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Apoptosis Induced by Progesterone in Human Ovarian Cancer Cell Line SNU-840

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Abstract Progesterone has been used as an ingredient of anticancer drug for patients with ovarian carcinoma. However, the mechanism of anticancer effects by progesterone has not been understood. In this study, the effects of progesterone on ovarian cancer cells, SNU-840, were investigated. After the incubation with progesterone, the viability of the cells was evaluated by MTT assay. As a result, 45% of the cells were viable after 48 h of incubation with 100 μM progesterone. In addition, [^{3}H]thymidine incorporation assay showed that the proliferation of the cells was completely inhibited by progesterone after 48 h of incubation at 100 μM concentration. Colorimetric TUNEL assay revealed the fragmentation of the chromosomal DNA, suggesting that the process of the cell death was apoptosis. The level of the p53 mRNA was determined by northern blotting assay, since many apoptosis processes are mediated by up-regulation of the p53 expression. The level of the p53 mRNA reached its maximum at 12 h and decreased after 24 h of incubation with progesterone. In conclusion, progesterone inhibits the proliferation and elicites apoptosis of SNU-840 cells. Also, it up-regulates the p53 mRNA transiently. J. Cell. Biochem. 82: 445–451, 2001.

Key words: apoptosis; MTT; ovarian cancer; p53; progesterone; TUNEL

Three common cancers in women — endometrial, breast, and ovarian — are related to sex hormones. In these cancers, the main effects of the hormones may be achieved through the control of cell proliferation and differentiation [Albanes and Winick, 1988; Cohen and Ellwein, 1990; Preston-Martin et al., 1990; MacMahon, 1993]. One of the sex hormones, progesterone, has complex effects in female sex organs [Graham and Clarke, 1997; Groshong et al., 1997]. In two of its major target organs, the uterus and the mammary gland, it strongly influences proliferation and differentiation. In the uterus, progesterone inhibits epithelial growth and induces differentiation [Clarke and Sutherland, 1990]. Also, progesterone is required for terminal growth and differentiation of the mammary gland [Clarke and Sutherland, 1990]. Therefore, knock-out mice lacking progesterone receptor have severely impaired mammary gland development [Lydon et al., 1995]. In animal model of mammary carcinogenesis, progesterone can either inhibit or promote tumor formation, depending on the regimen used [Graham and Clarke, 1997]. In animals with established progesterone receptor-positive mammary tumor, progesterone is usually proliferative and progesterone antagonists inhibit tumor growth [Horwitz, 1992]. Progesterone also have strong effects on human hormone-responsive endometrial and breast cancers [Clarke and Sutherland, 1990]. Second-line high dose progesterone therapy effectively suppresses estrogen-mediated growth of breast cancer [Owen et al., 1998].

It was previously reported that the ovary carcinoma risk may be reduced by use of combination-type oral contraceptives, which contain estrogen and a high dose of progesterone [Schneider and Birkhauser, 1995]. However, the effects of progesterone on ovarian cancer have not been clear. In this study, several experiments were performed to investigate the effects of progesterone on ovarian cancer cell

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line SNU-840. The growth inhibitory effects of progesterone were confirmed by MTT assay and [^{3}H]thymidine incorporation assay. Also, apoptosis in progesterone treated cells was proved by modified TdT-Mediated dUTP Nick End Labeling (TUNEL) assay. Northern blotting assay was performed to determine the level of p53 mRNA after progesterone treatment. These results showed that progesterone inhibits cell proliferation and induces apoptosis of SNU-840 cells. It was also proved that progesterone upregulated the p53 mRNA transiently.

MATERIALS AND METHODS

Cell Culture and Progesterone Treatment

Human ovarian carcinoma cell line SNU-840 (Korean Cell Line Bank, Korea) was used in all experiments. SNU-840 cells were established from the primary lesion of a malignant brenner tumor [Yuan et al., 1997]. The cells were maintained in RPMI1640 medium containing 10% heat-inactivated fetal bovine serum, streptomycin, and penicillin G, in a humidified atmosphere of 5% CO_2 at 37°C.

For progesterone treatment, SNU-840 cells were seeded at a density of 2.0×10^6 cells/dish in 100 mm culture dishes, and incubated in RPMI1640 medium supplemented with 10% FBS for 24 h before the addition of the progesterone. Progesterone was dissolved in ethanol at a concentration of 1 or 10 mM and filtered through 0.22 μm polycarbonate membrane. One hundred microliter of 1 or 10 mM progesterone in ethanol was added to each dish that contained 10 ml of RPMI1640 medium supplemented with 10% FBS. One hundred microliter of absolute ethanol was added to the control cells. Final concentration of ethanol was 1%(v/v). The cells were then incubated for a desired time at 37°C in a 5% CO_2 incubator.

MTT Assay

MTT was dissolved in 1× phosphate buffered saline (PBS) at 2 mg/ml, filtered through 0.22 μm polycarbonate membrane filters. SNU-840 cells were seeded in 96-well plates at a density of 2×10^4 cells/well and incubated for 24 h before the addition of the progesterone. After the addition of 1 μl of 1 or 10 mM progesterone, the cells were incubated for 6, 12, 24, or 48 h at 37°C in a 5% CO_2 incubator. One microliter of absolute ethanol was added to the control cells. All treatments were analyzed with six replicates. At the end of the incubation, the medium was replaced with 100 μl of fresh serum free RPMI1640. The medium for the control cells was also replaced with 100 μl of fresh serum free RPMI1640 medium. Twenty-six microliters of 2 mg/ml MTT solution in 1× PBS was then added. Plates were incubated for an additional 4 h at 37°C in a tissue culture incubator, then MTT-containing medium was aspirated off and 150 μl of DMSO was added to dissolve the formazan crystal formed by living cells. Absorbance at 570 nm was measured using a microplate reader (Molecular Devices, Sunnyvale, CA) within 1 h after the addition of DMSO. The percentages of the viable cells after the progesterone incubation were calculated by comparison with the control cells that were incubated with 1% ethanol for the same time periods as the progesterone incubated cells, according to the following equation:

$$\text{Cell viability}\,(\%) = \frac{\text{OD}_{570(\text{sample})}}{\text{OD}_{570(\text{control})}} \times 100$$

where the $\text{OD}_{570(\text{sample})}$ represents the measurement from the wells treated with progesterone and the $\text{OD}_{570(\text{control})}$ represents the measurement from the wells treated with ethanol only.

[^{3}H]Thymidine Incorporation Assay

SNU-840 cells were seeded in 96-well plates at a density of 2×10^5 cells/ml, 100 μl/well and incubated for 24 h before the addition of the progesterone. After the addition of 1 μl of 10 mM progesterone in ethanol or absolute ethanol, the cells were incubated for 6, 12, 24, or 48 h at 37°C in a 5% CO_2 incubator. All treatments were analyzed in triplicate. After the treatment, the medium was removed by aspiration. The cells were pulsed for 2 h with [^{3}H]thymidine (1 μM Ci/well, NEN, Boston, MA), harvested by precipitation with cold 5% trichloroacetic acid, and solubilized in 0.2 N NaOH. The amount of radioactivity was counted by liquid scintillation counting. The percentages of [^{3}H]thymidine incorporation were calculated by comparison with each control that was incubated with 1% ethanol for the same time period as progesterone incubated cells.

Colorimetric TUNEL Assay

DeadEnd colorimetric apoptosis detection system (Promega, Madison, WI) was used to detect apoptotic cell death in situ in the

progesterone-treated SNU-840 cells. Briefly, SNU-840 cells were attached to Poly-Prep slides (Sigma, St. Louis, MO) and treated with 100 μM progesterone or ethanol. The cells were fixed by immersing the slides in 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 equilibration 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 TdT (terminal deoxynucleotidyl transferase, 25 U) enzyme at 37°C for 1 h. The reaction was stopped by immersing the slides in 2× SSC (0.3 M NaCl and 30 mM sodium citrate, pH 7.0). The cells were incubated in 0.3% hydrogen peroxide and then with streptavidin horseradish-peroxidase (HRP) diluted in 1× PBS. The cells were incubated with diaminobenzidine (DAB) components, rinsed several times in deionized water, and mounted in an aqueous or permanent mounting medium. Each slide was observed under a light microscope.

Northern Blotting Assay

The progesterone treated SNU-840 cells were washed with 1× PBS twice, and total RNA was prepared by acid-guanidium thocyanate-phenol-chloroform extraction as described previously [Chomczynski and Sacchi, 1987], using RNawiz (Ambion, Ausin, TX). The concentration of RNA was measure by the absorbance at 260 nm. Northern blotting assay was carried out as described previously [Lee et al., 1998]. Briefly, 10 μg of RNA was denatured with formaldehyde–formamide and separated on a 1% agarose–formaldehyde gel. RNA was then transferred to nitrocellulose membrane (Sigma, St. Louis, MO). The blot was hybridized with nick-translated [α-^{32}P]dCTP (Amersham Life Science)-labeled human p53 cDNA probe at 42°C. Following multiple washing with 2× SSC, 0.1% SDS, and then with 0.25× SSC, 0.1% SDS, the membrane was subjected to autoradiography.

RESULTS

Cell Morphology and Viablity of SNU-840 Cells After the Treatment of Progesterone

The morphological changes of ovarian cancer cells, SNU-840 cells, were evaluated after the progesterone treatment. SNU-840 cells were incubated with 100 μM progesterone for 6, 12, 24, and 48 h. The morphology of the cells began to change after 12 h (Fig. 1C) and some of the cells were floating after 24 h of incubation (Fig. 1D). Since progesterone was dissolved in ethanol, the control cells were incubated with ethanol only. In all the experiments, the final concentration of ethanol was 1% (v/v). At this concentration, ethanol did not have any effect on the morphology of the cells (Fig. 1F). This result showed that the morphological change was due to progesterone and not due to ethanol. In addition, the morphology of the cells was not changed in the presence of 10 μM progesterone up to 48 h (data not shown).

The cell viability was evaluated by MTT assay (Fig. 2). SNU-840 cells were incubated with 10 or 100 μM progesterone for 6, 12, 24, and 48 h. The control cells were incubated with 1% ethanol. After progesterone treatment, the number of the viable cells was significantly decreased. Cell viability began to decrease after 12 h of incubation with 100 μM progesterone and 55% of the cells lost their viability after 48 h. However, in the presence of 10 μM progesterone, the cells did not lose their viability. Therefore, the effective concentration of progesterone to SNU-840 cells is 100 μM.

Inhibition of Cell Proliferation by Progesterone

To evaluate the cell proliferation, [^{3}H]thymidine incorporation assay was carried out. The level of [^{3}H]thymidine incorporation after progesterone treatment is shown in Figure 3. The control cells were incubated with 1% ethanol. [^{3}H]thymidine incorporation was decreased to 26% after 24 h and reached 0.6% after 48 h of incubation compared with the control cells. This result showed the proliferation was completely inhibited after 48 h of the incubation with 100 μM progesterone.

Detection of Apoptosis by Modified TUNEL Assay

Once triggered, apoptosis ultimately leads to activation of DNA endonuclease with fragmentation of DNA into about 180–200 bp units. In order to confirm that progesterone induces apoptosis of SNU-840 cells, modified TUNEL assay was carried out. In this assay, biotinylated nucleotides are incorporated at the 3′-OH DNA ends using the TdT enzyme. Streptavidin HRP is then bound to these biotinylated

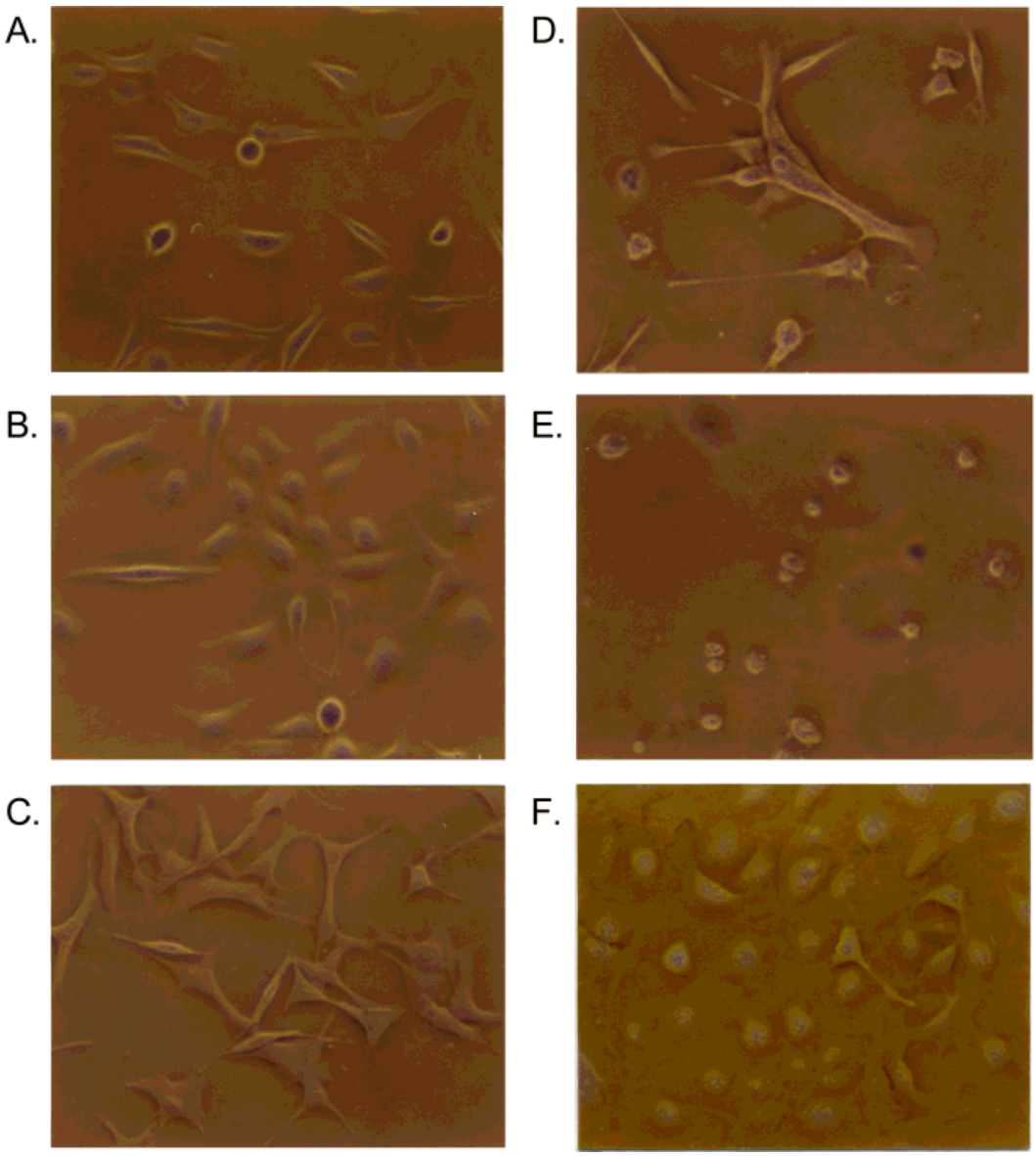

Fig. 1. The change of morphology after progesterone treatment. SNU-840 cells were incubated with 100 μM progesterone for 0 **(A)**, 6 **(B)**, 12 **(C)**, 24 **(D)**, or 48 h **(E)**. The control cells were incubated with 1% (v/v) ethanol for 48 h **(F)**. [Color figure can be viewed in the online issue, which is available at www.interscience. wiley.com.]

nucleotides, which are detected using the peroxidase substrate, hydrogen peroxide, and the stable chromogen, DAB. By this assay, apoptotic nuclei were stained dark brown. As shown in Figure 4, any apoptotic cells were not detected after 0, 6, or 12 h of incubation with progesterone. However, after 24 or 48 h of incubation, DNA fragmentation was detected. In the control cells incubated with 1% ethanol only, fragmentation was not detected up to 48 h. Therefore, progesterone induces the DNA fragmentation, suggesting that the cell death by progesterone is apoptosis.

Transient Up-Regulation of the p53 mRNA Level

Previous report showed that wild type p53 was expressed in SNU-840 cells [Yuan et al., 1997]. Since p53 has been identified as an inducer of apoptosis in many cells, the p53

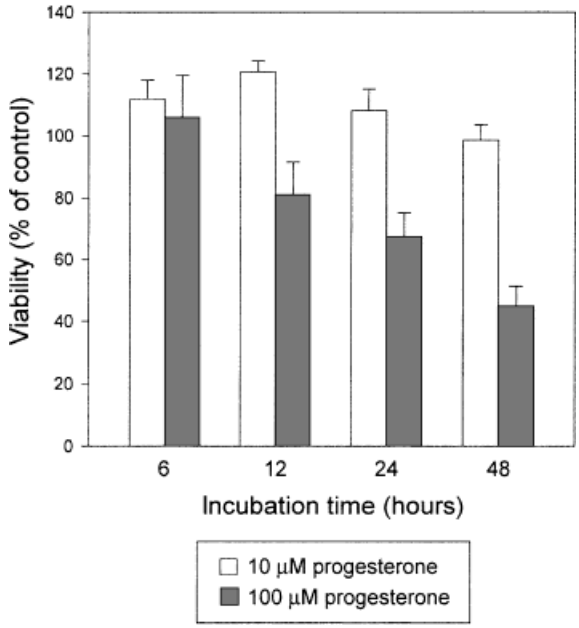

Fig. 2. Cell viability. SNU840 cells were incubated with 10 or 100 μM progesterone for indicated time. The percentage of the viability represents the mean of six different experiments. Standard error of the mean was indicated with bars.

mRNA level was measured by northern blotting analysis. Figure 5 shows that the level of the p53 mRNA was increased after 6 h of progesterone treatment and reached its maximum at 12 h. However, the mRNA levels began to decrease after 24 h. These results showed that the level of the p53 mRNA was transiently increased by progesterone treatment

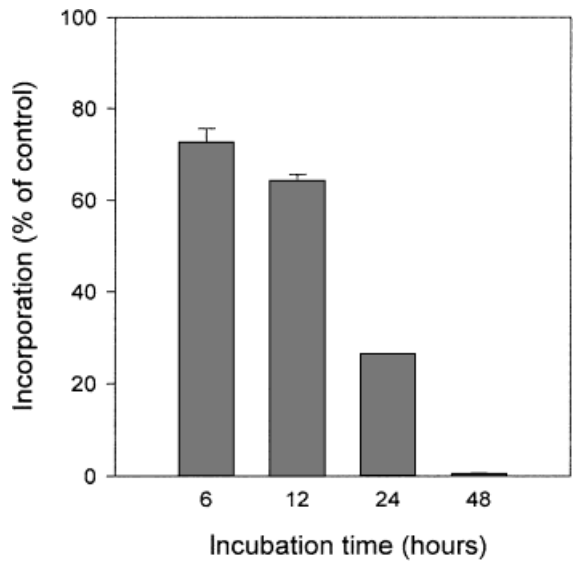

Fig. 3. Inhibition of cell proliferation. SNU-840 cells were pulsed with 1 μCi/well [^{3}H]thymidine and harvested 2 h later after the cells were incubated with 100 μM progesterone for the indicated periods of time. The percentage represents the mean of three different experiments. Standard error of the mean was indicated with bars.

DISCUSSION

Progesterone has been used as an ingredient of anticancer drug for ovarian cancer. However, the cellular mechanism of anticancer effects of progesterone to ovarian cancer cells has not been clear yet. Therefore, in this study the cellular mechanism of progesterone action was investigated.

The viability of SNU-840 cells was evaluated by MTT assays in the presence of 100 μM progesterone. The cell viability was significantly decreased in the presence of 100 μM progesterone. The cell viability reached 45% after 48 h of 100 μM progesterone treatment. Cell morphology was also changed by treatment of progesterone. Many cells were floating after 48 h of progesterone treatment (Fig. 1E), suggesting that the cells were under the process of cell death. In addition, [^{3}H]thymidine incorporation assay showed that DNA replication was completely inhibited after 48 h of progesterone treatment. These results show that progesterone inhibits cell proliferation and induce cell death of SNU-840 cells at 100 μM concentration.

This loss of cell viability by progesterone was identified as apoptosis. Apoptosis is a distinct form of cell death controlled by internally encoded suicide programs [Steller, 1995; White, 1996]. Cells undergoing apoptosis display a series of morphological characteristics including cytoplasmic shrinkage, chromatin condensation, membrane blebbing, DNA fragmentation, and generation of apoptotic bodies that are rapidly phagocytosed and digested by neighboring cells [Kerr et al., 1972]. To prove that a certain cell death is apoptosis, some criteria have been employed. One of them, which is the most feasible, is the DNA fragmentation. In the process of apoptosis, chromosomal DNA is digested by a nuclease and, as a result, 180–200 bp of DNA fragments are produced. To detect this DNA fragments, colorimetric TUNEL assay was carried out. After 24 h of progesterone treatment, DNA fragments began to be detected.

It has become clear that the apoptotic activity of p53 is central to its role as a tumor suppressor [Gottlieb and Oren, 1998]. Also, p53 has been

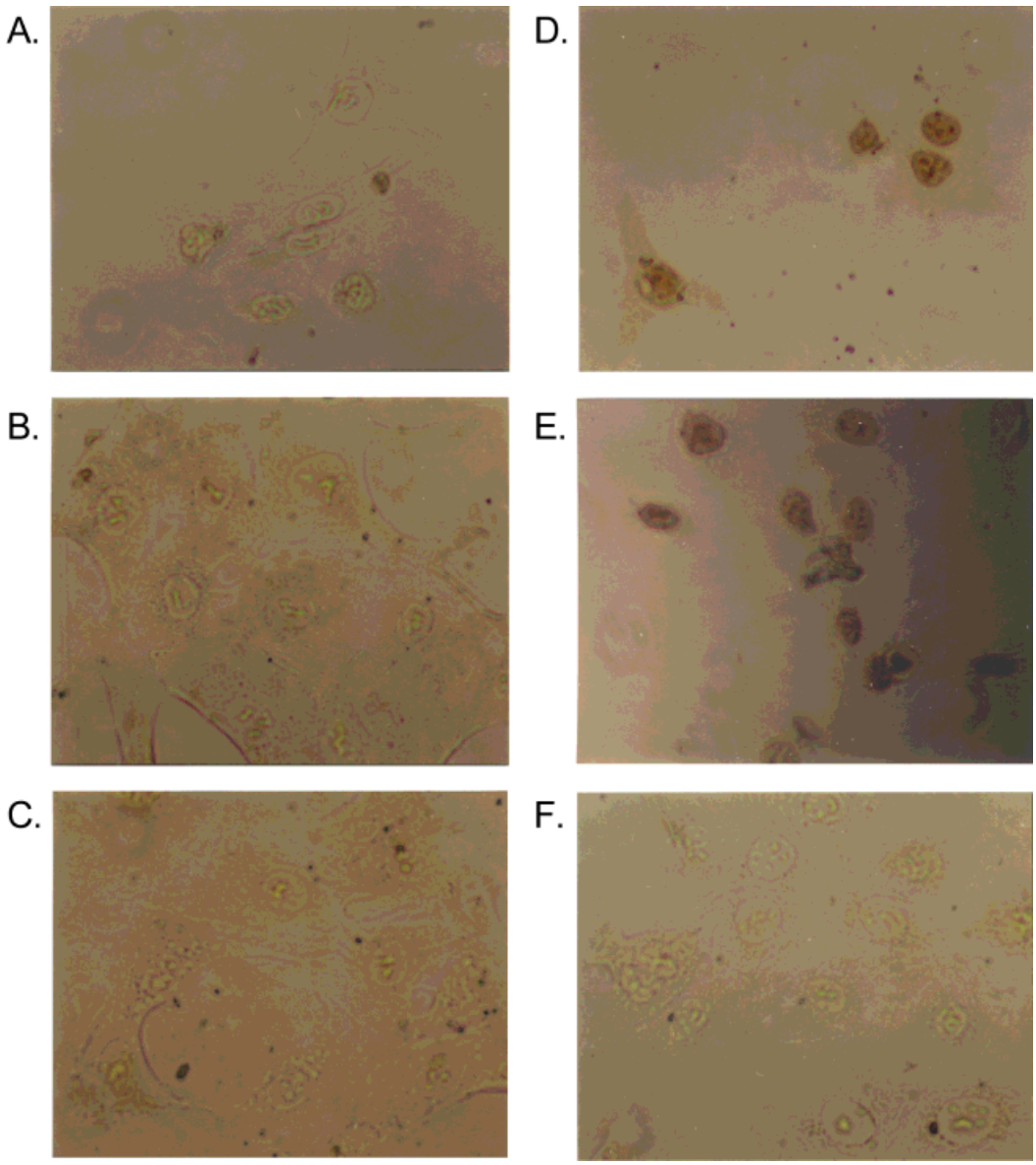

Fig. 4. Colorimetric TUNEL assay. SNU-840 cells were incubated with 100 μM progesterone for 0 **(A)**, 6 **(B)**, 12 **(C)**, 24 **(D)**, or 48 h **(E)**. The control cells were incubated with 1% (v/v) ethanol for 48 h **(F)**. Apoptotic cells were detected after the incubation with 100 μM progesterone for 24 (D) and 48 h (E). [Color figure can be viewed in the online issue, which is available at www.interscience. wiley.com.]

documented to induce either growth arrest or apoptosis when expressed in certain tumor cells lines [Yonish-Rouach et al., 1991; El-Deiry et al., 1993; Harper et al., 1993; Ko and Prives, 1996; Lassus et al., 1996; Desaintes et al., 1997]. Also, a previous report showed that wild type p53 is expressed in SNU-840 cells. Therefore, it is possible that the expression of the p53 gene precedes apoptosis. Our results of northern blotting revealed that expression level of the p53 gene reached its maximum at 12 h of progesterone treatment, which immediately preceded apoptosis and DNA fragmentation. However, the level of the p53 mRNA was markedly decreased after 24 h of progesterone treatment. These results suggest the possibility that apoptosis induced by progesterone undergoes p53-dependent pathway. Therefore, the study for p53 involvement in progesterone induced apoptosis is in progress.

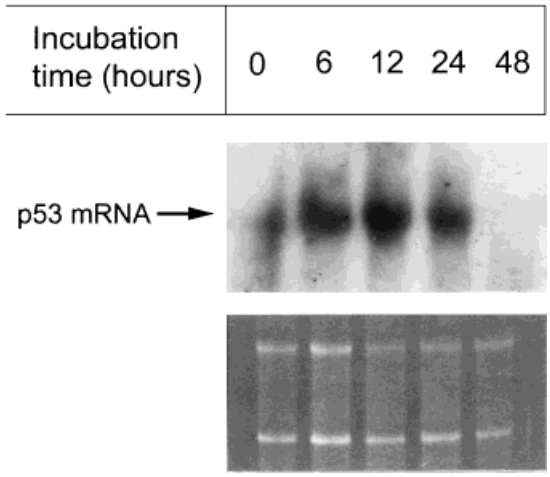

Fig. 5. Northern blotting analysis. After the incubation with 100 μM progesterone for indicated time, total RNAs were extracted and analyzed by northern blotting analysis. Upper panel, autoradiogram for northern blot; lower panel, 28 and 18S RNAs.

In summary, progesterone inhibited proliferation and induced apoptosis of ovarian cancer cells, SNU840 cells. In addition, progesterone induced the expression of the p53 gene transiently.

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