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Apoptosis induced by droloxifene and c-*myc*, bax and bcl-2 mRNA expression in cultured luteal cells of rats

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Abstract

Droloxifene is a tamoxifen derivative whose effects in the therapy of human breast cancer and postmenopausal osteoporosis have been studied widely. We had found that droloxifene could induce apoptosis of luteal cells of rat in vitro, but its mechanisms were unknown. In the present study, the expression of c-*myc*, bax and bcl-2 mRNA in cultured rat luteal cells during apoptosis induced by droloxifene was investigated and possible associations between these genes and apoptosis were analyzed. Cultured luteal cells of rats were incubated with droloxifene at various concentrations and with treatment durations. Occurrence of apoptosis was detected by terminal deoxyribonucleotidyl tranferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL), DNA staining and DNA electrophoresis. Expression of these genes' mRNA was determined by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). The results showed that the c-*myc* and bax mRNA levels increased as concentrations or treatment durations of droloxifene increased, while the bcl-2 mRNA level exhibited no changes. A marked increase of c-*myc* and bax mRNA appeared respectively with 12 and 24 h of treatment, while a clear increase of apoptosis of luteal cells was found at 18 h. These results suggested that droloxifene could induce apoptosis of luteal cells of rat in vitro. The increase of c-*myc* mRNA expression might be one of the initiating factors and the elevated ratio of bax/bcl-2 mRNA was also probably involved in this effect.

Keywords: Droloxifene; Luteal cell; Apoptosis; c-*myc*; bax; bcl-2; mRNA

1. Introduction

Apoptosis is a kind of physiological cell death that plays a key role in the maintenance of homeostasis of various animal species (Kerr et al., 1972; Raff, 1992). Recently, it was found to be involved in regression of the corpus luteum in many species, such as rat (Matsuyama et al., 1996), cattle (Juengel et al., 1993; Zheng et al., 1994), rabbit (Nicosia et al., 1995), ewe (Rueda et al., 1995), non-human primate (Young et al., 1997) and the human (Shikone et al., 1996; Yuan and Guidice, 1997). As an ovarian tissue that develops from a follicle after ovulation, corpus luteum synthesizes and secretes progesterone that is essential for the establishment and maintenance of pregnancy in mammals. If luteal function is blocked or disturbed, the implantation process and early pregnancy may be interfered with or even terminated. So, to study the compounds that induce apoptosis of luteal cells may contribute to the development of new agents for terminating implantation and early pregnancy.

Droloxifene, a tamoxifen derivative, is a novel antiestrogen (Eppenberger et al., 1991). It has theoretical advantages over tamoxifen in the therapy of human breast cancer. Based on preclinical data, these are higher affinity to the estrogen receptor, higher anti-estrogenic to estrogenic ratio, more effective inhibition of cell growth and division in estrogen receptor-positive cell lines, and less toxicity (Hasmann et al., 1994). Droloxifene may also be a potentially useful agent for the therapy of postmenopausal osteoporosis (Ke et al., 1995) because it can prevent estrogen deficiency-induced bone loss without causing uterine hypertrophy. The induction of apoptosis by droloxifene may be the common mechanism for both its estrogen agonist effects in bone and its antagonist effects in breast tissue (Grasser et al., 1997). In our previous study, we had

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found that droloxifene could induce apoptosis of rat luteal cells in vitro (Leng et al., 1999). Further studies showed that this drug had an anti-implantation effect and also could induce apoptosis of luteal cells of pre-implantation in pregnant rats (unpublished). However, the mechanisms are unknown.

Apoptosis is modulated by the expression of a number of regulatory genes, especially some oncogenes and tumor suppressor genes, such as p53, c-*myc*, bcl-2, and bax Staunton and Gaffney, 1998). The interaction between the proto-oncogene c-*myc* and members of the bcl-2 family may play an important role in the control of cell apoptosis (Bissonnette et al., 1992). These genes were also involved in the control of apoptosis of luteal cells (Fraser et al., 1995; Rueda et al., 1997). The localization of c-*myc* mRNA in human ovaries has been demonstrated (Piontkewitz et al., 1997), and it might interact with other factors to induce apoptosis during structural luteal regression in primates (Fraser et al., 1995). In the regressed corpus luteum of bovines, the bax mRNA level increases and might be associated with apoptosis of luteal cells (Rueda et al., 1997). In the human, the expression of BCL-2 protein has also been demonstrated in the corpus luteum, although its content remains unchanged throughout the luteal phase (Rodger et al., 1995).

Therefore, in order to elucidate the mechanisms of apoptosis induced by droloxifene, the objective of the present study was to observe the effects of droloxifene on the expression of c-*myc*, bax and bcl-2 mRNA in cultured rat luteal cells and to analyze the possible associations between these genes and apoptosis induced by droloxifene.

2. Materials and methods

2.1. Drugs and reagents

Droloxifene was synthesized by Prof XIA Peng (Department of Organic Chemistry, Shanghai Medical University). Collagenase (type II), dimethyl sulfoxide, 3′,3-diaminobenzidine, 4′,6-diamido-2-phenylindole hydrochloride (DAPI) were purchased from Sigma. McCoy's 5A medium, TRIZOL Reagent, Moloney murine leukemia virus reverse transcriptase and proteinase K were obtained from Gibco BRL. Programmed Cell Death Assay Kits, PCR Marks, dithiothreitol and ribonuclease inhibitor were purchased from Sino–American Biotechnology. *Taq* DNA polymerase, deoxyribonucleoside triphosphate and random hexamers were obtained from Promega.

2.2. Animals

Immature female Sprague–Dawley rats (22–25 days) were obtained from SIPPR/BK (Shanghai, China). The rats were housed in an air-conditioned room with controlled lighting (12 h light/dark) and allowed free access to water and rat chow. Each rat was injected subcutaneously with 65 IU of pregnant mare serum gonadotropin followed by 35 IU of human chorionic gonadotropin 65 h later. Five days after injection of human chorionic gonadotropin, rats were killed by cervical dislocation for collection of corpora lutea.

2.3. Luteal cell culture

Ovaries were excised from rats treated with pregnant mare serum gonadotropin and human chorionic gonadotropin. Corpora lutea were separated from ovaries under a dissection microscope, then stabbed with a needle to disperse luteal cells. The remaining tissues were cut into pieces and digested with 100 U/ml collagenase at 37°C for 20 min. Dispersed luteal cells were washed with McCoy's 5A medium four times, and seeded at a density of 5×10^5 cells/dish into 35-mm dishes in 1.5 ml of McCoy's 5A medium supplemented with 10% neonatal bovine serum. Cells were cultured in 5% CO_2 at 37°C. 24 h later, cultured cells were incubated with droloxifene, 0.001, 0.002 and 0.004 mM, for 24 h or treated with droloxifene, 0.004 mM, for 6, 12 and 18 h. In the control group, cells were incubated with medium containing 0.2% dimethyl sulfoxide, which had no effect on the cells (Leng et al., 1999). Apoptosis of luteal cells was detected in situ by terminal deoxyribonucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) and a DNA staining method. DNA and RNA of cells were extracted and analyzed.

2.4. Apoptosis assays

2.4.1. Apoptosis in situ detection:

Cultured luteal cells were incubated with droloxifene of 0.004 mM for different durations and apoptosis of cells was detected by TUNEL and a DNA staining method.

TUNEL detection was carried out according to the kit supplier's instructions. Briefly, cells were fixed with 10% neutral buffered formalin for 30 min and treated for 5 min at room temperature with 0.2% Triton X-100. After three washes with distilled water, endogenous peroxidase was blocked by incubation in 0.3% hydrogen peroxide for 20 min. Then, the cells were incubated with labeling buffer containing terminal deoxynucleotidyl transferase and biotin-11-deoxyuridine triphosphate (dUTP) for 1 h at 37°C in a moisture chamber. After three washes with phosphate-buffered saline (PBS, pH7.4), the cells were treated with horseradish peroxidase-conjugated avidin for 30 min at 37°C followed by four washes with PBS. Nuclei exhibiting DNA fragmentation were visualized by incubation in 3′,3-diaminobenzidine of 0.5 g/l for 10min. Finally, the cells were counterstained with hematoxylin and observed under light microscopy. The nuclei of apoptotic cells that contained DNA fragments were stained brown.

DNA staining was performed using the method of Aharoni (Aharoni et al., 1995). Luteal cells were fixed for 30 min at 24°C with 3% paraformaldehyde in PBS (pH7.4), washed intensively with PBS, and treated for 4 min with 1% Triton X-100/PBS followed by PBS washing. Then, cells were incubated for 60 min at 24°C with 0.5 mg/l DAPI in PBS. After intensive washing with PBS, the cells were observed using a fluorescent microscope (filter BP 436). Cells, which contained highly dense and irregular nuclear chromatin inclusions, were defined as apoptotic, while the non-apoptotic cells showed more homogeneous and moderate DNA staining throughout the nucleus.

2.4.2. DNA extraction and agarose gel electrophoresis

DNA was extracted from the luteal cells using a method modified from Rueda et al. (1995). Briefly, cells were lysed in lysis buffer (pH 8.0; 10 mM Tris–HCl, 100 mM EDTA, and 0.5% sodium dodecyl sulfate, w/v) at 37°C for 1 h and then- incubated with proteinase K (50 μg/ml, final volume) at 55°C for 21 h in a water bath. After incubation, each sample was cooled on ice for 60 min, and ice-cold saturated phenol/chloroform/isoamyl alcohol (25:24:1, v:v:v) was added. The samples were centrifuged at $8500 \times g$ for 10 min and the aqueous phase was extracted twice again. Then, the aqueous phase was recovered, and DNA was precipitated with −20°C 100% ethanol overnight. After two rinses with ice-cold 70% ethanol, each DNA sample was air-dried, and resuspended in Tris EDTA buffer (pH 8.0; 10 mM Tris–HCl, 1 mM EDTA). The isolated DNA was subjected to electrophoresis on 2% agarose gel for 4 h at 40 V using a 1 × TAE (40 mM Tris-acetate, 2 mM EDTA) running buffer. The gels were stained with ethidium bromide and photographed under UV light.

2.5. Total RNA isolation and semi-quantitative RT-PCR analysis

Total RNA of the luteal cells was extracted using TRIZOL reagent according to the supplier's instructions. RNA was quantitated by optical density measurements at 260 and 280 nm using a spectrophotometer, and integrity was confirmed by running 2 μg RNA on a 1% agarose gel.

Reverse transcription was performed in a volume of 20 μl reaction mixture containing 1 μg of total RNA, 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 20 units of ribonuclease inhibitor, 0.5 mM deoxyribonucleoside triphosphate, 0.5 μg random hexamers and 200 units of Moloney murine leukemia virus reverse transcriptase at 37°C for 60 min. Then, reverse transcriptase was inactivated by heating at 95°C for 5 min. Resulting reverse transcription products were stored at −70°C until use.

The PCR primers of β-actin and c-*myc* were synthesized according to the references (Ou et al., 1998; Nishimura et al., 1992). The PCR primers of bax and bcl-2 were designed strictly using OLIGO software according to the DNA sequence of bax and bcl-2 (Tilly et al., 1995; Sato et al., 1994). Table 1 shows the detailed sequences of the primers and the expected size of PCR products.

PCR was carried out in a volume of 50 μl reaction mixture which contained 1 μl reverse transcription product, 50 mM KCl, 10 mM Tris–HCl (pH 9.0), 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.3 mM deoxyribonucleoside triphosphate, 0.3 μM of each specific PCR primer, and 2 U of *Taq* DNA polymerase. The samples were overlaid with light mineral oil and heated to 94°C for 5 min. The cycles were 94°C for 45 s, 55°C for 45 s, and 72°C for 1.5 min. Final extension was for 7 min at 72°C. The cycles performed are listed in Table 1. PCR of β-actin that was chosen as an internal control was carried out in the same tubes as for the genes. All the cycles chosen were in the range of linear relationship between the PCR products and the number of cycles (data not shown). PCR products were run on a 1.5% agarose gel containing ethidium bromide and viewed under UV light. The PCR signal intensities were semi-quantified by scanning the gels using a UVP white/UV Transilluminator and analyzed with Gelworks 1D Intermediate software. All c-*myc*, bax and bcl-2 densitometric measurements were normalized with the internal control, β-actin.

Table 1
Sequences of primers and cycles used in RT-PCR analysis of c-*myc*, bax and bcl-2 mRNA

Gene	Sequences	Location	Size of PCR products (bp)	PCR cycles
c-*myc*	5'-AACTTACAATCTGCGAGCCA-3'	2407	342	30
	5'-AGCAGCTCGAATTTCTTCCAGATAT-3'	4305		
bax	5'-TGGCGATGAACTGGACAACAAC-3'	92	301	28
	5'-CCCGAAGTAGGAAAGGAGGC-3'	373		
bcl-2	5'-CACCCCTGGCATCTTCTCCT-3'	366	349	30
	5'-GTTGACGCTCCCCACACACA-3'	695		
β-actin[a]	5'-AACCGTGAAAAGATGACCCAG-3'	1671	741	28 for bax
	5'-CTCCTGCTTGCTGATCCACAT-3'	3067		30 for c-*myc* and bcl-2

[a] Internal control.

2.6. Statistical analysis

The data are expressed as the means ± S.E.M. Statistical differences were evaluated with Student's *t*-test. Differences were considered to be significant if $P < 0.05$.

3. Results

3.1. Occurrence of apoptosis in cultured rat luteal cells treated with droloxifene

Cultured luteal cells were treated with 0.004 mM droloxifene for 6, 12 or 18 h, and the occurrence of apoptosis was identified using the TUNEL method and DNA staining. In the control group, almost all the cells were normal and apoptotic nuclei which showed positive staining by TUNEL detection or condensed chromatin by DNA staining with DAPI were only seldom visible (Fig. 1A,B). After 6-h treatment with droloxifene, no difference could be observed between the treated and control groups (data not shown). Only a few of the cells that were incubated with droloxifene, 0.004 mM, for 12 h were apoptotic (Fig. 1C,D). However, a marked increase of apoptotic cells appeared after 18-h treatment with droloxifene. TUNEL detection showed many apoptotic nuclei which stained positively (Fig. 1E), and after being stained with DAPI, lots of apoptotic cells which contained condensed nuclei and fragmented chromatin appeared (Fig. 1F).

DNA was extracted from the cultured luteal cells that were treated with 0.004 mM droloxifene for 12 or 18 h, and the occurrence of apoptosis was detected by agarose gel electrophoresis. A typical "DNA ladder" which represents formation of oligonucleosomes was prominent in cells incubated with droloxifene for 18 h, while no "DNA

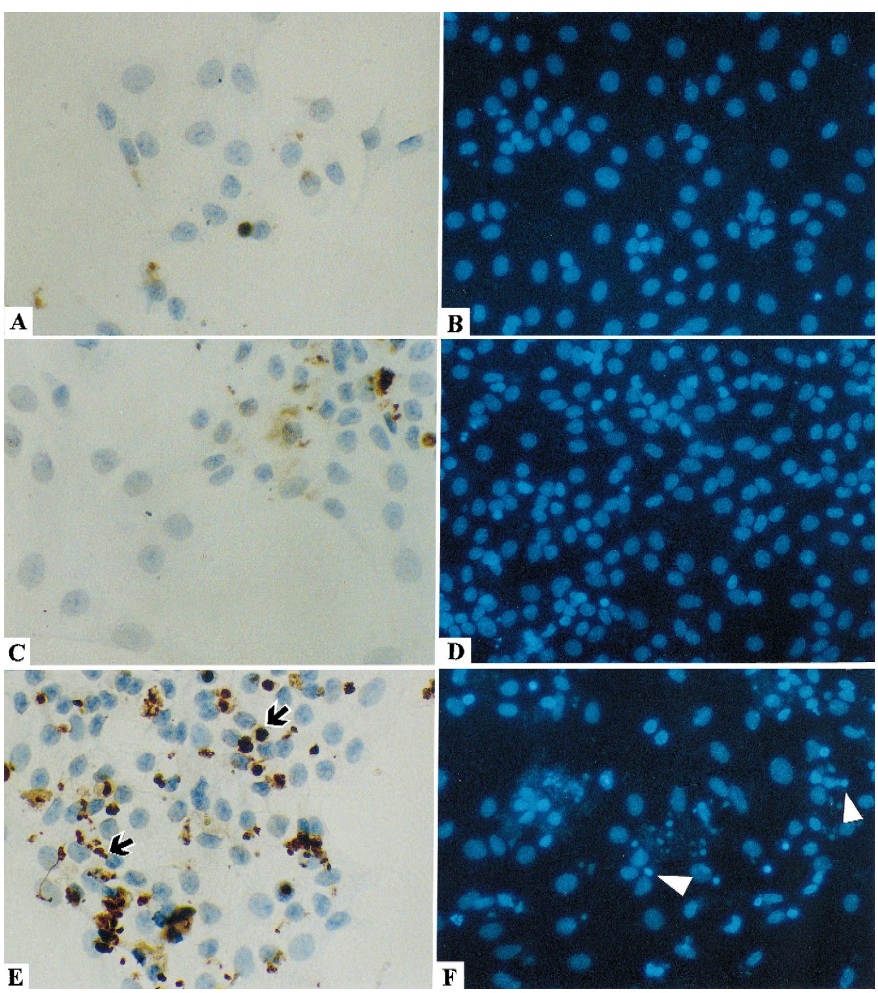

Fig. 1. Apoptosis of cultured luteal cells of rat induced by droloxifene. Cultured cells were incubated with droloxifene, 0.004 mM for 12 h (C, D), 18 h (E, F) or without droloxifene (A, B). TUNEL method (A, C, E) and DNA staining with DAPI (B, D, F) were used to identify the occurrence of apoptosis. Almost all cells in the control group were normal, and only a few apoptotic cells could be observed after 12-h incubation with droloxifene. However, lots of apoptotic cells appeared after 18-h treatment with droloxifene. The arrows and arrow heads indicate apoptotic cells identified by the TUNEL method or DNA staining respectively. Scale bars represent 20 μm.

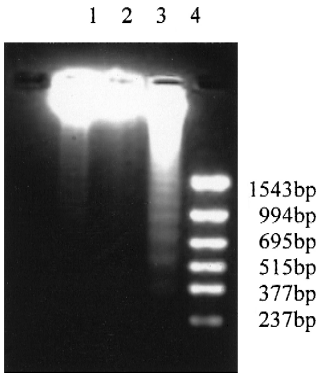

Fig. 2. Apoptosis of cultured luteal cells of the rat induced by droloxifene. Agarose gel electrophoretic analysis of DNA isolated from cells incubated with droloxifene, 0.004 mM, for 12 h (lane 2) and 18 h (lane 3) or without droloxifene (lane 1). Lane 4 was a DNA marker.

ladder" was found in the control or the 12-h incubation group (Fig. 2).

3.2. Expression of c-myc mRNA

Semi-quantitative RT-PCR analysis revealed a low level c-*myc* mRNA in the control group, while its expression increased greatly in cells treated with droloxifene at increasing concentrations or treatment durations (Fig. 3). A significant increase ($P < 0.05$) in c-*myc* mRNA expression could be observed in cells which were treated with 0.004 mM droloxifene for 12 h or longer. Cells treated with 0.004 mM droloxifene for 24 h exhibited an 8-fold increase in c-*myc* mRNA level compared with the control group.

3.3. Expression of bax mRNA

The expression of bax mRNA in cultured rat luteal cells was confirmed by semi-quantitative RT-PCR analysis. Like the changes of c-*myc* mRNA expression, the level of bax mRNA also increased with increasing droloxifene concentrations or treatment durations (Fig. 4). However, the increase in bax mRNA expression was obviously less than that of c-*myc*. Only in the rat luteal cells incubated with 0.004 mM droloxifene for 24 h, was a significant increase of bax mRNA observed, and there was only a 1.9-fold increase as compared to the control group.

3.4. Expression of bcl-2 mRNA

Semi-quantitative RT-PCR analysis also revealed the expression of bcl-2 mRNA in cultured rat luteal cells. However, there were no changes in bcl-2 mRNA expression in cells treated with droloxifene, using various concentrations and treatment durations (Fig. 5).

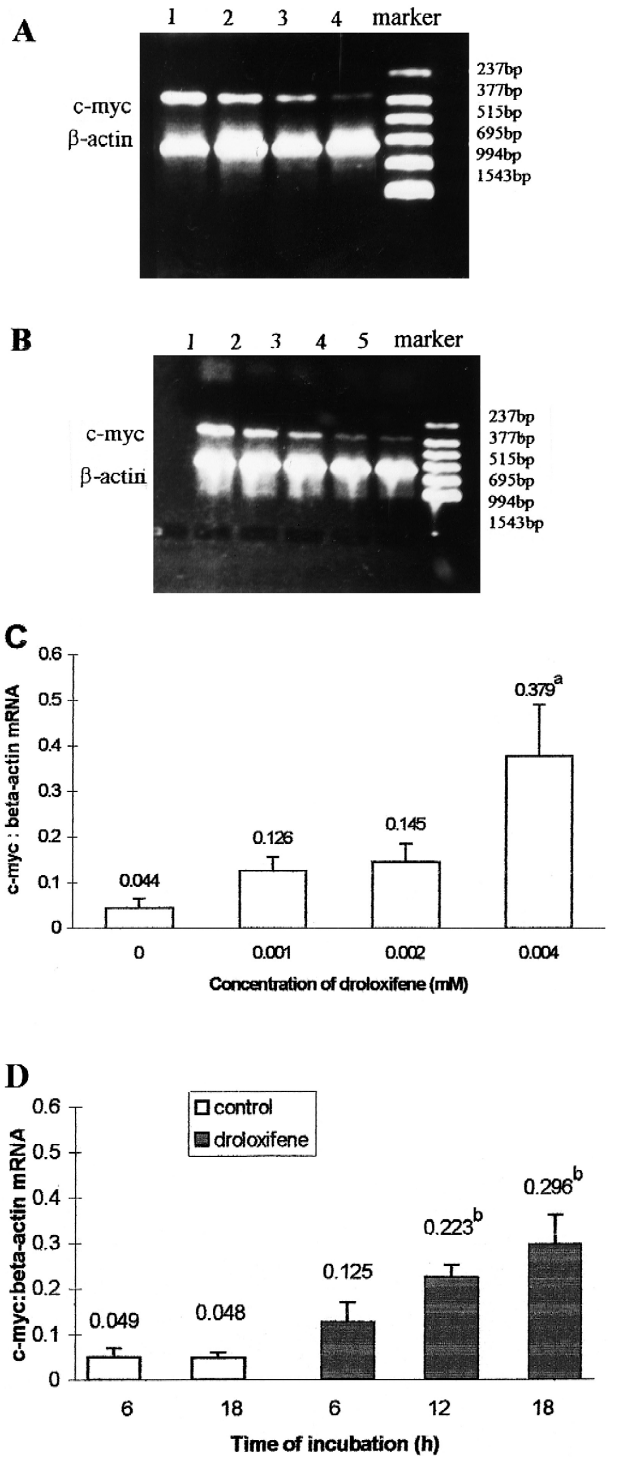

Fig. 3. Expression of c-*myc* mRNA in cultured rat luteal cells treated with droloxifene using various concentrations and durations. (A, B) Agarose gel eletrophoresis of RT-PCR product of c-*myc* and β-actin mRNA isolated from cultured rat luteal cells incubated with droloxifene of 0.004 mM (lane 1), 0.002 mM (lane 2), 0.001 mM (lane 3) and without droloxifene (lane 4) for 24 h (A) or treated with 0.004 mM droloxifene for 18 h (lane 1), 12 h (lane 2), 6 h (lane 3) and without droloxifene for 18 h (lane 4) or 6 h (lane 5) (B). (C, D) Semi-quantitative analysis of the c-*myc* mRNA level, using densitometric scanning of the eletrophoresis. Values represent means ± S.E.M. ($n = 3$ at each point) of the ratio of RT-PCR product of c-*myc* to β-actin. [a]$P < 0.05$ vs. control, [b]$P < 0.01$ vs. control.

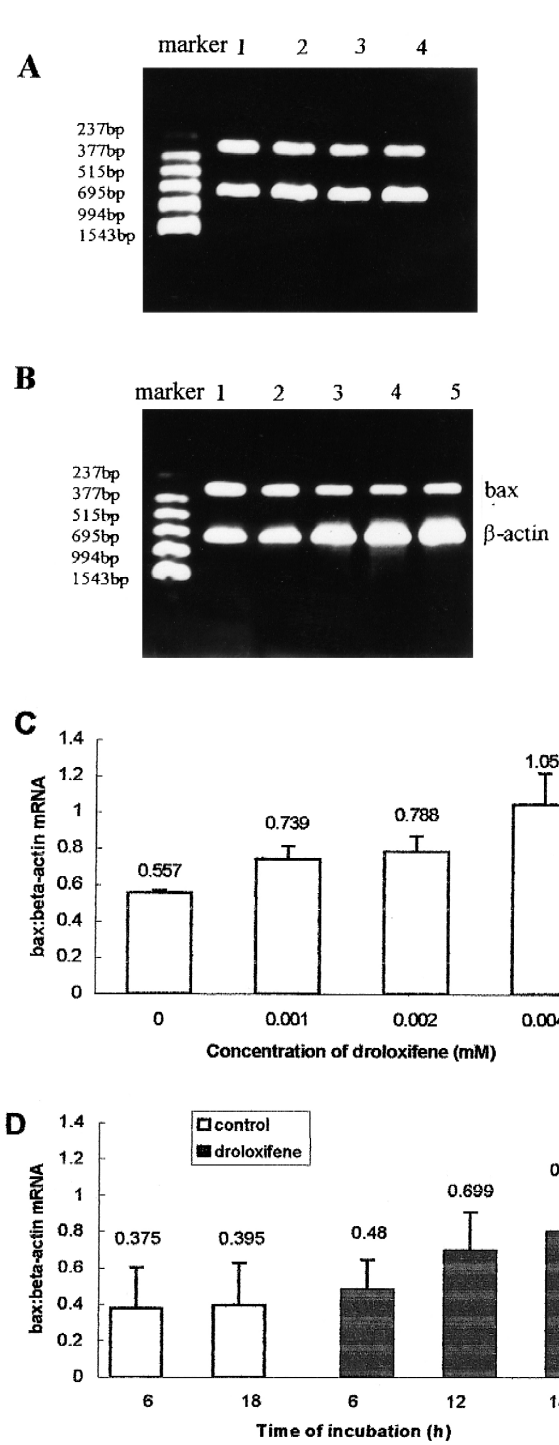

Fig. 4. Expression of bax mRNA in cultured rat luteal cells treated with droloxifene, using various concentrations and durations. (A, B) Agarose gel eletrophoresis of RT-PCR product of bax and β-actin mRNA isolated from cultured rat luteal cells incubated with droloxifene, 0.004 mM (lane 1), 0.002 mM (lane 2), 0.001 mM (lane 3) and without droloxifene (lane 4) for 24 h (A) or treated with 0.004 mM droloxifene for 18 h (lane 1), 12 h (lane 2), 6 h (lane 3) and without droloxifene for 18 h (lane 4) or 6 h (lane 5) (B). (C, D) Semi-quantitative analysis of bax mRNA level with densitometric scanning of the eletrophoresis. Values represent means ± S.E.M. ($n = 3$ at each point) of the ratio of RT-PCR product of bax to β-actin. [a] $P < 0.05$ vs. control.

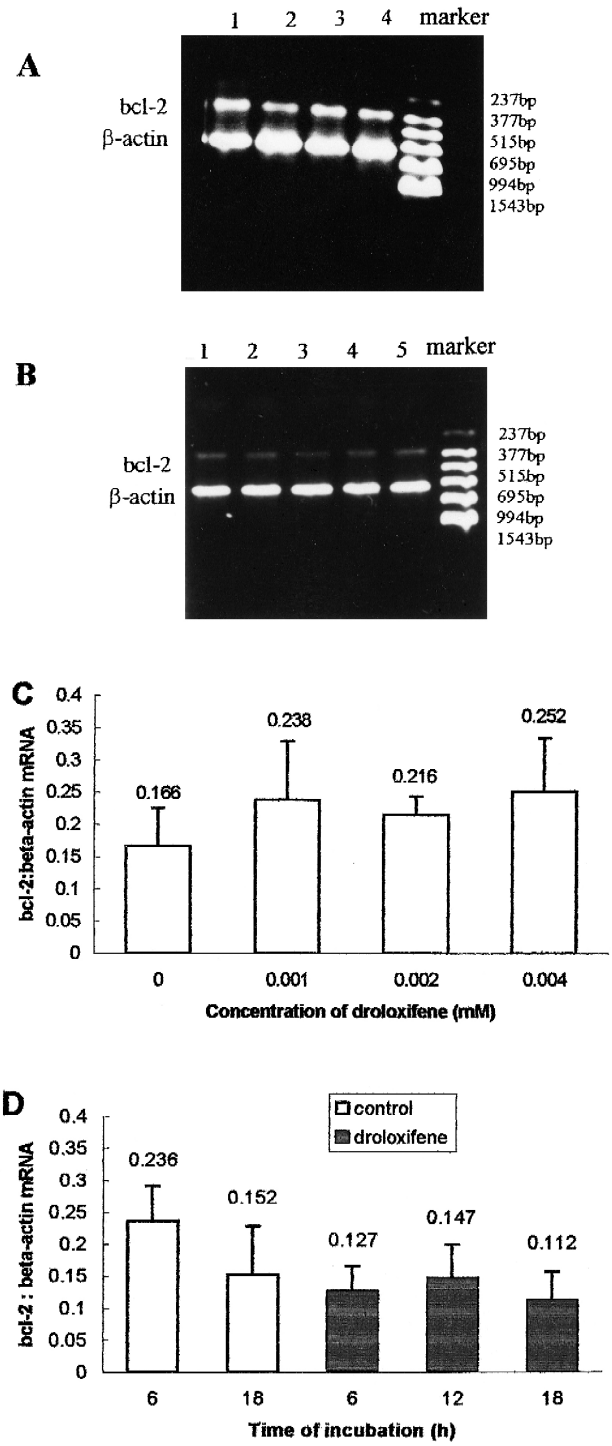

Fig. 5. Expression of bcl-2 mRNA in cultured rat luteal cells treated with droloxifene, various concentrations and durations. (A, B) Agarose gel eletrophoresis of RT-PCR product of bcl-2 and β-actin mRNA isolated from cultured rat luteal cells incubated with droloxifene of 0.004 mM (lane 1), 0.002 mM (lane 2), 0.001 mM (lane 3) and without droloxifene (lane 4) for 24 h (A) or treated with 0.004 mM droloxifene for 18 h (lane 1), 12 h (lane 2), 6 h (lane 3) and without droloxifene for 18 h (lane 4) or 6 h (lane 5) (B). (C, D) Semi-quantitative analysis of bcl-2 mRNA level with densitometric scanning of the eletrophoresis. Values represent means ± S.E.M. ($n = 3$ at each point) of the ratio of RT-PCR product of bcl-2 to β-actin.

4. Discussion

Corpus luteum plays a key role in the establishment and maintenance of pregnancy in mammals. In the human, production of progesterone by this gland is essential for the first 8 weeks of gestation (Auletta and Flint, 1988). Abnormal regression of corpus luteum will disturb or even terminate the implantation process and early pregnancy. It is of much importance to study drugs that can induce corpus luteum regression. Droloxifene is an anti-estrogenic compound whose effect in the therapy of human breast cancer and postmenopausal osteoporosis has been studied widely (Hasmann et al., 1994; Ke et al., 1995). Recently, we found that it could induce apoptosis of rat luteal cells in vitro after 24-h treatment, and apoptotic cell numbers increased with increasing concentrations of droloxifene. In the present study, luteal cell apoptosis induced by droloxifene was confirmed again, and possible relationships between the expression of c-*myc*, bax and bcl-2 mRNA and apoptosis of luteal cells were considered.

It has already been demonstrated that 0.004 mM droloxifene could induce apoptosis of luteal cells in vitro. We now found that the "DNA ladder," which represents formation of oligonucleosomes, appeared in cultured luteal cells after an 18-h treatment with 0.004 mM droloxifene, but not in cells treated for 12 h. TUNEL detection and DNA staining of DAPI also showed that the marked increase of apoptotic cells appeared with 18 h of treatment with droloxifene. So, all these results suggest that apoptosis of cultured luteal cells induced by 0.004 mM droloxifene occurred between 12 and 18 h of treatment.

The proto-oncogene, c-*myc*, has been shown to play a pivotal role in cell proliferation and DNA synthesis, but in some tissues its expression under conditions of growth arrest is associated with the initiation of apoptosis (Hoffman and Liebermann, 1998). The expression of the c-*myc* gene in ovaries of humans (Putowski et al., 1997; Li et al., 1994), primates (Fraser et al., 1995) and rats Piontkewitz et al., 1997; Delidow et al., 1990) had been confirmed. However, the exact contribution of this proto-oncogene to the apoptosis of luteal cells during regression of the corpus luteum is not clear. In non-human primates, the increase of c-Myc protein expression may be associated with the apoptosis of luteal cells induced by a prostaglandin $F_{2\alpha}$ analogue or gonadotropin releasing hormone (GnRH) antagonist (Fraser et al., 1995). Here, our results showed that c-*myc* mRNA was expressed in cultured rat luteal cells, and that its level increased with increasing concentrations of droloxifene after 24-h treatment. The tendency of c-*myc* mRNA level to increase corresponded with the increase of apoptotic cell numbers induced by increasing concentrations of droloxifene. The marked increase of the c-*myc* mRNA level ($P < 0.01$) induced by 0.004 mM droloxifene appeared after 12 h of treatment, while the clear increase in apoptosis of luteal cells was found at 18 h. Therefore, the increase of c-*myc* mRNA expression occurred earlier than did apoptosis, which suggested that the increase of c-*myc* mRNA level might be one of the initiating factors involved in the apoptosis of luteal cells induced by droloxifene in vitro.

The c-*myc* proto-oncogene has two coupled functions: proliferation and apoptosis. These opposing roles of c-*myc* require that other gene products should interact with c-*myc* to determine the final outcome of cells. A candidate for such a modifying gene is bcl-2 (Reed, 1994). Activated bcl-2 gene could prevent apoptosis induced by c-Myc (Bissonnette et al., 1992), and bax, another bcl-2 family gene was observed to be increased when c-Myc was over expressed (Sakamuro et al., 1995). BCL-2 is known to protect against apoptosis triggered by a wide range of factors. However, the inhibitory effect of BCL-2 on apoptosis is determined by the interaction with BAX, a 21kDa protein with a degree of homology to BCL-2. BCL-2 can form heterodimers with BAX and lose its protective effect. When BCL-2 is present in excess, cells are protected from apoptosis. However, when BAX is in excess and the homodimers of BAX dominate, cells are susceptible to programmed cell death. So, it appears to be the relative ratios of BCL-2 and BAX that determine the fate of a cell, rather than the absolute concentrations of either (Oltvai et al., 1993). We now found that both bax and bcl-2 mRNA are expressed in cultured luteal cells of rats. However, the changes in these two genes induced by droloxifene were different. The level of bax mRNA in cultured luteal cells of rat increased with increasing droloxifene concentrations or treatment durations. This finding was similar to the increased bax mRNA found during corpus luteum regression of bovines, and in contrast to the consistent expression of BAX protein during corpus luteum maintenance or regression in humans, which suggested that mechanisms leading to apoptosis of luteal cells might be different in different species. In contrast to the elevated bax mRNA levels, the expression of bcl-2 mRNA showed no apparent changes after treatment with droloxifene at various concentrations and durations. Therefore, according to the increased bax/bcl-2 ratio, the inhibitory effect of bcl-2 on c-*myc*- induced apoptosis might have been blocked, and excessive bax mRNA expression probably stimulated the apoptosis of luteal cells, although its marked increase occurred after the appearance of "DNA ladder."

Droloxifene could induce apoptosis of luteal cells of rat in vitro, and the expression of c-*myc*, bax and bcl-2 might be involved in it. However, the relationships between apoptosis or those genes' expression in luteal cells and the anti-estrogenic effect of droloxifene are not clear. Some previous reports suggested that the effect of estrogen on the function of corpus luteum was species-linked. In humans (Endo et al., 1998) and in rhesus monkeys (Karsch and Sutton, 1976), estrogen might produce a luteolytic action, while it was the primary luteotropic hormone (Bill and Keyes, 1983; Dharmarajan et al., 1991) and withdrawal of estradiol could result in luteal cell apoptosis

(Goodman et al., 1998) in rabbits. The estrogen could inhibit apoptosis of luteal cells in the pregnant mare serum gonadotropin and human chorionic gonadotropin-induced pseudopregnant rats (Zhang et al., 1998), but another report indicated that estradiol induced luteolysis in the intact pregnant rats (Tamura, 1983). More studies should thus be done to elucidate the possible associations between apoptosis induced by droloxifene and its anti-estrogenic effect. The results could contribute to evaluation of the possibility of this compound developing into a new kind of contraceptive.

In conclusion, droloxifene induced apoptosis of luteal cells of rat in vitro. The expression of c-*myc* and bax mRNA increased with increasing droloxifene concentrations or treatment durations, while the bcl-2 mRNA level showed no changes. These results suggested that the increase in c-*myc* mRNA might be one of the initiating factors and the elevated ratio of bax/bcl-2 mRNA was also probably involved in the apoptosis induced by droloxifene.

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