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Selective administration and consequent effects of dexamethasone on the proliferation and apoptosis of human ovarian cancer cells

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Abstract
Dexamethasone (DEX) has been routinely used as a pre-treatment in the clinical application of paclitaxel (PTX) to treat ovarian cancer. However, PTX-induced apoptosis might be inhibited by DEX. This study was undertaken to investigate the effects of DEX on the apoptosis induced by PTX.
Both of SKOV-3 and HO-8910 human ovarian cancer cells were divided into four groups: (1) untreated (Con); (2) treated with DEX (0.1 μM) alone; (3) treated with PTX (50 nM); and (4) pre-treated with DEX (0.1 μM), and 24 h later, treated with PTX (DEX + PTX). Cell proliferation was determined by the 3-(4,5)-dimethylthiahiazo (−z-y1)-3,5-di- phenytetrazoliumromide (MTT) dye uptake method, while cell apoptosis was analyzed by propidium iodide (PI) staining and flow cytometry. Then, reverse transcription polymerase chain reactions (RT-PCRs) were applied to semi-quantitative analysis, followed by western blot analysis. Statistical analysis was performed, with Fisher's least significant difference test.
Our results demonstrated that DEX can differentially inhibit SKOV-3 and HO-8910 cell proliferation induced by PTX and decrease the apoptosis rates in cancer cells. Pre-treatment with DEX could up-regulate the expressions of members of anti-apoptotic Bcl-2 family (Bcl-2 and Bcl-XL) and members of IAP family (survivin). The expression of cleaved caspase-3 was down-regulated by DEX, shown by semi-quantitative RT-PCRs and western blot analysis.

Keywords: dexamethasone, untreated, treated with DEX (0.1 μM) alone, treated with PTX (50 nM), pre-treated with DEX (0.1 μM)

1. Introduction
Ovarian cancer has the poorest prognosis of all gynecological cancers and is a leading cause of death in women with cancers [1, 2]. Recently, new chemotherapeutic agents, including taxanes, gemcitabine and irinotecan, have become clinically available to treat ovarian cancer. Among these newer drugs, paclitaxel (PTX) is the one used most frequently for the treatment of patients with ovarian cancer, especially the advanced ovarian cancer.
The exact mechanism of cell death induced by PTX is elusive, though it is widely known that the major cellular target of PTX is the tubulin/microtubule system in mitosis [3]. Dexamethasone (DEX) is routinely used as a pre-treatment in the clinical application of PTX to prevent hypersensitivity reactions and other adverse effects such as nausea, emesis and toxic reactions [4, 6].
However, recent studies have discovered that DEX selectively inhibits PTX-induced apoptosis in a number of carcinoma cell lines, including ovary, breast, testis, prostate, bladder, pancreas, kidney, liver, colon, brain, cervix, bone and skin [7, 14]. The inhibition of PTX-induced apoptosis by DEX may occur through enhancing DNA repair capacity, suppressing host antitumor immune responses or blocking apoptosis [15, 16]. The apoptosis of cancer cell may be antagonized by anti-apoptotic modulator proteins, such as survivin (a member of the IAP family), Bcl-2 and Bcl-XL (members of the Bcl-2 family). The death receptor and mitochondrial death pathways may be linked by Bcl-XL, which subsequently activates the proteolytic activation of effector caspases-3 to trigger the mitochondrial dysfunction and cytochrome c release [17-19]. Also, DEX pre-treatment has been recently reported to interfere with apoptotic death in brain tumour cells by the transcriptional activation of a Bcl-XL gene [20-26].
To determine whether DEX would inhibit PTX-induced apoptosis in ovarian cancer cells and reduce the therapeutic efficacy of PTX, we performed *in vitro* experiments with the human ovarian cancer SKOV-3 and HO-8910 cell lines to evaluate the effects and mechanisms of DEX on human ovarian cancer cell apoptosis induced by PTX. Surprisingly, DEX had a strong anti-apoptotic effect on the carcinoma cells and prevented PTX-induced cancer cell reduction and apoptosis. This result was due to the inhibition of key molecules of death receptor and mitochondrial apoptosis pathway, resulting in a blockade of caspase activity. The direct transfer of caspases restored the apoptosis sensitivity of DEX-treated carcinomas *in vitro*. These findings suggest that the pro-apoptotic effects of chemotherapy regimens in patients with ovarian cancer might be strongly antagonized by the anti-apoptotic effects of DEX.

2. Materials and methods
2.1 Cell culture and group
SKOV-3 and HO-8910 human ovarian cancer cells were obtained from Shanghai Cancer Institute, and cultured in RPMI-1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO) and 1% penicillin and streptomycin (Sigma Chemical Co., St. Louis, MO) in a 5% CO_2 and 37°C incubator. DEX (Sigma Chemical Co., St. Louis, MO) was dissolved in ethanol before added to culture medium. The final ethanol concentration was 0.1%. PTX (Sigma Chemical Co., St. Louis, MO) was dissolved in PBS (PH

7.4, Sigma Chemical Co., St. Louis, MO) with a concentration of 1 μM.
The SKOV-3 and HO-8910 cells were divided into four groups: (1) untreated (Con); (2) treated with DEX (0.1 μM) alone; (3) treated with PTX (50 nM); and (4) pre-treated with DEX (0.1 μM), and 24 h later, treated with PTX (DEX + PTX).

2.2 Cell proliferation assay
The proliferation of SKOV-3 and HO-8910 were determined by the 3-(4,5)-dimethylthiahiazo (−z-y1)-3,5-di-phenytetrazoliumromide (MTT) dye uptake method. Briefly, cells (10,000/well) were incubated in triplicate in a 96-well plate in a final volume of 0.1 mL for the indicated time periods at 37°C. Then, 0.025 mL of MTT solution (5 mg/mL in PBS) was added to each well. After 2 h incubation at 37°C, 0.1 mL dimethylformamide (Sigma Chemical Co., St. Louis, MO) was added, incubation was continued for 30 min at 37°C, and then the O.D. value was measured using a Bio-Rad (Model 550) microplate reader at 570 nm, take dimethylformamide as the blank.

2.3 Propidium Iodide (PI) staining by flow cytometry
Four groups of cells were harvested and centrifuged at 1,000 rpm for 5 min and then fixed in 5 mL 70% pre-refrigerated ethanol for 24 h at −20°C. After washing the cells with 10 μL PBS twice, 1 mg/mL RNase (Sigma Chemical Co., St. Louis, MO) was added and incubated for 30 min at 37°C. Then, cells were stained with 300 μL/50 μg/mL PI (Sigma Chemical Co., St. Louis, MO) in the dark for 30 min at 4°C. Cell apoptosis was analyzed by flow cytometry (FCM, FACScan, Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

2.4 Apoptosis assay
Four groups of cells were collected and washed twice with PBS. Cells were centrifuged at 1,000 rpm for 5 min and then were resuspended in reaction buffer (200 μL HEPES (2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid, Sigma Chemical Co., St. Louis, MO) buffer, 1 μL fluorescein isothiocyanate-labeled annexin V (annexin V-FITC), 2 μL/50 μg/mL PI, ApoAlert Annexin V–FITC Apoptosis Kit, CLONTECH) and incubated in dark for 10 min at room temperature. After 1 h, cell apoptosis was analyzed by flow cytometry.

2.5 Semi-quantitative analysis
To semi-quantify the mRNAs of survivin, Bcl-2, and Bcl-XL, reverse transcription polymerase chain reactions (RT-PCRs) were performed. Cells were washed with ice-cold PBS once, and total RNA was isolated with TRIzol (Invitrogen Life Technologies, San Diego, CA), according to the instructions of the manufacturer. Approximately 2 μg RNA was treated with ribonuclease-free deoxyribonuclease, and cDNA was synthesized by using Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA), and 2 μL of synthesized cDNA was subjected to 30 cycles of PCR that resulted in a single specific amplification product of the expected size. The PCR conditions were as follows: 30 sec denaturation at 94°C, 1 min annealing at 55°C, and 45 sec extension at 72°C. PCR primers used in this study were as follows: survivin, sense 5′-TTGGCAGGTGCCTGTTGAAT-3′ and antisense 5′-AGCCAGTCCCCCACAGCAT-3′; Bcl-2, sense 5′-GTGGAGGAGCSTCTTCAGGGA-3′ and antisense 5′-AGGCSACCCAGGGTGATGCSAA-3′; and Bcl-XL, sense 5′-TTGGACAATGGACTGGTTGA-3′ and antisense 5′-GTAGAGTGGATGGTCAGTG-3′. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control of the PCR. The primers for GAPDH were sense 5′-GGATTTGGTCGTATTGGG-3′ and antisense 5′-GGAAGATGGTGATGGGATT-3′.
To determine the specificity of RT-PCRs, we performed the amplifications three times and took the mean value of these three reactions. The RT-PCRs were in the linear range of amplification for the target mRNA, as well as for the control. Each RT-PCR product was demonstrated on 1.4% agarose gel stained with ethidium bromide (Sigma Chemical Co., St. Louis, MO). The bands were documented, scanned, and quantified using Quantity One software (PDI, New York, NY) and normalized with internal control (GAPDH).

2.6 Western blot analysis
Cells were lysed in ice-cold lysis buffer (10 mM Tris (tris (hydroxymethyl) aminomethane, pH 7.5), 0.1 nm EDTA (Ethylene Diamine Tetraacetic Acid), 0.1 nM EGTA (Ethylene Glycol Tetraacetic Acid), 0.5% SDS (Sodium Dodecyl Sulfonate), 0.1 mm β-mercaptoethanol, containing 2 μg/mL of each of the protease inhibitors: leupeptin, aprotinin, and pepstatin, Sigma Chemical Co., St. Louis, MO) for 10 min. Protein concentrations in cell lysates were measured using the Bio-Rad protein determination assay. Twenty micrograms of protein were separated by SDS-PAGE, transferred to nitrocellulose membranes (Schleicher and Schuell, Inc., Burlington, VT), and immunoblotted with various primary antibodies. Antibodies to cleaved caspase-3 and Bcl-XL were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The blots were further incubated with conjugated alkaline phosphatase.

2.7 Statistical analysis
Statistical analysis was performed using one-way ANOVA (One-factor Analysis of Variance), with Fisher's least significant difference test. Data were expressed as mean ± SD (Standard Deviation). Differences were considered significant, if $p < 0.05$.

3. Results
The effect of DEX on the growth of paclitaxel-induced cells was firstly examined by MTT assay. The growth of PTX-induced SKOV-3 cells and HO-8910 cells was promoted by DEX in a concentration-dependent manner. These results indicate that DEX can promote the proliferation of PTX-induced SKOV-3 and HO-8910 cells.
To investigate whether DEX could interfere with apoptosis, we treated SKOV-3 cells in the presence or absence of DEX pre-treatment. Twenty-four hours later, apoptosis was examined by staining of the cells with PI-staining and then analyzed by FCM. The apoptosis rates in human ovarian cancer cell line SKOV-3 induced by PTX, were 5.55 ± 0.53% if pre-treated with DEX, and 13.90 ± 1.62% if not treated with DEX (p (p value) < 0.01). We treated HO8910 cells in the presence or absence of DEX for 24 h as well. The apoptosis was examined by staining of the cells with annexin V-FITC/PI and then analyzed by FCM. The percentages of apoptosis in human ovarian cancer cell line HO-8910 induced by PTX, were 11.45 ± 0.945% if pre-treated with DEX, and 29.94 ± 1.49% if not treated with DEX ($p < 0.01$)

To determine whether anti-apoptotic modulator proteins, such as survivin, a member of the IAP family, and the Bcl-2 and Bcl-XL proteins of the Bcl-2 family were involved in DEX-induced apoptosis inhibition, HO-8910 and SKOV-3 cells were treated with PTX (50 nM) for 24 h with or without DEX pre-treatment (0.1 μM) to examine the expressions of Bcl-2, Bcl-XL, and survivin. As shown in, the expressions of Bcl-XL and survivin mRNAs were significantly up-regulated by the pre-treatment of DEX ($p < 0.01$), whereas the expression of Bcl-2 was un-affected ($p > 0.05$).

We detected the change of Bcl-XL proteins in SKOV-3 and HO-8910 cells by western blot analysis. As shown in the expressions of Bcl-XL protein were up-regulated by DEX pre-treatment in both HO-8910 and SKOV-3 cells ($p < 0.01$).

We detected the change of cleaved caspase-3 proteins in SKOV-3 and HO-8910 cells by western blot analysis. As shown in, the protein expression of cleaved caspase-3 was down-regulated by DEX pre-treatment in both cancer cell lines ($p < 0.01$).

4. Discussions

DEX has been introduced to tumor therapy because it has potent pro-apoptotic effects on lymphoid cells, thus effective in treating tumor-related edema, inflammation, pain, and electrolyte imbalances [26]. It can also reduce nausea, hyperemesis, and acute toxicity in normal tissue [16, 17]. However, more and more data have strongly recommended that the application of DEX renders the majority of malignant solid-tumor cells resistant to apoptosis and promotes proliferation following cytotoxic therapy [27, 28]. In addition, the anti-chemotherapeutic effect of DEX can be seen in anticancer drugs such as cisplatin, 5-fluorouracil, adriamycin, actinomycin D, doxorubicin and gemcitabine [29, 30]. It has also been known that DEX-induced resistance to chemotherapy agents is through enhancing the anti-apoptotic gene, such as Bcl-XL gene in glioma cells [31, 32] and other solid malignant cells [33-35].

In the present study, we demonstrated that DEX may protect human ovarian cancer cell lines HO-8910 and SKOV3 from PTX-induced apoptosis. These findings suggested that the use of DEX can prevent hypersensitivity in the therapy of ovarian cancer, but result in resistance to chemotherapy that reduces tumor growth. Bcl-XL, a member of the Bcl2 family, is an anti-apoptotic gene on mitochondria. Several transcription factors are present in the Bcl-XL promoter region, such as NF-kB, Stat3, Stat5, and AP-1. DEX has been shown to enhance NF-kB activity in MCF7 breast cancer cells [36]. In contrast, GCs also inhibit PTX-induced apoptosis by inhibition of NF-kB activation [37, 38].

Several studies have reported that the inhibitory effect of DEX on the therapeutic activity of PTX seems to be apoptosis-specific because PTX-mediated mitotic arrest or cell cycle distribution was unaffected or marginally affected by the application of steroid [39-41]. By using flow cytometry assays, western blot analysis and RT-PCR, we identified several genes of the death receptor and anti-apoptotic molecule pathways to be influenced by DEX. The expression of Bcl-XL and survivin mRNAs were up-regulated by the pre-treatment of DEX ($p < 0.01$). However, the mRNA of Bcl-2 mRNA was un-affected by DEX pre-treatment ($p > 0.05$). In term of protein expressions, Bcl-XL protein was up-regulated by DEX pre-treatment ($p < 0.01$). It has been shown, for fibrosarcoma cells, that glucocorticoids can increase the Bcl-XL level by inducing the transcriptional activation of the Bcl-XL promoter [42]. Therefore, one might speculate that the inhibitory effect of DEX on the therapeutic activity of PTX occurs by increasing the expression of anti-apoptotic genes, such as Bcl-XL and survivin.

It is well known that caspase −3 is the effector caspase in apoptosis and can be activated by irradiation, chemotherapeutics, or members of the tumor necrosis factor family [43-46]. Therefore, DEX could resist the PTX-induced expression of key elements of the cell death receptor pathway, such as cleaved caspase-3, in ovarian cancer. Our further experiment confirmed that the expression of cleaved caspase-3 was down-regulated by DEX pre-treatment ($p < 0.01$). Intriguingly, down-regulation of these pro-apoptotic genes was not observed in lymphoid cells. This observation is in line with other reports showing the up-regulations of pro-apoptotic genes, such as CD95, caspase-3, and caspase-4, or down-regulations of anti-apoptotic molecules, such as Bcl-2 and Bcl-XL, after DEX treatment in lymphoid cells [47-49]. These pro- and anti-apoptotic effects of DEX might be the reason for its inhibition of apoptosis in carcinoma cells but its activation of apoptosis in lymphoid cells.

It is well known that DEX acts through glucocorticoid receptors (GRs) [50], however, the expressions of GRs were not evaluated in this study. It is a limitation, because only with the knowledge of GRs changes, can the effect of DEX on the cell lines be thoroughly understood.

In conclusion, our data suggested that the application of DEX significantly inhibited the therapeutic activity of PTX in ovarian cancer cells. This result is in contrast to the effect of GCs in lymphoid cells and may involve cell type–specific regulation of survival molecules or anti-apoptotic molecules. Thus, our results may provide new evidence that the anti-apoptotic effect of DEX on human ovarian cancer cells is mediated by up-regulating Bcl-XL and surviving, and down-regulating caspase-3 activity. These findings have profound clinical meanings in term of proper application of DEX which might reduce side effects and sacrifice the efficacy of PTX on cancer cell death as well.

5. Conclusion

Our data gained invaluable insights of the antagonistic mechanisms of DEX on PTX-induced cancer cell death and may provide new methods of using DEX as antineoplastic drugs or agents in the clinical treatment for ovarian cancer patients.

6. Author's Contribution

Partha Majumder is Gold Medalist in Human Physiology, having expertise in the area of cultivation in recent Biomedical research and former Head of the Department of Applied Biotechnology and Bioinformatics, Sikkim Manipal University, CC:1637, Kolkata, India.

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