Effect of combined treatment with progesterone and tamoxifen on the growth and apoptosis of human ovarian cancer cells

JI-YOUNG LEE 3, JONG-YEON SHIN 4, HYUN-SEOK KIM 5, JEE-IN HEO 1, 2, YOON-JUNG KHO 2, HONG-JUN KANG 6, SEONG-HOON PARK 1, 5 and JAE-YONG LEE 1, 2

1 Department of Biochemistry and 2 Institute of Natural Medicine, College of Medicine, Hallym University, Chuncheon, Gangwon-do 200-702, Republic of Korea; 3 Department of Obstetrics and Gynecology, Sisters of Charity Hospital, State University of New York at Buffalo, Buffalo, NY 14214, USA; 4 Genomic Medicine Institute, Medical Research Center, Seoul National University, Seoul, Republic of Korea; 5 Molecular Radiation Oncology, Radiation Oncology Branch, Center for Cancer Research, NCI, NIH, Bethesda, MD 20892; 6 Genetic Disease Research Section, NIDDK, National Institutes of Health, Building 10, Room 9D11, Bethesda, MD 20892, USA

Received June 9, 2011; Accepted July 28, 2011
DOI: 10.3892/or.2011.1460

Abstract. Progesterone has a potential protective effect against ovarian carcinoma induced by estrogen. Progesterone is also known to cause apoptosis while tamoxifen induces growth arrest. Therefore, we attempted to determine whether combined treatment with progesterone and tamoxifen has a synergistic effect on anti-cancer activity. Although progesterone is known to cause apoptosis while tamoxifen induces growth arrest in many cancer cells, the detailed action of progesterone and tamoxifen and the anticancer effect of combined treatment have not been tested in ovarian cancer cells. Therefore, we tested the growth and apoptosis activity of progesterone and tamoxifen and the anticancer effect of combined treatment of progesterone and tamoxifen in ovarian cancer cells. Ovarian cancer cells, PA-1, were treated with progesterone, tamoxifen, or a combination of progesterone and tamoxifen. The anti-cancer effects were investigated by use of flow cytometry, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, DNA fragmentation analysis, and Western blot analysis. We found that 100 µM progesterone induced typical apoptosis in PA-1 cells. Treatment of PA-1 cells with 10 µM tamoxifen resulted in an increase in the levels of p21, p27, p16 and phospho-pRb, indicating typical G1 arrest. Co-treatment of PA-1 cells with 100 µM progesterone and 10 µM tamoxifen resulted in typical apoptosis, similar to that induced by treatment with 100 µM progesterone alone. These results indicate that progesterone caused apoptosis and tamoxifen induced G1 arrest. Combined treatment with tamoxifen and progesterone caused apoptosis similar to that induced by treatment with progesterone alone and had no additional anti-cancer effect in ovarian cancer cells.

Introduction

Ovarian carcinoma is one of the most common fatal gynecologic malignancies and is characterized by an insidious onset and a lack of early specific symptoms. About two-thirds of patients with ovarian carcinoma usually present with advanced ovarian carcinoma and have widespread tumor dissemination. Unfortunately, the most effective strategy for the management of ovarian carcinoma is yet to be determined. Although Taxol and platinum-based combination chemotherapy is a standard treatment for ovarian carcinoma and has achieved a high response rate, its success is limited by the development of drug resistance (1). Therefore, it is important to explore alternative treatment modalities that have favorable cost benefit ratios in terms of toxicity and do not lead to the development of drug resistance or disease relapse.

It has been reported that estrogen and progesterone are involved in the etiology and long-term survival of patients with ovarian carcinoma. In 1963, Long and Evans (2) suggested for the first time that ovarian carcinoma might be sensitive to hormones. According to their report, the use of diethylstilbestrol in 14 patients with advanced ovarian carcinoma led to a partial response in 4 (28%) patients. The incidence of ovarian carcinoma is increased among women after menopause because of lower levels of sex steroids. Estrogen replacement therapy in postmenopausal women does not reduce the risk of ovarian carcinoma. However, the risk may be reduced by use of combination-type oral contraceptives, which contain estrogen and a high dose of progesterone. Recent studies have reported the presence of estrogen, progesterone, and androgen receptors in varying concentrations and combinations in ovarian carcinoma (3). According to the results of many studies, although estrogen is proposed to facilitate the induction of ovarian carcinoma, progesterone has a potential protective effect (4, 5). Progesterone has strong effects on
human hormone-responsive endometrial and breast cancers. It has also been reported that progesterone significantly inhibits cell proliferation and reduces the risk of ovarian carcinoma (6). In particular, because progesterone has been reported to have antiproliferative and apoptotic effects on ovarian cancer cell lines, progesterone has been widely used in the clinical treatment of ovarian carcinoma. Van der Vange et al (7) reported that the response rate was approximately 7% in a series with an adequate number of patients and response criteria. In addition to progesterone, tamoxifen, which is a competitive estrogen antagonist, has been successfully used in the management of early and advanced breast cancer. The clinical activity of anti-estrogens in ovarian carcinoma was first reported by Myers et al (8). The reported response rates to tamoxifen were between 0 and 28%, and the overall response rate was approximately 8%. Although Marth et al suggested a better response for endometrioid adenocarcinomas of the ovary, a literature review revealed no apparent differences in histological subtype, grade of tumor, or hormone receptor values between responders and non-responders and no correlation between response rate and the presence of estrogen or progesterone receptors in the tumors were evident (9-11). However, the estrogen receptor-negative/progesterone receptor-positive phenotype predicts favorable tumor biology and long term survival, probably reflecting the functional effects on tumor proliferation, differentiation, and apoptosis (12). Tamoxifen causes cell cycle arrest (G$_1$/G$_0$ arrest) with up-regulation of both p21 and p27 levels in susceptible estrogen-receptor positive breast cancer cells (13). The exact molecular mechanisms of apoptosis or growth arrest of ovarian cancer cells induced by progesterone and tamoxifen are not clear. In addition, the effect of combined treatment of progesterone and tamoxifen has not been tested.

In this study, the molecular mechanisms of apoptosis and growth arrest of ovarian cancer cells induced by progesterone and tamoxifen were investigated. In addition, the effect of combined treatment of progesterone and tamoxifen was tested since combined treatment seems to have synergistic effects.

Materials and methods

Cell lines and cell cultures. PA-1, a human ovarian cancer cell line with wild-type p53, was obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). Cells were cultured in Dulbecco's modified essential medium (DMEM, Gibco-BRL, NY) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Inc.), streptomycin (100 µg/ml), and penicillin G (100 IU/ml), and maintained at 37°C in a humidified incubator with 5% CO$_2$.

RT-PCR. Total-RNA was isolated from 2x10$^6$ cells using RNA Plus™ (Quantum Biotechnologies, Inc., CA). cDNA was synthesized by incubation at 52°C for 20 min in a 20-µl reaction volume containing RNA (100 ng), 10 mM deoxynucleotide triphosphates (Boehringer Mannheim), 10X reaction buffer (provided by Takara Shuzo Co., Japan) and Avian Myeloblastosis Virus (AMV) reverse transcriptase (Takara). Primers for hormone receptor gene were designed that amplified mRNA between exons 7 and 8 of the estrogen receptor gene (5’-GCACCCTGAACTCTCTGGAA-3’, 5’-GACGGGTGACTGCAAACATCC-3’). The PCR reaction was performed as follows: incubation at 95°C for 8 min followed by 40 cycles of reaction (denaturation at 95°C for 1 min, annealing at different temperature for 1 min, extension at 72°C for 1 min), and a final extension at 72°C for 10 min. Annealing temperatures for the estrogen receptor and progesterone receptor were 55 and 65°C, respectively. The amplified PCR products were resolved by electrophoresis on 1% agarose gel and visualized by ethidium bromide staining.

Treatment of PA-1 cells with tamoxifen, progesterone, or both. Progesterone (Sigma, St. Louis, MO) and tamoxifen (Sigma) were dissolved in ethanol and kept at 4°C until use. Cells were plated and medium was changed to DMEM with 10% charcoal-stripped FBS and antibiotics (streptomycin and penicillin G) to deplete medium steroid hormones and estrogenic compounds for the remainder of the assay period. After 48 h, cultures were replenished with fresh charcoal-stripped DMEM, and then progesterone, tamoxifen, or both drugs were added to each dish.

Methyl thiazole tetrazolium (MTT) assay. Cells were seeded in 96-well plates at a density of 2x10$^3$ cells/well and incubated for 24 h. After treatment of drugs, the cell growth was assessed by staining with MTT dye at 37°C for 2 h. The staining medium was then replaced by phosphate-buffered saline (PBS) with 2.5% of protamin sulfate stock solution (Sigma), and the plates were incubated at 4°C overnight. The formazan was extracted with 250 µl of dimethyl sulfoxide (DMSO, 10%, Sigma) at room temperature for 4 h. Then 150 µl of the extract was transferred to a flat-bottomed 96-well plate for reading the absorbance at 570 nm in a microplate reader (Dynatech, UK).

Cell cycle analysis. Cells were harvested by trypsinization at 0, 12, 24, and 48 h after drug treatment, collected gently, and resuspended in 2 ml of PBS. Cells were then fixed by adding gradually 5 ml of 95% ethanol with vortexing. After incubation at room temperature for 30 min, cells were stored at 4°C. Cells were then collected by centrifugation and stained by adding 1 ml of 50 µg/ml propidium iodide solution (Sigma). RNase A (Sigma) was then added at a final concentration of 100 µg/ml and samples were incubated at room temperature for 30 min. The DNA contents of the cells were analyzed by a Becton-Dickinson FACScan flow cytometer.

TUNEL assay: terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling. PA-1 cells were attached to Poly-Prep slides (Sigma) and treated with progesterone 100 µM for 48 h. The cells were fixed with 10% buffered formalin and permeabilized by immersing the slides in 0.2% Triton X-100 solution. After the cells were washed in PBS, the cells were incubated in equilibrium buffer (200 mM potassium cacodylated, pH 6.6, 25 mM Tris-HCl, pH 6.6, 0.2 mM DTT, 0.25 mg/ml BSA and 2.5 mM cobalt chloride) containing biotinylated nucleotide mix and 25 units of terminal deoxynucleotidyl transferase (TdT) at 37°C for 1 h. The reaction was stopped by immersing the slides in 2X SSC (0.3 M NaCl and 30 mM sodium citrate, pH 7.0). The cells on slides were incubated in 0.3% hydrogen peroxide and then with strepta-
vidin horseradish-peroxidase (HRP) diluted in PBS. The cells were incubated with diaminobenzidine (DAB) components, rinsed several times with deionized water, and mounted in an aqueous or permanent mounting medium. Each slide was observed under a fluorescence microscope and photographs were taken at appropriate magnification. All batches of experiments were repeated at least three times.

Analysis of DNA fragmentation. Drug treated-cells were washed twice with PBS and resuspended in 25 µl PBS. Cells were lysed by the addition of 25 µl lysis buffer (60 mM Tris, pH 7.4, 50 mM ethylene diamine tetraacetic acid, and 1.6% sodium lauryl sarcosine) containing proteinase K, and incubation was carried out at 50°C for 3 h, and digestion with 200 µg/ml DNase-free RNase A for an additional 20 min. DNA from the cell lysates was then analyzed on a 2% agarose gel containing ethidium bromide, and visualized and photographed under ultraviolet light. All batches of experiments were repeated at least three times.

Detection of apoptosis-related proteins and cyclin-dependent kinase inhibitors. Cells were lysed in lysis buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, 10% glycerol, and 1% NP-40, 0.1 mM PMSF, 10 µg/ml each leupeptin, aprotinin, and pepstatin A). Equal amounts of proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes (Millipore, USA). PVDF membranes were incubated with primary antibodies and then with horseradish peroxidase-conjugated secondary antibodies. The resulting bands were visualized by ECL system (Amersham Biosciences). Primary antibodies used in this study were anti-caspase-3, anti-poly (ADP-ribose) polymerase (PARP), anti-cytochrome c, anti-BAX, anti-BCL-2, anti-p53, anti-p21, anti-p27, anti-p16 and anti-Rb. All batches of experiments were repeated at least three times.

Subcellular fractionation. Cell pellets were resuspended in sucrose-supplemented cell extract buffer (300 mM sucrose, 10 mM HEPES, pH 7.4, 50 mM KCl, 5 mM EGTA, 5 mM MgCl2, 1 mM DTT, and protease inhibitor mixture). The cells were homogenized on ice with a dounce homogenizer. Unbroken cells and nuclei were removed by centrifugation at 2,000 x g for 10 min. The post-nuclear supernatant was further collected at 10,000 x g for at 4°C 10 min. The purity of the mitochondria fraction was determined by the absence of cytosolic β-actin using Western blot analyses. The supernatant was further ultracentrifuged at 14,000 x g for 10 min and then filtered by passing through a 0.22-µm filter (Millipore) to generate purified cytosolic fraction.

Results

mRNA expression of estrogen and progesterone receptors in PA-1 cells. Before the effects of progesterone and tamoxifen were tested, the expression of estrogen and progesterone receptors in PA-1 cells, which have wild type p53, was first assessed by RT-PCR. The expression of progesterone and estrogen receptor mRNAs in PA-1 cells was evident when compared with the control samples without reverse transcriptase (Fig. 1). The estrogen receptor mRNA amplicon was 470 base pairs (bp), and the progesterone receptor mRNA amplicon was 460 bp.

Effects of progesterone and tamoxifen on cell proliferation and viability. After treatment with progesterone, tamoxifen, or both hormones, the viability of ovarian cancer cells was evaluated by using the MTT assay. Progesterone was added at final concentrations of 10, 30 or 100 µM to PA-1 cells growing in DMEM with charcoal-stripped FBS. The PA-1 cells were
incubated for 0, 12, 24, 48 or 72 h. The control cells were not treated with progesterone. Treatment with 10 µM progesterone slightly inhibited cell growth as compared to the control cells. Significant cell growth inhibition was observed at 100 µM of progesterone at 48 h of incubation (Fig. 2B).

Similarly, PA-1 cells were treated with 1, 3 or 10 µM tamoxifen for 0, 12, 24, 48 or 72 h, and the number of the viable cells was counted. As was observed with progesterone, the effects of tamoxifen were dependent on concentration. Treatment with 1 or 3 µM tamoxifen had little effect, but 10 µM of tamoxifen significantly decreased cell viability at 48 h (Fig. 2A). The effective concentrations of progesterone and tamoxifen for inhibition of cell growth were 100 and 10 µM, respectively, and these concentrations were used for subsequent experiments. Co-treatment with 10 µM tamoxifen and 100 µM progesterone resulted in less pronounced inhibition of growth than observed with either progesterone or tamoxifen treatment alone (Fig. 2C).

Cell cycle analysis. As 100 µM progesterone and 10 µM tamoxifen were most effective in inhibiting cell proliferation, these concentrations were used for cell cycle analysis. The nuclear DNA content of individual cells was analyzed by flow cytometry after treatment with 100 µM progesterone, 10 µM tamoxifen, or both to evaluate cell viability and cell cycle distribution. The peak representing cells in sub-G0 phase was evaluated after 0, 12, 24 and 48 h of incubation (Fig. 3). The number of cells in sub-G0 phase increased markedly after treatment with 100 µM progesterone as incubation time increased, whereas the fraction of cells in S-phase (M3 phase) decreased. When PA-1 cells were treated with 10 µM tamoxifen, most cells were in the G1 phase of the cell cycle. After co-treatment with tamoxifen and progesterone, the number of cells in the sub-G0 stage increased markedly as incubation time increased, but the fraction of cells in S-phase decreased. These results suggest that treatment with progesterone and co-treatment with progesterone and tamoxifen could be effective in inhibiting cell proliferation of ovarian cancer cells.
and tamoxifen caused apoptosis because of the increased cell population in the sub-G₀ phase. Treatment with tamoxifen resulted in G₁ arrest because of the increased number of cells in the G₁ phase.

**Analysis of progesterone-induced apoptosis.** To confirm progesterone-induced apoptosis in PA-1 cells, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, DNA fragmentation analysis, and Western blot analysis of apoptotic markers were performed. After treatment with 100 µM progesterone, 10 µM tamoxifen, or both for 48 h, the number of apoptotic cells with fragmented DNA were quantitatively determined by TUNEL assay. Apoptotic cells (fluorescent cells) were detected by use of a fluorescence microscope (Fig. 4A). Although apoptotic cells were not detected in the control cells and the cells treated with 10 µM tamoxifen, apoptotic cells were detected among the cells treated with progesterone and the combination of progesterone and tamoxifen. The results showed that progesterone induced apoptosis and DNA fragmentation.

Apoptosis ultimately induces the activation of DNA endonuclease, which cleaves DNA into fragments of approximately 180-200 bp. Agarose gel electrophoresis of DNA isolated from progesterone-treated cells showed the characteristic DNA fragmentation ladder, indicating that progesterone-treated cells had the typical characteristics of apoptosis (Fig. 4B). Apoptosis mediated by p53 is known to involve transcriptional repression of BCL-2 and activation of BAX. To test whether progesterone-induced apoptosis of PA-1 cells is facilitated by this mechanism, PA-1 cells treated with progesterone were subjected to Western blot analysis with anti-BCL-2 and anti-BAX antibodies. Changes in the levels of BCL-2 or BAX were not observed in cells treated with 100 µM of progesterone. Western blot analysis of p53 and p21 proteins showed a time-dependent up-regulation in p53 and p21 proteins. After incubation for 24 h, the intensities of the p53 and p21 bands reached their peaks (Fig. 5A).

To further explore the detailed molecular processes of progesterone-induced apoptosis, the expression of caspase-3, PARP, and cytochrome c was evaluated in progesterone-treated cells. Caspase-3 is an effector caspase that is critical in many apoptotic pathways. When caspase-3 is activated, it is cleaved into fragments of 11 and 20 kDa. Activated caspase-3 cleaves several key enzymes that are required for normal cell maintenance, including a DNA repair enzyme PARP (14). Western blot analysis of PARP showed that PARP is cleaved from its intact form (116 kDa) into fragments of 85 and 25 kDa. Western blot analysis also showed decreased intensity of the caspase-3 band after progesterone treatment (Fig. 5A). This result suggests that progesterone-induced apoptosis of PA-1 cells involves caspase-3-mediated apoptosis.

To examine the involvement of BAX and cytochrome C, the cell cytosolic and mitochondrial fractions were separated, and the content of BAX protein in each fraction was analyzed by Western blot analysis. The results showed that cytosolic BAX had mostly disappeared after 24 h and had completely disappeared after 48 h of progesterone treatment, while the mitochondrial content of BAX increased after 48 h (Fig. 5B). These results indicate that translocation of BAX from the cytosol to mitochondria mediates progesterone-induced
apoptosis, presumably through cytochrome C release from the mitochondria.

Apoptotic mechanisms were also observed after simultaneous treatment with tamoxifen and progesterone. Up-regulation of p53, p21 and p27 proteins was observed, but the levels of BAX and BCL-2 were unchanged. Cleavage of caspase-3 and PARP were confirmed by Western blot analysis (Fig. 5C).

Analysis of tamoxifen-induced cell growth arrest. Molecular changes induced by tamoxifen were analyzed by Western blot analysis. The p53, p21 and p27 protein levels increased within 24 h after tamoxifen treatment (Fig. 6). However, the levels of these proteins decreased after 24 h. Increased p16 and dephosphorylated Rb protein levels were observed 24 h after tamoxifen treatment. These results suggest that typical G1 arrest is involved in tamoxifen-induced cell cycle arrest.

Discussion

To investigate the effects of combined progesterone and tamoxifen treatment on ovarian carcinoma, the growth and death of epithelial ovarian cancer cells with wild-type p53 were tested. Progesterone has been reported to promote apoptosis in ovarian carcinoma cells (14) in a p53-dependent manner (15). Tamoxifen causes cell cycle arrest (G0/G1 arrest) with up-regulation of both p21 and p27 in susceptible estrogen receptor-positive breast cancer cells (13). However, whether combined treatment with progesterone and tamoxifen has synergistic anti-cancer effects has also not been investigated. In this study, we demonstrated that progesterone treatment of ovarian carcinoma cells triggers apoptosis and tamoxifen treatment induces growth arrest at G1. Combined treatment induced apoptosis similar to that induced by progesterone alone and had no synergistic effect on progesterone treatment alone. Flow cytometric analysis showed that progesterone treatment and combined treatment with both hormones induced apoptosis after 48 h while tamoxifen treatment alone induced mostly G0 arrest (Fig. 3). Furthermore, the TUNEL assay and DNA fragmentation analysis confirmed that progesterone treatment and combined treatment induced apoptosis (Fig. 4). Western blot analysis revealed the digestion of PARP and caspase-3 in both progesterone treatment and combined treatment (Fig. 5). Tamoxifen treatment resulted in G1 arrest-related induction of p21 and p16 and dephosphorylation of PRB (Fig. 6). Combined treatment showed no synergistic effect as compared to treatment with progesterone alone. We expected that combined treatment would induce more apoptosis or cell cycle arrest or cause these events to occur at a faster rate than either treatment alone. However, the results showed that this...
did not happen. Currently, we do not have an answer as to why this occurred.

One plausible explanation is the following. It has been proposed that the binding of p53 to specific p53 response elements differs greatly. Low-affinity sites appear to be associated with growth arrest-related genes, while high-affinity sites are more related to proapoptotic genes (16,17). Ubiquitination of p53 in response to mild damage is known to be associated with growth arrest-related genes, and acetylated and phosphorylated p53 proteins activate proapoptotic genes in response to severe damage. In addition, p53-interacting proteins like HZF interact directly with the p53 DNA-binding domain to activate p21 while proapoptotic genes are attenuated. In contrast, CAS is associated with p53 on the promoters of several proapoptotic genes. This relieves the inhibitory H3K27 methylation within the transcribed region of those genes, thereby increasing their transcription and facilitating apoptosis (18,19). Therefore, tamoxifen treatment appears to cause mild damage in which p53 induces growth arrest whereas progesterone treatment causes severe damage, which results in apoptosis. Combined treatment seems to induce a severe form of damage, resulting in apoptosis similar to the case of progesterone treatment.

We still do not know the detailed mechanism of how p53 decides between growth arrest and apoptosis in PA-1 cells subjected to these treatments. Combined treatment is used in many therapeutic cancer regimes since it sometimes results in synergistic effects on cancer. Some cell death-inducing agents that induce the death of cancer cells via different pathways will exert a synergistic effect. Even combined treatment with apoptosis-inducing agents will be advantageous if the two agents involve different apoptosis pathways. However, the combination of an anti-cancer medicine that induces growth arrest and another that induces apoptosis may not be recommended as the combination will not have an advantageous effect. Further detailed characterization of the mechanisms of action of these medicines in p53-deleted or p53-mutated ovarian cancer cells may provide a better understanding of these treatment methods.

Acknowledgements

This study was supported by Priority Research Centers Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-0029642).

References