Regulation of bcl-2 gene family members in human endometrium by antiprogesterin administration *in vivo*

H. O. D. Critchley¹, S. Tong¹, S. T. Cameron¹, T. A. Drudy¹, R. W. Kelly² and D. T. Baird¹

¹Department of Obstetrics and Gynaecology, The University of Edinburgh, 37 Chalmers Street, Edinburgh EH3 9ET, UK; and

²Medical Research Council Reproductive Biology Unit, Centre for Reproductive Biology, 37 Chalmers Street, Edinburgh EH3 9ET, UK

It is likely that the changes which occur in the endometrium throughout the menstrual cycle involve apoptosis, and that expression of associated genes, such as the bcl-2 family, are regulated by sex steroids. The aim of this study was to investigate the presence of bcl-2, Bax and oestrogen receptor proteins in secretory endometrium collected from ten patients with normal ovulatory cycles 4 or 6 days after the LH surge, and on the same days in a subsequent cycle in which the formation of secretory changes was inhibited by the administration of the antiprogesterin mifepristone (RU486) 2 days after the onset of the LH surge. Since some stromal cells display positive immunoreactivity, leucocyte subpopulations of macrophages (CD68-positive) and large granular lymphocytes (CD56-positive) were identified in serial sections. After administration of mifepristone on day 2 after the LH surge, a significant increase in bcl-2 immunoreactivity was observed in glandular and surface epithelium. A positive correlation (0.874) with nuclear oestrogen receptor immunoreactivity in endometrial glands was demonstrated. Subsets of stromal cells, identified as CD68-positive macrophages and CD56-positive large granular lymphocytes displayed positive immunoreactivity for the bcl-2 epitope, which was unaffected by mifepristone administration. Bax immunostaining was similar in control and antiprogesterin-treated endometrium. These data indicate that antiprogesterin administration inhibits progesterone downregulation of steroid receptors in endometrial glands, resulting in persistence of a proliferative endometrium and accompanying bcl-2 secretion.

Introduction

The term apoptosis describes the mode of cell death that is responsible for the deletion of cells in normal tissues (Wyllie *et al.*, 1980). The process is distinct from necrosis and displays characteristic features. Regulation of the apoptotic process is complex, and a family of related proteins, the bcl-2 gene family, have either a positive or negative role. bcl-2 protein is the prototype of the gene family that is associated with the inhibition of apoptosis (Wyllie, 1994). One mode of regulation is via the interaction of bcl-2-related apoptosis inhibitors and apoptosis promoters, for example, Bax protein (Oltavi *et al.*, 1993). Bax protein, also a bcl-2 family member, promotes cell death, possibly countering the anti-apoptotic action of bcl-2 through heterodimer interaction (Oltavi *et al.*, 1993).

The role and regulation of apoptotic events in human endometrium remains unknown. Kokawa *et al.* (1996) observed apoptosis in the functional layer of early proliferative and late secretory phase endometrium, although there was no evidence for apoptosis in the basal layers. These observations were consistent with much earlier

reports (Hopwood and Levison 1976) of a morphological increase in the number of apoptotic bodies in pre-menstrual and menstrual phase endometrium.

The predominance of bcl-2 in proliferative endometrium was reported by this group (Koh *et al.*, 1995) and confirmed by others (McLaren *et al.*, 1997; Tao *et al.*, 1997). Tao *et al.* (1997) also described maximal immunoreactivity in the basal endometrium. In contrast, the same group reported that Bax protein immunoreactivity was low in the proliferative phase, but increased markedly in secretory phase endometrial tissues, particularly in the functional epithelial layers. These results support a co-ordinated shift towards greater concentrations of the pro-apoptosis protein Bax in the glandular compartment of the secretory phase. In a study of Bax immunoreactivity in normal endometrium, McLaren *et al.* (1997) reported positive Bax immunostaining throughout the menstrual cycle, but did not specify whether this was in the functional or basalis layer. The endometrial tissues were collected by sharp curettage and hence were likely to include elements of the basalis.

It is highly possible that the expression of genes that encode for apoptosis-related proteins are regulated by sex steroid hormones. In this context, Otsuki *et al.* (1994) briefly reported a correlation between oestrogen and progesterone

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receptor expression and the cyclic variations in bcl-2 in human endometrium in glandular epithelial cells.

Mifepristone is an 11 β phenyl substituted steroid that is similar in structure to progesterone. The compound lacks the C-19 methyl group and the 2C side chain at C-17 of progesterone (Baulieu, 1997). Mifepristone binds strongly to the progesterone receptor and glucocorticoid receptor, but does not bind to the oestrogen receptor. Affinity for the glucocorticoid receptor and weak binding for the androgen receptor is not surprising in view of the structural homology among the family of steroid receptors. It has been demonstrated that, in women, mifepristone will induce endometrial bleeding in the luteal phase of the menstrual cycle and abortion in early pregnancy (Baird, 1993). A single dose of 200 mg given to women shortly after ovulation retards the development of a secretory endometrium and a number of progesterone-dependent products (Swahn *et al.*, 1990; Cameron *et al.*, 1997). Since apoptosis has been reported in secretory endometrium (Hopwood and Levison, 1976; Kokawa *et al.*, 1996) and bcl-2 immunoreactivity decreases in the secretory phase (Koh *et al.*, 1995; Tao *et al.*, 1997) steroids have been implicated in this process.

In the present study, endometrial tissue from healthy women exposed to antiprogesterin *in vivo* in the early secretory phase was examined and the possible regulation of bcl-2 gene family members in the human endometrium by antiprogesterin administration *in vivo* was considered. The objective was to investigate the hormonal control of apoptosis-related proteins by comparing the expression of bcl-2 and Bax in endometrium collected after the administration of mifepristone soon after ovulation to control mid-secretory endometrium.

Materials and Methods

A total of ten healthy women aged between 25 and 38 years (mean age 32) with regular menstrual cycles (25–35 days) were included in the study. The characteristics of the patients have been reported (Cameron *et al.*, 1997). The mean body mass index of subjects was 25.5 kg m⁻² (range 21.3–35.5). All subjects had a normal gynaecological examination. No subject had used hormonal contraception in the preceding 3 months and those women who were not surgically sterilized used a barrier method of contraception throughout the study period. The protocol was approved by the local Ethical Subcommittee and each woman provided written informed consent.

Subjects were studied over a control cycle and a treatment cycle. In the treatment cycle, subjects received 200 mg mifepristone on day 2 after the onset of the LH surge in urine. The timing of the LH surge was detected by the subjects themselves using a commercially available home urine LH detection kit (Conceive; Quidel, San Diego, CA). Each women tested her urine twice a day at 08:00 h and 18:00 h from day 10 of the menstrual cycle (10 days after the start of menstruation) in both control and treatment cycles. Detection of the urine LH surge was confirmed by radioimmunoassays. An endometrial biopsy was collected with a pipelle suction curette (Prodimed, Neuilly-en-Thelle)

from each subject 4 or 6 days after the LH surge on the same day of both control and treatment cycles. In the treatment cycle, the biopsy was thus taken 2 or 4 days after mifepristone ingestion.

All endometrial biopsies were fixed in 4% (w/v) paraformaldehyde for histological assessment and immunohistochemical analysis. The histological appearances after haematoxylin and eosin staining have been reported (Cameron *et al.*, 1997) and dated according to the criteria of Noyes *et al.* (1950).

Immunohistochemistry

Sections (5 μ m) of endometrial biopsies were examined for immunohistochemical localization of (i) the bcl-2 epitope using the technique of Koh *et al.* (1995), (ii) the Bax epitope, (iii) CD68-positive macrophages, (iv) CD56-positive large granular lymphocytes, and (v) the oestrogen receptor. Commercially available monoclonal and polyclonal antibodies were used for the detection of bcl-2 (DAKO Ltd, High Wycombe; Pezzella *et al.*, 1990), Bax (Pharmingen, San Diego, CA), macrophages (DAKO, CD68 code M876), CD56-positive large granular lymphocytes (Zymed Laboratories, San Francisco, CA) and oestrogen receptors (DAKO, ER1D5).

Pretreatment of sections for bcl-2, Bax, oestrogen receptor and CD56 immunohistochemistry

Histoclear (National Diagnostics, Atlanta, GA) was used to remove the paraffin wax from the tissue sections, which were then rehydrated through a descending ethanol series and washed in 0.1 mol Tris-buffered saline (TBS) I⁻¹ for bcl-2 and CD56, and 0.1 mol PBS I⁻¹ for Bax and oestrogen receptor for 2 $\times$ 5 min. bcl-2, oestrogen receptor and CD56 epitopes were exposed by microwaving at high power in 0.01 mol sodium citrate buffer I⁻¹, pH 6.0 for 2 $\times$ 5 min (oestrogen receptor), 3 $\times$ 5 min (bcl-2) or 4 $\times$ 5 min (CD56). The Bax epitope was exposed by microwaving at high power in distilled water for 2 $\times$ 5 min. After treatment, all sections were allowed to stand for 20 min in the microwave oven. After another wash in TBS or PBS, non-specific endogenous peroxidase activity was blocked by treatment with 3% (w/v) hydrogen peroxide in distilled water for 10 min at room temperature. All tissue sections were exposed to a non-immune block with either normal horse serum (for bcl-2, oestrogen receptor and CD56) or normal goat serum (for Bax) for 20 min at room temperature and then incubated with the appropriate primary antibody.

Immunolocalization of bcl-2

Tissue sections were incubated with monoclonal mouse anti-human bcl-2 antibody at a dilution of 1:40 for 60 min at 37°C. Thereafter, tissue sections were labelled with an avidin-biotin peroxidase detection system (Vectastain; Vector Laboratories, Peterborough) that included the

secondary biotinylated horse anti-mouse antibody. Tissue sections were then incubated with diaminobenzidine (DAB solution, DAB Kit; Vector Laboratories) for 10 min. Finally, the sections were counterstained with haematoxylin, dehydrated, cleared in xylene and mounted in Pertex mounting medium (Cellpath plc, Hemel Hempstead). Negative controls were performed by replacing the primary antibodies with mouse immunoglobulin at the same concentration as the primary antibody.

Immunolocalization of CD56

Tissue sections were incubated with monoclonal mouse anti-human CD56 antibody at a dilution of 1:250 for 60 min at 37°C and labelled as described earlier.

Immunolocalization of macrophages (CD68)

Endometrial tissue sections were incubated with monoclonal mouse anti-human CD68 antibody at a dilution of 1:50 for 60 min at room temperature and labelled as described earlier. An antigen retrieval step was necessary to localize the CD68 epitope. Therefore, the tissue sections were subjected to an enzyme digestion in 0.1% (w/v) trypsin and 0.1% (w/v) calcium chloride, pH 7.8. The digestion was conducted at 37°C for 15 min and enzyme activity was then destroyed by washing the sections in tap water.

Immunolocalization of Bax

After pretreatment, tissue sections were incubated with polyclonal rabbit anti-human Bax antibody at a dilution of 1:1200 overnight at room temperature. Staining was again detected using an avidin–biotin peroxidase system. Negative controls were performed by replacing the primary antibody with non-immune rabbit immunoglobulin. First trimester villi provided a positive control and positive immunoreactivity was clearly visible in the cytotrophoblast layer (Lea *et al.*, 1997a).

Immunolocalization of oestrogen receptor

After pretreatment, tissue sections were incubated with monoclonal mouse anti-human oestrogen receptor antibody at a dilution of 1:25 for 60 min at 37°C. Thereafter, staining was detected using an avidin–biotin peroxidase system as described earlier.

Data analysis

Immunoreactivity was assessed using light microscopy and the intensity of immunostaining for bcl-2 and Bax proto-oncogenes in all tissue sections was scored semi-quantitatively on an arbitrary four point scale, indicating the intensity and proportion of positively stained cells.

A score of 3 indicated intense staining of the majority of each cell type, that is, stromal and glandular epithelium or presumed leucocyte components. A score of 0 indicated an absence of staining and scores of 1 and 2 indicated weak or moderate intensity of immunoreactivity, respectively. Both the immunohistochemistry and the scoring of positively stained cells were performed by a single individual without prior knowledge of the phase of the menstrual cycle. Each subject in the study underwent a single endometrial biopsy in each of the control and treatment cycles. Duplicate sections were analysed for each biopsy. One section was exposed to the primary antibody and the other section served as the negative control. Immunostaining in the whole tissue section was taken into consideration in the assessment; at least ten glands were evaluated per section. Immunoreactivity was fairly homogenous for bcl-2, Bax and oestrogen receptor. Immunohistochemistry is associated with variability in immunostaining and hence each antibody was applied to each series of tissue sections in a single run. It is important to note that the immunolocalization of CD56- and CD68-positive leucocytes was undertaken to identify the characteristics of the presumed leucocyte subtype with positive immunostaining of the bcl-2 epitope. Serial sections were used for this purpose.

ANOVA was used to evaluate whether there was any significant difference in immunoreactivity recorded in the semi-quantitative system described earlier. Assignment of significance of differences was determined by Fisher's protected least significant difference test. Correlations were derived from, and correlation matrix was obtained using, Statview 4.5.

Results

In control endometrium in the secretory phase on days 4 and 6, mild positive immunoreactivity for bcl-2 was observed in both the glandular and surface epithelium (Figs 1a and 2a). There was a significant increase in positive immunoreactivity ($P < 0.01$) in glandular and surface epithelium after administration of mifepristone (Figs 1a and 2b). There was no significant change in bcl-2 stromal immunoreactivity in endometrial biopsies collected on day 4 or day 6 after the LH surge and in the treatment cycle after administration of mifepristone (Fig. 1a).

Isolated stromal cells showed positive immunoreactivity for the bcl-2 epitope. However, there was no significant change in immunostaining in these stromal cells before and after administration of mifepristone. Examination of serial sections with the cell surface markers CD68 and CD56 indicated that the isolated stromal cells were likely to be either CD68-positive macrophages or CD56-positive large granular lymphocytes (Fig. 3).

Low immunoreactivity for the Bax epitope was observed in glandular and surface epithelium in all control biopsies (collected on days 4 and 6 after the LH surge) (Fig. 2c). There was no significant change in positive immunostaining after administration of mifepristone (Figs 1b and 2d). Positive immunoreactivity was also noted in stromal tissues in both

Fig. 1. Immunostaining scores for (a) bcl-2, (b) Bax and (c) oestrogen receptor in human endometrium with and without administration of mifepristone on day 2 after the onset of the LH surge. □, glandular tissue; ■, stromal tissue; ▨, surface epithelium; ▩, white blood cells. Endometrial biopsies for control and treated groups were collected on day 4 or day 6 after the LH surge. Values are mean $\pm$ SEM.

the control and treated endometrium (Figs 1b and 2c,d), but this was less pronounced than in the glandular and surface epithelium.

A significant increase in oestrogen receptor immunoreactivity was observed in both the glandular and stromal endometrial tissue after administration of antiprogesterin (Fig. 1c). A positive correlation (0.874) was demonstrated between bcl-2 immunoreactivity in glandular tissue and the positive nuclear immunoreactivity for glandular oestrogen receptors.

Discussion

The results of the present study confirm that bcl-2 and Bax proteins are localized mainly to the glandular and surface epithelium of secretory endometrium. After administration of the antiprogesterin mifepristone in the early luteal phase, the development of secretory changes in the endometrium was inhibited (Swahn *et al.*, 1990; Cameron *et al.*, 1997). Antiprogesterins inhibit the expression of a number of progesterone-dependent proteins, including prostaglandin dehydrogenase, glycodein, 17 β steroid dehydrogenase and leukaemia inhibitory factor. In addition, the downregulation of oestrogen and progesterone receptors that normally occurs during the luteal phase is prevented (Cameron *et al.*, 1997). The increase in immunostaining for bcl-2 protein in the glands of women administered mifepristone indicates that the expression of this gene may be stimulated by oestrogen and downregulated by progesterone.

An increase in bcl-2 concentrations in glandular tissue would decrease apoptosis and extend the life span of the epithelial cells. Since an increase in survival of epithelial cells in the colon has been associated with an increased risk of carcinoma (Tsujii and DuBois, 1995; Tsujii *et al.*, 1998), the increase in bcl-2 after treatment with antiprogesterin reinforces the protective role of progesterone in an oestrogen-dominated uterus.

The isolated cells in the stromal compartment that stained strongly for bcl-2 also showed clear staining for CD68 and CD56, indicating that these cells may be subpopulations of macrophages or large granular lymphocytes. The fact that the degree of staining in the cells was not influenced by administration of mifepristone indicates that they are not under the influence of progesterone.

Oestrogen induces proliferation and growth of glandular and stromal cells by interacting with oestrogen receptors. It is thought that the anti-proliferative effect of progesterone is due to its ability to downregulate expression of the oestrogen receptor. Mifepristone prevents this action of progesterone and hence a large number of oestrogen receptors (and progesterone receptors) persists in the endometrium of women administered this compound in the early luteal phase (Cameron *et al.*, 1997). It is possible that the upregulation of bcl-2 that occurs after treatment with mifepristone is due to the persistence of oestrogen receptors. However, in spite of the high concentration of oestrogen receptors and a marked increase in Ki67 expression (Cameron *et al.*, 1996), the endometrium shows little or no evidence of mitosis after administration of mifepristone. The anti-proliferative action of mifepristone is not fully understood, but is thought to be due to a product of the mifepristone-progesterone receptor complex that prevents the cells from completing the S phase of the cell cycle (Cameron *et al.*, 1996).

The immunoreactivity of the Bax epitope was constant both in control endometrium and in endometrium after administration of antiprogesterin. This is consistent with the observations of McLaren *et al.* (1997) and Tao *et al.* (1997) of Bax immunostaining in glandular epithelium. McLaren *et al.* (1997) indicated that this finding implies a lack of hormone regulation of this epitope. The observations of this group are

Fig. 2. *bcl-2* immunoreactivity in luteal phase human endometrium on day 4 after the LH surge (a) without and (b) with administration of mifepristone on day 2 after the LH surge. *Bax* immunoreactivity in luteal phase human endometrium (c) without and (d) with administration of mifepristone on day 2 after the LH surge; results from (c) day 6 and (d) day 4 after the LH surge. (e) Negative control, *bcl-2* primary antibody replaced with mouse immunoglobulin at equivalent concentration. (f) Positive control for *Bax* immunostaining, first trimester villi with positive immunoreactivity (brown) in the cytotrophoblast; (g) negative control for *Bax* immunoreactivity, first trimester villi absent immunoreactivity. Scale bars represent (a-e) 100 µm, (f,g) 50 µm.

Fig. 3. Photomicrographs of serial sections of luteal phase human endometrium on day 4 after the LH surge after administration of mifepristone on day 2 after the LH surge. Sections are immunostained for (a) bcl-2; (b) CD56-positive large granular lymphocytes and (c) CD68-positive macrophages. Arrowheads indicate the same cells in serial sections. Positive bcl-2 immunoreactivity in subsets of CD56- and CD68-positive leucocytes is shown. Scale bar represents 50 µm.

in contrast to those of Tao *et al.* (1997), since the latter report does consider Bax expression to be hormone regulated. In the present study, there was no difference in Bax immunostaining between control endometrium and tissues collected after mifepristone administration.

The observation of bcl-2 positive immunoreactivity in a

subpopulation of isolated stromal cells in the control tissues collected in the secretory phase and in the endometrium after mifepristone administration is of interest. This group (Koh *et al.*, 1995; Lea *et al.*, 1997b) has identified bcl-2 immunopositive cells in non-pregnant endometrial stroma and in first trimester decidua. Furthermore, co-localization with the CD56-positive large granular lymphocytes was reported. In the present study macrophages were observed in the endometrial biopsies. This is consistent with a population of bcl-2 positive but Bax negative tissue macrophages in ectopic endometrial tissues (McLaren *et al.*, 1997). McLaren *et al.* (1997) report the presence of such subpopulations only in ectopic endometrial tissues, and suggest that the presence of bcl-2 and absence of Bax contributes to the survival of the macrophages.

In conclusion, the findings of the present study in antiprogesterin-treated human endometrium support earlier reports of steroid regulation of at least bcl-2 expression and possible modulation via the oestrogen receptor (Otsuki *et al.*, 1994; Teixeira *et al.*, 1995). Blocking the progesterone receptor with mifepristone on day 2 after the onset of the LH surge resulted in upregulation of oestrogen receptors in endometrial glands, a retardation of the development of secretory differentiation, and continued bcl-2 expression in the glandular epithelium. The presence of bcl-2 immunoreactivity in a subset of macrophages and large granular lymphocytes indicates continued survival of these leucocyte subpopulations in endometrium after antiprogesterin exposure. These observations are consistent with morphological changes in the endometrium throughout the menstrual cycle, and support steroid regulation of expression of at least the bcl-2 protein in the uterine epithelium.

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