Changes in the ratio of Bax and Bcl-2 mRNA expression and their cellular localization throughout the ovulatory cycle in the human oviduct

Christine Briton-Jones,^{1,2,3} Ingrid Hung Lok,^{1,4} Alice Lai See Po,^{1,2} Che Kwok Cheung,^{1,2}
Tony T. Y. Chiu,^{1,4} and Christopher Haines^{1,2,5}

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Purpose: To examine changes in the ratio of Bax and Bcl-2 mRNA expression throughout the ovulatory cycle in the ampullary region of the human oviduct.

Methods: The mucosal layer was isolated from the human oviduct tissue and semiquantitative reverse-transcriptase polymerase chain-reaction (RT-PCR) analysis of mRNA of Bax and Bcl-2 was performed. Immunohistochemistry provided the cellular localization of the Bax and Bcl-2 proteins. The ratio of expression of Bax and Bcl-2 mRNA was examined in the ampullary region of the oviduct in samples obtained in the follicular, periovulatory, and luteal phases of the ovulatory cycle.

Results: Bax expression was constant in the follicular and periovulatory phase but showed a significant increase in the luteal phase. The Bax protein was present in all oviduct mucosal epithelial cells and the intensity of staining increased in luteal phase samples. Bcl-2 was expressed at a relatively constant level throughout the ovulatory cycle. The Bcl-2 protein was present in some but not all mucosal epithelial cells and the proportion of positive cells remained constant throughout the ovulatory cycle.

Conclusion: The proapoptotic gene Bax shows a significant increase in mRNA expression in the luteal phase of the ovulatory cycle while the expression level of the antiapoptotic gene Bcl-2 remains constant throughout the ovulatory cycle. The ratio of Bax:Bcl-2 increases significantly in the luteal phase consistent with cells undergoing apoptosis.

KEY WORDS: Apoptosis; Bax; Bcl-2; human oviduct.

INTRODUCTION

The oviduct is the site of oocyte collection, gamete transport, fertilization, early embryo cleavage, and

embryo transport. Despite being the site of critical events in early reproduction the cellular physiology and pathology of the oviduct is poorly understood. Advancement in the knowledge of cell development and differentiation of the oviduct mucosa may lead to new treatment or diagnostic strategies for infertility, assisted reproductive technology, and fallopian tube carcinoma.

While the cycles of cell growth differentiation, degeneration, and regeneration in the endometrium (1) is well characterized in the literature, studies describing cell development in the oviduct are limited. The incidence of oviduct carcinoma is considerably lower than in the endometrium (2), which may suggest less

¹ The Department of Obstetrics and Gynaecology, The Prince of Wales Hospital, Shatin, New Territories, Hong Kong, People's Republic of China.

² Department of Obstetrics and Gynaecology, The Chinese University of Hong Kong, Hong Kong, People's Republic of China.

³ Present address: The Fertility Institutes, 16030 Ventura Boulevard, Suite 404, Encino, CA 91346.

⁴ Department of Obstetrics and Gynaecology, The Hospital Authority of Hong Kong, The Prince of Wales Hospital, Shatin, New Territories, Hong Kong, People's Republic of China.

⁵ To whom correspondence should be addressed; e-mail: cjhaines@cuhk.edu.hk.

dynamic cell growth and regeneration. In contrast, many studies using morphological observations have demonstrated continual cellular changes in the oviduct mucosal cells throughout the ovulatory cycle.

During the follicular phase, the mucosal epithelium develops a multilayered appearance due to a greater degree of cell crowding (3) however, in the luteal phase the epithelium has a single-layered, simple appearance. Epithelial height has also been shown to increase significantly during the follicular phase (4). Therefore, in the follicular phase of the ovulatory cycle the oviduct mucosal cells increase in number and size.

Postovulation the surface of the nonciliated mucosal cells display irregular protrusions, some of which are higher than the cilia of adjacent cells (3). Some of the nonciliated cells at the time of ovulation appear to be dissociated or completely separated from the underlying cell matrix, resulting in the release of cell fragments into the oviduct lumen. The release of surface cytoplasmic fragments is also accompanied by extrusion of larger cell fragments including nuclei and some whole cells (3). In the early to midluteal phase there is a reversion to a flatter appearance with little or no secretion (3). Therefore postovulation morphological studies show a loss of oviduct mucosal cells into the lumen of the oviduct and a return to a less crowded more flat appearance of the mucosal surface.

Lok *et al.* (5) showed the presence of telomerase, a marker of stem cells in selected oviduct mucosal epithelial cells. By examining telomerase mRNA expression and protein localization in the same samples Lok *et al.* (5) showed that while the mRNA expression was present in all samples the protein was not, with the protein being associated with the presence of estradiol. This study demonstrates that the oviduct mucosa is capable of regulated cell growth and regeneration. A prerequisite factor in regulated tissue regeneration is apoptosis.

Apoptosis is an essential mechanism in cellular homeostasis and plays an important role as a defense system to remove damaged, infected, or mutated cells. Cells undergoing apoptosis are characterized as displaying shrinking of the cell leading to loss of contact with extracellular matrix, chromatin condensation, DNA fragmentation, and plasma membrane blebbing (6). To date two major pathways of apoptosis have been described: the death receptor pathway and the mitochondrial pathway [reviewed in (7)]. The mitochondrial pathway of apoptosis is regulated

by a complex balance of pro and antiapoptotic genes of the Bcl-2 family acting within a cell (8). Two genes from the Bcl-2 family are the proapoptotic gene Bax and the antiapoptotic gene Bcl-2. The ratio of Bax to Bcl-2 gene expression in a tissue can determine whether cells will be protected from apoptosis or will die from it (9).

In summary, the human oviduct shows similarities to the endometrium with cellular changes in growth and degeneration in a cyclical pattern reflecting the function to secrete embryotrophic factors. However, unlike the endometrium there are no obvious signs of cellular degeneration by apoptosis. This study proposes that the human oviduct undergoes cyclical periods of apoptosis postovulation. The specific hypothesis tested that the ratio of Bax to Bcl-2 expression increases in the luteal phase to reflect a tissue undergoing apoptosis.

MATERIALS AND METHODS

Collection of Oviduct Tissue

Oviduct tissue ($N = 15$) was obtained from women who were undergoing either a laparoscopy for tubal sterilization or a hysterectomy for a benign uterine condition. The stage of the cycle was determined by the date of commencement of the last menstrual period and was confirmed by appropriate serum concentrations of luteinizing hormone (LH), progesterone (P), and estradiol (E_2). All oviduct samples showed normal morphology.

This study was approved by the Clinical Research Ethics Committee of the Chinese University of Hong Kong, project number FM/C/13.

Excised oviduct tissue was immediately placed in 20 mL of HEPES buffered Quinn's Human Tubal Fluid (Irvine Scientific, Santa Ana, CA, USA). This medium was used for all tissue manipulation. The oviduct was rinsed to minimize blood cell contamination. The lumen of the oviduct was exposed by incising along the antimesenteric border and the mucosal folds were dissected off macroscopically. The samples were taken from the ampullary region of the tube as this is the site of fertilization and early embryo cleavage. Only two of the investigators performed the tissue dissection and the technique was closely monitored to ensure accurate replication.

Semiquantitative RT-PCR Analysis

mRNA was extracted from the oviduct mucosal tissue using the Oligotex direct mRNA kit (Qiagen, Hilden, Germany). All mRNA samples were treated with 1 U of DNase (Boehringer Mannheim/Hoffman-La Roche, Basel, Switzerland). The DNase was inactivated by incubating for 5 min at 90°C. One hundred ng of mRNA was used for cDNA synthesis with Multiscribe reverse transcriptase (PE Biosystems, Foster City CA, USA). All the resultant cDNA was then used for PCR with Ampli-taq Gold DNA polymerase (PE Biosystems, Foster City, CA, USA). Each PCR cycle consisted of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. Thirty cycles were performed, followed by a final extension at 72°C for 4 min. The RT-PCR assay was validated by establishing that the coamplified product for the (experimental) Bax or Bcl-2 and (internal standard) β -actin primer sets were linear and parallel with increasing amounts of start cDNA and the cycle number and primer concentrations were optimized in the exponentially increasing phase of the detectable product.

Forward and reverse primers specific to Bax were derived from Laffon *et al.* (8). The sequences of the primers were: forward—ATG GAC GGG TCC GGG GAG reverse—ATC CAG CCC AAC AGC CGC (Mwgag Biotech, Ebersberg, Germany). Forward and reverse primers specific to Bcl-2 were derived from Laffon *et al.* (8). The sequences of the primers were: forward—AAG CCG GCG ACG ACT TCT reverse—GGT GCC GGT TCA GGT ACT CA (Mwgag Biotech, Ebersberg, Germany). β -actin was coamplified with Bax or Bcl-2 to provide a semiquantitative internal control for RNA quantity and PCR reaction efficiency. β -actin is commonly used as a standard when comparing samples under different hormonal conditions, as it is constitutively expressed (10). Forward and reverse primers specific to β -actin were derived from published primer sequences (10). The sequences of the β -actin primers were: forward—ATC GTG GGG CGC CCC AGG CAC and reverse—CTC CTT AAT GTC ACG CAC GAT TTC (Mwgag Biotech, Ebersberg, Germany).

Twenty percent of each PCR reaction was separated by gel electrophoresis on a 2% agarose gel with 0.5 μ g/mL ethidium bromide in TBE buffer. The separated PCR products were visualized under Ultra Violet illumination. A video camera sent the UV illuminated gel image to a computer where the software package Gel Doc enabled an image of the gel to be

recorded. The integrated optical density (IOD) was determined for each PCR product by the image analyzer, Gel Doc System. The IOD ratio between the PCR amplified Bax or Bcl-2 product with its simultaneously amplified control, β -actin was obtained for each sample.

Statistics

The nonparametric Wilcoxon sign test was used to determine whether significant differences were present between the ratio of Bax and Bcl-2 mRNA expression in different ovulatory cycle phases. A probability value was considered significant when $P < 0.05$.

Immunohistochemistry

Ninety tissue sections consisting of three replicates of the ampullary region of the 15 oviducts were analyzed for the presence of Bax or Bcl-2 by specific immunohistochemical staining. Tissue sections fixed on glass slides were dewaxed in graded xylenes, rehydrated through graded alcohols, and washed in phosphate buffered saline (PBS).

Sections were stained for Bax or Bcl-2 with mouse monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The secondary antibody and chromogenic substrate addition was performed using the DAKO Catalyzed Signal Amplification System reagents and protocol (Dako, Glostrup, Denmark). Negative controls were performed by omitting addition of primary antibody. Staining was detected under light microscopy.

RESULTS

Six samples were collected from the follicular phase, five from the periovulatory phase, and four from the luteal phase. The periovulatory phase was distinguished from the follicular phase by the presence of an elevated serum LH concentration.

A PCR product of the appropriate size was obtained from both the Bax and Bcl-2 PCR primers in samples collected from all three cycle phases. There was no significant difference between the expression of Bax mRNA in the follicular compared to the periovulatory phase (Fig. 1A). However there was a significantly higher expression of Bax in the luteal phase where $P < 0.0001$ (Fig. 1A). There was

Fig. 1. (A) A bar graph showing the mean $\pm$ standard error for Bax/beta-actin mRNA expression in the follicular, periovulatory, and luteal phases of the ovulatory cycle. * $P < 0.0001$. (B) A bar graph showing the mean $\pm$ standard error for Bcl-2/beta-actin mRNA expression in the follicular, periovulatory, and luteal phases of the ovulatory cycle.

no significant difference between the expression of Bcl-2 mRNA in the three different cycle phases (Fig. 1B).

Ratio of Bax:Bcl-2 mRNA Expression

There was no change in the ratio of Bax:Bcl-2 expression between the follicular and periovulatory phases. However, there was significantly higher Bax:Bcl-2 mRNA expression in the luteal compared to the follicular and periovulatory phases where $P < 0.0005$ (Fig. 2).

Localization of Bax and Bcl-2 in the Ampullary Region of the Oviduct by Immunohistochemistry

Strong and specific cytoplasmic Bax staining was observed in the mucosal epithelial cells at all stages of the ovulatory cycle (Fig. 3). However, more intense staining was observed in the luteal phase samples (Fig. 3D). Specific cytoplasmic Bcl-2 staining was observed in some epithelial cells with a similar staining intensity at all stages of the ovulatory cycle (Fig. 4).

DISCUSSION

Bax mRNA expression was similar in the follicular and periovulatory phases of the ovulatory cycle but showed a significant increase in expression level in the luteal phase. In comparison Bcl-2 expression remained constant throughout the different cycle phases. This study is the first to report relative levels of expression of Bax and Bcl-2 in the human oviduct. Studies in chick oviduct confirm our results for Bcl-2. Monroe *et al.* (11) found that the expression of Bcl-2 remained constant in the chick oviduct with exposure and removal of estradiol in culture.

The ratio of Bax:Bcl-2 remains the same in the follicular and periovulatory phases but significantly increases in favor of the proapoptotic gene Bax in the luteal phase. Therefore the ratio of Bax:Bcl-2 in our results predicts a protection from apoptosis in the follicular and periovulatory phases and a change to activation of apoptosis in the luteal phase. The follicular and periovulatory phases are characterized by growth and differentiation of the oviduct mucosal cells to prepare for the presence of gametes and embryos. The luteal phase is characterized by postovulatory secretion from the oviduct mucosa with cell debris in the lumen of the oviduct and

Fig. 2. A bar graph showing the mean $\pm$ standard error for Bax/Bcl-2 relative expression in the follicular, periovulatory, and luteal phases of the ovulatory cycle. * $P < 0.0005$.

Fig. 3. Immunolocalization of Bax in human ampullary region oviduct by mouse monoclonal antibody (*brown*) with counterstaining (*blue*). (A) Negative control with PBS and 1.5% fetal bovine serum substituted for the primary anti-Bax antibodies. (B) Bax staining in the follicular phase. (C) Bax staining in the periovulatory phase. (D) Bax staining in the luteal phase.

a return to a more flattened appearance of the mucosa (4). Therefore, our results indicative of active apoptosis in the luteal phase conform to the pattern seen in earlier morphological studies of the oviduct mucosa cell growth and degeneration (3).

This is the first published data on the localization of Bax in human oviduct mucosa and shows the presence of the protein in the cytoplasm of the mucosal epithelial cells. An earlier study showed that Bcl-2 is only present in secretory cells in human oviduct mucosa (12). Our results confirm this study in that some mucosal epithelial cells were positive while oth-

Fig. 4. Immunolocalization of Bcl-2 in human ampullary region oviduct by mouse monoclonal antibody (brown) with counterstaining (blue). (A) Negative control with PBS and 1.5% fetal bovine serum substituted for the primary anti-Bcl-2 antibodies. (B) Bcl-2 staining in the follicular phase. (C) Bcl-2 staining in the periovulatory phase. (D) Bcl-2 staining in the luteal phase.

ers remained negative. While it is most likely that this is showing the difference between secretory and ciliated epithelial cells as we did not do concurrent staining for ciliated cell markers this can only be hypothesized. It is interesting to note that the percentage of Bcl-2 positive cells did not change in the different phases of the cycle which may indicate that the percentage of ciliated cells remains con-

stant which is in agreement with (3,13) but in contrast to (4,14).

CONCLUSIONS

The data collected in this study supports our hypothesis that the ratio of Bax to Bcl-2 expression

increases in the luteal phase indicative of a tissue undergoing apoptosis. This therefore supports the proposal that the human oviduct undergoes cyclical periods of apoptosis postovulation. The questions that are raised by this study relate broadly to oviduct health and disease and to the environment created for optimum gamete and early embryo development. Disorders in the local control of apoptosis may lead to oviduct disease, increased susceptibility to ectopic pregnancy, or to oviduct carcinoma. A deficit in apoptosis protection may lead to inadequate follicular phase response and lower accumulation of oviduct secretory proteins.

The future extension of this study of cell development of the human oviduct mucosa in normal physiological conditions and in pathology could lead to new insights in infertility diagnosis and to refinements of in vitro conditions for embryos in assisted reproductive treatment.

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