Fas ligand, Fas antigen and Bcl-2 expression in human endometrium during the menstrual cycle

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In this study, we investigate Fas ligand expression in the human endometrium during the menstrual cycle in relation to Fas antigen and Bcl-2 expression, using immunoelectron microscopy and Western blotting. Endometrial samples were obtained from 54 pre-menopausal non-pregnant women who underwent laparotomies for benign diseases. The Fas ligand, as well as the Fas antigen, were expressed on the surface of endometrial glandular cells throughout the menstrual cycle, whereas Bcl-2 showed a cyclic expression pattern, peaking during the late proliferative phase. A noteworthy finding was that both the Fas ligand and the Fas antigen were localized on Golgi apparatuses and vesicles, in addition to the cell membranes, during the late proliferative phase. These results indicate that the Fas ligand and Fas antigen which are localized on Golgi apparatuses and vesicles during the late proliferative phase are incorporated into the cell membranes during the secretory phase, and are co-expressed on the cell membranes of endometrial glands throughout the menstrual cycle. The factors regulating Fas-mediated apoptosis in the human endometrium, including the level of expression of the Fas ligand and Bcl-2 are discussed.

Key words: apoptosis/Bcl-2/Fas antigen/Fas ligand/human endometrium

Introduction
Historically, menstruation was generally regarded as being associated with ischaemic necrosis of a functional layer of the endometrium caused by contraction of the spiral arteries, and dependent on sex hormone concentration (Markee, 1940; Bartelmez, 1957). In contrast to past studies, recent electron microscopic studies have revealed the presence of apoptotic bodies in human endometrial epithelial cells during the late secretory phase (Hopwood and Levison, 1976; Otsuki et al., 1994). It is well known that Bcl-2, which inhibits apoptosis, promotes the survival of various cells (Tsujimoto, 1989; Garcia et al., 1992). Bcl-2 was initially isolated from human follicular lymphoma cells having the t(14;18) chromosomal translocation (Tsujimoto and Croce, 1986), and is a member of the Bcl-2 family, along with Bax, Bcl-xlong, and Mcl-1. We previously reported (Otsuki et al., 1994) that Bcl-2 is expressed in human endometrial glandular cells during the proliferative but not the secretory phase, and that it plays an important role in inhibiting apoptosis in the human endometrium during the proliferative phase. However, factors promoting apoptosis in the human endometrium remain to be clarified.

Recent studies have indicated that several proteins such as the Fas antigen (Fas)/tumour necrosis factor receptor (TNFR), the Fas ligand (FasL)/TNF, p53, and Myc, in addition to the proteins of the Bcl-2 family, are involved in the regulation of apoptosis. In particular, Fas, which is a member of TNFR family and a type-I membrane protein (Itoh et al., 1991), induces apoptosis via cross-linking with the Fas antibody or FasL in various cells and tissues. Watanabe et al. (1997) have reported that Fas is expressed in the eutopic human endometrium throughout the menstrual cycle. In contrast, FasL expression has not yet been demonstrated, whereas Bcl-2 and Fas expression has been clearly demonstrated in human endometrium throughout the menstrual cycle (Otsuki et al., 1994; Watanabe et al., 1997).

The aim of this study was to identify the pattern of expression of FasL in the human endometrium during the menstrual cycle, in relation to the expression of both Bcl-2 and Fas.

Materials and methods

Patients
Endometrial samples were collected after obtaining informed consent from 54 pre-menopausal non-pregnant women (age range, 35–53 years) who underwent laparotomies for benign diseases. All the women had a history of regular menstrual cycles and did not take contraceptives for at least 3 months prior to surgery. The endometrial specimens containing myometrium (~1×2×0.5 cm) were obtained from the corpora of the uteri at random and were divided into five categories by histological dating with reference to menstrual history: menstrual phase (n = 8), early proliferative phase (n = 12), late proliferative phase (n = 12), early secretory phase (n = 6), and late secretory phase (n = 16).

Antibodies
The following antibodies were used: polyclonal rabbit antibodies against the extracellular domain of recombinant human Fas (Fas D, a gift from Dr Takehiko Koji, Nagasaki University School of Medicine, Nagasaki, Japan) (Koji et al., 1994) and monoclonal antibodies against FasL (Transduction Laboratories, KY, USA) and human Bcl-
2 (Ab-1; Oncogene Research Products, MA, USA). The appropriate dilutions for these antibodies were determined in preliminary experiments.

**Immunohistochemistry for light microscopy**

Portions of the endometrial specimens were embedded in Tissue-Tek (Miles, Elkhart, IN, USA) and frozen in dry ice-acetone for light microscopic analysis. The frozen blocks were cut into 6 µm thick sections with a cryostate (Microm, Heidelberg, Germany) at −20°C. Serial sections were mounted on poly-l-lysine-coated glass slides, and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.4) for 10 min. The sections were then exposed to 0.1% NaN3 and 0.3% H2O2 in distilled water and treated with BlockAce (Dainippon Pharmaceutical Co, Osaka, Japan). Subsequently they were stained using an indirect immunoperoxidase technique. Each primary antibody was used at a dilution of 1:200 with 0.01% phosphate-buffered saline (PBS). After overnight incubation with the primary antibody at 4°C, the sections were further incubated with goat anti-rabbit secondary antibody conjugated with horseradish peroxidase for Fas D, or with goat anti-mouse antibody for FasL (Dako, Glostrup, Denmark), diluted 1:100 with 0.01 M PBS, for 1 h. Peroxidase activity was analysed by exposure of the sections to a solution containing 0.05% 3', 3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma, St Louis, MO, USA) and 0.01% H2O2 in Tris–HCl buffer (DAB solution) at pH 7.6 for 5 min. Sections were finally counterstained with 1% Methyl Green and examined by light microscopy. Some of the serial sections were stained with haematoyxlin and eosin to verify the structural integrity of the tissues.

Heterogeneous Fas and FasL staining patterns were noted among the individual glands and even among glandular cells forming a gland at each menstrual phase. Therefore, the expression of Fas or FasL in individual glandular cells at each phase of the menstrual cycle was assessed in terms of the intensity of immunostaining and scored based on the following four grades (scores: 0 = none, 1 = weak, 2 = distinct, and 3 = strong). The histochemical score (HSCORE) (Leone et al., 1991) was calculated using the following equation: HSCORE = Σ Pi(i + 1), where i = 0, 1, 2, or 3, and Pi is the percentage of cells exhibiting the relevant staining intensity, varying from 0 to 100%. The statistical analysis of the differences among the different menstrual phases was performed using the Bartlett test and one-way analysis of variance. P < 0.05 was considered to be statistically significant.

The slides showing various immunostaining patterns of Fas or FasL were independently interpreted and confirmed by two observers.

**Immunoelectron microscopy**

For immunoelectron microscopy, portions of the endometrial tissues were fixed in a solution containing 4% paraformaldehyde, 0.05% glutaraldehyde and 0.2% picric acid in 0.1 M PB, and then sequentially immersed in 0.1 M PB containing 5% sucrose for 1 h, 10% sucrose for 1 h and 20% sucrose until the tissues sank to the bottom of the container. The specimens were then cut into 60 µm thick serial sections with a microscaler at 4°C and the sections were stained using the indirect immunoperoxidase technique, as described above for the cryosections. After exposure to DAB solution, the sections were fixed with 1% osmium tetroxide in 0.1 M PB for 1 h, stained further with 1% uranyl acetate in 70% ethanol for 40 min, and then dehydrated and embedded in epoxy resin. Ultrathin sections (60–80 nm thick) were cut using an ultramicrotome equipped with a diamond knife. All samples were observed under an electron microscope (H-7100, Hitachi Ltd, Tokyo, Japan).

Rabbit immunoglobulin (IgG3) for Fas and mouse IgG3 for FasL, both diluted 1:100 respectively were used as negative controls for immunohistochemical analysis by light microscopy and transmission electron microscopy.

**Western blotting**

The remaining endometrial tissues were homogenized in lysis buffer [1% NP-40, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulphate (SDS), 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 0.1 µM pepstatin A in PBS, pH 7.4]. The protein concentration was determined by a DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Protein (10 µg) was loaded into each lane, separated by SDS–polyacrylamide gel electrophoresis using a 10 or 12% polyacrylamide gel, and electroblotted onto a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). Equal loading of proteins in each lane was confirmed by staining the membrane with a BLOTFastStain kit (Geno Technology Inc, St. Louis, MO, USA). The membrane was blocked with Block Ace (Dainippon Pharmaceutical Co, Osaka, Japan) at room temperature for 1 h and incubated overnight at 4°C with mouse anti-Fas or anti-Bcl-2 monoclonal antibody diluted at 1:1000. The membrane was further incubated for 2 h with biotin-conjugated goat anti-mouse IgG antibody (Kirkiegaad Perry Laboratories, MD, USA), and horseradish peroxidase-conjugated streptavidin (Zymed Laboratories, CA, USA) diluted at 1:3000. The immunoblot was developed using DAB as chromogen. Jurkat cells (Ohtsu et al., 1997) and HMV-1 cells were used as positive standards for Bcl-2 and FasL respectively.

**Results**

**Immunohistochemistry**

None of the controls exhibited Fas or FasL (Figure 1A) immunoreactivity. Endometrial glands are characterized by their straight tubular shape and increase in number during the proliferative phase, which begins at the end of menstruation. Fas and FasL immunoreactivities were observed only on the apical membrane of the endometrial glandular cells facing the lumen, with weak immunostaining during the early proliferative phase (Figure 1B). Fas and FasL immunoreactivities on the apical membranes gradually increased as the cycle progressed (Figure 1C). The most characteristic finding was the granular staining pattern for Fas and FasL in the supranuclear region of glandular cells during the late proliferative phase (Figures 1C, D). During the secretory phase, the glands become more tortuous and ultimately undergo marked sacculation, resulting in the formation of relatively wide lumina of irregular shape. The glandular cells often protrude into the lumen. The cell bodies showed the strongest Fas and FasL immunoreactivities during the late secretory phase (Figure 1E). The strong Fas and FasL immunoreactivities on the apical membranes were observed until menstruation, although the granular staining pattern in the supranuclear region was no longer seen during the secretory phase. These changes in immunoreactivities of Fas and FasL in the human endometrial glands were similar to each other throughout the menstrual cycle. Endometrial stromal cells, and vascular and myometrial smooth muscle cells exhibited no Fas and FasL expression at any stage during the menstrual cycle.

Differences in the staining intensity for Fas between the...
Figure 1. Expression of Fas and the Fas ligand in the human endometrium. (A) No Fas ligand is exhibited on the cell membranes of glandular cells during the early proliferative phase in the control slide. (B) Fas ligand is expressed only on the apical membranes (arrows) of glandular cells during the early proliferative phase. The immunostaining of Fas ligand is weak and heterogeneous. (C) Fas ligand is strongly expressed on apical membranes in the late proliferative phase. Note the presence of Fas ligand in the supranuclear region (arrows). (D) Fas is present not only on the apical membranes of the glandular cells but also in the supranuclear region (arrows) during the late proliferative phase. (E) FasL is strongly expressed on the glandular cells (arrows) near the glandular lumen in the late secretory phase. Bars = 10 µm.

early proliferative and late proliferative, early secretory and late secretory phases, and the late secretory and the menstrual phases were statistically significant ($P < 0.05$). The highest mean HSCORE for Fas was observed during the late secretory phase (Figure 2A). With regard to the mean HSCORE for FasL, differences between the early proliferative and menstrual phases, and the early proliferative and late secretory phases were also significant ($P < 0.05$) (Figure 2B).

**Immunoelectron microscopy**

The general ultrastructural features of human endometrial glandular cells during the menstrual cycle noted in this study were in good agreement with those reported by other investigators (Armstrong *et al.*, 1973; Gordon, 1975; Inoki *et al.*, 1997). In brief, the glandular cells possessed non-curved oval nuclei with an even distribution of chromatin and a few prominent nucleoli during the early proliferative phase. Glandular cells during the late proliferative phase were characterized by an increased level of heterochromatin. Few glandular cells showing morphological characteristics of necrosis or apoptosis could be identified during the proliferative phase. During the secretory phase, endometrial glandular cells showed a reduced volume of cytoplasm and a decrease in number of their junctional apparatuses such as desmosomes, causing the widening of the intercellular spaces. As the cycle progressed, some glandular cells possessed nuclei with chromatin con-
Fas ligand expression in human endometrium

Figure 2. Statistical analysis of HSCOREs of Fas and Fas ligand in human endometrial glandular cells during the menstrual cycle. (A) Fas during the early proliferative phase (E.P.) (170 ± 64), late proliferative phase (L.P.) (248 ± 53), early secretory phase (E.S.) (301 ± 7), late secretory phase (L.S.) (359 ± 32) and menstrual period (M.) (208 ± 53). (B) Fas ligand during the E.P. (175 ± 23), L.P. (203 ± 60), E.S. (232 ± 79), L.S. (287 ± 55), and M. (294 ± 23) (mean ± SD).

Figure 3. An apoptotic body (arrow), characterized by peripheral masses of chromatin, observed in the glandular lumen (GL) during the late secretory phase. Note the widened intercellular space between adjacent endometrial glandular cells (GC). Bar = 1 µm.

densation. Finally, apoptotic bodies, characterized by peripheral masses of chromatin, appeared both near the luminal surface and in the glandular lumen during the late secretory and menstrual phases (Figure 3).

Fas was mainly localized on the apical membranes and on the surfaces of microvilli and cilia throughout the menstrual cycle. The most noteworthy finding was the presence of Fas on the surface of vesicles beneath the apical membranes and on Golgi apparatuses (Figure 4) localized in the supranuclear region of the glandular cells during the late proliferative phase.

FasL was present on Golgi apparatuses in some glandular cells during the late proliferative phase, as was Fas. FasL was localized on the apical membranes, more or less throughout the menstrual cycle, and the most intense expression was noted during the late secretory (Figure 5) and menstrual phases.

Figure 4. Fas is often localized on the Golgi apparatuses (arrows) of glandular cells during the late proliferative phase. Bar = 1 µm.

Analysis of endometrial FasL and Bcl-2 by Western blotting

To determine whether the endometrial proteins labelled by anti-FasL and anti-Bcl-2 antibodies were indeed FasL and Bcl-2, the endometrial proteins were subjected to Western blot analysis. Equal loading of proteins in each lane was confirmed after electroblotting (data not shown). The molecular weights of FasL and Bcl-2 were consistent with those known for FasL and Bcl-2 (37 and 26 kDa respectively) (Tsujimoto and Croce, 1986; Suda et al., 1993). FasL expression in the endometrium was confirmed during all phases of the menstrual cycle, and was more intense during the secretory phase than during the proliferative phase (Figure 6A). On the other hand, Bcl-2 exhibited a cyclic expression pattern with a peak during the late proliferative phase, and its expression level markedly decreased during the secretory phase (Figure 6B).

Discussion

Hopwood and Levision (1975) reported detecting apoptosis in the human endometrium, following the advancement of the
To date, stimuli inducing apoptosis in the human endometrium have not yet been clarified, although Bcl-2 has been reported to inhibit apoptosis in the human endometrium during the proliferative phase. Therefore, it is difficult to explain if human menstrual shedding depends on Fas-mediated apoptosis. It is generally recognized that expression of Fas alone does not guarantee activation of Fas-mediated apoptosis. Recently, Nagata and Goldstein (1995) proposed that the level of expression of FasL would determine whether considerable loss of endometrial glandular cells due to apoptosis was during the late secretory, premenstrual and menstrual phases, and to a lesser extent during the proliferative phase of the menstrual cycle.

To date, stimuli inducing apoptosis in the human endometrium have not yet been clarified, although Bcl-2 has been reported to inhibit apoptosis in the human endometrium during the proliferative phase (Otsuki et al., 1994). Attention has recently been focused on apoptosis mediated by the Fas-Fas ligand system. There are some reports of Fas expression in the human endometrium throughout the entire menstrual cycle (Tabibzadeh et al., 1995; Watanabe et al., 1997). The expression pattern of Fas in this study agrees well with that reported previously. However, until now, the changes in the subcellular localization of Fas in human endometrial glandular cells had not been noted, because the morphological analysis in previous studies depended on light microscopy and not on electron microscopy. In this study using immunoelectron microscopy, it was noted that Fas, as well as FasL, are mainly localized on Golgi apparatus and vesicles during the late proliferative phase. Both Fas and Fas ligand are transmembrane glycoproteins. It is well documented that proteins are initially synthesized in membrane-bound ribosomes and then transported from the rough endoplasmic reticulum to the Golgi apparatus. Some proteins are modified by the addition of sugar residues in the trans-Golgi network containing a variety of glycosyltransferases, and then sorted into specific membrane-bound vesicles for subsequent incorporation into a membrane (Farquhar and Palade, 1981; Griffiths and Simons, 1986). Therefore, our immunoelectron microscopic analysis shows that both Fas and the Fas ligand in human endometrial glandular cells are mainly localized on Golgi apparatuses and vesicles during the late proliferative phase, and that they are incorporated into cell membranes during the secretory phase. These findings are consistent with the results of a study of Fas immunostaining on human endometrial glandular cells (Watanabe et al., 1997), in which Fas expression was determined to be slightly stronger during the secretory phase than during the proliferative phase.

Recently, French et al. (1996) reported on the co-expression of Fas and FasL in various organs of adult mice, such as the thymus, lung, spleen, small intestine, large intestine, seminal vesicle, prostate, and uterus. This report leads to the question, ‘Why does menstrual shedding not occur in adult mice, despite the co-expression of both Fas and FasL?’ The question may be answered in terms of the functional differences between the soluble forms of FasL in mice and humans, because Tanaka et al. (1995) and Suda et al. (1996) reported that soluble FasL from mouse is inactive or very unstable, in contrast to the biologically active form of FasL in humans. Moreover, Kayagaki et al. (1995) and Mariani et al. (1995) suggested that the active form of soluble FasL in humans is released from the cell surfaces after processing by matrix metalloproteinase (MMP). In the human endometrium, Fas may be activated by soluble FasL secreted from endometrial glandular cells in an autocrine or paracrine manner and in turn transmit signals for the induction of apoptosis into the cytoplasm. This assumption that the human endometrium, co-expressing Fas and FasL, undergoes self-regulation by apoptosis has already been noted in chorionic cytotrophoblasts and amnionic epithelial cells of human fetal membranes at term (Runic’ et al., 1998).

In this study, Fas and FasL were consistently co-expressed in the human endometrium throughout the menstrual cycle, although menstrual shedding of human endometrium does not occur during the proliferative phase. Therefore, it is difficult to explain if human menstrual shedding depends on Fas mediated apoptosis. It is generally recognized that expression of Fas alone does not guarantee activation of Fas-mediated apoptosis. Recently, Nagata and Goldstein (1995) proposed that the level of expression of FasL would determine whether...
the Fas–FasL apoptotic pathway is activated. In addition, it is of note that human colonic carcinoma and leukaemia cells do not undergo ‘suicide apoptosis’, although both exhibit concomitant expression of Fas and FasL. (O’Connell et al., 1996; Tanaka et al., 1996). It is well known that Bcl-2 inhibits Fas-mediated apoptosis via inactivation of an ICE-like protease (Enari et al., 1995; Shimizu et al., 1996) which is located downstream of Fas/FasL in the apoptotic pathway. Our results based on Western blotting of Bcl-2 clearly demonstrate that Bcl-2 is expressed cyclically in the human endometrium, showing a peak during the late proliferative phase, and this has been supported by the results of other immunohistochemical studies (Otsuki et al., 1994; Koh et al., 1995; Watanabe et al., 1997). Therefore, factors such as the level of expression of FasL and Bcl-2, may also be involved in the regulation of Fas-mediated apoptosis.

Recently, attention has been called to the fact that Fas/Fas signalling has an important role as ‘immune privilege’, especially in cornea and testis (Douglas and Carl, 1997; Griffith and Ferguson, 1997); when activated inflammatory lymphocytes enter the cornea and testis, the cells bearing Fas undergo apoptosis due to binding to FasL, which is expressed on the epithelial cells of these tissues. In human placenta, Uckan et al. (1997) reported that the syncytiotrophoblast layer demonstrate consistent immunoreactivity for FasL and apoptotic T-lymphocytes in term placenta and speculated that the presence of FasL in the trophoblast contributes to immune privilege at the fetal–maternal site, perhaps by mediating apoptosis of activated Fas-expressing lymphocytes of maternal origin. Therefore, FasL expressed on human endometrial glandular cells may contribute not only to self-regulation by apoptosis but also to immune privilege by inducing apoptosis of activated Fas-bearing lymphocytes, thereby protecting the spermatozoa and the zygote.

In conclusion, our results document for the first time, the expression of FasL in the human endometrium. Further studies will be needed to elucidate the roles of Fas/FasL signalling in association with Bcl-2 in physiological and pathological triggered apoptosis in the human endometrium.

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