Differential susceptibility to 9-nitrocamptothecin (9-NC)-induced apoptosis in clones derived from a human ovarian cancer cell line: possible implications in the treatment of ovarian cancer patients with 9-NC

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We have investigated whether variability in the apoptotic pathway may account for the differential susceptibility to apoptosis-induction by 9-nitrocamptothecin (9-NC) in cell subpopulations derived from the human ovarian cancer cell line, SKOV-3. Quantitative differences in the apoptotic fractions of cells were assessed by flow cytometry, whereas major regulatory and executing components of the apoptotic machinery were investigated by Western blot analysis using specific antibodies. The results indicate that indeed the apoptotic pathway was activated by 9-NC in some, but not all, cells of the SKOV-3 cell line, suggesting that 9-NC alone may partially be effective for treatment of patients with ovarian cancer. *Anti-Cancer Drugs* 14:427–436 © 2003 Lippincott Williams & Wilkins.

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Introduction

Despite advances in the surgical and medical management of patients with ovarian cancer, the overall prognosis is still poor, with a 5-year survival rate of only 20–30% in patients with advanced ovarian cancer [1]. Standard treatment for ovarian cancer consists of platinum-based drugs, cisplatin and carboplatin, in combination with paclitaxel, cyclophosphamide (nitrogen mustard) and doxorubicin [2]. Resistance to platinum-based chemotherapy in ovarian cancer has been associated with absence of functional tumor suppressor protein, p53 [3,4], whereas the loss of functional p53 has been correlated with increased cisplatin cytotoxicity in a human cancer ovarian cell line [5]. In this regard, overexpression of the apoptosis effector Bax in human ovarian cancer cells lacking functional p53 enhanced cell death in response to paclitaxel in a p53-independent manner; however, the sensitivity to etoposide was unchanged [6]. Also, p53-knockout ovarian cancer cells were resistant to cisplatin, doxorubicin and Ara-C, but they retained their sensitivity to taxol and camptothecin [4]. Therefore, it appears that functional p53 is associated with sensitivity of cancer ovarian cells to several chemotherapeutic agents, but there are still other drugs, including camptothecin analogs, that can induce apoptosis of these cells in a p53-independent manner [7].

The caspases are proteolytic enzymes currently considered as the central executioners of all forms of the apoptotic pathway and, therefore, the ability of various anticancer drugs to induce activation of caspases is fundamental for induction of apoptosis in cancer cells. All cells express several members of the caspase family as inactive precursors in the cytosol, but whether all are functionally required for a single apoptotic pathway still remains somewhat obscure. During the process of apoptosis the initiator caspases are activated first, and then target and process the precursors of effector caspases to yield active effector caspases, which in turn target and cleave a set of nuclear and cytosolic proteins and ultimately result in the apoptotic death of the cells. In general, there are two major apoptotic pathways, the 'extrinsic' and the 'intrinsic'. The extrinsic apoptotic pathway is initiated by stimulation of 'death receptors' on the cell surface and requires activation of caspase-8. The intrinsic apoptotic pathway is characterized by release of mitochondrial cytochrome *c* into the cytosol, where it binds to Apaf-1 and procaspase-9, and leads to activation of caspase-9. In either apoptotic pathway, activated

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caspase-8 and/or caspase-9 can activate the effector caspases-3 and -6 or -7, which in turn target and cleave diverse cellular molecules that are important for the cell's survival including lamin, fodrin, gelsolin, PARP, topoisomerase (Topo) I and other molecules as well, thus leading to cell death. In general, the expression or activation of the initiator caspase-8 has been associated with the extent of susceptibility to apoptosis of many cancer cells treated with chemotherapeutic agents including camptothecin congeners [7–11], whereas other anticancer drugs induce apoptosis of cancer cells via the mitochondrion-dependent mechanism [for recent reviews on caspases, see 12–14].

The anticancer drug, 9-nitrocamptothecin (9-NC), like the parental compound, camptothecin, interferes with the Topo I-mediated process of the chromatin DNA breakage repair [15,16], an event ultimately leading to cell death by apoptosis. Induction of apoptosis by 9-NC is preceded by arrest of the cancer cells in the S-phase of the cell cycle [for reviews, see 17–19]. A phase II clinical trial of 9-NC was conducted in patients with heavily refractory ovarian cancer and the results showed that of the 26 evaluable patients, two exhibited partial remission and 13 exhibited stable disease [20]. Although these results were encouraging, no complete response was observed and this has raised questions about the efficacy of 9-NC used as a single agent in the therapy of ovarian cancer.

In this study we wanted to determine the effectiveness of 9-NC in a cell model derived from advanced ovarian cancer and characterize the apoptotic pathway that kills these cells. The human ovarian adenocarcinoma SKOV-3 cell line was chosen for this study because of the absence of p53 due to a deletion in the *p53* gene, and demonstration of resistance to the apoptotic effect of cisplatin and doxorubicin, all characteristics of advanced and refractory ovarian tumors [21].

Materials and methods

Reagents

Clinical grade 9-NC was provided by SuperGen (Dublin, CA) and used as a fine suspension in polyethylene glycol-300 (JT Baker, Phillipsburg, NJ). Fetal bovine serum, EDTA, phenylmethylsulfonyl fluoride, dimethylsulfoxide and reagents for SDS–PAGE were purchased from Sigma (St Louis, MO). Giemsa-stain solution containing glycerol and methanol were from JT Baker. Protein quantification reagents were purchased from Bio-Rad (Hercules, CA).

Cells

The human ovarian adenocarcinoma SKOV-3 cell line [21] was obtained from ATCC (Manassas, VA) and maintained in RPMI 1640 medium supplemented with 10% FBS at 37°C in a humidified incubator under 5% CO_2. To derive clones, a suspension of trypsinized SKOV-3 cells was diluted to 0.5 cells/ml and seeded in 96-well plates (100 µl/well). Twenty-eight cell clones were derived, expanded, treated with 25 nM 9-NC, and classified as 'early', 'intermediate' and 'late' responders by comparing the percent of apoptotic cells after 72 h of drug treatment. Assays were performed in triplicate. In the studies described in this report, we utilized an ER (early responder) and a LR (late responder) clone.

Drug treatments

Exponentially growing cells were collected after trypsinization, re-seeded at equal numbers in 100-mm dishes and then allowed to adhere overnight in the 37°C incubator. Then the media was replaced with fresh media containing various 9-NC concentrations and incubation continued for various lengths of time. Control cultures received PEG alone. The final PEG concentration in the cultures was 0.025% (v/v) or less. Attached and non-attached cells were collected at the desired times of treatment and processed for microscopy observations, and biochemical and flow cytometry analyses.

Microscopy and flow cytometry

For microscopy and flow cytometry studies, identical cell cultures at low density received fresh media containing 25 nM 9-NC. For microscopy observations, cells attached to the plastic substrate were either placed in PBS and photographed under an inverted microscope (Zeiss Axiovert 25) or were stained with Giemsa, and then examined under a Nikon microscope connected to a computer equipped with imaging software. For flow cytometry studies, the percentage of untreated and drug-treated cells in each phase of the cell cycle and in apoptosis was determined by analysis of the relative DNA content in the cells using a FACScan flow cytometer (Becton Dickinson, Plymouth, UK) and the ModFit LT program (Becton Dickinson). We have previously described microscopy and flow cytometry methodologies [22–24].

Immunoblotting

Whole-cell lysates were prepared and subjected to immunoblot analysis as described [25]. The monoclonal antibodies used in this report recognized human topoisomerase I, cytochrome *c*, Bcl-2, Bcl-x_L and Bak (BD Biosciences, San Jose, CA); caspase-8 (Sigma-Aldrich, St Louis, MO); Bax and caspase-9 (Santa Cruz Biotechnology, Santa Cruz, CA); and PARP (BioMol, Plymouth Meeting, PA).

Cytochrome *c* efflux assay

This assay has been described [26]. Briefly, cells were collected and washed twice in PBS, and then suspended in 10 mM HEPES buffer, pH 7.5, containing 50 mM KCl

in the presence of protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN) and 1 mM PMSF. The cell suspension was forced to pass through a 25-gauge needle 5–6 times and the resulting cell lysate was divided to two equal volumes—one volume was used for preparation of the mitochondrial fraction and one volume for preparation of the cytosolic fraction [26]. Subsequently, both fractions were subjected to immunoblot analysis to detect cytochrome *c* using a specific antibody to cytochrome *c* and enhanced chemiluminescence reagents.

Transfection

SRα-*Bcl-2* and pGK-Hygro DNAs were prepared with the aid of a Qiagen Plasmid Maxi Kit (Qiagen, Valencia, CA). After digestion with *Ssp*I and *Hin*dIII, linear SRα-*Bcl-2* and pGK-Hygro DNAs were transiently transfected into human ER cells using SuperFect reagent (Qiagen). Cells were seeded 1 day prior to experimentation and reached 50% confluence at the time of transfection. For each six-well dish, 5 μg SRα-*Bcl-2*, 0.5 μg pGK-Hygro and 20 μl SuperFect reagent were used. After 48 h of incubation in fresh cell culture medium, cells were selected with 500 μg/ml Hygromycin B (Calbiochem, La Jolla, CA). Single colonies were removed after 2 weeks and propagated before they were tested for overexpression of Bcl-2 using immunoblotting and Bcl-2 antibody. The ER cells overexpressing Bcl-2 were designated ER/Bcl-2.

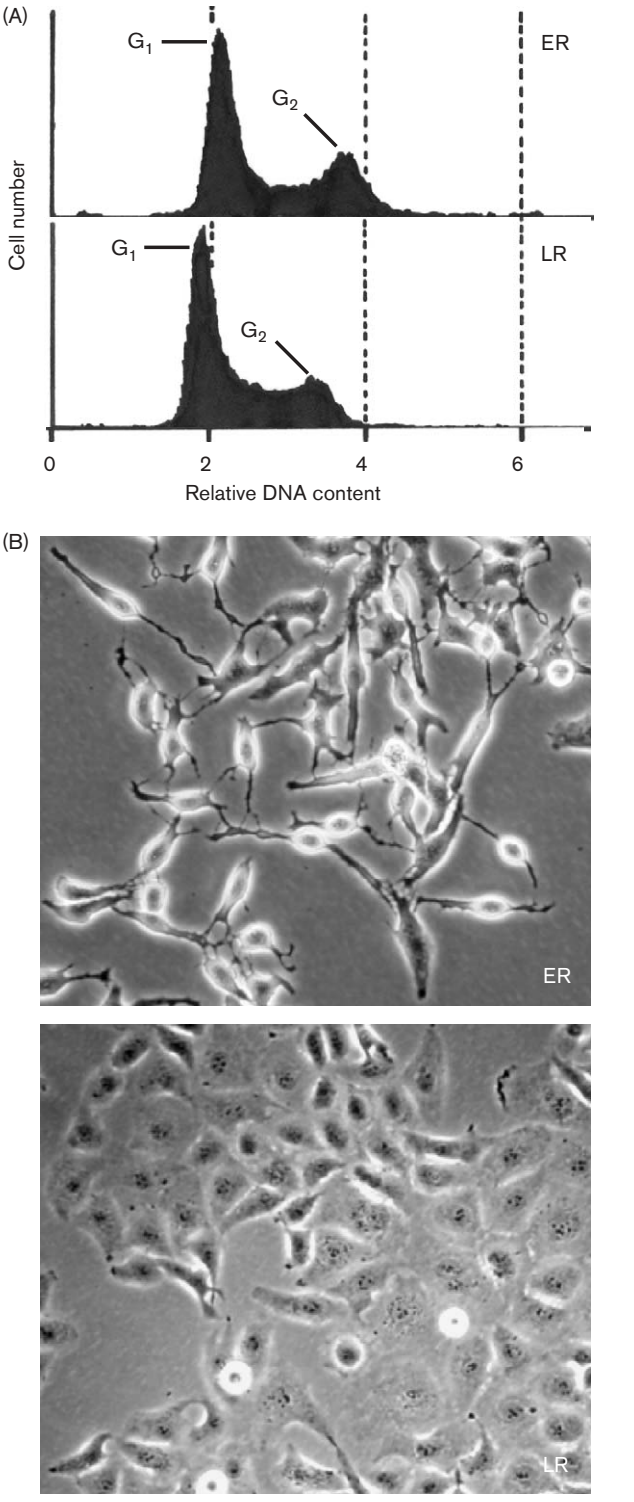
Results

Partial characterization of cell clones, and morphological and cell cycle changes after upon 9-NC treatment

Exponentially growing ER and LR cells were analyzed by flow cytometry for distribution in various stages of the cell cycle. Alignment of the histograms indicated a distinct difference in the relative DNA content of the ER and LR cells in the G_1 fraction, i.e. DNA index (DI) (Fig. 1A). More specifically, the ER cells appeared to be more aneuploid than LR cells as indicated by the relative localization of the corresponding G_1 (i.e. $G_0 + G_1$) peaks of the histograms. The $G_0 + G_1$ fractions were estimated to be 62 ± 6 and $51 \pm 4\%$ for the ER and LR cells, respectively. The doubling times of ER and LR cells were 25 ± 3 and 23 ± 3 h, respectively. Further, differences were observed in the shape, size and mode of adherence of the ER and LR cells as shown in the photomicrographs of Fig. 1(B). However, the percent of ER and LR cells in a culture of SKOV-3 cells cannot be estimated by direct microscopic observations.

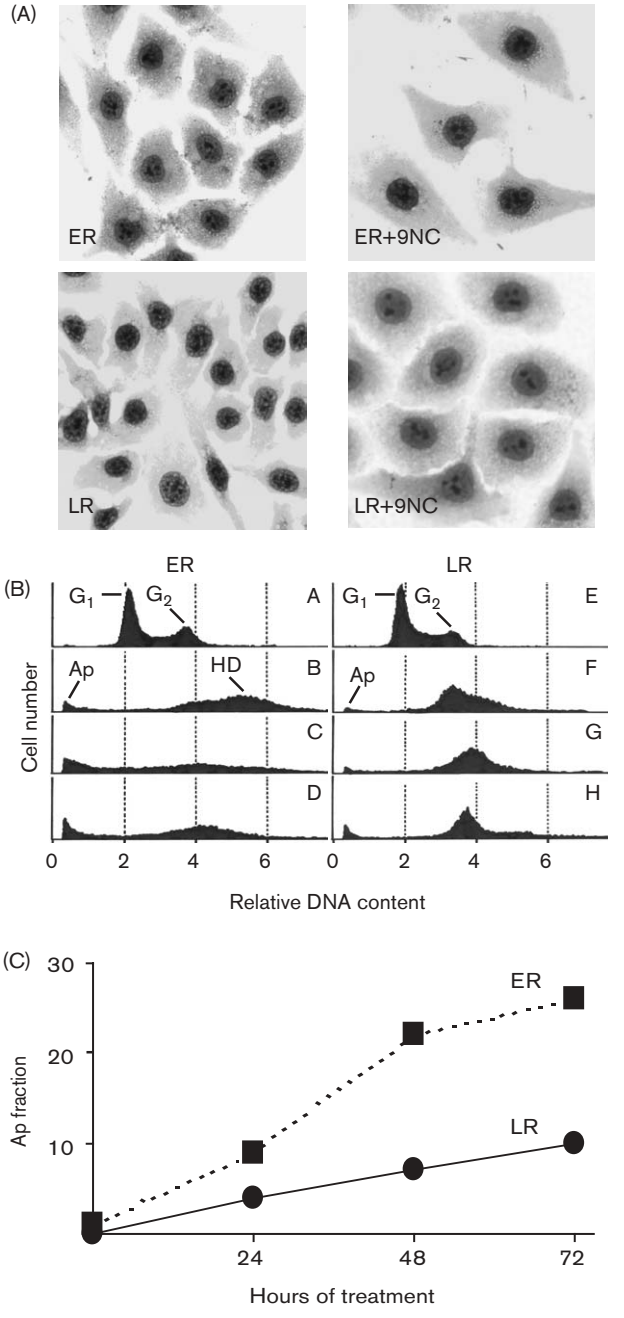
ER and LR cells were treated with 25 nM 9-NC for various periods of time, and morphological changes and cell cycle perturbations were monitored by microscopy of stained cells and flow cytometry, respectively. Photomicrographs of stained cells, shown in Fig. 2(A), indicate that the process to stain the cells resulted in morphological features different than those observed in unstained

Fig. 1



Flow cytometry analysis and microscopy of cells. Exponentially growing ER and LR cells were either harvested and subjected to flow cytometry analysis (A) or were observed and photographed under an inverted microscope, while they remained attached to the culture container (B). G_1, G_0 + G_1 cells; G_2, G_2 + M cells.

Fig. 2



Cell cycle perturbations in the morphology and cell cycle of cells treated with 9-NC. Exponentially growing cells were treated with 25 nM 9-NC. (A) Untreated and 9-NC-treated, for 72 h, ER and LR cells attached to the substrate were stained with Giemsa and photomicrographed under the same magnification; (B) Cells were treated with 9-NC for various periods of time, and then collected and analyzed for cell cycle changes by flow cytometry. (C) Apoptotic fractions in the cell cultures were estimated from the histograms of Fig. 2(B) and then plotted as a function of the period of 9-NC treatment. Treatment of cells with 9-NC (in Fig. 2B) was for 0 h (A and E), 24 h (B and F), 48 h (C and G) and 72 h (D and H). Ap, apoptotic cells; HD, hyperdiploid cells

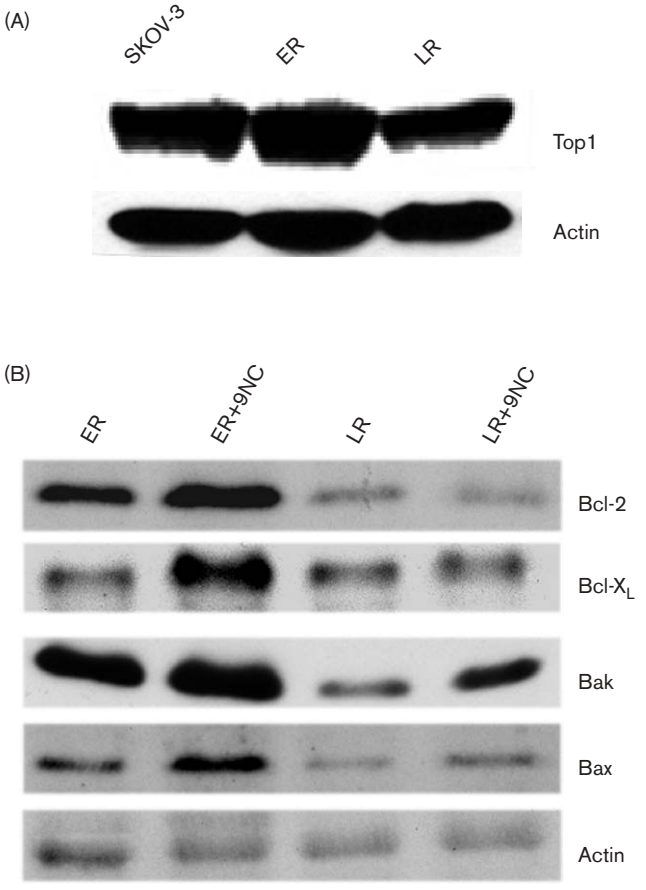
cells. Following treatment with 9-NC for 72 h, both ER and LR cells ceased to proliferate, and increased in size. The size of the nuclei was also increased. It should be noted that only attached cells were examined in Fig. 2(A) and that the number of detached, presumably dead cells was much larger in cultures of ER than LR cells after 9-NC treatment.

Cell cycle perturbations and apoptosis were monitored by analysis of changes in the relative DNA content (Fig. 2B). In this study, both attached and non-attached cells in the culture were pooled and subjected to analysis. After 24 h of 9-NC treatment no ER cells were present in the G_1 and S phases, while most cells had become hyperdiploid and a small fraction had entered apoptosis (histogram B). In contrast, at 24 h of treatment, the majority of LR cells were arrested in late S/G_2 phase and some cells had become hyperdiploid, while a small fraction was in the apoptotic fraction (histogram F). It is also noticeable that there were no cells in the G_1 fraction, like in the drug-treated ER cells (see histograms B and F). The difference in the response of ER and LR cells to 9-NC was more dramatic at 48 h of treatment (histograms C and G). The apoptotic fraction had increased in the culture of ER cells apparently because of an increase in the number of dying hyperdiploid cells, whereas the apoptotic fraction remained relatively unchanged in the 9-NC-treated LR cells, which appeared to contain higher DNA content (histogram G). Finally, at 72 h of treatment, there was only a small increase in the apoptotic fraction of LR cells, while the majority of the cells were still accumulated at the G_2/ hyperdiploid phases (histogram H). In contrast, the hyperdiploid ER cells continued to die and as a result the apoptotic fraction continued to increase (histogram D). In actuality, the apoptotic fraction was underestimated from histogram D, which does not show that a portion of the dead cells was degraded/fragmented as observed by microscopy. The flow cytometry methodology analyzes only intact cells. In contrast, no cell fragments were observed in the culture of LR cells treated with 9-NC for 72 h. Further, the dramatic difference in sensitivity of ER and LR cells to 9-NC-induced apoptosis is better depicted in Fig. 2(C), which shows the percent of apoptotic ER and LR cells estimated at various times of 9-NC treatment. These results were derived from analysis of the histograms shown in Fig. 2(B).

Expression of Topo I and Bcl-2 family proteins

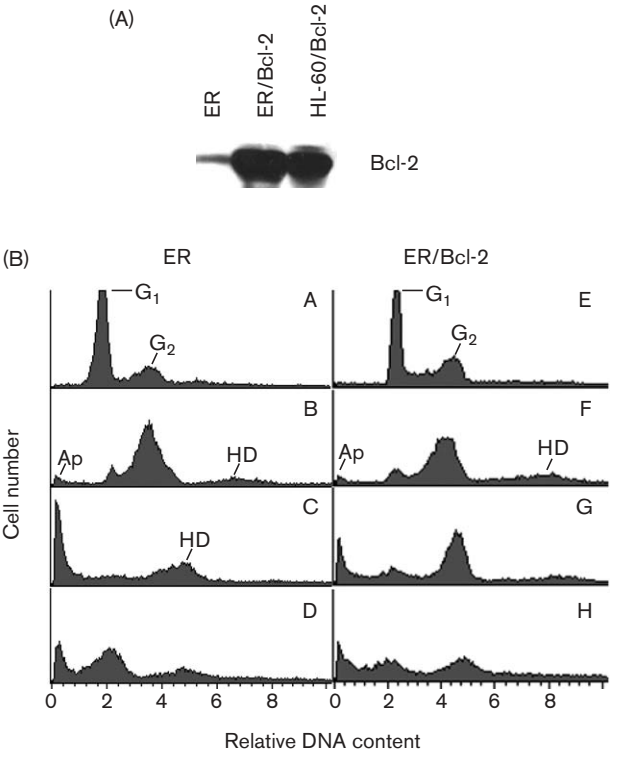
It is well established that Topo I is required for the camptothecin analogs to exert their apoptotic action on cells. Therefore, we examined whether the differential susceptibility of ER and LR cells to 9-NC was the result of a differential expression of Topo I in these cells. Western blot analysis of whole ER and LR cell extracts using a specific antibody to Topo I indicated that

Fig. 3



Expression of Topo I and Bcl-2 family proteins. Whole extracts were prepared from untreated and 9-NC-treated cells, and subsequently subjected to immunoblot analysis of Topo I, Bcl-2, Bcl-x_L, Bak, Bax and actin (control protein) expression using specific antibodies. 9-NC treatment was for 72 h.

Fig. 4



Overexpression of Bcl-2 does not block 9-NC-induced apoptosis. (A) Detection of Bcl-2 expression by immunoblot analysis in whole extracts from ER, ER/Bcl-2 and HL-60/Bcl-2 (control) cells. (B) Flow cytometry detection of cell cycle perturbations and apoptosis in ER and ER/Bcl-2 cells treated with 25 nM 9-NC for 0 h (A and E), 24 h (B and F), 48 h (C and G) and 72 h (D and H).

exponentially grown ER and LR cells express robust levels of Topo I, but LR cells contained somehow less Topo I than ER cells (Fig. 3A). Also, Topo I was readily detected in the whole extract of the parental SKOV-3 cells. Detection of nearly identical amounts of the household protein, actin, confirmed that similar amounts of total cell protein were subjected to comparative analysis. Subsequently, the cell extracts were investigated for expression of the Bcl-2 family proteins, Bcl-2, Bcl-x_L, Bax and Bak, that have been associated with regulation of caspase activity and therefore apoptosis induced by anticancer drugs. The results of the immunoblot analysis exhibited no significant differences in the expression of Bcl-2, Bak and Bax in ER and LR cells after 9-NC treatment, whereas Bcl-x_L was expressed at a higher level in 9-NC-treated ER, but not 9-NC-treated LR cells (Fig. 3B). In general, Bcl-2 family proteins were much less expressed in LR than ER cells as deduced by the fact that equal amounts of total cell protein were analyzed as confirmed by the expression levels of the control protein, actin.

Overexpression of Bcl-2 does not block 9-NC-induced apoptosis

To study whether Bcl-2 overexpression can confer resistance to 9-NC treatment in ER cells, we initially transfected ER cells with a *bcl-2* cDNA (thereafter these cells were termed ER/Bcl-2) and Bcl-2 expression was compared to those of non-transfected ER cells as well as to a control HL-60/Bcl-2 cell line, that was previously shown to stably overexpress Bcl-2 [26]. Using a specific antibody to Bcl-2, we detected the presence of Bcl-2 in whole-cell extracts containing the same amounts of total protein (Fig. 4A). It is apparent that ER/Bcl-2 cells

expressed very high levels of Bcl-2, when compared to non-transfected ER cells, and the Bcl-2 content was even higher than the content detected in the control HL-60/Bcl-2 cells.

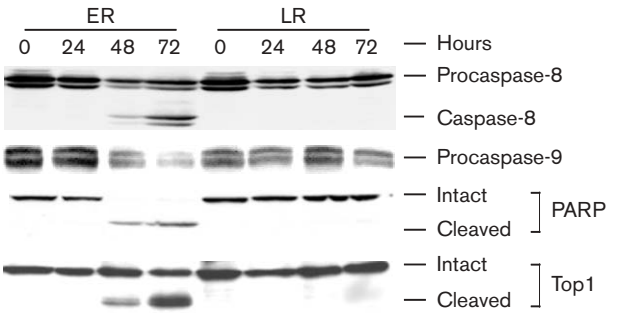
Subsequently, ER and ER/Bcl-2 cells were compared for sensitivity to treatment with 25 nM 9-NC for various periods of time by monitoring changes in the cell cycle using flow cytometry (Fig. 4B). Treatment with 9-NC for 24 h (histograms B and F), 48 h (histograms C and G) and 72 h (histograms D and H) resulted in very similar patterns of perturbations in ER (A–D) and ER/Bcl-2 (E–H) cells, indicating that Bcl-2 overexpression had practically no inhibitory effect on 9-NC-induced apoptosis. Finally, 9-NC resulted in the appearance of hyperdiploid cells in ER/Bcl-2 cells like in ER cells.

Activation of caspases-8 and -9 in 9-NC-treated ER and LR cells

The susceptibility of ER, but not LR, cells to 9-NC-induced apoptosis, observed by flow cytometry analysis, prompted us to investigate the ability of this drug to activate the initiator procaspases-8 and -9 in these cells. For this, we investigated whether the inactive forms, i.e. procaspases-8 and -9, were converted to their active counterparts, caspases-8 and -9, in ER and LR cells treated with 9-NC. Time-dependent activation of caspases was monitored by analyzing aliquots of whole extracts of cells, containing the same amount of total protein, after various periods of 9-NC treatment (Fig. 5). Cleaved (i.e. active) caspase-8 in ER cells was first detected at 48 h of drug treatment, and a dramatic conversion of procaspase-8 to caspase-8 was initially observed at 72 h of treatment. In contrast, no conversion of procaspase-8 to caspase-8 was detected in LR cells treated with 9-NC up to 72 h. Similarly, a time-dependent decrease in procaspase-9 was observed in drug-treated ER, but not in drug-treated LR cells (Fig. 5). The decrease in procaspase-9 is indicative of its processing (i.e. activation) to caspase-9. It should be noted that the antibody to procaspase-8 also recognizes caspase-8, whereas the antibody to procaspase-9 does not recognize caspase-9.

Activated caspase-8 and/or -9 target and activate the effector caspase-3, which subsequently targets and cleaves several cellular macromolecules including Topo I and PARP, though at different recognition sites [27]. Accordingly, we investigated whether PARP and Topo I were cleaved in 9-NC-treated ER cells subsequent to caspase-8 and -9 activation. The hypothesis was that no PARP and Topo I cleavage should be observed in 9-NC-treated LR cells lacking activated caspases-8 and -9. For these studies, we used antibodies that specifically recognize intact and cleaved products of PARP and Topo I. Indeed, there was virtually no intact, but only cleaved PARP at 48 h of 9-NC treatment in ER cells. Also, cleaved

Fig. 5



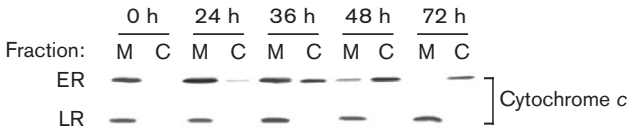
Activation of caspases and cleavage of specific substrates in 9-NC-treated cells. Cells were treated with 25 nM 9-NC for 0, 24, 48 and 72 h, and whole-cell extracts were subjected to immunoblot analysis for detection of procaspases/caspases-8 and -9, and the specific substrates PARP and Topo I targeted and cleaved by caspase-3.

Topo I was first detected in the ER cells at 48 h of 9-NC treatment and the amount of the cleaved product was dramatically increased at 72 h (Fig. 5). In contrast, only intact PARP and Topo I were present in the 9-NC-treated LR cells. Therefore, these results indicated that activation of caspases-8 and -9 in ER cells, or reversibly, lack of activation of these caspases in LR cells, was the key event(s) accounting for the sensitivity and resistance of ER and LR cells, respectively, to the apoptotic effect of 9-NC.

Release of mitochondrial cytochrome *c* in 9-NC-treated cells

A prerequisite for the activation of caspase-9 is the release of mitochondrial cytochrome *c* in the cytoplasm, where it binds to Apaf-1 and procaspase-9, and thus triggers auto-activation of caspase-9. Since caspase-9 is activated in 9-NC-treated ER, but not 9-NC-treated LR, cells, it would be expected that cytochrome *c* is released from the mitochondria of ER, but not of LR cells after 9-NC treatment. We investigated this hypothesis by assaying the presence of cytochrome *c* in the mitochondrial and cytosolic fractions of 9-NC-treated cells. Cytochrome *c* was identified by a specific antibody used in immunoblot analysis. The results demonstrated the absence of cytochrome *c* in the cytosolic fraction of ER cells after 24 h of 9-NC treatment, whereas cytochrome *c* was readily detected at 36 h of treatment (Fig. 6). At 48 h, no cytochrome *c* was detected in the mitochondrial, but only in the cytosolic fraction. At 72 h, the amount of cytochrome *c* released in the cytosol was greatly decreased, apparently because of further degradation. In contrast, no mitochondrial cytochrome *c* was released in the cytoplasm of LR cells treated with 9-NC for 72 h (Fig. 6). These results re-assessed our observation that 9-NC can induce activation of caspase-9 in ER but not LR cells.

Fig. 6



Release of mitochondrial cytochrome *c* into cytoplasm. ER and LR cells were treated with 25 nM 9-NC for the indicated times, and then collected to prepare mitochondrial (M) and cytosolic (C) fractions, which were subsequently subjected to immunoblot analysis to detect the presence of cytochrome *c*.

Discussion

In this report, we have presented experimental findings to show that we have selected two cell clones from the human ovarian cancer SKOV-3 cell line with differential susceptibility to the apoptotic effect of the anticancer drug, 9-NC. The parental SKOV-3 cell line does not express p53, and is resistant to cisplatin and doxorubicin. Thus, the SKOV-3 cell line exhibits all the characteristics of advanced and refractory ovarian tumors [21]. The ER and LR cells differ dramatically in their response to the apoptotic effect of 9-NC as assessed by flow cytometry studies. Several other clones, also isolated from the SKOV-3 cell line, responded to 9-NC, but the extent of the response was intermediate of that exhibited by the ER and LR cells.

We initially assessed by Northern blot analysis that both cell clones expressed no p53, like the parental SKOV-3 cells (results not shown). Therefore, all findings reported hereafter are independent of p53. The differential sensitivity of ER and LR cells to 9-NC treatment was visually monitored by the number of detached cells in culture as well as by the patterns of perturbations in the relative DNA content, i.e. cell cycle phases, detected by flow cytometry. Although 9-NC induced hyperdiploidy and eventually resulted in apoptosis, the hyperdiploid ER cells entered apoptosis much faster that the hyperdiploid LR cells.

The enzyme Topo I mediates the apoptotic effect of 9-NC [15,16] and, therefore, the level of Topo I expression may be a parameter associated with the extent of apoptosis induced by 9-NC. Our results indicated that the LR cells contained somehow less Topo I than the ER cells, and therefore 9-NC may have resulted in more extensive DNA damage in ER than LR cells, and consequently a larger apoptotic fraction in the 9-NC-treated ER cells. On the other hand, it has been postulated that cleavable complex formation rather than Topo I level is more important for cell killing by camptothecin [28,29]. However, we have not investigated the extent of functionality of Topo I in ER and LR cells.

We subsequently investigated the expression levels of the anti-apoptotic proteins Bcl-2 and Bcl-x_L. This investigation was prompted by a previous suggestion that expression of Bcl-x_L and abrogation or absence of p53 cooperate to allow rapid and progressive polyploidization following mitotic spindle damage [30]. In this regard, our results indicated that ER and LR cells, both lacking p53, contained elevated amounts of Bcl-2 and Bcl-x_L (in ER cells) or similar amounts of these proteins after 9-NC treatment. Elevation of Bcl-2 and Bcl-x_L in 9-NC-treated ER cells should have had increased resistance to apoptosis if the latter was solely dependent on mitochondria. However, this was not the case. Therefore, the differential sensitivity of ER and LR cell clones to 9-NC cannot be explained by quantitative differences in Bcl-2 or Bcl-x_L in absence of p53. It has also been reported that overexpression of either Bcl-2 or Bcl-x_L can protect tumor cells from a wide variety of apoptotic stimuli and confers a multi-drug resistance phenotype [31–33]. Overexpression of Bcl-2 prevents initiation of apoptosis by preventing the efflux of cytochrome *c* from the mitochondria to the cytoplasm [34,35]. However, contradictory findings have been reported on the apoptotic effect of camptothecin on human malignant cells ectopically expressing Bcl-2. Thus, ectopic Bcl-2 expression in malignant lung cells conferred resistance to camptothecin-induced apoptosis [36], whereas ectopic Bcl-2 expression in malignant glioma cells had no inhibitory effect on camptothecin-induced apoptosis [37]. This discrepancy in the reported findings has yet to be clarified. Further, our studies of ER/Bcl-2 cells (i.e. ER cells overexpressing Bcl-2) treated with 9-NC indicated that Bcl-2 was unable to inhibit the apoptotic action of 9-NC. Therefore, induction of apoptosis in ER cells treated with 9-NC was independent of Bcl-2.

We also investigated the presence of the pro-apoptotic proteins Bax and Bak in ER and LR cells. Our results indicated a very small increase in the expression of Bax and Bak in both ER and LR cells after 9-NC treatment. In this context, it has been shown that accumulation of Bax and Bak in the mitochondria increases the susceptibility of cells to various apoptotic stimuli [38–41], whereas cells lacking both Bax and Bak, but not lacking only one of these components, are completely resistant to cytochrome *c* release and apoptosis [42]. Therefore, the concurrent low expression of Bak and Bax may have contributed to the inability of mitochondria to release cytochrome *c*, and therefore to the increased resistance to apoptosis in 9-NC-treated LR cells.

Since caspases are essential in the execution of the extrinsic (i.e. mitochondria-independent) and intrinsic

(i.e. mitochondria-dependent) mechanisms of apoptosis, we examined the activation of representative caspases in order to elucidate which apoptotic pathways are associated with the differential susceptibility of ER and LR cells to 9-NC. In general, biochemical processing (i.e. cleavage) of the initiator caspase-8 or -9 is an event indicative of activation of the extrinsic or intrinsic mechanism, respectively [13,14]. In this regard, our results indicated that both caspase-8 and -9 were activated in 9-NC-treated ER, but not LR, cells. Further, activation of caspase-8 or -9 eventually leads to activation of the executioner caspases-3, -6 and -7 that target and cleave various cellular substrates, thus resulting in apoptosis [13,14]. The caspase-cleaved substrates include proteins involved in DNA metabolism and repair such as PARP and Topo I, with Topo I cleavage being a relatively late event in apoptosis [13,14]. Again, our findings demonstrated that 9-NC resulted in cleavage of both PARP and Topo I in ER cells, whereas only the intact forms of PARP and Topo I were detected in 9-NC-treated LR cells. Further, to confirm that 9-NC induced caspase-9 activation in ER, but not LR, cells, we investigated the presence of cytochrome *c* in the mitochondrial and cytosolic fractions of the cells before and after treatment with 9-NC. The rationale for this investigation was that upon activation of the mitochondria-dependent (i.e. intrinsic) apoptotic pathway, the mitochondrial cytochrome *c* is released in the cytoplasm, where it binds to Apaf-1 and procaspase-9 resulting in processing, i.e. activation, of caspase-9 [13,14]. Consistent with the presence of caspase-9 processing in 9-NC-treated ER cells, mitochondrial cytochrome *c* was released in the cytosol of these cells, and the released amount was dependent on the period of drug treatment. On the other hand, no mitochondrial cytochrome *c* was released in the cytosol of 9-NC-treated LR cells, consistent with our finding that caspase-9 remained intact. Thus, it appears that 9-NC induced apoptosis in ER and LR cells by different pathways. Detection of caspase-8 and -9 indicated that both the extrinsic and intrinsic apoptotic pathways were activated in ER cells treated with 9-NC. A possible connection between extrinsic and intrinsic pathways has been reported, i.e. in addition to directly cleaving other caspases, caspase-8 may activate other caspases through the use of the cytochrome *c*/Apaf-1/caspase-9 signaling pathway [43] by cleaving the pro-apoptotic Bcl-2 family member Bid that is localized on the mitochondria, thus triggering release of mitochondrial cytochrome *c* [44–46].

In conclusion, 9-NC-treated ER cells exhibited well-characterized events of apoptosis including activation of caspases-8 and -9, release of mitochondrial cytochrome *c*, and cleaved PARP and Topo I. Interestingly, these events were not observed in 9-NC-treated LR cells at the time apoptosis was already in progress as assessed by microscopy and flow cytometry (see Figs 2B and 5). However, the apoptotic events described above were observed in both ER and LR cells treated with another anticancer drug, etoposide (results not shown), indicating that the relative resistance of LR cells to 9-NC was drug-specific. Also, the fact that 9-NC-treated LR cells exhibited altered morphology and a delayed onset of apoptosis induction indicates the presence of functional Topo I in these cells. At present, we have hypothesized that the relative resistance of LR cells to 9-NC is associated with an apoptotic mechanism independent of caspases and mitochondria. Such a possibility has been reported for certain anticancer drugs including camptothecin [47,48], but we have not investigated this possibility in this report. Alternatively, the delayed apoptosis in 9-NC-treated LR cells may be due to development of premature senescence, which is then followed by apoptotic death of the senescent cells. It has been shown that DNA-damaging agents, including camptothecin, not only promote apoptosis, but could also induce cellular senescence [49–51]. Development of differentiated or senescent LR cells treated with various 9-NC concentrations is currently under investigation in this laboratory.

In this report, we have demonstrated that the human ovarian cancer cell line, SKOV-3, consists of at least two cell subpopulations with regard to susceptibility to the anticancer drug, 9-NC. The SKOV-3 cell line was derived from an ovarian tumor, which apparently also consisted of cell subpopulations with differential susceptibility to 9-NC. It is our speculation that other human ovarian tumors similarly consist of cells with differential susceptibility to 9-NC. Accordingly, we hypothesize that such tumors treated with 9-NC will initially remain stable or regress, because of apoptotic death of the 9-NC-sensitive cells, but subsequently the tumor growth will resume because of the 9-NC-resistant cells that will continue to proliferate. This hypothesis is corroborated by results of a clinical trial phase II of ovarian cancer patients treated with 9-NC [20]. In that clinical trial, 9-NC administration was followed by appearance of stable disease and partial regression in some patients, but subsequently the disease progressed again while exhibiting insensitivity to 9-NC treatment. Of course, an additional parameter that could confer insensitivity to 9-NC is the presence of cells with reduced expression or functional capability of Topo I, although this does not appear to be the case in the SKOV-3 cell line. Therefore, ovary and other tissue cancer types with partial or limited response to 9-NC should be treated with a combined protocol of 9-NC and another anticancer agent. This way, 9-NC treatment will initially eliminate the 9-NC-sensitive cancer cells, and subsequently, the 9-NC-resistant cells will be eliminated after treatment with an appropriate other anticancer drug.

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