to both apoptotic as well as necrotic cell death must be considered when defining the susceptibility of individual gastric cancers to cisplatin.

Acknowledgement

This work was supported by grants from Chinese National Natural Science Foundation of (30572118) and Natural Science Foundation of Anhui, China. X.D.Z. is a recipient of a Career Development and Support Fellowship of The Cancer Research Institute, New South Wales, Australia.

References

1 Edinger L, Thompson CB. Death by design: apoptosis, necrosis and autophagy. *Curr Opin Cell Biol* 2004; **16**:663–669.
2 Adams JM. Ways of dying: multiple pathways to apoptosis. *Genes Dev* 2003; **17**:2481–2495.
3 Majno G, Joris I. Apoptosis, oncosis, and necrosis. An overview of cell death. *Am J Pathol* 1995; **146**:3–15.
4 Hersey P, Zhang XD. Overcoming resistance of cancer cells to apoptosis. *J Cell Physiol* 2003; **196**:9–18.
5 Soengas MS, Lowe SW. Apoptosis and melanoma chemoresistance. *Oncogene* 2003; **22**:3138–3151.
6 Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. *Science* 1998; **281**:1322–1326.
7 Shimizu S, Narita M, Tsujimoto Y. Bcl-2 family proteins regulate the release of apoptogenic cytochrome *c* by the mitochondrial channel VDAC. *Nature* 1999; **399**:483–487.
8 Zong WX, Ditsworth D, Bauer DE, Wang ZQ, Thompson CB. Alkylating DNA damage stimulates a regulated form of necrotic cell death. *Genes Dev* 2004; **18**:1272–1282.
9 Gillespie SK, Zhang XD, Hersey P. Ingenol 3-angelate induces dual modes of cell death and differentially regulates tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in melanoma cells. *Mol Cancer Ther* 2004; **3**:1651–1658.
10 Jiang CC, Chen LH, Gillespie S, Kiejda KA, Mhaidat N, Wang YF, *et al*. Tunicamycin sensitizes human melanoma cells to tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis by up-regulation of TRAIL-R2 via the unfolded protein response. *Cancer Res* 2007; **67**: 5880–5888.
11 Zhang XD, Wu JJ, Gillespie S, Borrow J, Hersey P. Human melanoma cells selected for resistance to apoptosis by prolonged exposure to tumor necrosis factor-related apoptosis-inducing ligand are more vulnerable to necrotic cell death induced by cisplatin. *Clin Cancer Res* 2006; **12**: 1355–1364.
12 Walsh GM, Dewson G, Wardlaw AJ, Levi-Schaffer F, Moqbel R. A comparative study of different methods for the assessment of apoptosis and necrosis in human eosinophils. *J Immunol Methods* 1998; **217**:153–163.
13 Gonzalez VM, Fuertes MA, Alonso C, Perez JM. Is cisplatin-induced cell death always produced by apoptosis? *Mol Pharmacol* 2001; **59**:657–663.
14 Roos WP, Kaina B. DNA damage-induced cell death by apoptosis. *Trends Mol Med* 2006; **12**:440–450.
15 Martinou JC, Green DR. Breaking the mitochondrial barrier. *Nat Rev Mol Cell Biol* 2001; **2**:63–67.
16 Chen L, Willis SN, Wei A, Smith BJ, Fletcher JI, Hinds MG, *et al*. Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. *Mol Cell* 2005; **17**:393–403.
17 Fenoglio-Preiser CM, Wang J, Stemmermann GN, Noffsinger A. TP53 and gastric carcinoma: a review. *Hum Mutat* 2003; **21**:258–270.
18 Ferrer M, Izeboud T, Ferreira CG, Span SW, Giaccone G, Kruyt FA. Cisplatin triggers apoptotic or nonapoptotic cell death in Fanconi anemia lymphoblasts in a concentration-dependent manner. *Exp Cell Res* 2003; **286**:381–395.
19 Shibuya H, Kato Y, Saito M, Isobe T, Tsuboi R, Koga M, *et al*. Induction of apoptosis and/or necrosis following exposure to antitumour agents in a melanoma cell line, probably through modulation of Bcl-2 family proteins. *Melanoma Res* 2003; **13**:457–464.
20 Leist M, Jaattela M. Four deaths and a funeral: from caspases to alternative mechanisms. *Nat Rev Mo Cell Biol* 2001; **2**:589–598.
21 Hersey P, Zhang XD. Current strategies in overcoming resistance of cancer cells to apoptosis melanoma as a model. *Int Rev Cytol* 2006; **251**:131–158.