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Fas, FasL, Bcl-2, and Bax in the Endometrium of Rhesus Monkey During the Menstrual Cycle

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ABSTRACT To study possible role and regulation of apoptosis occurred in primate endometrium, the expression of apoptosis-related molecules, Fas, FasL, B cell lymphoma/leukaemia-2 (Bcl-2), and Bax were analyzed in relation to occurrence of apoptosis and proliferation in the cycling endometrium of the rhesus monkey using immunohistochemistry and Western blot. The cell apoptosis and proliferation were evaluated by means of in situ 3′-end labeling and Ki67 immunostaining, respectively. The results showed that the expressions of Fas, Fas ligand (FasL), Bcl-2, and Bax were co-localized predominantly in the epithelial cells of the endometrium. Modest Fas staining with no obvious change was detected throughout the menstrual cycle, while the levels of FasL and Bax protein in the epithelial cells increased in the secretory phase when apoptosis was most prevalent. In contrast, epithelial immunostaining for Bcl-2 was maximal during the proliferative phase and decreased in the secretory phase. Bcl-2 immunoreactivity was also detected in some immunocytes. The coordinated expression of Fas, FasL, Bcl-2, and Bax in the cycling endometrium of the rhesus monkey suggests that the cyclic changes in endometrial growth and regression may be regulated by the balance of these factors under the action of ovary steroids. *Mol. Reprod. Dev. 70: 478–484, 2005.* © 2005 Wiley-Liss, Inc.

Key Words: apoptosis; proliferation; endometrium; menstrual cycle; rhesus monkey

INTRODUCTION

For preparation of embryo implantation, endometrium undergoes cyclic changes of proliferation and differentiation under regulation of estrogen and progesterone secreted by ovary. Endometrial regeneration may involve apoptosis, proteolysis, and angiogenesis, these processes have been demonstrated in the implantation site of the rhesus monkey during the early pregnancy (Feng et al., 2000, 2001; Gao et al., 2001a,b).

Apoptosis is a major phenomenon in regulating turnover of the endometrium during menstruation and implantation (Von Rango et al., 1998; Chatzaki et al., 2001, 2003). The cells undergoing apoptosis have characteristic structural changes in nucleus and cytoplasm. The nuclear disintegration involves DNA cleavage into oligonucleosomal length DNA fragments (Fehsel et al., 1991; Compton, 1992; Gavrieli et al., 1992), and the DNA fragments can be detected by terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end-labeling (TUNEL) technique. Expressions of apoptotic regulatory molecules, such as Fas, FasL, P53, and the proteins of B cell lymphoma/leukaemia-2 (Bcl-2) family, have been reported in human endometrium (Tabibzadeh, 1995; Tao et al., 1997; Watanabe et al., 1997; Ioffe et al., 1998; Selam et al., 2001). The Fas–FasL system is a major pathway for induction of apoptosis in cells and tissues (Nagata and Golstein, 1995). Fas (APO-1 or CD95) is a member of tumor necrosis factor receptor (TNFR) family which induces apoptosis via cross-linking to FasL, a member of TNF superfamily (Itoh et al., 1991; Suda et al., 1993; Yamashita et al., 1999). Bcl-2 was first discovered in the human follicular lymphoma and then regarded as a cell death repressor (Reed, 1994; Yang and Korsmeyer, 1996). Bax is a Bcl-2 family member that promotes cell death susceptibility, possibly by countering the effect of Bcl-2 on cell survival through heterodimer interaction (Ozakawa et al., 1996). Bax to Bcl-2 "rheostat" may be a critical factor in regulating apoptosis in human endometrium during the normal menstrual cycle. Members of the Bcl-2 family, such as the apoptosis-inhibiting proteins, Bcl-2 and Bcl-x_L, and the apoptosis-promoting homologues, Bax and Bak, have also been found in human endometrium (Gompel et al., 1994; Tabibzadeh, 1995; Tao et al., 1997, 1998).

The aim of the present study was to investigate the localization and possible role of Fas, FasL, Bcl-2, and Bax in the cycling endometrium of rhesus monkey.

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MATERIALS AND METHODS

Animals

Healthy, adult female rhesus monkeys (*Macaca mulatta*) from the monkey colony of the Primate Research Center, Kunming Institute of Zoology, Chinese Academy of Sciences (CAS) were used for this study. All experimental work was approved by the Animal Ethics Committees of both the Institute of Zoology and the Kunming Institute Primate Research Center, CAS. The animals were caged individually and were evaluated daily by visual examination of the perineum for menses, with the onset of menses defined as Day 1 of the menstrual cycle. Adult female monkeys with regular menstrual cycles of approximately 28 days were chosen for the experiment. The monkeys were anesthetized by an injection of ketamine, and the uteri were removed surgically and cut to pieces at menstrual cycle day (MD) 5, 10, 16, 20, and 25, respectively (three animals each group) and the specimens were quickly washed in cold phosphate-buffered saline (PBS) to remove adherent blood, then placed in cold 4% paraformaldehyde fixative for 24 hr at 4°C and further processed through graded dehydration, clearing, and embedding in paraffin for immunohistochemistry and in situ 3′-end-labeling analysis. Part of specimens was cryopreseved at −70°C for Western blot analysis.

Reagents

Primary antibodies including rabbit anti-human Fas (SC-716), rabbit anti-human FasL (SC-834), mouse anti-human Bcl-2 (SC-7382), and rabbit anti-human Bax (SC-493) were obtained from Santa-Cruz (Santa Cruz, CA); mouse anti-Ki67 (ZM0166) was purchased from Zymed (San Francisco, CA); biotin labeled secondary antibodies, alkaline phosphate-conjugated (AP-) avidin and Vector-red substrates were from Vector (Vector Laboratories, Burlingame, CA). Digoxigenindideoxy-UTP (ddUTP), TdT, blocking reagent, anti-DIG AP-antibody and 5-bromo-4-chloro-3-indoxyl phosphate/nitro-blue tetrazolium chloride (BCIP/NBT) were purchased from Roche (Mannheim, Germany). Proteinase K was purchased from Merck-Schuchardt (Darmstadt, Germany). Ficoll, levamisole, and dextrasulphate were purchased from Sigma (St. Louis, MO). SuperSignal® West Pico substrate was from Pierce (Rockford, IL).

TUNEL

Apoptotic cells were identified by using the terminal TUNEL technique. The procedure was slightly modified based on Gao et al. (2001b) as the following. Deparaffinized and hydrated 4 μm sections were first treated with 10 μg/ml proteinase K at 37°C for 20 min, and then subjected to 3′-end-labeling of the DNA with 1 μM ddUTP and 1 U/μl TdT at 37°C for 1 hr. The sections were washes three times in Tris buffer, and incubated with blocking buffer (100 mM Tris, 150 mM NaCl, pH 7.5, and 1% blocking reagent) for 30 min at room temperature. Next, sections were incubated with the primary AP-conjugated anti-DIG antibody (1:500 in 1% blocking reagent, 100 mM Tris, and 150 mM NaCl, pH 7.5) at room temperature for 2 hr, and then washed with Tris buffer. Staining was developed using the standard substrates NBT (337.5 μg/ml) and (BCIP 175 μg/ml). Negative controls were similarly processed with the omission of TdT. To estimate the percentage of apoptotic events, the number of apoptotic cells was counted in 10 high-power field areas. The number of apoptotic events was divided into three groups according to the percentage of occurrence.

Immunohistochemistry

Serial 4 μm sections of tissue were deparaffinized, and rehydrated through degraded ethanol. Antigen retrieval was performed by incubating the sections in 0.01 M citrate buffer (pH 6.0) at 98°C for 20 min and cooling at room temperature for 20 min. Non-specific binding was blocked with 10% (v/v) normal goat serum in PBS for 1 hr. The sections were incubated with the primary antibodies specific for Ki67 (4 μg/ml), Fas (2 μg/ml), FasL (2 μg/ml), Bax (1 μg/ml), and Bcl-2 (4 μg/ml), respectively in 10% goat serum at RT for 2 hr. Sections were then washed three times with PBS (10 min each) and incubated with the biotinylated secondary antibody (goat anti-rabbit IgG for Fas, FasL, and Bax; horse anti-mouse IgG for Ki67 and Bcl-2, RT, 30 min). Successive washes (3 × 10 min) were followed by incubation with avidin–alkaline phosphatase complex (1:200, RT, 20 min), sections were developed with standard substrates (337.5 μg/ml NBT and 175 μg/ml BCIP) or Vector Red substrates, according to the manufacturer's protocol after another three washes. Endogenous alkaline phosphatase activity was inhibited by supplement of 1 mM levamisole into substrate. Sections incubated with normal IgG instead of primary antibody served as negative controls. For assessment of staining in cells of different compartments, semi-quantitative subjective scoring was done blinded by three investigators. For Ki67, the percentages of immunoreactive cells were assessed on 4,000 cells/tissue section at least.

Western Blot

Immunoblot was done as previously described (Zhou et al., 2002) to verify the specificity of the antibodies used in this study with the monkey tissue and examine the difference in expression levels between proliferative and luteal phase in the tissue of MD5, MD10, MD20, and MD25. The tissues were homogenized in lysis buffer (5 mmol/L phosphate buffer, pH 7.2, containing 0.1% Triton X-100, 1 mmol/L phenylmethylsulfonylfluoride, 1 mg/L chymostatin), respectively and the protein content of the supernatant after centrifugation was determined by spectrophotometer, using bovine serum albumin as a standard. Sample lysates were mixed with the loading buffer (final concentration, 62.5 mmol/L, 1,4-dithiothreitol, 5% sodium dodecyl sulfate (SDS), and 10% glycerol), boiled for 8 min, separated by SDS–polyacrylamide gel electrophoresis (50 μg total protein/lane). After electrophoretically transferred to the polyvinylidene difluoride membrane, the membranes were blocked with

5% nonfat milk/PBS for 1 hr, followed by incubation at 20°C for 1 hr with the primary antibodies for Fas, FasL, Bcl-2, and Bax, respectively (IgG, 0.2 μg/ml) in 5% milk/PBS. The membranes were washed three times, 5 min for each, in 5% milk/PBS and incubated with HRP-conjugated horse anti-mouse IgG (0.2 μg/ml, for Bcl-2) or goat anti-rabbit IgG (0.04 μg/ml, for Fas, FasL, and Bax) in 5% milk/PBS for 1 hr, respectively. The membranes were washed in PBS three times 5 min for each, followed by 10 min of incubation with SuperSignal® West Pico substrate, then exposed on X-ray film. For negative controls, the primary antibodies were replaced with normal IgG at the same concentration and origin.

Microscopic Assessment

Endometrial samples from three individual monkeys for each group were analyzed. Experiments were repeated at least three times, and one representative from at least three similar results was presented. The mounted sections were examined using a Nikon microscope.

RESULTS

Apoptosis in the Endometrium of Rhesus Monkey

The TUNEL technique was used to identify apoptotic cell types in the endometrium at the time points of the monkey menstrual cycle on D5, D10, D16, D20, and D25. Apoptotic cells were identifiable after TUNEL staining of the endometrium during all phases of the cycle and predominantly localized in the epithelium (Fig. 1A–E). The number of apoptotic cells in the endometrium was low on MD5, MD10, and MD16, relatively high on MD20, and reached its maximum at the late secretory phase (MD25). No obvious staining was detected in the negative control.

Proliferative Activity Revealed by Ki67 Immunostaining

As shown in Figure 1F–J the Ki67-positive cells were found mainly in the epithelium, and also in the stroma and the blood vessels in less extent during the proliferative phase (MD5, MD10). The number of Ki67-positive cells in the epithelium decreased during the early secretory phase (MD16) and diminished in the endometrium of MD20 and MD25.

Expression of Fas and FasL

During the menstrual cycle, expressions of Fas (Fig. 2A–E) and FasL (Fig. 2F–J) were predominantly localized in the epithelial cells of the endometrium. Modest Fas staining with no obvious change was detected throughout the menstrual cycle, while epithelial FasL protein level increased in the secretory phase (Fig. 2H–J), but not in the proliferative endometrium (Fig. 2F,G). No obvious staining was detected in the negative controls.

Expression of Bcl-2 and Bax

The epithelial and stromal immunostaining for Bcl-2 (Fig. 3A–E) was observed from MD5 (Fig. 3A) to MD16 (Fig. 3C), with the maximum staining at MD10 (Fig. 3B), and almost no positive staining was detected in the epithelium of MD20 (Fig. 3D) and MD25 (Fig. 3E). Certain Bcl-2 positive immunocyte-like cells were found throughout the cycle with no obvious change in number and degree of the staining. The staining in some of these cells was localized to granules in the cytoplasm (Fig. 3F) under the higher magnification. Bax immunoreactivity was observed in the epithelium (Fig. 3G–K) throughout the cycle and gradually increased in a time-dependent

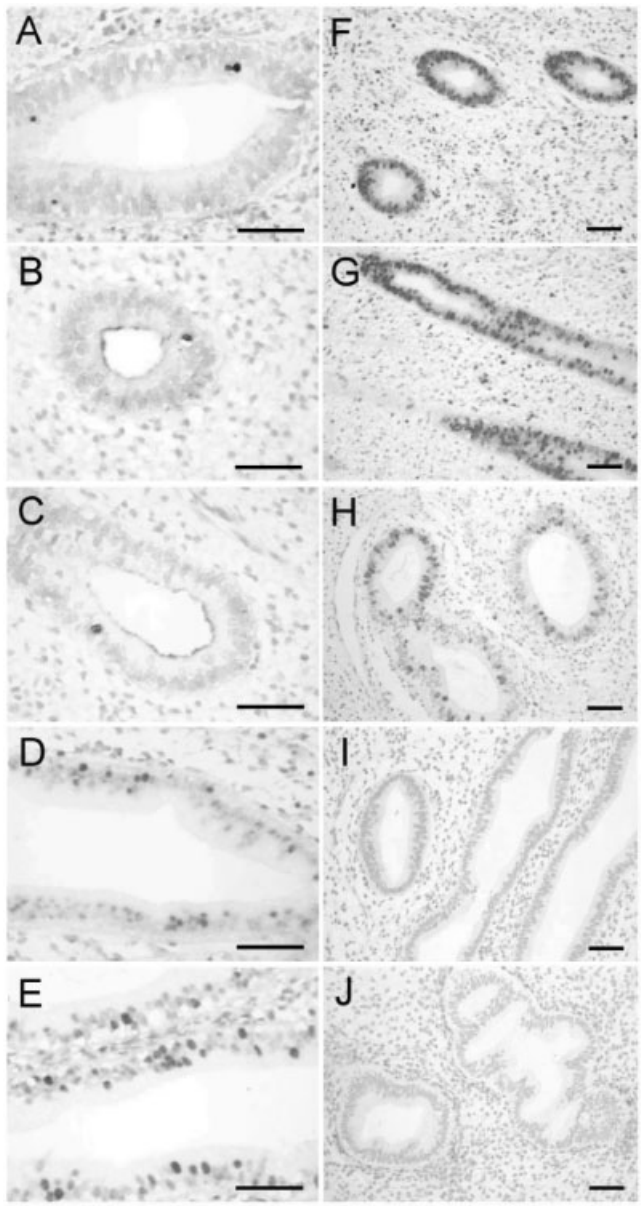

Fig. 1. The apoptosis detected by terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end-labeling (TUNEL) assay (**A**–**E**, positive nuclei are dark blue) and proliferation revealed by Ki67 immunostaining (**F**–**J**, positive nuclei are red) in the functional layer of endometrium of rhesus monkey during the menstrual cycle. A/F, MD5; B/G, MD10; C/H, MD16; D/I, MD20; E/J, MD25. Scale bars represent 50 μm.

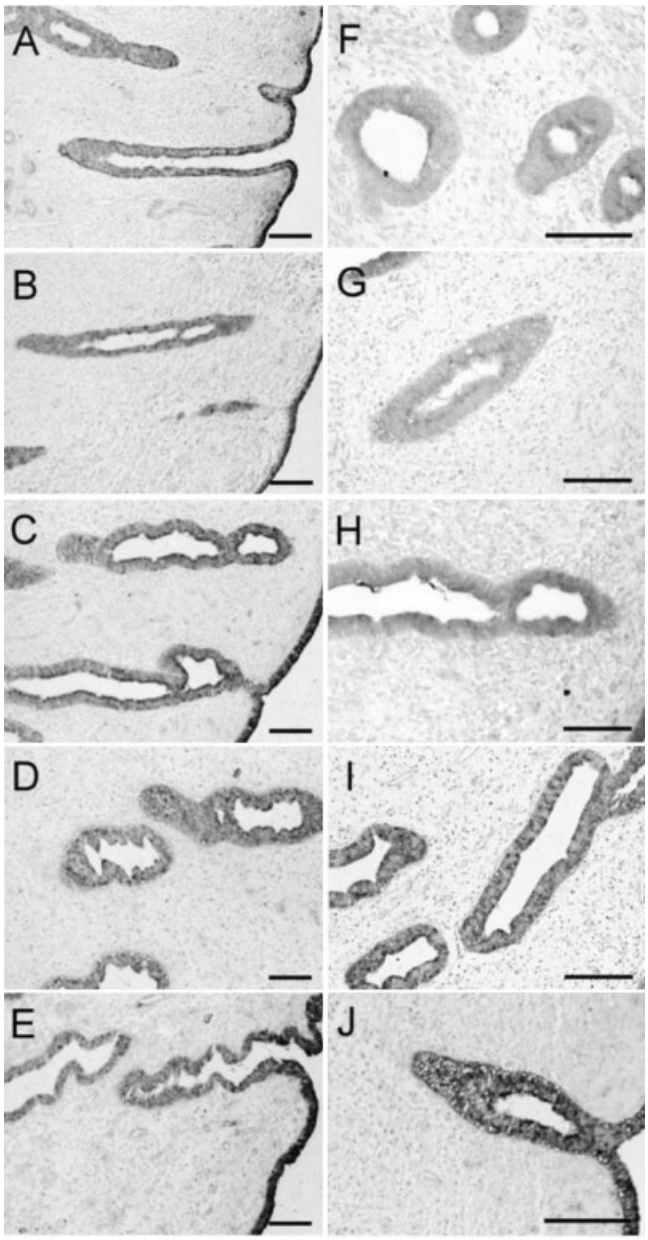

Fig. 2. Immunohistochemical staining for Fas (**A**–**E**) and FasL (**F**–**J**) in the endometrium of rhesus monkey during the menstrual cycle. Positive staining is brown. A/F, MD5; B/G, MD10; C/H, MD16; D/I, MD20; E/J, MD25. Scale bars represent 100 μm.

manner and reached the maximum on MD20 and 25 (Fig. 3J,K). A strong staining for Bax protein was also observed in some immunocytes within some blood vessels. In addition, slight staining for Bax was also detected in the stroma and blood vessels. No obvious staining was detected in the negative controls (Fig. 3L).

Western Blot

The result of Western blot assay confirmed the expression changes of these factors between proliferative and luteal phase in the cycling endometrium as demonstrated by immunohistochemistry. As shown in Figure 4, only slight difference of Fas staining (Fig. 4A) between the proliferative phase (MD5, MD10) and secretory phase (MD20, MD25) was detected, whereas the FasL staining (Fig. 4B) was stronger in the secretory phase as compared with proliferative phase. The protein level of Bcl-2 (Fig. 4C) in proliferative phase was higher than that in the secretory phase. An intense staining of Bax (Fig. 4D) was observed in the secretory phase. No obvious band was detected in all the negative controls (data not shown).

DISCUSSION

The primate endometrium undergoes characteristic proliferative, secretory, and menstrual phases under the control of steroid hormones secreted by the ovary. In the present study, we examined the cycle-dependent variation in the expression of the apoptosis-related molecules, in correlation with cell proliferation and apoptosis in the functional layer of endometrium of the rhesus monkey.

TUNEL analysis showed a relatively high frequency of apoptosis occurred in the endometrium at late secretory phase, and only limited apoptotic cells was observed in other phases. On the other hand, the Ki67 immunohistochemistry showed that an active proliferation in the endometrium took place in proliferative phase, and dramatically decreased in the secretory phase. These results are in agreement with the previous reports in human endometrium (Kokawa et al., 1996), and indicating the morphological similarities between the two species. It was reported in human that estrogen secreted by ovary stimulated endometrial proliferation and reached its maximum at the time of ovulation (Ferenzy and Guralnic, 1983). Progesterone, on the other hand, inhibited the endometrium for further proliferation and its withdrawal may induce a commitment to cell death and apoptosis at the end of menstrual cycle. The existed well established data tempted us to raise the possibility that both the Fas/FasL pathway and the molecules of Bcl-2 family may play an essential role in the processes of apoptosis in the endometrium of rhesus monkey during the menstrual cycle.

In this study, the expression patterns of Fas and FasL detected by immunohistochemistry and Western blot were well consistent with the reports from human endometrium (Watanabe et al., 1997; Yamashita et al., 1999; Chatzaki et al., 2001). Yamashita et al. (1999) demonstrated a translocation of Fas and FasL proteins from the Golgi apparatuses and vesicles during the proliferative phase to cell membrane during the secretory phase of human endometrial cells, therefore, the epithelial Fas and FasL during proliferative phase might be the storage of these two proteins and may play an active role in the secretory phase. In the endometrium, Fas may be activated by FasL secreted from endometrial glandular cells in an autocrine or paracrine manner and in turn transmits signals into the cytoplasm to induce apoptosis, thus the level of FasL would determine whether the Fas–FasL apoptotic pathway

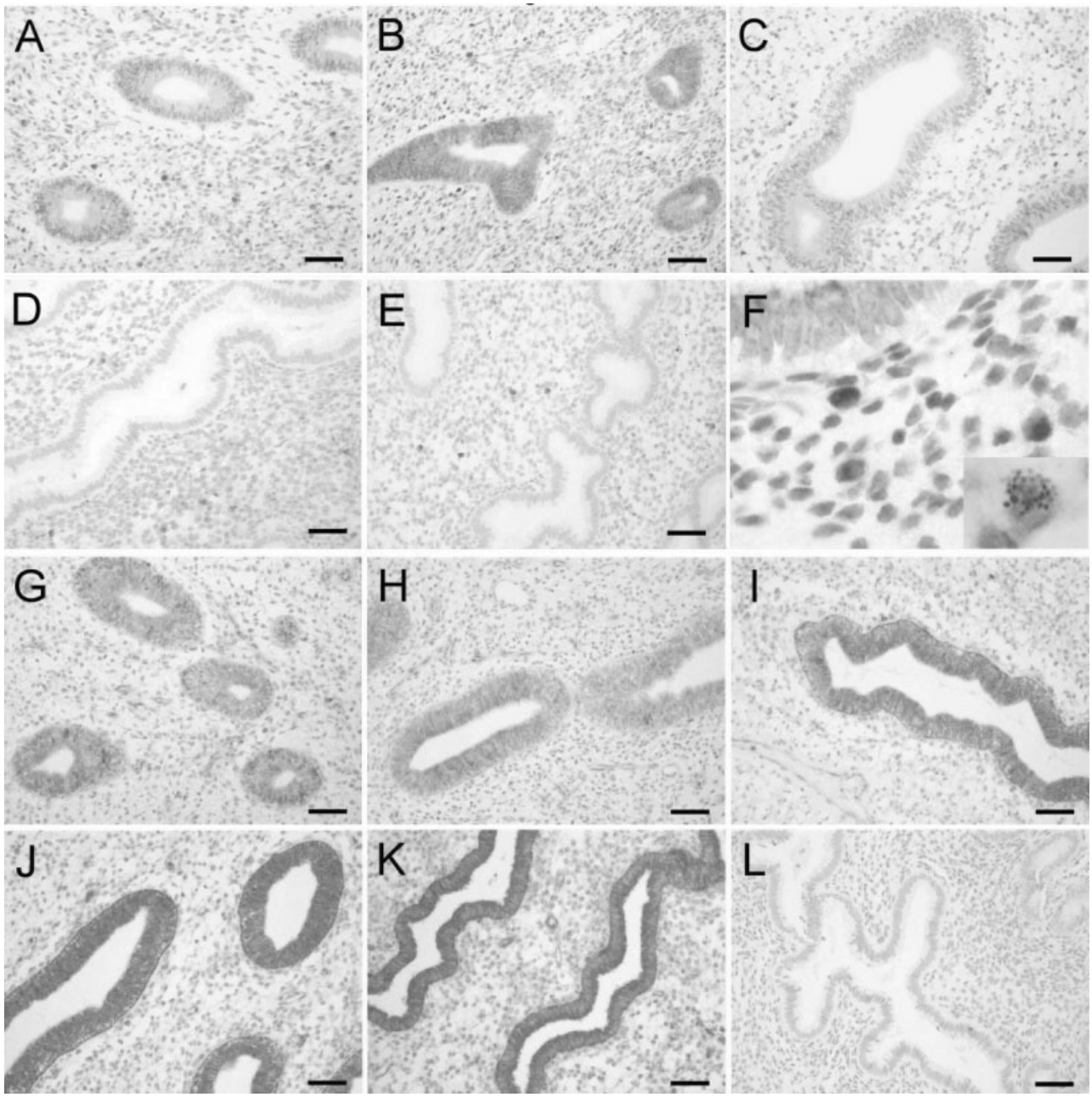

Fig. 3. Immunohistochemical staining for B cell lymphoma/leukaemia-2 (Bcl-2) (**A**–**F**) and Bax (**G**–**L**) in the endometrium of rhesus monkey during the menstrual cycle. Positive staining is red, and nuclear counterstain blue. A/G, MD5; B/H, MD10; C/I, MD16; D/J, MD20; E/K, MD25; F, Bcl-2 in the endometrium of MD5 with arrowhead indicating immunocyte; L, negative control for Bax in the endometrium of MD25. Scale bars represent 50 μm.

is activated (Nagata and Golstein, 1995). In the present study, the relative higher levels of FasL in the endometrium of rhesus monkey during the secretory phase further suggested that the epithelial apoptosis may be mediated by the Fas–FasL pathway in an autocrine or paracrine manner (French et al., 1997). The cyclic expression of FasL also implies a regulation by the steroid hormones secreted by the ovary, and this is in agreement with some results of in vitro studies (Song et al., 2002). The regulation of Fas expression or translocation in the endometrium is still unclear. Steroids and tumor suppresser genes, such as p53 and c-myc (Fuchs et al., 1997; Roberts et al., 1997) may be involved. In addition, transforming growth factor beta 1, PDGF, and IL-8 may be also involved in the regulation of the Fas/FasL pathway (Garcia-Velasco et al., 1999; Selam et al., 2002; Song et al., 2002, Chatzaki et al., 2003).

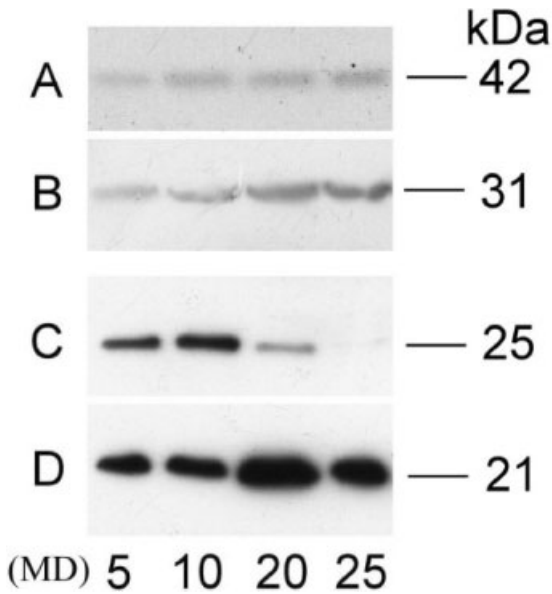

Fig. 4. Western blot analysis of Fas (**A**), FasL (**B**), Bcl-2 (**C**), and Bax (**D**) in the rhesus monkey endometrium during the menstrual cycle. A: Only slight difference of Fas staining is detected between the proliferative phase (MD5, MD10) and secretory phase (MD20, MD25). B: The staining of FasL is more intense in the secretory phase as compared with proliferative phase. C: The protein level of Bcl-2 in proliferative phase is higher than in the secretory phase. D: The staining of Bax is more intense in the secretory phase as compared with proliferative phase.

It is of note that concomitant expression of Fas and FasL is not sufficient for the occurrence of apoptosis (Yamashita et al., 1999). As it is suggested, tumor survival factor Bcl-2 may inhibit Fas/FasL-mediated apoptosis in human endometrium during the proliferative phase (Otsuki et al., 1994; Shimizu et al., 1996; Yamashita et al., 1999). Therefore, members of Bcl-2 family may play important roles in the regulation of endometrial apoptosis. Coordinated shifts in the balance between the anti-apoptotic (Bcl-2 and Bcl-x_L) and pro-apoptotic (Bax, Bak, and Bcl-x_S) homologues may be responsible for the cyclic changes of endometrial growth and regression (Tao et al., 1997). The opposing expression pattern of Bcl-2 and Bax in our study demonstrated that the ratio of these two proteins cyclically expressed in glandular epithelial cells during the cycle paralleled with the cycle-related changes in epithelial cell apoptosis as detected by TUNEL. This conclusion is supported by some of the previous results from human (Gompel et al., 1994; Tabibzadeh et al., 1995; McLaren et al., 1997), and provides another evidence that the Bax to Bcl-2 "rheostat" may be a critical factor in regulating apoptosis in primate endometrium during the normal menstrual cycle. As suggested by the present and other in vitro or in vivo studies (Gompel et al., 1994; Tao et al., 1997; Critchley et al., 1999; Bozdogan et al., 2002), the cyclic expression of Bcl-2 and Bax in the endometrium of rhesus monkey may be regulated by ovarian sex steroids.

In our study, strong Bcl-2 staining was also observed in certain immunocytes in the monkey endometrium. A similar observation in human endometrium demonstrated that CD68 and CD56 was localized to the Bcl-2 positive immunocytes, and indicated that these cells might be subpopulations of macrophages or large granular lymphocytes (Critchley et al., 1999). As the endometrial stromal immunocytes expressing Bcl-2 do not undergo apoptosis (Jones et al., 1998), the presence of Bcl-2 may contributes to the survival of these cells. It is interesting to note that the degree of the staining in these cells was not influenced by the menstrual cycle phases in the present study, suggesting the lack of regulation by the steroid hormones. However, in human endometrium, an increasing numbers of leucocytes were found in the luteal phase, which proliferate and express Bcl-2 (Jones et al., 1998), the reason for the difference is unclear.

CONCLUSION

We reported for the first time that the proliferation, apoptosis, and the expression of related apoptotic molecules in the endometrium of rhesus monkey, and demonstrated a coordinated expression pattern of Fas, FasL, Bcl-2, and Bax in relation to cell apoptosis and proliferation during the cycle. These data suggest that cyclic changes in endometrial growth and regression may be precisely regulated by shifts in the ratio and balance of these apoptosis-related factors.

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