Dexamethasone enhances cell resistance to chemotherapy through increasing adhesion to extracellular matrix in human ovarian cancer cells

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Running Title: Dex resists chemotherapy by enhancing adhesion
Abstract

Glucocorticoids (GCs) are widely used as co-medication in therapy of solid malignant tumors to relieve some of the side effects of chemotherapy drugs. However, recent studies have shown that GCs could render cancer cells more resistant to cytotoxic drug-induced apoptosis, but the mechanism is largely unknown. In the present study, we found that the treatment of human ovarian cancer cell lines HO-8910 and SKOV3 with synthetic GCs Dexamethasone (Dex) significantly increased their adhesion to extracellular matrix (ECM) and their resistance to apoptosis induced by cytotoxic drugs cisplatin and paclitaxel. Dex also increased the protein levels of adhesion molecules integrin β1, α4 and αV in HO-8910 cells. The neutralizing antibody against integrin β1 prevented Dex-induced adhesion and significantly abrogated the protective effect of Dex toward cytotoxic agents. We further found that TGF-β1 alone not only increased cell adhesion and cell survival of HO-8910 cells in the presence of cisplatin, but also has synergistic pro-adhesion and pro-survival effects with Dex. Moreover, TGF-β1 neutralizing antibody that could block TGF-β1-induced cell adhesion and apoptosis resistance markedly abrogated the synergistic pro-adhesion and pro-survival effects of Dex and TGF-β1. Finally, we further demonstrated that Dex could up-regulate the expression of TGF-β receptor type II (TβRII) and enhance the responsiveness of cells to TGF-β1. In conclusion, our results indicate that increased adhesion to ECM through enhancing integrin β1 signaling and TGF-β1 signaling plays an important role in chemoresistance induced by GCs in ovarian cancer cells.

Keywords: Dexamethasone, chemotherapy, cell viability, cell adhesion, integrin β1, TGF-β1
**Abbreviations**

Dex, dexamethasone; GCs, glucocorticoids; GR, glucocorticoid receptor; TGF-β1, transforming growth factor-β1; TβRI, TGF-β receptor type I; TβRII, TGF-β receptor type II; FN, fibronectin; ECM, extracellular matrix; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; SDS, sodium dodecyl sulfate; DCC-CBS, dextran-coated charcoal stripped calf bovine serum.

**Introduction**

Glucocorticoids (GCs) are being used in the treatment of childhood leukemia for several decades (Frei et al. 1965) and have become one of the common drugs in almost all chemotherapy protocols for lymphoid malignancies due to their ability to induce apoptosis in the majority of hematological cells and efficiently kill lymphoid cells (Schmidt et al. 2004). Nowadays, GCs are also widely used as co-medication in cancer therapy of solid malignant tumors because of their effectiveness in the treatment of the malignant tumor or treatment-related edema, inflammation, pain, electrolyte imbalance, as well as due to their ability to stimulate appetite, to prevent nausea, emesis, and toxic reactions caused by cytotoxic treatment (Rutz 2002, Rutz & Herr 2004). GCs are also given before, during and after chemotherapy of solid malignant tumors to reduce acute toxicity, particularly hyperemesis and to protect normal tissue, e.g. bone marrow progenitor cells, of cancer patients against the long-term effects of genotoxic drugs (Kriegler et al. 1994).

While GCs strongly induce apoptosis in cells of the hematological lineage, they also
support survival in several nonhematologic tissues such as mammary gland, liver, lung, glioma and subcutaneous adipocytes (Bailly-Maitre et al. 2002, Mikosz et al. 2001, Moran et al. 2000, Schorr & Furth 2000, Webster et al. 2002, Zhang et al. 2001). More recent data indicates that GCs can inhibit apoptosis induced by chemotherapy not only in established cancer cell lines and tumor xenografts, but also in the freshly isolated cell from surgical resections from tumors of various origins, including ovary, breast, prostate, pancreas, liver, colon, brain, cervix, bone, skin and nervous system (Herr et al. 2003, Sui et al. 2006, Zhang et al. 2006a, Zhang et al. 2006b, Zhang et al. 2006c, Zhang et al. 2006d, Zhang et al. 2006e). In addition, the anti-chemotherapy effect of GCs can be seen in several anticancer drugs including cisplatin (Gassler et al. 2005, Wolff et al. 1996, Zhang et al. 2006a, Zhang et al. 2006b, Zhang et al. 2006d, Zhang et al. 2007), paclitaxel (Sui et al. 2006, Wu et al. 2004, Wu et al. 2005), 5-fluorouracil (Zhang et al. 2006a, Zhang et al. 2007), adriamycin (Weller et al. 1997), actinomycin D (Wolff et al. 1996), doxorubicin (Wu et al. 2004) and gemcitabine (Gassler et al. 2005, Zhang et al. 2007). The GC-induced pro-survival effects should be of important clinical relevance when they interfere with the effect of chemotherapeutics. Recent studies suggest that GC-conferred cellular resistance to cancer therapy may occur through multiple mechanisms, for example, by enhancing DNA repair capacity, suppressing host anti-tumor immune responses, and blocking apoptosis (Herr et al. 2003, Rutz 2002, Rutz and Herr 2004). Some Dex-induced proteins, such as inhibitors of apoptosis (cIAP-2, X-IAP, Bcl-XL and Bcl-2), mitogen-activated protein kinase phosphatase-1 (MKP-1), as well as GC-induced serum and GC inducible protein kinase-1 (SGK-1) may contribute to the prevention of chemotherapy-induced apoptosis by GCs (Herr et al. 2003, Herr et al. 2007, Runnebaum et al. 2005, Webster et al. 2002, Wu et al. 2004, Wu et al. 2005). However,
the molecular mechanisms underlying the anti-apoptotic effect of GCs in epithelial cells are still largely unknown.

In our previous work, we found that Dex could dramatically prolong the detachment time of the cells digested with trypsin in human ovarian cancer cell line HO-8910, suggesting that Dex may be able to increase the cell adhesion ability to ECM. Since cell adhesion to ECM is pivotal for survival and growth of most of the solid cancer cells derived from epithelium (Pinkse et al. 2004, Rozzo et al. 1997), we hypothesized that the increase of cell resistance to cytotoxic therapy-induced apoptosis by Dex may due to its promotion effect in cell adhesion to ECM. Therefore, in the present study, we investigated the effect of Dex on cell adhesion to ECM of two human ovarian cancer cell lines, HO-8910 and SKOV3, and examined the relationship between Dex’s effect of enhancing adhesion and Dex-induced cell resistance to chemotherapeutic agents. We further explored the mechanism of Dex action and mainly focused on the adhesion molecule integrin β1 subfamily, as well as the TGF-β1 signaling. Our results provide new evidence that Dex’s role of pro-adhesion through enhancing integrin β1 signaling and TGF-β1 signaling is one of the basic mechanisms responsible for Dex-induced apoptotic resistance against chemotherapy in ovarian cancer cells.

Materials and Methods

Cell culture

Human ovarian cancer cell HO-8910 was described previously (Chen et al. 2006). Human ovarian cancer cell SKOV3 was cultured in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin (Huashun Technology, Inc., Shanghai, China) at 5% CO₂ and 37°C.
**Semi-quantitative RT-PCR**

Total RNA was extracted with the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). After reverse transcription, the cDNA products were amplified by PCR with the annealing temperature at 58°C (integrin β1) or 55°C (TβRII, GAPDH). The amplifying cycles and templates were 24 cycles and 75 ng cDNA for integrin β1, 24 cycles and 40 ng cDNA for TβRII, and 20 cycles and 20 ng cDNA for GAPDH according to the results of optimization. The primers were sense 5'-GCACGTTCAGAAGTCGGTT-3' and antisense 5'-AGATATGCAACAATCCAGTGGT-3' for TβRII, (467 bp) (Li et al. 2006), sense 5'-ACACGTCTCTCTCTGTCG-3', antisense 5'-CAGTTGTTCGGCAGCCTCTCT-3' for integrin β1 (157bp) (Kappert et al. 2000), sense 5'-TTCATTGACCTCAACTACATG-3', antisense 5'-GTGGCAGTGTGACATGGAC-3' for GAPDH (443 bp). PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining.

**Western blotting**

Total cell lysates were prepared with 1×SDS lysis buffer containing 0.1 mM β-mercaptoethanol, 2 µg/ml of each of the protease inhibitors leupeptin, aprotinin, and pepstatin. After electrophoresis, the protein was transferred to a nitrocellulose membrane, blocked with 5% nonfat milk, and probed overnight with primary antibodies against integrin β1 (Upstate, 1/500), integrin α4, αV, (Chemicon, 1/500), TβRII (Santa Cruz, CA, 1/500 ), cleaved caspase-3 (Asp175) (Cell Signaling, 1/500), β-actin (Sigma-Aldrich Chemicals, 1/10000). The blots were then washed, exposed to HRP-conjugated secondary antibodies (Rockland Immunochemicals, 1/5000) for 2 h, and finally detected by ECL chemiluminescence (Pierce, Rockford, IL, USA).
Transient transfection and luciferase assay

Twenty-four hours before transfection, HO-8910 cells were plated in triplicate into a 24-well plate at a density of 5×10^4 cells/well. Cells were transiently transfected with 1 µg/well of p3TP-Luc driven luciferase reporter plasmid (kindly provided by Dr. J. Massagué) using Lipofectamine plus transfection reagent (Invitrogen Life Technologies, Carlsbad, Calif, USA). In order to normalize the transfection efficiency, pRL-TK-Renilla-luc (30 ng/well, Promega, Madison, WI, USA) was co-transfected into the cells. Cells were grown for 12 h and then treated with 10^{-7} M Dex (Sigma-Aldrich Chemicals, St. Louis, MO, USA) and/or 10 ng/ml TGF-β1 (Peprotech, Rocky Hill, NJ, USA) for 24 h. The luciferase activities were determined using the dual luciferase assay system. Values were normalized by renilla luciferase and presented as fold-induction over control.

Analysis of viability

Cells were seeded at 2×10^3 cells per well in 96-well culture plates in triplicate, and cultured in RPMI-1640 containing 5% charcoal-dextran stripped CBS (CD-CBS). Cisplatin (Sigma-Aldrich Chemicals, St. Louis, MO, USA), Paclitaxel (a gift from Prof. Zhu Minghua, Department of Pathology, Changhai hospital, Second Military Medical University) or/and Dex were added and refreshed every other 24 h. For blockade assay, 10~20 µg/ml of integrin β1 antibody, 10 µg/ml of TGF-β1 antibody or control IgG was also added to the medium. At indicated time, the viable cells were determined by MTT assay as described before (Chen et al. 2006).

Measurement of apoptosis

Cells were cultured in appropriate media containing 5% CBS with each agent for the indicated time. Cells were trypsinized and stained with FITC-conjugated annexin V
and propidium iodide (BD Biosciences, Heidelberg, Germany) according to the manufacturer’s instructions. The apoptotic cells were analyzed by flow cytometric analysis.

**Cell adhesion assay**

Cell adhesive ability to ECM was determined by cell adhesion assay (Chen et al. 2004). Cells were incubated in the medium containing the agents for indicated time and then digested and counted. 8×10^4 cells were seeded into 96-well plates precoated with 10 µg/ml FN (Calbiochem, Darmstadt, Germany) and incubated at 37 °C for 1 h. The plates were gently washed three times with PBS to remove unattached cells. The remaining cells in 96-well plates were determined by MTT assay.

For blockade experiment, the 96-well plates were precoated with 10 µg/ml of FN for 1 h, followed by 1% BSA for 45 min at 37 °C. 2×10^4 resuspended cells were incubated with 10~20 µg/ml of integrin β1 antibody, 10 µg/ml of TGF-β1 antibody or control IgG for another 45 min at 37 °C before seeding into wells to detect the cell adhesion ability with the same procedure as described above.

**Statistical analysis**

Data are expressed as mean±SD of at least three determinations. Statistical significance between experimental groups was analyzed by analysis of variance, and the significance level was set at p<0.05.

**Results**

**Dex increases cell viability by inhibition of chemotherapy drug-induced apoptosis in ovarian cancer cells HO-8910 and SKOV3**

We first investigated the effects of cisplatin or paclitaxel alone or plus Dex on
survival of HO-8910 cells. As shown in Figure 1A, cisplatin could significantly decrease the survival cell number in a dose-dependent manner. Co-treatment of cells with Dex and different doses of cisplatin significantly increased the survival cell number. $10^{-7}$ M Dex increased the survival cell number by 53.8% (p<0.01) in the presence of 2 µg/ml of cisplatin, a dose commonly used in the clinic. A similar protective effect of Dex was also seen when cells were treated with paclitaxel, another chemotherapy drug (Figure 1C). These findings were confirmed in SKOV3, a different human ovarian cancer cell line (Figure 1B, 1D).

Since $10^{-7}$ M Dex alone led to a slight inhibition of cell growth by about 20% (Figure 1A), the increase in cell survival number when co-treated cells with Dex and cisplatin or paclitaxel was not due to the acceleration of cell growth, but most probably due to Dex-induced attenuation of cell apoptosis caused by cytotoxic drugs. To test this probability, we further examined the change of apoptosis of HO-8910 cells treated with cisplatin (2 µg/ml) or Dex ($10^{-7}$ M) alone or combination of Dex and cisplatin for 48 h by flow cytometry. As shown in Figure 1E, cisplatin resulted in remarkable cell apoptosis with 18.38% (p<0.05). However, co-treatment of cells with Dex and cisplatin significantly diminished the apoptosis cells to 10.1% (p<0.01). Moreover, Dex could also inhibit the activation of caspase-3 protein by cisplatin in HO-8910 cells (Figure 1F). These results indicated that Dex increases cell viability through protecting cells against apoptosis induced by chemotherapy drugs.

**Dex increases cell adhesion ability of ovarian cancer cells in the absence or presence of cisplatin**

Our preliminary studies found that HO-8910 cells treated with Dex were more resistant to trypsin digestion than cells treated with vehicle control. The detachment time of cells treated with or without $10^{-7}$ M Dex for 24 h followed by digestion with
0.25% trypsin was 16 ± 3.5 and 4 ± 0.5 min, respectively. This phenomenon was also observed in SKOV3 cells, suggesting that the cell adhesion to ECM may be strengthened by Dex. We further investigated the effect of Dex on cell adhesion ability. As shown in Figure 2A and 2B, Dex could significantly increase the cell adhesion ability to FN coated culture plates in a time- and dose- dependent manner in both HO-8910 and SKOV3 cells. In addition to the increased cell adhesion ability, Dex treatment also induced a fibroblast-like change in cell morphology (data not shown).

Based the above data we proposed that Dex-increasing adhesion to ECM may be involved in its protecting cells from apoptosis induced by chemotherapy drugs. To test this hypothesis, we investigated the effect of cisplatin on cell adhesion in the absence or presence of Dex. As shown in Figure 2C, in the absence of Dex, treatment of cells with cisplatin for 24h dramatically decreased the adhesion cell number by 51% (p<0.05) as compared with that of control. However, when cells were treated with both cisplatin and increasing concentrations of Dex, the adhesive cell number gradually increased. There was a 2-fold increase in the adhesive cell at 10⁻⁷ M Dex compared to cells treated with cisplatin alone.

**Dex up-regulates protein expressions of integrin α4β1 and αVβ1 in HO-8910 cells**

As one of the receptors of ECM proteins, adhesion molecular integrin β1 subfamily plays a crucial role in regulating cell adhesion to ECM (Hynes 1992). All members of integrin β1 subfamily are heterodimers formed by one common integrin β1 subunit and different kind of α subunits (Hynes 1992). We investigated the effect of Dex on the expression of integrin β1 as well as two kinds of α subunit, α4 and αV, which are primary receptors of fibronectin in HO-8910 cells. As shown in Figure 3, though Dex could not change the expression of integrin β1 in mRNA level (data not shown), it
could induce the expression of integrin β1, α4 and αV at protein levels in a time- and dose-dependent manner.

**Blocking integrin β1-mediated cell adhesion attenuates the protective effect of Dex on chemotherapy drug-induced apoptosis in HO-8910 cells**

In an effort to elucidate the role of Dex-induced expression of integrin β1 in protection cell against chemotherapeutic agents, we further examined the influence of the integrin β1 blocking antibody on the cell adhesion and cell survival. As shown in Figure 4A, preincubation of cells with integrin β1 blocking antibody significantly blocked Dex-induced cell adhesion in a dose-dependent manner. As compared with Dex plus IgG group, addition of 10 µg/ml and 20 µg/ml integrin β1 blocking antibody reduced the attached cell number by 25% (p<0.05) and 67.3% (p<0.01), respectively. Meanwhile, the cell survival protective effect of Dex was also decreased when cells were treated with Dex combined with integrin β1 blocking antibody. As shown in Figure 4B, 20 µg/ml of integrin β1 blocking antibody could reduce the protective effect of Dex from cisplatin by 61.5% (p<0.05). A similar but stronger reduction of the protective effect of Dex could be seen when paclitaxel was used in the experiments (Figure 4C). These results indicate that the enhancement of cell adhesion to ECM which mediated mainly by integrin β1 subfamily plays an important role in GC protecting cells against chemotherapeutic agents in HO-8910 cells.

**Dex and TGF-β1 have a synergistic effect on enhancing cell adhesion and resistance to cisplatin in HO-8910 cells**

TGF-β1 is the most important growth factor that facilitates cell adhesion to ECM by increasing matrix production and accumulation, as well as enhancing expression of cell adhesion molecules (Dawes et al. 2007). Considering that ovarian epithelial cells
can secrete TGF-β1 (Peng 2003), we therefore tested whether TGF-β1 signaling pathway is involved in Dex’s role of pro-adhesion and pro-survival.

As shown in Figure 5A, adhesion ability of cells treated with $10^{-7}$ M Dex, 10 ng/ml of TGF-β1 alone or both of the two agents for 24 h was 1.89-fold (p<0.01), 1.4-fold (p<0.05) and 2.64-fold (p<0.01), respectively compared to control cells, indicating that TGF-β1 not only increased adhesion of HO-8910 cells alone, but also has a synergistic pro-adhesion effect with Dex. Moreover, 10 µg/ml of TGF-β1 neutralizing antibody that could significantly block the pro-adhesive effect of TGF-β1 markedly inhibited the synergistic effect of Dex and TGF-β1 on cell adhesion. The similar synergistic pro-survival effect was also observed when co-treatment of cells with Dex and TGF-β1 in the presence of cisplatin. And such a synergistic effect was also markedly abrogated by TGF-β1 neutralizing antibody as well (Figure 5B). These results indicate that TGF-β1 signaling pathway is involved in Dex-induced cell adhesion to ECM as well as cell resistance to cisplatin in HO-8910 cells.

**Dex enhances the responsiveness of HO-8910 cells to TGF-β1 by increasing the expression of TβRII**

The synergistic effect of Dex and TGF-β1 on cell adhesion as well as cell resistance to cisplatin in HO-8910 cells suggested that there is a positive crosstalk between GC and TGF-β1 signaling pathways. Therefore we examined the effect of Dex on the expression of TGF-β1 and its two types of receptor (TβR-I and TβR-II). The results demonstrated that the expressions of TGF-β1 and TβR-I remain unaffected (data not shown), but TβR-II expression in HO-8910 cells was significantly up-regulated by $10^{-7}$ M Dex at both mRNA and protein levels (Figure 5C).

It is well known that binding of TGF-β1 to constitutively active TβR-II leads to recruitment and interaction of TβR-I, which phosphorylates downstream Smad
proteins that activate target genes in the nucleus. We then examined the response of cells to TGF-β1 signaling by transient transfection of cells with p3TP-luc, a TGF-β1 responsive reporter gene. As shown in Figure 5D, TGF-β1, Dex and TGF-β1 plus Dex induced a 2- (p<0.01), 1.5- (p<0.05) and 2.8- (p<0.01) fold increase in luciferase activity, respectively. These results indicate that Dex may enhance the responsiveness of cells to TGF-β1 by up-regulating the expression of TβR-II.

Discussion
Recently more and more data from preclinical, and to some extent clinical studies, have strongly recommend a GC-conferred resistance to cancer therapy in the majority of malignant solid tumors – irrespective of tumor origin and the nature of specific anticancer drugs (Gassler et al. 2005, Herr et al. 2003, Runnebaum et al. 2005, Sui et al. 2006, Webster et al. 2002, Weller et al. 1997, Wolff et al. 1996, Wu et al. 2004, Wu et al. 2005, Zhang et al. 2006a, Zhang et al. 2006b, Zhang et al. 2006c, Zhang et al. 2006d, Zhang et al. 2006e, Zhang et al. 2007). In this study we have demonstrated that Dex could protect human ovarian cancer cell lines HO-8910 and SKOV3 from apoptosis induced by chemotherapy drugs, including cisplatin and paclitaxel, as well as adriamycin and actinomycin D (data not shown).
These findings indicate that the use of GCs as co-medication in the therapy of ovary carcinomas and other solid tumors may result in desensitization to chemotherapy, ultimately leading to faster tumor growth. Therefore it should be careful to administer GCs in routine chemotherapy in the cancer patients. The protection of normal tissue by GCs may be good for patients, but the protection of cancer cells may impair the effect of chemotherapy. Thus, it is understandable that the appeal for reevaluating the administration of GCs in the management of solid cancer patients has emerged
recently. After performing an overall statistical analysis of a large screening study, 
Zhang et al. also suggested the replacement of GCs by non-steroidal anti-emetic 
agents, which do not induce therapy resistance (Zhang et al. 2007).

In addition to chemotherapy resistance, we also noticed that Dex alone led to an 
inhibition of cell growth in HO-8910 and SKOV3. The similar results were also 
reported in other several solid tumor cells such as glioblastoma cells and 
osteosarcoma cells (Mattern et al. 2007). The effects of GC may reflect its important 
role in maintaining the homeostasis of cell number. It inhibits the proliferation of cell 
under physiological condition, however enhances cell survival under the pressure of 
cytotoxic drugs and other harmful stimuli. Moreover, it is known that cell cycle arrest 
may reduce the sensitivity of cells to the chemotherapy drugs. So, Dex-induced cell 
growth inhibition and cell cycle arrested in G1 may also be one of mechanisms of its 
anti-apoptotic effect on cancer therapy. Mattern et al. held the similar opinion that the 
inhibition of cell growth by inducing cell cycle arrest may be crucially involved in 
switching the balance of several interacting pathways to survival upon treatment with 
GCs (Mattern et al. 2007).

For anchorage dependent monolayer culture, attachment of cells to ECM is one of the 
prerequisites for cell survival, growth and differentiation. As reported, Dex increased 
cell adhesion ability in lung fibroblasts (Brenner et al. 2001) and lens epithelial cells 
(Sawhney 2002), but decreased in amnion epithelial cells (Guller et al. 1995a) and 
placental cells (Guller et al. 1995b), indicating that Dex plays a role in regulating cell 
adhesion to ECM in a cell-specific manner. Our study adds new data that Dex 
significantly promotes adhesion of human ovarian cancer cells HO-8910 and SKOV3 
to ECM, and demonstrates for the first time that the enhancement of adhesion by Dex 
is associated with its effect of promoting cell viability to chemotherapy.
It is well known that adhesion molecular integrin β1 subfamily plays a crucial role in regulating cell adhesion to ECM proteins, such as collagen, fibronectin and laminin (Hynes 1992, Jin & Varner 2004, Rozzo et al. 1997). It has been reported that Dex shows cell-type specific regulation on the expression of integrin β1. For example, the expressions of integrin β1 mRNA and protein were reduced in human skin fibroblast (Zoppi et al. 1998) and human cytotrophoblast (Ryu et al. 1999), while were induced in human gastric carcinoma cells (Murakami et al. 1998) by Dex. But there is no information about how Dex affects the expression of integrin β1 in ovarian cancer cells. Our further experiment demonstrated that expressions of integrin β1 as well as two kinds of α subunit, α4 and αV were up-regulated by Dex at protein level in human ovarian cancer HO-8910 cells. And the regulation of Dex on integrin β1 seems to occur at post-transcriptional level, since this regulation was not seen at mRNA level (data not shown). Moreover, we demonstrated that blocking cell adhesion to ECM with neutralizing antibody of integrin β1 significantly attenuated the protection of Dex from chemotherapy in HO-8910 cells, indicating that cell adhesion to ECM mediated by integrin β1 adhesion molecule is responsible for the effect of Dex against chemotherapeutic agents. It should be pointed out, however, that the neutralizing antibody of integrin β1 could not completely block the Dex-induced cell adhesion to ECM, suggesting other adhesion molecules besides integrin β1 and/or other mechanisms may also be involved in the action of Dex. Our recent results found that Dex could enhance cell adhesion by increasing the expression of some ECM components, such as collagen I and hyaluronic acid in HO-8910 cells (unpublished...
data). This finding is in line with the latest report that the enhanced ECM deposition may play a direct role in primary chemoresistance in ovarian carcinoma (Etemadmoghadam et al. 2009).

Several studies have reported that there is a synergistic effect between GC and TGF-β1 signaling pathways to regulate a variety of physiological and pathologic processes, such as modulating cell differentiation in osteoclast (Takuma et al. 2003) or cell growth in U937 cells (Kanatani et al. 1996). Since TGF-β1 is the most important growth factor that facilitates cell adhesion to ECM by increasing matrix production and accumulation, and ovarian epithelial cells can secrete TGF-β1 (Peng 2003), we therefore hypothesized that TGF-β1 signaling pathway may be involved in Dex’s role of pro-adhesion and pro-survival. The results in this experiment showed that TGF-β1 alone has the pro-adhesion and pro-survival effects although the effects are weaker than that of Dex, and combination of Dex and TGF-β1 showed the obvious synergistic effect on cell adhesion as well as cell resistance to cisplatin. Furthermore, the neutralizing antibody of TGF-β1 could not only significantly block the pro-adhesive and pro-survival effects of TGF-β1 itself, but also obviously inhibit the synergistic effect of Dex and TGF-β1. These results suggested that there is a positive crosstalk between GC and TGF-β1 signaling pathways. Our previous studies found that Dex induced the expression of TGF-β receptor type II (TβR-II) and enhanced growth-inhibitory effect of TGF-β1 on androgen independent human prostate cancer PC-3 cells (Li et al. 2006). In this experiment we also found that Dex up-regulated TβR-II expression, but did not affect the expression of TGF-β1 and TβR-I. We indeed demonstrated subsequently that Dex could enhance the responsiveness of HO-8910 cells to TGF-β1 by a TGF-β1 responsive reporter gene assay. Therefore, the induction of TβR-II expression by Dex may be an explanation for Dex-enhanced
TGF-β1-induced cell adhesion to ECM and cell resistance to cisplatin in HO-8910 cells. Since we only observed the effect of Dex on TGF-β1 signaling in this study, whether TGF-β1 signaling can enhance the effect of Dex in turn is unclear now and is worth for further study.

In summary, using human cancer cell line which is commonly used in the elucidation of the mechanisms responsible for the biological activity, we provide new evidence that Dex’s role of pro-adhesion to ECM is one of the basic mechanisms of Dex-induced apoptotic resistance against chemotherapy. The pro-adhesion action of Dex is achieved at least by enhancing protein levels of members of integrin β1 subfamily and TGF-β1 signaling pathway. It will be of considerable interest to ask whether this effect of GC is involved in its cell protection against apoptosis induced by other harmful stimuli, such as hypoxia and ischemia. Furthermore, our studies are performed on human ovarian cancer cell line, whether the conclusions could be applied to cancer cells from other epithelial origins is unknown. Additional experiments will be required to extend our conclusions.

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**Titles and legends to figures:**

**Figure 1.** Dex protects cell survival in response to cytotoxic drugs in human ovarian cancer cells HO-8910 and SKOV3. (A)–(D), The detection of cell survival by MTT assay. HO-8910 (A, C) and SKOV3 (B, D) cells were cultured in the medium containing ethanol or various dose of cisplatin (A, B) or paclitaxel (C, D) as indicated in the absence or presence of $10^{-7}$ M Dex for 4 days, and then the viability
was monitored. Values plotted are mean ± S.E. *p<0.05, **p<0.01 vs. corresponding dose of cytotoxic drugs. (E) and (F). Dex protects cell apoptosis against cisplatin in HO-8910 cells. HO-8910 cells were cultured in medium containing ethanol or 2 µg/ml of cisplatin in the absence or presence of $10^{-7}$ M Dex for 48 h. Apoptosis was analyzed by staining of cells with annexin-FITC and FACS-analysis (E). The expression of cleaved caspase-3 was determined by Western blotting (F). Relative densitometric units of cleaved caspase-3/β-actin are shown in the upper panel, with the density of the control bands set at 1.0. Experiments were performed three times with similar results. Values plotted are mean ± S.E. # p<0.05 vs. control, * p<0.05, **p<0.01 vs. cisplatin.

Figure 2. Dex enhances cell attachment to fibronectin and antagonizes the impairment of cisplatin on cell adhesion. Cells were pretreated with 0~1 µM Dex for 24 h (A) or $10^{-7}$ M Dex for 0~48 h (B), or treated with ethanol, 2 µg/ml cisplatin, or 2 µg/ml cisplatin combined with 1~$10^{-7}$ M Dex for 24 h (C). 8×10^4 cells were seeded into 96-well plate coated with 10 µg/ml fibronectin. 60 min later cells were washed three times with PBS, and the remaining attached cell number was determined by MTT assay. Data were summarized from three independent sets of experiments. * p<0.05 and ** p<0.01 vs control in A and B. * p<0.05, ** p<0.01 in C.

Figure 3. Dex induces the expressions of integrin β1, α4 and αV protein in HO-8910 cells. HO-8910 cells were maintained in RPMI-1640 medium containing 5% DCC-CBS for 24 h and then treated with $10^{-7}$ M Dex for different time intervals (A) or with different concentrations of Dex for 24 h (B). Cells were then harvested for
detection of integrin β1, α4 and αV by Western blotting. β-actin was also detected for loading control. Relative densitometric units of integrin/β-actin for time intervals and different concentrations are shown in the lower panel, with the density of the control bands set at 1.0. The blots represent one of three independent experiments. * p<0.05, ** p<0.01 vs. the control of corresponding integrin subunits.

**Figure 4.** Dex-enhanced cell attachment and cell survival are partially reversed by integrin β1 blocking antibody. (A) Cells were cultured in medium in the absence or presence of 10^{-7} M Dex for 24 h and were digested and counted. 2×10^4 cells were incubated with IgG, 10 µg/ml or 20 µg/ml integrin β1 blocking antibody for 45 min, and then seeded into 96-well plate coated with 10 µg/ml FN. 60 min later the cell adhesion ability was assayed. (B) and (C), 2×10^3 cells were cultured in medium containing chemotherapy drugs, chemotherapy drugs plus 10^{-7} M Dex, combined with either integrin β1 blocking antibody or IgG for 4 days, and cell survival was detected by MTT assay. Data were representative of three independent sets of experiments. * p<0.05, ** p<0.01.

**Figure 5.** Dex enhances cell adhesive and survival responses to TGF-β1 in **HO-8910 cells.** (A) Dex enhances cell adhesive ability induced by TGF-β1. HO-8910 cells were treated with the indicated agents for 24 h and cell adhesion ability was assayed. (B) Dex enhances TGF-β1-induced cell apoptosis resistance to cisplatin. Cells were incubated with the indicated agents for 4 days and the survival cell number was determined with MTT assay. (C) Dex increases the expression of TβR-II mRNA and protein in HO-8910 cells. Cells were treated with 10^{-7} M Dex for different times.
and TβR-II mRNA and protein levels were determined by semi-quantitative RT-PCR and Western blotting, respectively. Relative densitometric units of TβR-II/GAPDH and TβR-II/β-actin are shown in the upper panel, with the density of the control bands set at 1.0. The blots represent one of three independent experiments. (D) Dex enhances the luciferase activity induced by TGF-β1. Cells were transfected with the reporter plasmid p3TP-Luc, and then incubated with agents as indicated. 16 h later, cells were harvested and assayed for luciferase activity. All of the results shown above are representative of three independent experiments. # p<0.05, ## p<0.01 vs. control (or cisplatin), * p<0.05.
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Fig 1
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Fig 2
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Fig 3

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Fig 4
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Fig 5