Bcl-2, Fas and caspase 3 expression in endometrium from levonorgestrel implant users with and without breakthrough bleeding

Peter A.W.Rogers[1,3], Fiona Lederman[1], Debbie Plunkett[1] and Biran Affandi[2]

[1]Department of Obstetrics and Gynaecology, Monash University, Monash Medical Centre, 246 Clayton Road, Clayton, Victoria 3168, Australia and [2]Department of Obstetrics and Gynaecology, Faculty of Medicine, University of Indonesia, Jakarta 10430, Indonesia

[3]To whom correspondence should be addressed at: Department of Obstetrics and Gynaecology, Monash University, Monash Medical Centre, 246 Clayton Road, Clayton, Victoria 3168, Australia. E-mail: peter.rogers@med.monash.edu.au

Women using the progestin-only contraceptive Norplant® often suffer from unpredictable bouts of breakthrough bleeding, which usually occurs from a thin atrophic endometrium. The role of cellular apoptosis in the endometrial response to Norplant has not been investigated. The aim of the present study was to use immunohisto-chemistry to produce semi-quantitative scores for expression of the apoptosis-related proteins Bcl-2, Fas and caspase 3 in endometrium from 16 controls and 42 women using Norplant with minimal or major breakthrough bleeding problems. The results showed no difference in endometrial immunostaining for any of the three proteins between Norplant users with and without breakthrough bleeding. There was also no evidence of endometrial endothelial cell immunostaining for any of the proteins. Bcl-2 was the only protein to show a cyclical pattern, with higher expression in the proliferative compared to secretory glands. All three proteins showed different expression levels in control functionalis versus basalis, with the survival protein Bcl-2 being higher in basalis, and the death receptor Fas and the proteolytic enzyme caspase 3 being higher in the functionalis. Overall, the results suggest that apoptosis is regulated differently in functionalis compared to basalis, and that atrophic Norplant-exposed endometrium appears more like functionalis than basalis with respect to expression of Fas and caspase 3. There was no evidence for a role for apoptosis in the mechanisms that underlie progestin-induced endometrial breakthrough bleeding.

Key words: Bcl-2/caspase 3/endometrium/Fas/Norplant

Introduction

The human endometrium undergoes regular cycles of growth and regression throughout the reproductive life of the female. During times of remodelling and regression, significant amounts of programmed cell death or apoptosis occur (Hopwood and Levison, 1975; Tabibzadeh, 1995; Kokawa et al., 1996). To date there has not been a quantitative analysis of the cell types, the location within the endometrium, and the stage of the menstrual cycle at which apoptosis occurs in the human. Apoptotic bodies first appear around day 20 of a standardized 28 day cycle, and reach a maximum around day 24, about 4 days before the commencement of menstruation. It seems clear that apoptosis is widespread throughout many cells types just preceding and during menstruation (Hopwood and Levision, 1975; Kokawa et al., 1996). Perhaps surprisingly, apoptosis is also seen in the basalis layer of the endometrium during the secretory phase, despite the fact that this layer is not shed during menstruation and acts as the source of remaining endometrial cells from which the functionalis grows in the following proliferative phase.

Regulation of apoptosis is complex, with an increasing number of factors being shown to have a role in the process of programmed cell death. Some cells have what are termed death receptors on their surface. Such cells detect extracellular signals and in response rapidly move into the apoptotic pathway. The best-characterized death receptors are Fas (also called CD95 or Apo1) and tumour necrosis factor receptor 1 (TNFR1, also called p55 or CD120a) (Ashkenazi and Dixit, 1998). Within cells, a number of proteins play key roles in regulating the apoptotic pathway. These include Bcl-2, which promotes cell survival by inhibiting adapters needed for the activation of proteases that dismantle the cell (Adams and Cory, 1998). Molecules with homology to Bcl-2, such as Bax, Bak and Bad promote apoptosis through a range of mechanisms that probably include displacing adapters from pro-survival proteins such as Bcl-2. Thus for many but not all of the apoptotic signals, the balance between Bcl-2 family members plays a key role in determining whether apoptosis or cell survival is a final outcome.

Further along the apoptotic pathway is a proteolytic system which results in protein cleavage and the breakdown of the cell. Proteases termed caspases play a central role in this cell breakdown (Thornberry and Lazebnik, 1998). The overall picture of how caspases contribute to apoptosis is not fully understood. However, it is known that caspases are highly specific and can only cleave proteins at an aspartic acid site. Thus caspase activation results in cleavage of specific proteins within the cell, including apoptosis protective proteins such as Bcl-2. One of the key caspases involved in apoptosis is caspase 3 (also termed CPP32, Yama or apopain), which becomes activated during the early stages of apoptosis. In its active form, caspase 3 proteolytically cleaves and activates other caspases, as well as relevant targets in the cytoplasm. Apoptosis is usually terminated with phagocytosis of the affected cell by surrounding cells, in a total sequence that can be completed within 30–60 min.

Given the spontaneous and cyclical high levels of apoptosis in human endometrium, it is not surprising that there have been many studies of the expression of apoptosis related factors in this tissue (Gompel et al., 1994; Otsuki et al., 1994; Koh et al., 1995; Tabibzadeh et al., 1995; Tao et al., 1997; Watanabe et al., 1997; McLaren et al., 1997; Jones et al., 1998; Tao et al., 1998; Critchley et al., 1999; Yamashita et al., 1999). From these papers, a general picture of Fas and Bcl-2 immunoexpression throughout the menstrual cycle has been developed. Bcl-2 is most prominent in the surface epithelium and glands during the proliferative stage of the cycle, showing a significant post-ovulatory reduction. Stromal activity is weaker without the same evidence for a cyclical pattern. Stromal staining in the basalis appears less than in the functionalis. Fas is expressed throughout the cycle and is more prominent in glands than stroma. There is no apparent cyclical pattern in Fas expression in either stroma or glands. In contrast to Bcl-2 and Fas, caspase 3 expression has not been investigated in human endometrium.

Given the cyclical nature of apoptosis in human endometrium, it seems likely that oestrogen and progesterone must directly or indirectly regulate the signals that ultimately result in apoptosis in this tissue. Thus, it might be predicted that apoptosis levels will be influenced under differing steroid regimens. Despite this, there has only been limited work to date on the role of oestrogen and progesterone in regulating apoptosis in endometrial cells. In a study of five samples of pseudodecidualized endometrium from women receiving exogenous progesterone treatment (either norethisterone or medroxyprogesterone acetate) high concentrations of Bcl-2 were reported with no evidence of apoptosis (Jones et al., 1998). In another study it was concluded that antiprogesterone treatment with mifepristone (RU486) inhibits progesterone downregulation of steroid receptors in endometrial glands, thus resulting in persistence of a proliferative endometrium with elevated Bcl-2 concentrations (Critchley et al., 1999).

There have been no studies to date investigating expression of Bcl-2, Fas and caspase 3 in endometrium from women using Norplant (levonorgestrel). The aim of the present study was to use immunohistochemistry to produce semi-quantitative scores for expression of Bcl-2, Fas and caspase 3 in endometrium from women: (i) during the normal menstrual cycle, (ii) using Norplant with minimal

breakthrough bleeding problems, and (iii) using Norplant with major breakthrough bleeding problems. A sub-aim was to specifically look for expression of Bcl-2, Fas and caspase 3 in endometrial endothelial cells to establish if any of these apoptotic-related proteins may correlate with breakthrough bleeding in Norplant users.

Materials and methods

Control and Norplant-exposed endometrial tissues

Full thickness normal endometrial tissue ($n = 16$) were obtained from reproductive age (i.e. not menopausal and with normal menstrual cycle lengths) women in Melbourne undergoing hysterectomy for a variety of reasons including: fibroids, menorrhagia, adenomyosis, prolapse and tubal metaplasia. Tissue samples were only selected from areas of the uterus that appeared normal by gross pathological examination, and this normal appearance was also confirmed by routine histology. Endometrial biopsies were also collected by Pipelle suction curette (Prodimed 60530; Neuilly-en-Thelle, France) or Karman cannula (Rocket, London, UK) (Hadisaputra *et al.*, 1996) from Indonesian women ($n = 42$) attending Raden Saleh Clinic in Jakarta with between 3 and 12 months exposure to Norplant contraception. Each subject maintained a daily menstrual record card recording either no bleeding or spotting/bleeding. From the menstrual diary, biopsies were categorized as being from bleeders ($n = 22$) or non-bleeders ($n = 20$) based on an assessment of the 90 days prior to the time of biopsy. Bleeders were defined as subjects with spotting or menstrual bleeding for ≥ 25 out of the 90 days and non-bleeders as having spotting and bleeding for ≤ 10 days.

All biopsies were routinely fixed in 10% buffered formalin for 4 h, processed and embedded in paraffin. Sections (5 μm) were cut and stained with haematoxylin and eosin. Control cycle endometrial tissues were classified by an experienced histopathologist using established criteria (Noyes *et al.*, 1950) into five menstrual cycle stages (menstrual, early proliferative, late proliferative, early secretory, late secretory).

Ethical approval for this study was obtained from Monash Medical Centre Human Ethics Committee, The Human Ethics Committee of the Faculty of Medicine at the University of Indonesia, and The World Health Organization.

Bcl-2, Fas and caspase 3 immunostaining

Paraffin sections, 5 μm thick, were cut from each biopsy and mounted on aminopropyltriethoxysilane (APES; Sigma, St Louis, MO, USA)-coated slides. The sections were air-dried overnight at 37°C. Tissue sections for caspase 3 were immunostained within 24 h of cutting following air-drying. The primary antibodies used were polyclonal, goat anti-human caspase 3 (p20{N-19}; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), polyclonal rabbit anti-human Fas (C-20; Santa Cruz) and monoclonal mouse anti-human Bcl-2 (clone Bcl-2/100/D5; IgG1; Novocastra, Newcastle, UK). Sections that required antigen retrieval were adhered to the slide by incubating at 60°C in air for 1 h prior to dewaxing. Slides were dewaxed to dH_2O and washed in phosphate-buffered saline (PBS; pH 7.4). Washing in PBS at room temperature (RT) was performed between all steps unless otherwise stated. Sections for Fas and Bcl-2 immunostaining underwent microwave antigen retrieval in 0.01 mol/l tri-sodium citrate (pH 6.0) for 3×5 min (defrost cycle; 500 W) and were then cooled for 20 min. Endogenous peroxidase was blocked with 3% hydrogen peroxide in 50% methanol for 10 min at RT. Slides were incubated with serum-free protein blocking agents (PBA; Lipshaw Immunon; Pittsburgh, PA, USA; or Dako block; Dako; Carpinteria, CA, USA) for 10 min, directly followed by incubation with the primary antibody. The primary antibody diluent was 1% bovine serum albumin in PBS. Caspase 3 (2 μg/ml) and Bcl-2 (0.29 μg/ml) antibodies were incubated at 4°C overnight and Fas (1 μg/ml) antibody for 1 h at 37°C. The Dako LSAB+ Peroxidase kit was used for the biotinylated secondary antibody and streptavidin–peroxidase complex. These were applied sequentially for 15 min at RT for all sections. Aminoethylcarbazole (AEC) red chromogen (Zymed; San Francisco, CA, USA) was applied for 10 min at RT, then washed with dH_2O and the sections mounted in Clearmount (Zymed) and air-dried overnight. Negative controls were

performed, substituting the primary antibodies with species, concentration and isotype-matched IgG controls.

Evaluation and scoring of immunostaining

Full thickness control endometrium was divided into three zones: basalis, functionalis and surface epithelium. These were defined morphologically with the assistance of a haematoxylin and eosin-stained section. Within the basalis and functionalis, endometrial glands and stroma were assessed separately. Blood vessels, smooth muscle, decidu- lized cells and other stromal components were noted if staining occurred. Norplant samples have no defined basalis and functionalis, therefore 'typ- ical' endometrial glands, stroma and surface epithe- lium were assessed for each biopsy. A score of 0, 1, 2 or 3 (0 = negative; 1 = weak; 2 = moderate; 3 = strong staining) was given to each component of the tissue section based on the intensity, distribu- tion and proportion of cell staining.

Tissue sections from each biopsy were not always available for evaluation of immunostaining performed under each of the three staining proto- cols. In addition, some individual sections, or tissue compartments within sections (e.g. surface epithelium, glands etc.), could not be scored for a range of reasons.

For comparisons between independent groups, statistical analysis was undertaken using the Mann– Whitney U-test (SPSS for MS Windows release 6.1). Comparisons between functionalis and basalis from the same sample were treated as related pairs and analysed by the Wilcoxon matched-pairs signed-ranks test (SPSS for MS Windows, release 6.1). $P < 0.05$ was taken as the value for statistical significance.

Results

Photographs giving examples of immunostaining for Bcl-2, Fas and caspase 3 are shown in Figures 1, 2 and 3 respectively. Graphical representation of the data for immunostaining of each separate tissue compartment is given in Figure 4.

Summary of Bcl-2 immunostaining

Bcl-2 immunostaining was cytoplasmic with cell nuclei being clear (Figure 1). Bcl-2 immuno-

staining varied between different tissue compart- ments and across the different stages of the menstrual cycle in full thickness endometrium (Figure 1a–d). Overall, the glands and surface epithelium stained more strongly than the stroma. In both the functionalis and basalis the glands stained more intensely in the proliferative phase of the cycle than the secretory phase ($P = 0.0462$ for functionalis and $P = 0.0359$ for basalis). In particular, functionalis gland staining was very weak by the late secretory stage. The stroma did not vary in staining intensity between proliferative and secretory stages of the cycle.

Basalis gland immunostaining was stronger than that seen in the functionalis, although this only became statistically significant when proliferative and secretory samples were combined and basalis compared with functionalis using a Wilcoxon matched-pair analysis ($P = 0.0384$). In the stroma the functionalis was nearly always negative whereas the basalis showed low, but significantly elevated, levels of staining in the proliferative ($P = 0.0253$) and secretory ($P = 0.0339$) phases. There was no staining of blood vessels or deciduaized cells. The myometrial smooth muscle was positive in almost all specimens examined.

Among Norplant users, there was no difference in Bcl-2 immunostaining between biopsies from women with or without excessive breakthrough bleeding. Heterogeneous cytoplasmic staining in the glandular and surface epithelium was present in ~ a third of the specimens (Figure 1e). Stroma stained very weakly or was negative, with the occasional single leukocyte positive. There was some heterogeneity of staining, with tissues that were positive varying from weak to strong intensity. There was no identifiable vascular staining.

Overall, Norplant tissue immunostaining levels did not match any particular compartment or cycle phase of control endometrium. Norplant glands were weaker than proliferative phase basalis glands ($P = 0.0094$), similar to proliferative functionalis and secretory basalis glands, and stronger than secretory functionalis glands ($P = 0.0444$). Norpl- ant stroma immunostaining was weaker than con- trol cycle basalis staining ($P = 0.0104$), but stronger than control cycle functionalis staining ($P = 0.0296$).
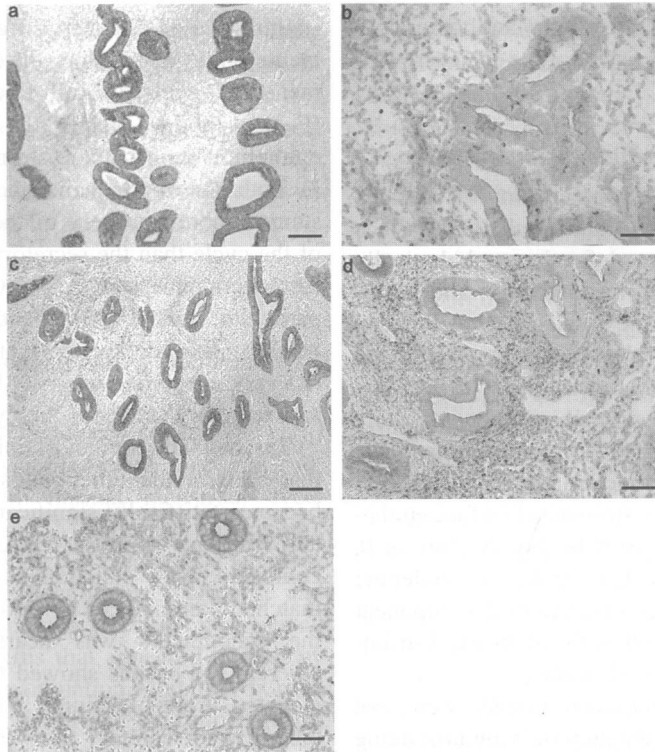
Figure 1. Human endometrium immunostained to show Bcl-2. (**a**) Proliferative phase functionalis. (**b**) Secretory phase functionalis. (**c**) Proliferative phase basalis. (**d**) Secretory phase basalis. (**e**) Tissue from a Norplant user. Note significant increase in proliferative phase functionalis and basalis glands compared to equivalent secretory phase sections. For (**a**) and (**c**) scale bar = 100 μm, for (**b**), (**d**) and (**e**) scale bar = 50 μm.

Summary of FAS immunostaining

Fas immunostaining usually occurred throughout the whole cytoplasm of cells (Figure 2) and was present at all stages of the menstrual cycle (Figure 2a–d). There was no correlation between the cycle stage and staining pattern or intensity. Staining intensity increased from the basalis to the functionalis in both glands ($P = 0.0095$) and stroma ($P = 0.0047$), with the surface epithelium often being the most intense. The endometrial stroma had weaker staining than the glands, and was more intense towards the surface epithelium. Pronounced staining of cilia-like structures on endometrial gland cells and surface epithelium was present in ~50% of specimens (Figure 2d). Blood vessel staining was not obvious in any of the specimens. A feature present mainly in the myometrium, but also in the endometrial stroma, was aggregates of very intense staining. These may possibly have been leukocytes. The myometrium often showed weak staining of the smooth muscle cells.

Among Norplant users, there was no difference in Fas immunostaining between biopsies from women with or without excessive breakthrough bleeding. Almost all samples had staining of endometrial glands and/or surface epithelium. There was some heterogeneity between glands within some sections, and staining ranged from very low to very intense between sections (Figure 2e). There was strong cilia-like staining in some glandular epithelial cells and surface epithelium. There were also scattered positive cells and cell aggregates in many sections, possibly leukocytes. Some decidual-like cells were also positive. The blood vessels showed no obvious staining in any of the sections.

Overall, the Fas immunostaining in Norplant biopsies was of similar intensity to that in control functionalis glands and stroma, and stronger than
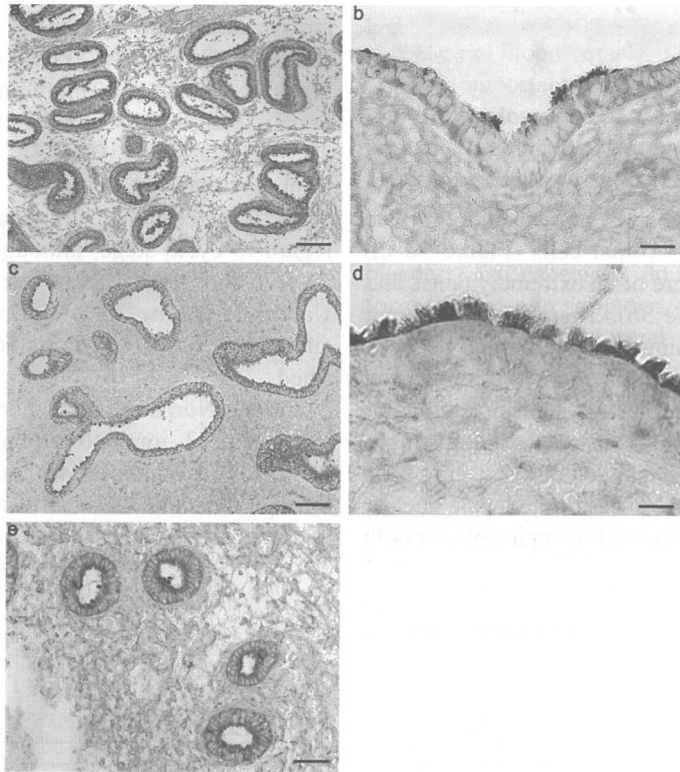
Figure 2. Human endometrium immunostained to show Fas. (**a**) Proliferative phase functionalis. (**b**) Proliferative phase surface epithelium. (**c**) Proliferative phase basalis. (**d**) High power of surface epithelium showing strong immunostaining of cilia-like structures. (**e**) Tissue from a Norplant user. Scale bars: (**a**) and (**c**) 100 μm, (**b**) 25 μm, (**d**) 10 μm, (**e**) 50 μm.
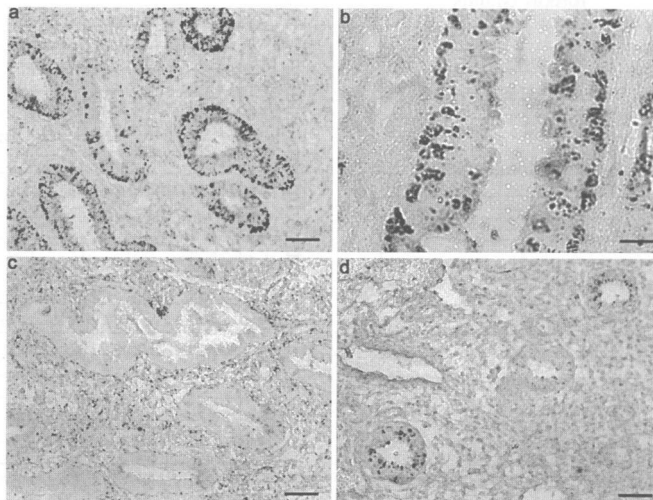

Figure 3. Human endometrium immunostained to show caspase 3. (**a**) Proliferative phase functionalis. (**b**) High power of proliferative phase glandular epithelium showing granular pattern of immunostaining. (**c**) Proliferative phase basalis. (**d**) Tissue from a Norplant user. Scale bars: (**a**), (**c**) and (**d**) 50 μm, (**b**) 10 μm.

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that in control basalis glands ($P = 0.0071$) and stroma ($P = 0.0027$).

Summary of caspase 3 immunostaining

Cellular immunostaining for caspase 3 was primarily confined to small dense granules within the cytoplasm of epithelial cells, but was more diffuse and often nuclear in stromal cells (Figure 3a–c). Epithelial granules were often extremely dense and dark in colour (Figure 3b). There was significant heterogeneity of staining both within and between biopsies. In some sections there was either an evenly distributed cytoplasmic stain or a granular stain within the stroma. This occasionally highlighted individual large single cells that may have been macrophages. There was limited, pale nuclear staining in some endometrial stromal cells, smooth

muscle cells of blood vessels within myometrium, and some myometrial cells. Granular and diffuse immunostaining was more intense in the functionalis than basalis in both glands ($P = 0.0455$) and stroma ($P = 0.0143$), with the strongest staining often just below the surface epithelium. There was no statistically significant pattern with regard to cycle stage, although the dark granular stain in the glands below the surface epithelium was most intense in late secretory samples. No endothelial caspase 3 immunostaining was identified.

Among Norplant users, there was no difference in caspase 3 immunostaining between biopsies from women with or without excessive breakthrough bleeding. Cellular distribution, and within and between biopsy heterogeneity of immuno-
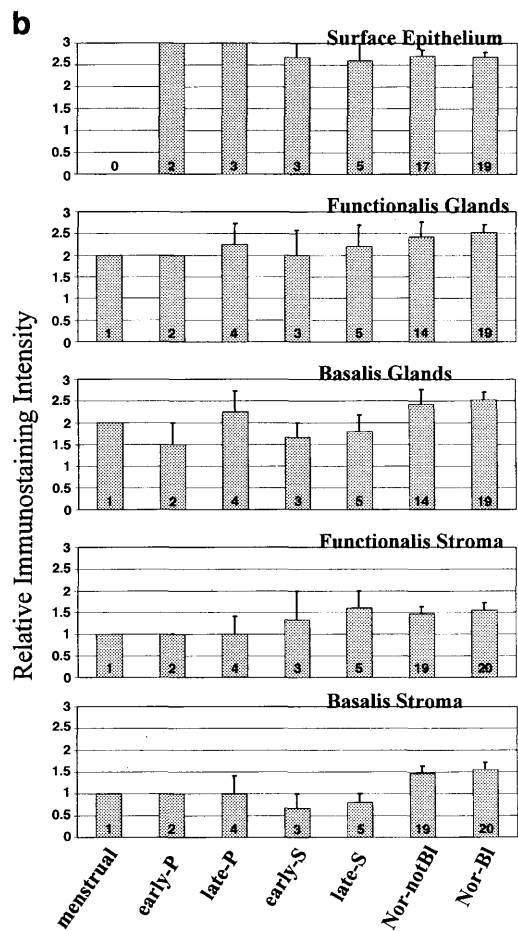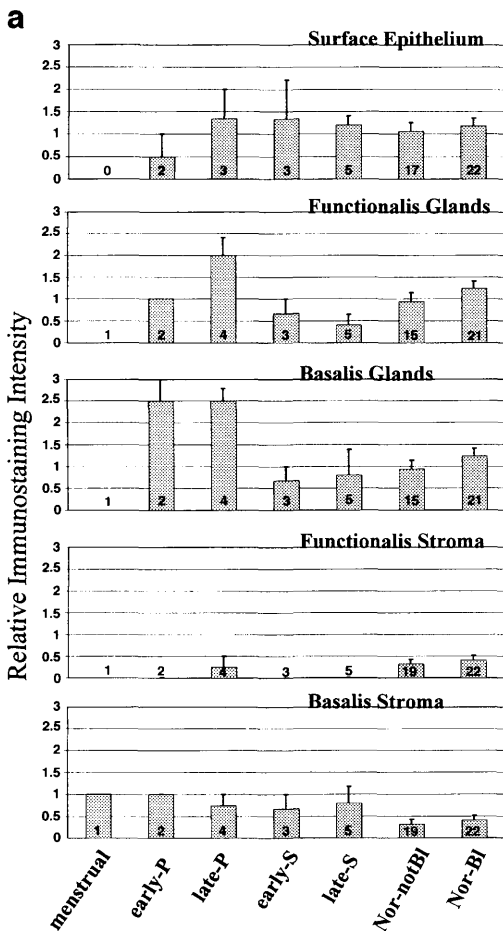

Figure 4a, b. For legend see facing page.

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staining was similar to that seen in controls (Figure 3d). Overall, the distribution and staining intensity of caspase 3 in biopsies from Norplant users was similar to control functionalis and stronger than basalis glands ($P = 0.0204$) and stroma ($P = 0.0017$).

Discussion

In this study we have described the immunolocalization of Bcl-2, Fas and caspase 3 in endometrial biopsies taken during the control menstrual cycle and from Norplant users with and without breakthrough bleeding. The findings of the study are essentially negative in relation to two of the major aims. There was no difference in immunostaining for any of these three apoptotic pathway-related markers between endometrial biopsies from women with and without breakthrough bleeding. Furthermore, no evidence was obtained for expression of any of these three proteins in endothelial cells of endometrial blood vessels. These negative findings make an important contribution to our understanding of the local mechanisms that may or may not underlie progestin-only induced endometrial breakthrough bleeding. Data from the current study do not provide any support for altered apoptotic events in the endometrium of bleeders versus non-bleeders, and especially do not provide evidence for differences in apoptosis of vascular cells.

The findings for normal cycle biopsies in the present study primarily agree with those of earlier workers who investigated the immunolocalization of Bcl-2 and Fas (Gompel et al., 1994; Otsuki et al., 1994; Koh et al., 1995; Tabibzadeh et al., 1995; McLaren et al., 1997; Tao et al., 1997; Watanabe et al., 1997; Jones et al., 1998; Tao et al., 1998; Critchley et al., 1999; Yamashita et al., 1999). The present study provides the first
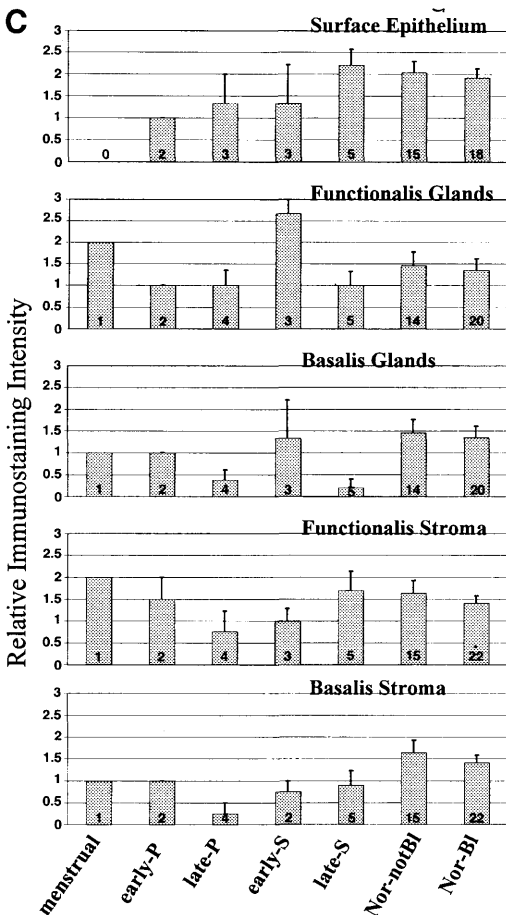

Figure 4. Graphs showing relative endometrial immunostaining intensity (mean ± SEM) for surface epithelium, and glands and stroma from functionalis and basalis at different stages of the menstrual cycle and in subjects using Norplant. (a) Bcl-2 immunostaining; (b) Fas, and (c) caspase 3. Early-P = early proliferative; late-P = late proliferative; early-S = early secretory; late-S = late secretory; Nor-notBl = Norplant user with minimal breakthrough bleeding problems;
Nor-Bl = Norplant user with significant breakthrough bleeding problems.

description of the immunolocalization of caspase-3 in normal endometrium. Our finding that immuno-expression is higher in glands and stroma of the functionalis compared to the basalis fits with the observation that apoptosis is generally more common in the functionalis than the basalis, particularly in the later part of the cycle.

Our observation that immunostaining for each of these three apoptosis pathway proteins is significantly different in the functionalis compared to the basalis, highlights the importance of dividing the endometrium into functional zones and not treating it as a homogeneous tissue. Overall our results fit well with the functional biology of the control endometrium. The survival protein Bcl-2 is elevated in the basalis compared to the functionalis, while Fas and caspase 3 are elevated in the functionalis compared to the basalis. The functionalis undergoes cyclical growth differentiation and shedding, and during these processes increased levels of apoptosis are seen. In contrast, the basalis remains relatively constant throughout the menstrual cycle and it is this tissue which provides the cells from which the new functionalis regenerates following menstruation. Apoptosis, although reported in the basalis, is less common in this tissue than in the functionalis.

One of the features of Norplant-exposed endometrium is its thin and atrophic appearance. Although Norplant biopsies can have differing histological appearances from subject to subject, in general terms the limited amount of endometrium within each biopsy has a consistent appearance. Thus it is not possible to divide the biopsies into clearly demarcated basalis or functionalis layers. Given that cyclical changes in histological appearance no longer occur in Norplant-exposed endometrium, and that the endometrium is usually very thin, it is possible that the remaining endometrial cells represent basalis rather than functionalis. However, it is also possible that the long-term progestin exposure has resulted in an abnormally differentiated functionalis-type endometrium. In the present study we compared the cellular localization and immunostaining intensity of three apoptosis pathway markers in Norplant endometrium, with the distribution seen in functionalis and basalis of control endometrium. For Fas and

caspase 3 there was no difference between control functionalis glands and stroma and the Norplant tissues. In contrast, Norplant immunostaining was significantly stronger than that seen in the basalis of control endometrium for both Fas and caspase 3. When comparing Norplant biopsy immunostaining for Bcl-2 with that seen during the control cycle, the pattern was more confusing. This is in part due to the fact that in control glands Bcl-2 immuno-staining varies across the cycle with higher levels seen in the proliferative phase. For Bcl-2, Norplant glands were weaker than proliferative phase basalis glands, similar to proliferative functionalis and secretory basalis glands, and stronger than secretory functionalis glands. Norplant stroma immuno-staining was stronger than functionalis but weaker than basalis. Ignoring the Bcl-2 results, these data suggest that Norplant endometrium is more typical of functionalis than basalis, at least in terms of the distribution of Fas and caspase 3.

Despite the fact that Norplant endometrium is atrophic, thin, and in the case of women with breakthrough bleeding may show focal degenerative changes, the levels of apoptosis pathway markers were substantially similar to those seen in the functionalis of controls. It is possible that levels of apoptosis are elevated during early use of Norplant as the endometrium changes from normal thickness to an atrophic appearance. Our data suggest that once a woman has used Norplant for between 3 and 12 months, the endometrium has reached a relatively uniform state where levels of apoptosis are not different to those seen in normal functionalis.

In conclusion, we have shown no difference in the cellular localization or levels of immunoexpression of three different apoptosis pathway markers in endometrium taken from Norplant users with and without increased breakthrough bleeding. Our study provides no evidence for a role for altered apoptosis mechanisms in progestin-only induced endometrial breakthrough bleeding. Furthermore, we have found no evidence of apoptosis in endometrial endothelial cells from controls or Norplant users. We have shown significant differences in expression of apoptosis pathway markers between control functionalis and basalis, and overall our study suggests that Norplant-exposed endometrium is more like control cycle functionalis than basalis.

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