EJGO European Journal of Gynaecological Oncology

Effect of exemestane on the invasive growth of endometrial carcinoma HHUA cells

S.G Wang, X.M. Meng, Y.Z. Dong

Department of Gynecology, Yuhuangding Hospital, Medical College of Qingdao University, Yantai (China)

Summary

Objective: To investigate the effect of exemestane on HHUA human endometrial carcinoma cells. *Materials and Methods:* The HHUA human endometrial carcinoma cells were treated with various concentrations of exemestane, and its effects on cell growth and apoptosis were investigated in vitro. The cell apoptosis was analyzed by flow cytometry and RT-PCR was used to investigate the expression of CD44s. The invasion ability of HHUA human endometrial carcinoma cells which treated with exemestane were assessed using transwell chamber model. *Results:* At increasing doses of exemestane, a simultaneous increase in apoptotic subpopulations was detected when compared with group A ($p < 0.05$); the CD44s expression was found to be suppressed after the exemestane treatment. The decrease was a dose-dependent with exemestane treatment. *Conclusion:* 6×10^{-8} mol/L exemestane is an optimal dose to inhibit the expression of CD44s mRNA and inhibit the invasive growth of the endometrial carcinoma HHUA cells.

Key words: Exemestane; Aromatase; Endometrial neoplasms; Therapy.

Introduction

Endometrial carcinoma is the most common malignant tumor of the female genital tract, and its incidence has increased in recent years [1, 2], Furthermore, the search for agents effective in the treatment of either advanced or recurrent endometrial cancer has proved to be disappointing [2, 3]. Therefore, innovative approaches are required for the treatment for endometrial cancer.

A positive association between the exposure to estrogens and the proliferation and invasion of endometrial carcinoma cells has been described in several reports [4-7]. Moreover, recent studies have indicated that aromatase inhibitors, such as exemestane, induced the apoptosis and inhibited the invasion of endometrial carcinoma cells by inactivating the aromatase and reducing the biosynthesis estrogens [8]. Exemestane, a non-steroidal aromase inhibitor that shuts down estrogen synthesis and paclitaxel, an antineoplastic drug, inhibiting microtubule formation, and interfering with the cells potential to proliferate, are well established treatments for metastatic breast cancer [9, 10]. Exemestane is a treatment for hormone-sensitive tumors in post-menopausal women with more favorable prognosis. In the current study, the authors investigated the anti-tumor activity of exemestane in endometrial carcinoma cell line HHUA cell on cell proliferation and apoptosis. In addition, they examined the effect of exemestane on endometrial carcinoma cell line HHUA cell. The present data will provide the theoretical basis for the clinical application of exemestane in the treatment of endometrial carcinoma.

Materials and Methods

Cell culture and groups

Endometrial carcinoma cell line HHUA cell were offered by Dr. Qiushi Zhang's Lab at the Women and Children Medical Center Hospital, Guangzhou. The HHUA cells were maintained in Dulbecco's Modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/L streptomycin. The cells were incubated in an atmosphere of 5% CO_2 at 37°C.

For detecting the exemestane significantly inhibited HHUA cells proliferation in a dose-dependent manner, exemestane were divided in four groups: group A: control group (no exemestane treated); group B: exemestane at a concentration 6×10^{-9} mol/L; group C: exemestane at a concentration 6×10^{-8} mol/L - the concentration was the highest blood drug concentration in the body; group D: exemestane at a concentration 6×10^{-7} mol/L.

Apoptosis assay

HHUA cells were seeded at a density of $5x10^5$ cells in 50-ml culture bottle. The medium was replaced in second day with five-ml new culture medium and then treated with exemestane for 72 hours. After 72 hours, 1×10^7 cells were collected by centrifugation and then resuspended cells in PBS twice, added five ul of Annexin V-FITC into the cells, and were incubated at room temperature for 15 minutes in the dark, and then added five ul propidium iodide (PI), and incubated at room temperature for five minutes in the dark. The cells were analysised by FACS. Cells that stained positive for PE and negative for PI were undergoing apoptosis. Cells that stained positive for both PE and PI were either in the end of apoptosis, were undergoing necrosis, or were already dead.

RNA extraction and reverse transcription-PCR (RT-PCR)

Total RNA was extracted from the cell lines and tissue samples using TRIzol reagent. RNA was synthesized into cDNA using M-

Revised manuscript accepted for publication June 29, 2015

Table 1. — *Cell apoptosis changes in human endometrial cancer HHUA cells.*

Groups	Apoptosis (%)	q value	*p* value
Group A (control)	4.22 ± 0.58		
Group B (6×10^{-9} mol/L)	3.98 ± 0.63	3.51	> 3.51
Group C (6×10^{-8} mol/L)	23.12 ± 4.66	9.13	< 0.05
Group D (6×10^{-7} mol/L)	29.85 ± 4.35	16.25	*< 0.05*

Table 2. — *HHUA cell proliferation inhibitory effect of different concentrations of exemestane.*

Groups	The number of invasive cell (%)	q value	*p* value
Group A (control)	59.65 ± 2.87		
Group B (6×10^{-9} mol/L)	52.0 ± 6.64	3.54	> 0.05
Group C (6×10^{-8} mol/L)	29.87 ± 5.12	8.54	< 0.05
Group D (6×10^{-7} mol/L)	23.14 ± 3.45	6.98	*< 0.05*

MLV reverse transcriptase. Real-time PCR was conducted on an instrument, using the following protocol: 94°C for five minutes and then 30 cycles of 94°C for one minute, 58°C for one minute, and 72°C for one minute. The PCR product was separated in 2% agarose gel and analysised using gel image analysis system. The expression of was normalized by the absorbance ratio of CD44s and β-actin. Primers used for quantification were as follows: CD44s forward primer, 5'- GTAATGGGTTCTGCATA TTTA-3', CD44s reverse primer, 5'- CTGTGATGATGGTTAAATACACCCTGTGCTG -3', b-actin forward primer, 5'- GTGGGGCGCCCCAGGCACCA-3' b-actin reverse primer, 5'-CTCCTTAATGTCACCGCACGATTTC -3'.

Cell invasion assay

The cells which were treated with different concentrations of exemestane were harvested and resuspended in culture medium, Then the cells (2×10^5/well) were suspended in 100 µl of serum-free medium and placed into the upper chamber of the insert with an eight-µm micro-porous membrane coated with Matrigel. Medium containing 20% fetal bovine serum was added into the lower chamber. Following 24 hours of incubation at 37°C, the cells remaining on the upper surface of the membrane were removed with a cotton swab, and the migrated cells on the lower surface were fixed with 95% methanol for ten minutes and stained with 0.1% crystal violet. The stained cells were counted in five randomly selected fields per membrane under an inverted phase-contrast microscope (×100 magnification). Each experiment was performed in triplicate wells and repeated three times.

Statistical analysis

All data were assayed in three independent experiments and were performed with Statistical Package for the Social Sciences (SPSS) 13.0 software. The results were displayed as the mean ± standard deviation (SD). One-way ANOVA test and *SNK-q* test were used to determine the difference between control and treatment groups. A *p*-value less than 0.05 was considered statistically significant.

Results

Apoptotic changes in human endometrial cancer HHUA cells treated with exemestane

To assess the ability of the HHUA cells to undergo apoptosis in response to exemestane and to distinguish between the different types of cell death, the authors double-stained the exemestane-treated cells with Annexin V and PI and analyzed the results using flow cytometry. Annexin V binding combined with PI labeling was preformed for the distinction of early apoptotic (AnnexinV+/PI-) and necrotic (AnnexinV+/PI+) cells. At increasing doses of exemestane, a simultaneous increase in apoptotic subpopulations was detected when compared with group A (Table 1).

Growth inhibition of human HHUA *cells by exemestane*

The effects of exemestane on the proliferation of human HHUA cells were determined using a transwell chamber model. HHUA cells were treated with different doses of exemesane for 72 hours. A dose-dependent decrease in the cell invasive was observed (Table 2). Hence the 6×10^{-8} mol/L of exemestane was an optimal dose.

Effect of exemestane on CD44s expression in human endometrial cancer HHUA cells

CD44s, has been causally linked to cancer cell proliferation, motility, and invasiveness. Therefore, in this study, the authors analyzed the mRNA expression level of CD44s in HHUA cells using RT-PCR (Figure 1). The CD44s expression was found to be suppressed after the exemestane treatment. The decrease is a dose-dependent with exemestane treatment.

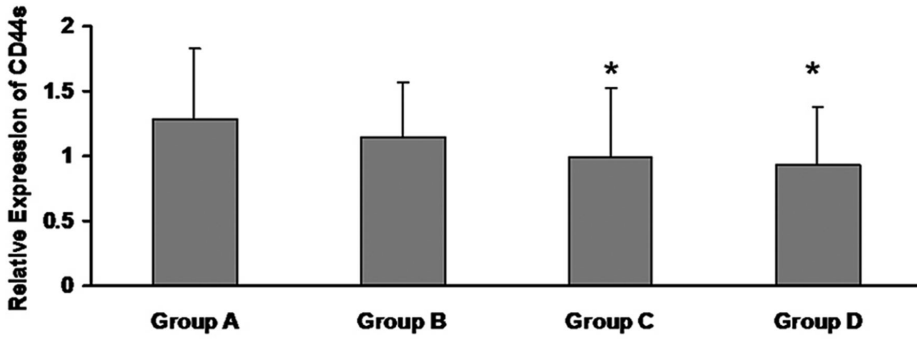

Figure 1. — Effects of exemestane on expression of CD44s in HHUA cells. HHUA cells were treated with different doses of exemestane. After, treatment cells were harvested and lysed, and the mRNA were analyzed for CD44s expression using RT-PCR. *$p < 0.05$ as compared with control treatment.

Discussion

Endometrial carcinoma is one of the most cancer worldwide. At present in many countries, the incidence of endometrial carcinoma has exceeded cervical cancer and is the first of the female genital tract malignant tumor. However, the etiology of endometrial carcinoma is not completely clear. Many studies have demonstrated that a majority of endometrial carcinoma cases are estrogen-dependent while estrogens can stimulate the growth of the endometrium. All these estrogen associated factors are relevant to endometrial carcinogenesis. Many clinical and experimental researches are actively exploring the hormone treatment of endometrial carcinoma; aromatase regulation is gaining more attention. Moreover, recent studies have indicated that aromatase inhibitors, such as exemestane, induced the apoptosis and inhibited the invasive of endometrial carcinoma cells by inactivating the aromatase and reducing the biosynthesis estrogens.

Aromatase, a member of the cytochrome-P450 superfamily, plays an important role in the conversion of androstenedione and testosterone to estrone and estradiol. Aromatase is a key enzyme that catalyzes the biosynthesis of estrogens from androgens. In humans, aromatase is encoded by the gene CYP19. Interfering with the activity of this gene or inhibiting its products can block aromatase biosynthesis. A polypeptide chain of 503 amino acid residues and a heme group constitute the basic structure of aromatase. Exemestane is irreversible non-steroidal aromatase inhibitors, chemical name is 6 - methylene male steroid - 1, 4-2-3 in 2 - diketone, relative molecular mass is 296 KD, and the largest blood drug concentration is 6 x 10^8 mol/L. Exemestane are analogs of the natural aromatase substrate androstenedione. It binds covalently to the substrate-binding site of aromatase and hereby irreversibly inactivates the enzyme [11]. The exemestane can induce cell apoptosis. Clinical data have indicated that the activity of aromatase could reduce 97.7% after four to six weeks with oral exemestane 25 mg per day [12].

The growth and metastasis of the malignant tumor is a complex process in which multiple genes are involved. Several evidences indicate that deregulation of CD44s contributes to human carcinogenesis [13]. CD44s is located on chromosome 11 and contains 20 highly conservative exons. and the introns split the gene into different areas. The protein which composes 341 amino acid residues standard peptides encoded by this gene is a cell-surface glycoprotein involved in cell-cell interactions, cell adhesion, and migration. The molecular weight is 85-130KD. There are two types of molecules: standard CD44 (CD44s) and variant CD44 (CD44v) [14]. The protein CD44s is a receptor for hyaluronic acid (HA) and can also interact with other ligands, such as osteopontin, collagens, and matrix metalloproteinases (MMPs). This protein participates in a wide variety of cellular functions including lymphocyte activation, recirculation and homing, hematopoiesis, and tumor metastasis: (1) involved in HA, chondroitin sulfate, and collagen fiber adhesion protein, extracellular matrix adhesion effect. The combination of CD44s and hyaluronic acid can promote cell anchor to the extracellular matrix; (2) participate the activation of T lymphocytes, strengthen the immune surveillance function, regulation cell proliferation, and aggressive growth; (3) participating the cell signaling pathways, CD44s protein which has a molecular structure similar to the GTP protein, can be combined with integrin to activate intracellular signal transduction system by phosphorylating tyrosine. Therefore, the abnormal expression of CD44s has been suggested to play a critical role in regulating both cell proliferation and invasion growth [15, 16].

The present results showed that the apoptosis rate was increased in the endometrial carcinoma HHUA cells after treatment with a 6×10^{-8} mol/L and 6×10^{-7} mol/L of exemestane, at which the mRNA level of CD44s is reduced. The results demonstrated that the exemestane inhibited the cell proliferation and invasion in the endometrial carcinoma HHUA cells. The present results also demonstrated that the exemestane suppressed HHUA cells invasion growth in a dose-dependent manner. At a dose of 6×10^{-9} mol/L treatment with exemestane, the expression of CD44s and the cell growth were not decreased in HHUA cells in comparison with control group. When HHUA cells were treated with 6×10^{-8} mol/L of exemestane, which is the highest blood drug concentration, the expression of CD44s was significantly decreased and the cell proliferation was completely arrested. However, no obvious changes in the expression level of CD44s and the invasion growth of HHUA cells were observed after treatment with 6×10^{-8} mol/L of exemestane. The results suggested that exemestane has a saturation dose. At the dose of 6×10^{-8} mol/L, treatment with exemestane significantly inhibited the expression level of CD44s. The dose of 6×10^{-8} mol/L was the best inhibition concentration to inhibit the invasion growth of HHUA cells. Further increases in drug concentration did not strengthen its effects on cancer cell invasion. The present data will provide the theoretical basis for the clinical application of exemestane on treatment of endometrial carcinoma.

References

[1] Reinhardt M.J.: "Gynecologic tumors. Recent results". *Cancer Res.*, 2008, *170*, 141.
[2] Obel J.C., Friberg G., Fleming G.F.: "Chemotherapy in endometrial cancer". *Clin. Adv. Hematol. Oncol.*, 2006, *4*, 459.
[3] Hill E.K., Dizon D.S.: "Medical therapy of endometrial cancer: current status and promising novel treatments". *Drugs*, 2012, *72*, 705.
[4] Mendivil A., Schuler K.M., Gehrig P.A.: "Non-endometrioid adenocarcinoma of the uterine corpus: a review of selected histological subtypes". *Cancer Control*, 2009, *16*, 46.
[5] Musa F., Huang M., Adams B., Pirog E., Holcomb K.: "Mucinous histology is a risk factor for nodal metastases in endometrial cancer". *Gynecol. Oncol.*, 2012, *125*, 541.
[6] Schwartz A.M., Silverberg S.G., Fu Y.S., Nozawa S., Huang S.,

Tsukasaki K., Williams K.R.: "Use of monoclonal antibodies MSN-1 and B72.3 in the prediction of the natural history of endometrial hyperplasia". *Int. J. Gynecol. Pathol.*, 1993, *12*, 253.

[7] Zhong B., Cai X., Yi X., Zhou A., Chen S., Su B.: "In vitro and in vivo effects of a cyclooxygenase-2 inhibitor nimesulide analog JCC76 in aromatase inhibitors-insensitive breast cancer cells". *J. Steroid Biochem. Mol. Biol.*, 2011, *126*, 10.

[8] Zhong B., Cai X., Yi X., Zhou A., Chen S., Su B.: "In vitro and in vivo effects of a cyclooxygenase-2 inhibitor nimesulide analog JCC76 in aromatase inhibitors-insensitive breast cancer cells". *J. Steroid Biochem. Mol. Biol.*, 2011, *126*, 10.

[9] Smith I.E., Dowsett M.: "Aromatase inhibitors in breast cancer". *N. Engl. J. Med.*, 2003, *348*, 2431.

[10] Smith I.E., Dowsett M., Yap Y.S., Walsh G., Lønning P.E., Santen R.J., Hayes D..: "Adjuvant aromatase inhibitors for early breast cancer after chemotherapy-induced amenorrhoea: caution and suggested guidelines". *J. Clin. Oncol.*, 2006, *24*, 2444.

[11] Gurates B., Sebastian S., Yang S., Zhou J., Tamura M., Fang Z., *et al.*: "WT1 and DAX-1 inhibit aromatase P450 expression in human endometrial and endometriotic stromal cells". *J. Clin. Endocrinol. Metab.*, 2002, *87*, 4369.

[12] Buzdar A., Howell A.: "Advances in aromatase inhibition: clinical efficacy and tolerability in the treatment of breast cancer". *Clin. Cancer Res.*, 2001, *7*, 2620.

[13] Li F., Devi Y.S., Bao L., Mao J., Gibori G.: "Involvement of cyclin D3, CDKN1A (p21), and BIRC5 (CD44s) in interleukin 11 stimulation of decidualization in mice". *Biol. Reprod.*, 2008, *78*, 127.

[14] Auzenne E., Ghosh S.C., Khodadadian M., Rivera B., Farquhar D., Price R.E., *et al.*: "Hyaluronic acid-paclitaxel: antitumor efficacy against CD44 (+) human ovarian carcinoma xenograft-s". *Neoplasia*, 2007, *9*, 479.

[15] Afify A., Pang L., Howell L.: "Diagnostic utility of CD44 standard, CD44v6, and CD44v3-10 expression in adenocarcinomas presenting in serous fluids". *Appl. Immunohistochem. Mol. Morphol.*, 2007, *15*, 446.

[16] Ponta H., Sheman L., Herrlich P.A.: "CD44: from adhesion molecules to signaling regulators". *Nat. Rev. Mol. Cell. Biol.*, 2003, *4*, 33.

Address reprint requests to:
S.G. WANG, M.D.
No.20 Yuhuangding East Street,
Yantai, Shandong 264000 (China)
e-mail: shaoguangwang2015@163.com