Progesterone Induces Apoptosis and Up-Regulation of p53 Expression in Human Ovarian Carcinoma Cell Lines

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BACKGROUND. Progesterone (PROG) has been shown to reduce the risk of developing ovarian carcinoma in postmenopausal women who have undergone estrogen and progestogen replacement therapy, and it has been clinically used to treat some types of ovarian tumors. It is not yet clear whether or not the antitumor activity of progestogen is due to its ability to induce apoptosis in precarcinomatous and carcinomatous ovarian cells. The apoptosis-related genes p53, *bcl*-2, and c-*myc* have important roles in the regulation of programmed cell death, and thus may be involved in the process of the suspected PROG-induced apoptosis.

METHODS. Antiproliferation effects of PROG on 3AO and AO ovarian carcinoma cells were determined by ^{3}H-thymidine incorporation. Apoptosis of the PROG-treated cells was determined by DNA laddering analysis and was quantitated by both nuclear condensation and flow cytometry after cells were stained with propidium iodide. Cell cycle analysis was also performed by flow cytometry. The expression of p53, *bcl*-2, and c-*myc* after 72 hours of PROG treatment was detected by Northern blot analysis.

RESULTS. In both 3AO and AO cell lines, cells proliferation was maximally inhibited by PROG after 72 hours of treatment at 10 μM concentration. Under the same conditions, more than 50% of PROG-treated cells had undergone apoptosis, whereas less than 3% of the cells were apoptotic in untreated cell cultures. After exposure to PROG for 72 hours, cells were arrested in the G_1 phase of the cell cycle, and the levels of p53 mRNA were remarkably increased in both cell lines. No changes in expression of *bcl*-2 or c-*myc* were detected.

CONCLUSIONS. PROG significantly inhibited cell proliferation and induced apoptosis in both of the ovarian carcinoma cell lines tested in this study. PROG treatment markedly up-regulated p53 expression in these cells, indicating involvement of p53 in PROG-induced apoptosis. ***Cancer* 1997;79:1944–50.**
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KEYWORDS: progesterone, all-*trans*-retinoic acid, apoptosis, cell cycle, ovarian carcinoma, p53 gene.

Ovarian carcinoma is one of the most common fatal gynecologic malignancies in the world.[1] The incidence of this disease rises after women reach menopause due to lower levels of sex steroids.[2] Estrogen replacement therapy in postmenopausal women does not reduce ovarian carcinoma risk.[3] However, the risk may be reduced by use of combination-type oral contraceptives (COCs),[4] which contain estrogen and a high dose of progestogen. In addition, progestogen has been widely used in the clinical treatment of ovarian carcinomas.[5] However, the molecular mechanism of the anticancer effect of progestogen is not yet fully understood.

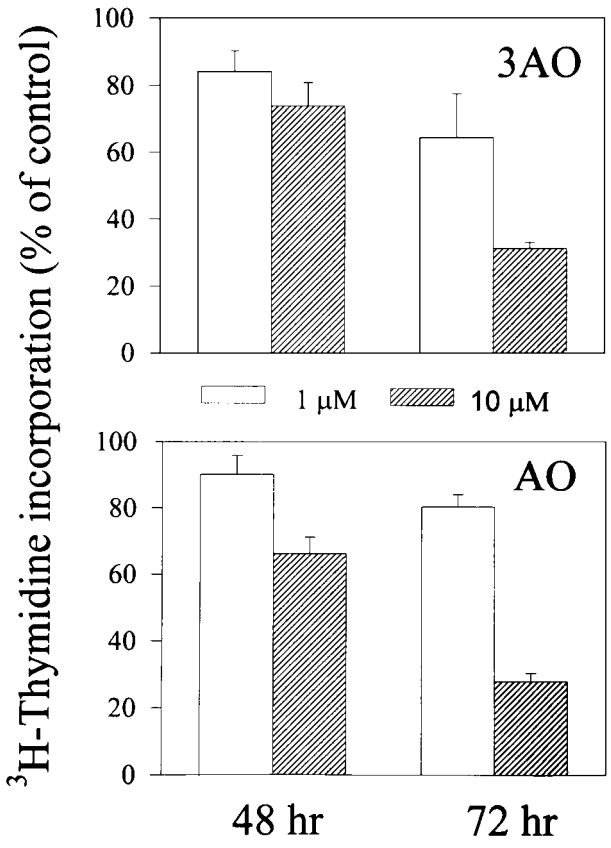

FIGURE 1. The effects of progesterone on ^{3}H-thymidine incorporation of human ovarian carcinoma cell lines 3AO and AO are shown. The cells were cultured for the indicated times treated with progesterone (1 μM and 10 μM, respectively). The percentages represent the mean of three different experiments. Bars represent standard error of the mean.

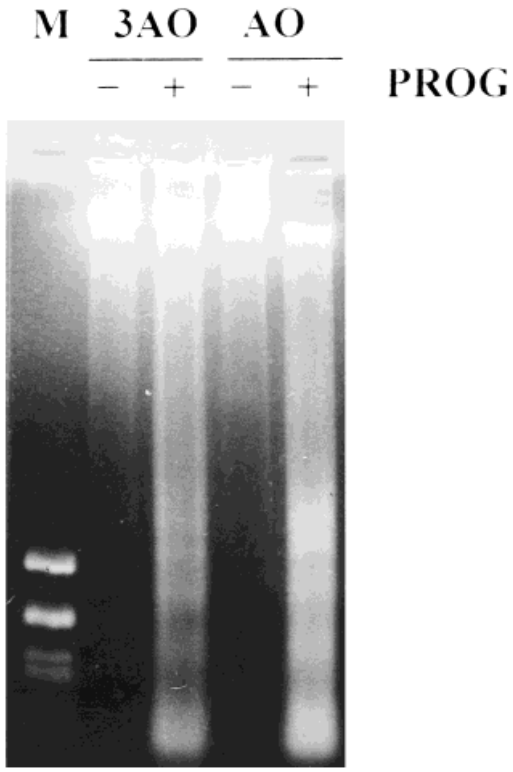

FIGURE 2. Progesterone (PROG)-induced DNA fragmentation was detected by gel electrophoresis. DNA samples from control or PROG-treated 3AO and AO cells were analyzed on a 1.2% agarose gel containing ethidium bromide. Lane 1: molecule-size markers (pGEM 7zf (+) DNA/Hae III markers); Lane 2: sample from 3AO control cells; Lane 3: sample from 3AO cells treated with 10 μM PROG for 72 hours; Lane 4: sample from AO control cells; Lane 5: sample from AO cells treated with 10 μM PROG for 72 hours.

It has been reported that many anticancer agents exert at least part of their effects by triggering programmed cell death,[6] and the induction of apoptosis in tumor cells has become a therapeutic objective. For example, recent data show that *N*-(4-hydroxyphenyl)-all-*trans*-retinamide, a member of the superfamily of nuclear receptor legends that includes progestogen and estrogen, is effective against human ovarian carcinoma transplanted in mice.[7] The retinoid in vitro suppresses human breast carcinoma cell growth by inducing apoptosis.[8] Therefore, induction of apoptosis may be one of the key mechanisms mediating the therapeutic effect of progesterone (PROG) in treatment of ovarian carcinoma.

Apoptosis, which plays a key role in normal development and oncogenesis, is a process genetically controlled by a number of genes,[9] including p53, *bcl*-2, and c-*myc*.[10,11] Among them, p53 is one of the most frequently investigated tumor-suppressing genes.[12] It has been shown that wild-type p53 can induce apoptosis in variety of cell types,[13] and any reagent that induces overexpression of wild-type p53 might promote apoptosis. p53 protein has a critical role in G_1 cell cycle arrest,[14,15] and p53-mediated apoptosis and cell cycle arrest have been used as indicators of wild-type and functional p53.[16] In this study, our objective was to determine whether PROG could induce apoptosis in ovarian carcinoma cells and whether any apoptosis-related genes were involved in the process.

MATERIALS AND METHODS

Cell Cultures and Drug Treatment

Human ovarian carcinoma cell lines, 3AO and AO, are estrogen- and progestogen-dependent. They were obtained from the Cell Bank at the Chinese Academy of Sciences for this study.[17–19] Both cell lines were grown in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (Evergreen, Hang Zhou, China), 100 units/mL penicillin,

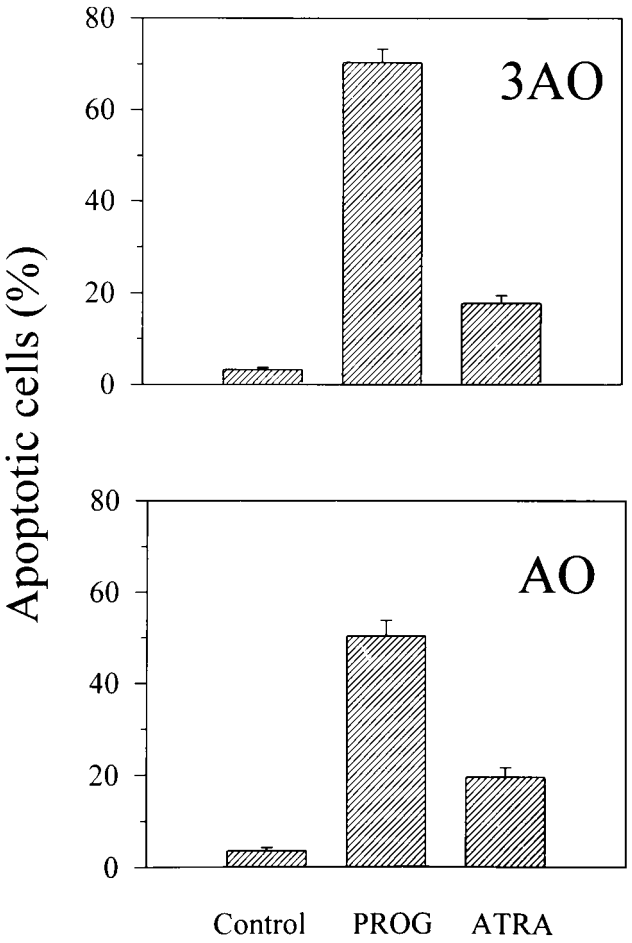

FIGURE 3. Percentages of apoptotic 3AO and AO cells after progesterone (PROG) or all-*trans*-retinoic acid (ATRA) treatment are shown. 3AO and AO cells were treated with indicated concentrations of 3AO and AO. Chromatin staining of the nucleus was performed by incubation with 30 μg/mL propidium iodide solution in phosphate-buffered soline for 15 minutes. The percentage of apoptotic cells was determined by counting approximately 500 cells. Independent experiments were carried out at least three times, and the data were given as the means $\pm$ standard error of the mean.

100 μg/mL streptomycin, and 2 mM glutamine. Exponentially growing 3AO and AO cells (5×10^4 cells/mL) were treated with different concentrations of PROG (Sigma, St. Louis, MO) for 48 hours and 72 hours, respectively.

Assay for Inhibition of ^{3}H-Thymidime Incorporation

3AO and AO cell lines were plated in sextuplicate wells of 96-well microtest plates are treated as described.[20] At various intervals after PROG was added, the plates were pulsed with 2 μCi ^{3}H-thymidine/well (specific activity, 22 Ci/mmol; Shanghai Institute of Nuclear Sciences, Chinese Academy of Sciences), trypsinized, and harvested on strips of fiberglass filter paper with the use of multiple automated sample harvesters. The radioactivity of individual samples was measured in a liquid scintillation counter.

Analysis of DNA Fragmentation by Gel Electrophoresis

Drug-treated and untreated 3AO and AO cells (1×10^6) were washed twice with phosphate-buffered saline (PBS) and resuspended in 25 μL PBS. The 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 1 mg/mL proteinase K, incubated for 3 hours at 50 °C, and digested with 200 μg/mL DNAse-free RNAse A for an additional 20 minutes. DNA from the cell lysates was then analyzed on a 1.2% agarose gel containing ethidium bromide, and visualized and photographed under ultraviolet light.[21]

Quantitative Analysis of Apoptosis

After being treated with 10 μm PROG and all-*trans*-retinoic acid (ATRA, Sigma, St. Louis, MO) for 72 hours, cells were centrifuged, and the pellets were gently resuspended in propidium iodide solution (PI; 50 μg/mL in 0.1% sodium citrate plus 0.15 Triton X-100; Sigma, St. Louis, MO).[22] Random fields of each treated cell culture were observed under a microscope through a $\times$40 objective lens in fluorescent mode. Apoptotic cells had condensed nuclei, and the percentage of apoptotic cells was calculated by counting approximately 500 cells.

PROG-treated and untreated 3AO and AO cells (2×10^6) were washed twice with PBS containing 0.1% glucose and then fixed in 1 mL ice-cold ethanol overnight at 4 °C. The fixed cells were pelleted and resuspended in 0.5 mL of PBS containing 0.1% glucose, 30 μg/mL PI, and 1 mg/mL RNAse A (Sigma, St. Louis, MO). The DNA contents of the cell were analyzed by flow cytometry (Becton-Dickinson, San Jose, CA) as described.[23]

Cell Cycle Analysis

Cell cycle distribution was determined by DNA content, as assayed by propidium iodide staining. The percentage of cells in each phase of the cell cycle was determined with the Cellfit software provided by Becton-Dickinson (San Jose, CA) as described.[24]

Northern Blot Analysis

Extraction of total RNA and Northern blot analysis were performed as described.[25] The human DNA probes used in this study were from the Pst I cDNA insert of pMG-WAF1 plasmid for wild-type p53,[26] the *Eco*RI/Hind III cDNA insert of plasmid pFL1 for *bcl*-2,[27] and the *Eco*RI cDNA insert of plasmid pGDSV7 for

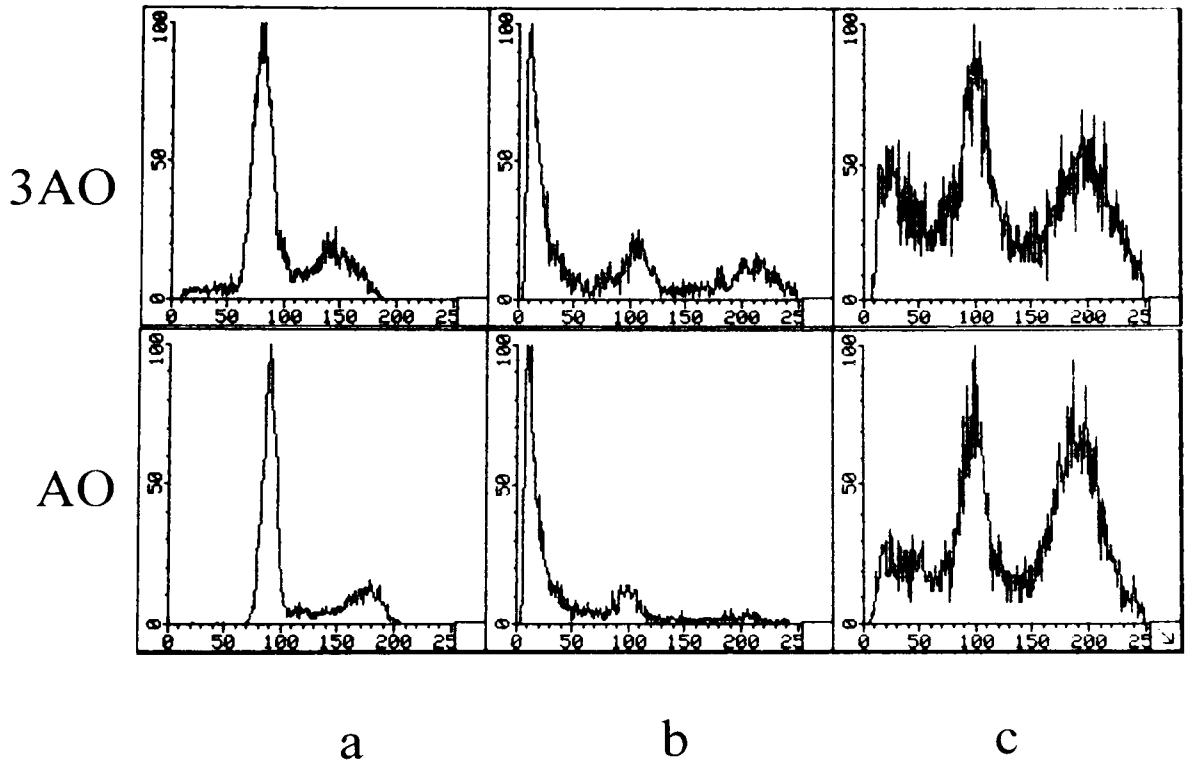

FIGURE 4. Fluorescence histograms are shown of 3AO and AO cells after treatment with 10 μM progesterone (PROG) or 10 μM all-*trans*-retinoic acid (ATRA) for 72 hours. Representative histograms of DNA analysis by flow cytometry (n = 3) were shown as indicated for control cells (a), PROG-treated cells (b), and ATRA-treated cells (c). Chi-square analysis showed that the drug-induced apoptosis as measured by the area under sub-G_1 peak in the histograms were significantly higher than that in the control. The X-axis represents fluorescence intensity; the Y-axis represents relative cell numbers.

c-*myc*.[28] Thirty μg of total RNA were loaded in each lane of a 1% agarose gel containing 3% formaldehyde and transferred to nylon membranes. Blots were hybridized to the probes radiolabelled to specific activity of $1-2 \times 10^9$ cpm/μg with α-^{32}P dATP (Amersham, Buckinghamshire, United Kingdom). Then the blots were exposed to X-ray films (Kodak, New York, NY) for 3–5 days. The membranes were rehybridized with a β-actin cDNA probe to serve as an internal control.

RESULTS

PROG Inhibition of Cell Proliferation

Cell proliferation was dramatically inhibited by PROG treatment in both ovarian carcinoma cell lines (Fig. 1). After 72 hours, 1 μM PROG inhibited ^{3}H-thymidine incorporation by 23% and 21% and 10 μM PROG by 69% and 73% in 3AO and AO cell lines, respectively. In contrast, the inhibitions after 48 hours of treatment with both concentrations of PROG were less significant than those after 72 hours of treatment. Therefore, PROG treatment at 10 μM for 72 hours was chosen for the subsequent experiments.

Analysis of PROG-Induced Apoptosis

After 72 hours of 10 μM PROG treatment, as shown in Figure 2, agarose gel electrophoresis of DNA from the apoptotic cells showed the characteristic DNA fragmentation ladder. Under the same conditions, the percentage of apoptotic cells reached 70 $\pm$ 9% in 3AO cells and 49 $\pm$ 7% in AO cells, as measured by the counting method (Fig. 3). Under the same conditions, cell apoptosis was approximately 76 $\pm$ 6% in 3AO cells and 55 $\pm$ 6% in AO cells, as determined by the sub-G1 peak in the flow cytometry histograms (Fig. 4). The two independent assays in this study gave similar results, and thus clearly demonstrated that PROG could indeed promote apoptosis in the ovarian carcinoma cells tested. The ability of PROG to induce apoptosis is apparently higher than that of ATRA (also at 10 μM for 72 hours), which is reported to induce apoptosis of breast carcinoma cells.[29]

Cell Cycle Analysis

The nuclear DNA content of individual cells in each population was determined by flow cytometry. The

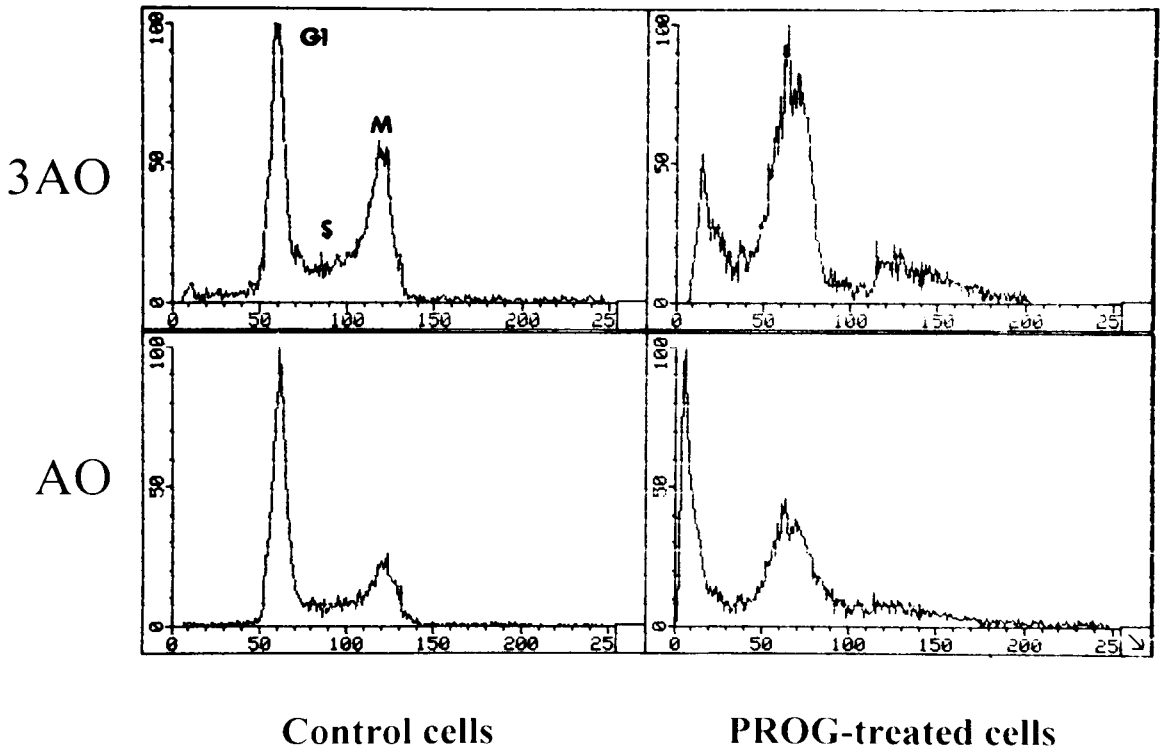

FIGURE 5. Apoptosis induced by progesterone (PROG) is preceded by arrest in G_1 of the cell cycle. PROG (10μM) was added to two proliferating 3AO and AO cells. Seventy-two hours after feeding, the cells were fixed, stained with propidium iodide, and analyzed by flow cytometry. Cell cycle profiles are shown.

results are presented in Figure 5. Seventy-two hours after treatment with PROG, the peak representing 3AO cells with G_1 DNA content had increased from 37.0 to 72.5%, and from 53.5 to 73.8% in AO cells, whereas the fraction of cells in S-phase had decreased from 31.9 to 11.3% in 3AO cells and from 26.6 to 13.4% in AO cells.

PROG Induces Up-Regulation of p53 Expression

Among the apoptosis-related genes we tested, after the cells were treated with 10 μM PROG for 72 hours, only the level of p53 mRNA markedly increased in both 3AO and AO cells as detected by Northern blot analysis (Fig. 6). The mRNA levels of *bcl*-2 and c-*myc,* however, were not significantly changed by the PROG treatment in the two cell lines (data not shown).

DISCUSSION

In the clinical treatment of ovarian carcinoma, PROG is usually used at a concentration 10–100 times higher than its physical concentration during luteal phase (about 50 nM).[30] Molecular mechanisms of PROG antitumor activity, though commonly believed to induce differentiation or growth inhibition, are not yet fully understood. In this study, our data clearly established that PROG can promote apoptosis in ovarian carcinoma cells besides its inhibition effects on cell growth. PROG at 10 μM is more potent in inducing the apoptosis than at 1 μM, indicating its dose-dependent manner.

It was previously reported that the use of COCs to treat postmenopausal women reduces ovarian carcinoma risk during estrogen replacement therapy.[4] Our results suggest that antitumor activity of COCs may come from its main component, progestogen, since PROG has been shown to induce apoptosis in the ovarian carcinoma cells here.

Retinoids, which belong to the same superfamily of nuclear receptor ligands, have been also widely used in the clinical treatment of some types of cancers. Recently, there have been some reports that retinoids can induce apoptosis in different cell lines, such as leukemia,[31] breast carcinoma,[32] and neuroblastoma cells lines.[29] Our study shows that ATRA can induce apoptosis in both ovarian carcinoma cell lines tested, suggesting that the retinoids may be applied to treat ovarian carcinomas clinically. However, the apoptotic effect of ATRA, in the parallel experiments in this

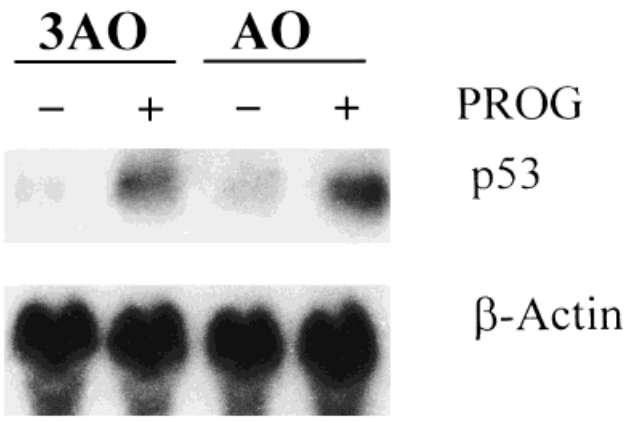

FIGURE 6. Progesterone (PROG) induced changes of the mRNA levels of p53 in 3AO and AO cells are shown. Northern blot analysis was carried out as described in ''Materials and Methods.'' After 72 hours of treatment with 10 μM PROG for 72 hours, the total RNA were extracted from both cells, and the equal amount of RNAs was loaded in each lane. β-actin mRNA was used as an internal control. Two independent experiments gave similar results.

study, was not so powerful as that of PROG. This is probably because both 3AO and AO cells are estrogen- and progestogen-dependent, so they are more sensitive to PROG than to ATRA.

As reported, the programmed cell death or apoptosis is a physiologic and genetically controlled multistep process.[33] Among the genes which regulate apoptosis, p53 and c-*myc* are primary apoptosis-promoting genes, and *bcl*-2 is a major apoptosis-suppressing gene. A pivotal role for p53 in the control of apoptosis has been demonstrated by experiments that the wild-type form of this protein induces rapid programmed cell death in leukemic cells.[34,35] Studies with thymocytes of p53 knockout mice further reveal the existence of two apoptotic pathways, one initiated by DNA damage, which requires p53, and the other stimulated by glucocorticoids and Ca^{2+} ionophores, which are p53-independent.[36] Wild-type p53 can trigger G_1 cell cycle arrest[37,38] and regulate a set of genes playing a role in the passage from G_1 to S.[39] In this study, the level of p53 mRNA increased in response to PROG, whereas that of c-*myc* and *bcl*-2 mRNA did not change. Our results thus suggest that p53 may be involved in PROG-induced apoptosis in 3AO and AO cells.

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