Effects of Estrogen and Progestogen on the Growth and Apoptosis of Human Cervical Cancer Cells

Huilan Wang[1], Fang Yuan, Xianghua Huang, Heling Zhang, Sujuan Yang, and Bin Shi

Department of Obstetrics and Gynecology, the Second Hospital of Hebei Medical University, Shijiazhuang, Hebei, 050000, Peoples Republic of China.

Abstract **Objectives** To investigate the effects of estrogen and progestogen on the proliferation and apoptosis of human cervical cancer cells. **Methods** The expression of estrogen receptor (ER) and progestogen receptor (PR) of in−vitro cultured human cervical cancer cell line Hela was examined by immunocellularchemistry; and then the effects of esteradiol (E_2), progesterone (P), and the combination of esteradiol and progesterone (E_2+P) on the proliferation of Hela cells were determined by methyl thiazolyl tetrazolium (MTT) method; furthermore, the effects on the cell cycle, apoptotic percentage, and the intracellular Bcl−2 expression were detected by flow cytometry, moreover, the cellular morphology changes both by light microscopy and electronic microscopy were verified. **Results** Hela cell expresses both ER and PR, and E_2 dose−dependently induces the Hela cell proliferation; progesterone, however, inhibits the cell proliferation; moreover, E_2+P combination has stronger inhibition effects. In contrast to estradiol treatment, progesterone and E_2+P treatments showed increased proportion of G0/G1 phase and apoptotic rate, decreased S phase the proportion and intracellular bcl−2 expression. Apoptosis morphology changes were observed in progesterone groups. **Conclusions** Estradiol could enhance the proliferation and inhibit the apoptosis occurrence in cervical cancer Hela cell line, however, progesterone could inhibit its proliferation and induce apoptosis. The combination of estradiol and progesterone has remarkably stronger inhibition effect than that of progesterone alone, indicating that enough progesterone can not only block the proliferation enhancing effect of estradiol, but also more potently inhibit the cell proliferation.

Key Words Cervical neoplasms; Estradiol; Progesterone; Proliferation; Apoptosis.

Cervical cancer is a leading gynecological malignancy worldwide, the occurrence age tends to be younger in recent years. The classical treatment strategy includes operation, radiotherapy and post−operational hormone therapy. Rational post −operational hormone replacement therapy (HRT) can not only benefit the treatment effectiveness but also greatly improve the life quality, especially for the younger patients[1,2]; however, the major concerns about the HRT have been focusing on the effectiveness on the cervical cancer and its potential carcinogenesis. In order to address this concern, we investigated that the estradiol, progesterone, and estradiol and progesterone combination on the cell growth and apoptosis in human cervical cancer Hela cell line, we also studied the expressions of estrogen and progestogen receptor, and the expression of bcl−2, an important anti−apoptosis gene.

1To whom correspondence and reprint requests should be addressed at: Department of Obstetrics and Gynecology, the Second Hospital of Hebei Medical University, No. 215 Heping West Road. Shijiazhuang, Hebei, 050000, P.R China.
Tel: 86−311−7222921/7222925.
Email:wanghuilan20002000@yahoo.com.cn

MATERIALS AND METHODS

Materials

Human cervical cancer Hela cell line was generously provided by the department of cyto−biology of Hebei Medical University, ATCC number CCL−2. Estradiol was purchased from Xian−Ju Pharmacetical (Zhejiang, China). Progesterone, mouse anti− human estrogen receptor, progestogen receptor, and bcl−2 monoclone antibodies were purchased from Zhongshan biological company (Beijing, China).

Cell culture

Hela cell was cultured in RPMI 1640 media, which

Table 1. Estradiol promotes the Hela cell proliferation

Group		Absorbency (A, $\overline{X}\pm s$)	Proliferation enhancement(%)
Control		0.923±0.080	0
Estradiol (nM)	0.50	0.996±0.110	7.84%
	1.00	1.094±0.080★	18.40%
	5.00	1.219±0.084★○	31.93%
	10.00	1.333±0.104★▲■	44.24%

Compare with control, ★P<0.05
Compare with 0.50 nM estradiol, ○ P<0.05; ▲ P<0.01
Compare with 5.00 nM estradiol, ■ P<0.05

Table 2. Progesterone inhibits the Hela cell proliferation

Group		Absorbency (A, $\overline{X}\pm s$)	Proliferation enhancement(%)
Control		0.984±0.027	0
Progesterone (μM)	0.01	0.948±0.034▲	3.68%
	0.10	0.699±0.047★▲	28.96%
	1.00	0.526±0.042★▲	46.50%
	10.00	0.414 ±0.036★▲	57.09%

Compare with control, ★ P<0.01; ▲ P<0.05

is supplemented with 10% fetal calf serum, 100 units/ml penicillin and 100 g/ml streptomycin, at 37℃, 5% CO_2 cell culture incubator. When the cell grows to around 70% confluent, subcultured by 0.02% EDTA digestion.

ER and PR expression

Hela single cell suspension is dropped onto polylysine pre-treated slides, air-dry, and fixed with 4℃ cold acetone. The ER and PR expression on Hela cell is detected by immunocellularchemistry (SP method) according to the immunocytochemical assay for the detection of ER and PR kit procedures by Zymed Laboratories (South San Francisco, CA). The result evaluation: two slides were prepared for each condition, five random high-magnification fields were chosen in every slides, depending on the color intensity (i) of nuclear brown particles to classify the scales into 0 to 3: 0 without staining; 1 pale brown staining; 2 reddish brown staining; 3 dark brown staining. Pi indicates the percentage of certain intensity staining cells out of the whole cell population (from 0%-100%). H-score=$\sum$Pi(i+1), the full score is 4 [3].

Cell proliferation experiment

In a 96-well flat bottom plate, each well inoculate 6×10^3 Hela cells in 200μl culture media, and cultured overnight for cell attaching. The next day, transfer the cell culture to 4℃ for 1h so as to come to synchronization. And then the cell culture was treated with conditioned media containing 0.50, 1.00, 5.00 or 10.00 mM estradiol (E_2 group), 0.01, 0.10, 1.00 or 10.00 μM progesterone (P group), or identical concentrations of estradiol and progesterone combination as E_2 or P alone (E_2+P group). The same concentration of E_2/P solvent (ethanol) was added as the control group. 72 h later, 20μl of 5 mg/ml MTT working solution was added to each well and cultured for 4 h. After that, discard the supernatants, and add 200 μl Dimethyl Sulphoxide (DMSO), vortexed, and then read the absorbance at the wavelength of 492 nm. Each concentration set eight parallel wells, and three independent experiments were carried out to decrease the experiment variation.

Cell cycle and apoptotic rate measurement

3×10^6 Hela cells were cultured in condition media containing 10 nM E_2, 10 μM P, or 10 nM E_2 + 10 μM P, and cultured for 72 h, after that single cell suspension was prepared, fixed with 70% 4℃ cold ethanol, and then stained with PI for flow cytometry for the cell cycle analysis, meanwhile, the subdiploid peak indicates the apoptotic cells.

Cell morphology observation

(1) Light microscopy: Hela single cell suspension was inoculated into 6-well plate pre-setting a cover slide

Table 3. E2+P combination inhibits the Hela cell proliferation

Group		Absorbency (A, $\overline{X}$±s)	Proliferation enhancement(%)
Control		0.923±0.08	0
E2+p (nM)	0.50+0.01	0.848±0.052★▲	8.18%
	1.00+0.10	0.571±0.083★▲	38.16%
	5.00+1.00	0.463±0.105★▲	49.94%
	10.00+10.00	0.304±0.055★▲	67.03%

Compare with control, ★ P<0.01; ▲ P<0.05

for each well, and treated with the same concentration of E_2, P, or E_2+P for 72 h, and then staining with Giemsa staining solution for light microscope observation. (2) Hela cells with the same treatment, and fixed with 5% glutaraldehyde, embedded and section, staining, and observation under JEM−1230 transperent electromicroscope.

Bcl-2 expression

Hela cells were treated with E_2, P, or E_2+P for 72 hr, after that single cells were prepared, fixed with 70% 4℃ cold ethanol, adjusted the cell concentration to 1×10^6/ml, add appropriate diluted mouse anti −human bcl−2 monoclone antibody, incubate for 1h at 4℃, after washing, FITC−labeled sheep anti−mouse IgG was added, incubated for 30 min at 4℃, and then washing 3 times, and resuspended the stained cell in PBS for flow cytometry assay immediately.

Statistical analysis

Data are showed in mean standard deviation ($\overline{x}$± SD), students t−test was employed for the comparison for ER and PR. Square analysis and Spearman correlation analysis was used for Multi −group comparison. Counting data were treated with X^2 test. SAS software was used for the statistic analysis, P<0.05 was considered as statistically significant.

RESULTS

Expression of ER and PR in Hela cells

Hela cells express both ER and PR, the relative quantification of ER is 0.99 ±0.30, PR, however, is 2.57 ±0.09. Compared with ER, PR is significantly higher (P<0.01).

Effect of E_2, P, and E_2+P on proliferation of Hela cells

MTT method is conventional for detecting cell proliferation. We employed it to approach and investigate the effect of E_2, and P on the Hela cell growth in−vitro. The results showed that with increasing of the concentration, E_2 enhances the Hela cell proliferation, moreover, the cell proliferation (MTT absorbance val-

Table 4. E2, P and E2+P on the regulation of cell cycle distribution and apoptosis

Group	Cell cycle distribution(%)		G2/M	Apoptotic rate (%)
	G0/G1	S		
Control	64.52±0.86	22.97±1.19	12.51±0.81	8.30±0.92
Estradiol	62.76±1.01	29.86±1.23★	7.38±0.47★	4.12±0.70★
Progesterone	76.00±0.65★	12.36±1.41★	11.64±0.56	20.75±0.56★
E2+P	78.20±0.52★▲	10.27±1.83★▲	11.53±0.96	24.65±0.77★▲

Compare with control, ★P<0.01

Compare with E2 alone, P<0.01; ▲ P<0.05

Compare with P alone, ▲ P<0.05

ue) is positive correlated with the E_2 concentration (Table 1), which indicates that E_2 dose−dependently promotes the proliferation of Hela cells. However, progesterone and E_2+P treatment strongly inhibit Hela cells proliferation, furthermore, the P concentration is correlated with the inhibitory rate significantly, which demonstrated that P, on the contrary, dose−dependently inhibits the Hela cell proliferation. When the concentration of P is at 10 μM, the E_2+P combination has significantly higher inhibition effect than that of P alone, although the 10 nM E_2 alone has proliferation−enhancing effects (Table 2 and 3), the result suggested that high dose of P can completely block the proliferation effect of E2, and actually E2 benefits P to play its role.

E_2, P and E_2+P on the effect of cell cycle distribution and apoptosis

In contrast with the control group, E_2 treatment increases the S−phase cell percentage, and decreases the apoptotic rate ($P<0.01$); P, or E_2+P treatment, however, increases the G_0/G_1−phase percentage, decreases the S−phase percentage, and increase the apoptotic rate ($P<0.01$); An obvious subdiploid apoptotic peak was monitored. Furthermore, compare with the P treatment alone, E_2+P increases the G_0/G_1−phase percentage, decreases the S−phase percentage, and increases the apoptotic rate ($P<0.01$), indicating that E_2+P inhibits the cell proliferation and induces cell apoptosis. (Table 4)

Cellular Morphology Changes

Apoptosis was originally defined by a sequence of morphologic features. Despite recent progress in apoptosis research at the biochemical and molecular levels, morphological changes are still considered the "gold standard" for apoptosis. Under light microscopy, Hela cells treated with E_2 are in good morphology, pink cytoplasm, purple nuclear with 2−3 nuclei, the cell membrane and nuclear membrane are intact, mitosis can be easily found, with rarely seeing cells with nucleus condensation around the nuclear membrane. However, Hela cells treated with P, E_2+P showed cell crimple, nucleus condensation, dark staining, nuclear chromatin segregated against the nuclear membrane or in the shape of crescent. Under electronic microscopy, Hell cells treated with E_2 rarely seen apoptotic cells, the cellular organelles are intact; in contrast, those treated with P or E_2+P showed cell volume shrinkage, cytoplasm condensation and dark−staining, rough endoplasmic reticulum expanded in various degrees; fragmentation of nucleus, aggregation of dense masses of chromatin beneath the nuclear membrane, and protrusion for the cell surface or formation of apoptotic bodies.

Bcl–2 Expression in Hela Cell

Compare with the control, E_2 treatment upregulates the bcl−2 expression, and P or E_2+P, however, downregulates the bcl−2 expression. When the concentration of P is at 10 μM, E_2+P has stronger inhibition effect on bcl−2 expression than that of P alone ($P<0.05$).

DISCUSSION

Effects and mechanisms of estrogen and progestogen on the growth and apoptosis of cervical carcinoma Hela cell

Both the uterus and cervical are the target organs of estrogen and progestogen, and in−vitro [4]and in−vivo[5] experiments have indicated that cervical cells response to both estrogen and progestogen. White et al.[6] found that E_2 can stimulate the growth of cervical cancer cell line HOG−1. Guzeloglu et al.[7]also demonstrated that E_2 could promote the growth of endometrial and breast cancer cell lines. Franke et al.[8] reported that P can inhibit the growth of E_2−induced progesterone receptor breast cancer cell line. Therefore, our study first investigated the ER and PR expression in Hela cells, and demonstrated that Hela cell has both ER and PR expression, and PR level is higher than that of ER. Then we studied the effects of estradiol and progesterone on the cell growth in in−vitro cultured Hela cells. Our results showed that E_2 and P have opposite effect on Hela cell growth, E_2 enhances the growth, moreover, with the increasing of E_2, the growth−promotion effect presents dose−dependently. P and E_2+P, however, impede the growth, the inhibition rate is positively correlated with the concentration of P. Interestingly, E_2+P has the stronger inhibition effect than that of P alone.

It is reported that steroid hormone can regulate cell

apoptosis. Mabuchi et al.[9] reported that E_2 could inhibit the apoptosis of ovarian cancer cells. Syed et al. [10] also demonstrated that progesterone at the concentration of 10^{-6}M can induce the apoptosis of immortalized human normal or malignant ovarian epithelial carcinoma cells, and the effect depends on the exposure time, further study indicated that it related with the level of FasL expression. Our study demonstrated that E_2 could induce the Hela cell proliferation and prevent the occurrence of apoptosis; P, or E_2+P, on the contrary, decrease the cell proliferation and induce the apoptosis. Both light microscopy and electronic microscopy observed typical apoptosis morphology changes: nucleus condensation, dark staining, chromatin condensasion and segregated against the nuclear border, and apoptosis bodies. Although the morphological features are considered to be the most reliable criteria in defining apoptosis, it does not provide accurate quantitative data and thus cannot be used to compare apoptosis between experiment conditions. However, flow cytometry analysis cell cycle distribution and sub$-G_1$ peak can provide quantitative data for apoptosis occurrence. Our data indicated that E_2 treatment decrease the apoptosis, and shift the cell cycle− increase the S−phase, decrease the G_2/M−phase, these results are in agreement with the reported result that E_2 induced the aggregation of S/G_2/M−phase cells in monocytes [11]. P and E_2+P, however, increased the apoptosis, meanwhile, P treatment showed G_0/G_1−phase cell increase, and S−phase decrease, suggesting that P blocked the progression of cell cycle from G_1 to S, and induced apoptosis, and therefore control the proliferation of tumor cells. Taken together, E_2 has proliferation promotion and anti−apoptosis effects on Hela cell, progesterone, however, has the anti−proliferation and apoptosis−induction effects. E_2 and P combination enhanced the effects of P for its anti−proliferation and apoptosis−induction.

Multiple genes control the cell proliferation and apoptosis. One of which, bcl−2, an apoptosis inhibition gene, engage in anti−apoptosis activities by increasing the resistance to variety of apoptosis inducing factors, however, it does not affect the cell proliferation. Bcl−2 level is higher in malignant tumors than that in benign tumors; moreover, bcl−2 level is correlated with the clinical staging, which indicats that bcl−2 is linked with the tumor progression[12]. It is reported that the mechanism by which E_2 and P play their roles is through their respective receptors and modulate the anti−apoptosis gene expression[13]. Our results indicated that E_2 induced bcl−2 expression, however, P and E_2+P down−regulated the expression, suggesting that E_2 and P on the regulation of Hela cells might be through their function on the regulation of bcl−2 protein expression.

The Comparison between Progesterone and E2+P Combination on the Cellular Proliferation and Apoptosis of Hela Cell

As mentioned above, Hela cells have both ER and PR expression, and the proliferation inhibition and apoptosis−promotion effects of E_2+P are stronger than that of P treatment alone, which indicats that the effect of E_2+P combination treatments on cervical carcinoma cell proliferation is dominated by the inhibition effect of P. There are a few interpretations: first, the PR level is higher than ER, this might be the reason that E_2+P combination is dominated by the inhibition effect of P; second, E_2 may be through up−regulating the PR expression [14], therefore increase the potency of P for its anti−proliferation and apoptosis−induction effect. Since one major concern for the post−operation HRT on its proliferation−enhancing effect in cervical cancer cells, here, our results demonstrated that E_2+P combination can completely eliminate its proliferation−promoting effect of E_2, actually E_2+P combination facilitated the anti−proliferation and apoptosis−induction effects of P, suggesting post−operational or −radiotherapy HRT is not only effective but safe. Clinical observation study indicated that post−operational HRT did not increase the mortality [15]; moreover, questionnaire investigation showed HRT improved the life quality[16]. Since survival rate and life quality are the two major parameters for evaluating a certain tumor therapy approach, our study will benefit the choice of post−operational HRT.

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