Expression of survivin and Bcl-2 in the normal human endometrium

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Survivin is a novel inhibitor of apoptosis. It has been reported that survivin is expressed during fetal development and in cancer tissues, but its expression has not been reported in adult tissues. We investigated the expression of survivin in the endometria of women with regular menstrual cycles using reverse transcription–polymerase chain reaction (RT–PCR) and immunohistochemistry, and compared these findings with Bcl-2, an apoptosis inhibitor. Survivin mRNA was detected by RT–PCR in all samples (nine of nine) of endometrium during the secretory phase, but in only four out of seven samples from endometrium during the proliferative phase, and in none of the atrophic endometrium. Immunohistochemistry demonstrated a survivin protein expression that was strongest in the nuclei of glandular epithelial cells during the late secretory phase. In the proliferative phase, glandular epithelial cells were not stained for survivin. The cyclic changes of survivin and Bcl-2 showed an inverse relationship, with Bcl-2 expression being strongest in the proliferative phase and survivin expression being strongest in the secretory phase. The up-regulation of survivin expression may be due to the concurrent rise in progesterone concentrations during the normal menstrual cycle. Moreover, survivin could play an important role independent of Bcl-2 in physiological homeostasis in the normal endometrium.

Key words: Bcl-2/endometrium/immunohistochemistry/RT–PCR/survivin

Introduction

Regulation of programmed cell death (apoptosis) preserves normal homeostasis, and tissue and organ morphogenesis (Vaux et al., 1994; Nagata, 1997). Aberrations of this process participate in human diseases and may contribute to carcinogenesis by prolonging cell viability resulting in the accumulation of transforming mutations (Thompson, 1995).

Survivin is a new inhibitor of apoptosis protein (IAP) expressed during development and in human malignancy. The survivin gene was recently identified by hybridization screening of human genomic libraries with the cDNA from a factor Xa receptor, effector cell protease receptor-1 (EPR-1) (Altieri, 1995; Ambrosini et al., 1997).

Survivin can bind specifically to the terminal effector cell death proteases, caspase-3 and -7 in vitro, and inhibit caspase activity and apoptosis in cells exposed to diverse apoptotic stimuli (Tamm et al., 1998). Survivin has been shown to be expressed in 60 cancer cell lines, including breast, colon, brain, leukaemia/lymphoma, lung, melanoma, ovarian, prostate and renal cancer (Tamm et al., 1998).

Survivin expression has been shown to be present during fetal development. In contrast, survivin transcripts were undetectable in terminally differentiated adult tissues, including peripheral blood leukocytes, lymph node, spleen, pancreas, kidney, skeletal muscle, liver, lung, brain, and heart (Ambrosini et al., 1997), reminiscent of the expression of another apoptosis inhibitor, Bcl-2, in these adult organs (LeBurn et al., 1993).

Menstruation has been recognized to be the result of ischaemic necrosis in the endometrium (Speroff and Vande Wiele, 1971). However, some authors (Hopwood and Levinson, 1975) reported apoptotic bodies in the human endometrium by light and electron microscopy. Recently, it has been reported that apoptosis appears in the human endometrium during the menstrual cycle by DNA gel fragmentation and in-situ apoptosis analysis techniques (Kokawa et al., 1996).

In addition, cyclic Bcl-2 expression in the human endometrium has been demonstrated by several investigators (Gompel et al., 1994; Otsuki et al., 1994; Tabibzadeh et al., 1995; Koh et al., 1995; Jones et al., 1998). Endometrial glandular cells express Bcl-2 during the proliferative phase, but not during the late secretory phase. The disappearance of Bcl-2 expression during the late secretory phase is consistent with the appearance of apoptotic cells during this same phase. Bcl-2 expression in glandular cells may also be regulated by steroid hormones (Gompel et al., 1994; Otsuki et al., 1994; Koh et al., 1995; Tabibzadeh et al., 1995).

To our knowledge, there has been no investigation of the expression of survivin in normal human endometrium during the menstrual cycle. Thus, the role of survivin and its mechanism during menstruation in normal human endometrium is unknown. In this study, we examined the expression of survivin and compared it with Bcl-2 in the normal human endometrium during the menstrual cycle by immunohistochemistry and reverse transcription–polymerase chain reaction (RT–PCR).
Materials and methods

Immunohistochemistry

Tissue samples of endometria for immunohistochemistry were obtained from 20 women (age, mean ± SD, 44.8 ± 3.09, range 40–50 years; four early proliferative, three mid-proliferative, three late proliferative, four early secretory, four mid-secretory, two late secretory phase) with regular menstruation (Table I). They underwent hysterectomy with the diagnosis of cervical intra-epithelial neoplasia at Tohoku University Hospital, Sendai, Japan, after informed consent was obtained. They had received no hormone treatment prior to surgery. Formalin-fixed and paraffin-embedded samples were prepared for histological diagnosis and immunohistochemical analysis. There were no pathological findings related to the endometrium during post-operative pathological examination of all samples used in the present study. Endometrial dating was performed according to Noyes’ criteria (Noyes et al., 1950).

Immunohistochemistry was performed using the LSAB 2 Kit (Dako Japan, Kyoto, Japan) and an antigen retrieval method. The primary antibodies were polyclonal rabbit anti-human survivin (Surv11; Alfa Diagnostic International, San Antonio, TX, USA) and monoclonal mouse anti-human Bcl-2 (Dako Japan). After deparaffinization, sections were treated with methanol/hydrogen peroxide for 5 min to block endogenous peroxidase. To retrieve masked antigens, the slides were immersed in citrate buffer (pH 6.0), and heated in an autoclave for 5 min at 120°C. The slides were incubated for 60 min with the primary antibody, followed by a 30 min incubation with the biotinylated secondary antibody, and then with peroxidase-labelled streptavidin for 30 min. Negative control slides in the absence of primary antibody were included for each staining. Finally, 3,3′-diaminobenzidine was used to develop the colour, and haematoxylin was used for counterstaining.

For the semi-quantitative evaluation of survivin and Bcl-2 expression, we used a scoring method modified by Sinicrope and Lu (Lu et al., 1998). The mean percentage of positive glandular epithelial cells in the functional layer of the endometrium was determined in at least five areas at ×400 magnification and assigned to one of the following categories: 0 = <5%; 1 = 5–25%; 2 = 25–50%; 3 = 50–75%; and 4 = >75%. The immunostaining intensity (I) was scored as follows: 1 (weak), 2 (moderate), or 3 (intense).

Table I. Immunohistochemical staining scores of survivin and Bcl-2 in human endometrium

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Survivin Score (P×I)</th>
<th>Survivin Positive Intensity (P)</th>
<th>Survivin Intensity (I)</th>
<th>Bcl-2 Score (P×I)</th>
<th>Bcl-2 Positive Intensity (P)</th>
<th>Bcl-2 Intensity (I)</th>
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<tr>
<td>Early proliferative phase</td>
<td>45</td>
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<td>0</td>
<td>9</td>
<td>3</td>
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<td>Mid-proliferative phase</td>
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<tr>
<td>Late proliferative phase</td>
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<td>Early secretory phase</td>
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<tr>
<td>Mid-secretory phase</td>
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<td>Late secretory phase</td>
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Positivity (P) was scored as follows: 0 = <5%; 1 = 5–25%; 2 = 25–50%; 3 = 50–75%; and 4 = >75%.

RT-PCR

A total of 19 normal endometrial samples were used in this study, including 16 samples from women with regular menstrual cycles (age, mean ± SD, 44.6 ± 5.21, range 33–51 years; proliferative phase, seven cases; secretory phase, nine cases) and three samples from post-menopausal women (age, mean ± SD, 52.7 ± 6.35, range 49–60 years). These tissues were obtained after hysterectomy in patients diagnosed with cervical intraepithelial neoplasia, following informed consent. They had normal regular menstrual cycles and had received no hormone treatment prior to surgery. The endometrial tissues collected were assessed for histopathological diagnosis according to previously described criteria (Noyes et al., 1950), and the remaining portions of the samples were frozen at –80°C until assay.

RNA extraction and first strand cDNA synthesis using oligo(dT)

Total RNA was isolated from frozen human endometrial tissue using a commercially available extraction method (Isogen, Nippon Gene Inc, Tokyo, Japan). First-strand cDNA was synthesized from 3–4 µg of total RNA using an Oligo(dT)12–18 primer and the Superscript™
Survivin and Bcl-2 expression in normal human endometrium

Figure 1. Expression of survivin in endometrial glandular epithelial cells throughout the menstrual cycle. P1 = early proliferative phase; P2 = mid-proliferative phase; P3 = late proliferative phase; S1 = early secretory phase; S2 = mid-secretory phase; S3 = late secretory phase. Staining score was calculated as described in the text. Error bar ± SD. Kruskal–Wallis test, P = 0.0096. P values of Scheffe’s F-test are indicated above.

Figure 2. Expression of Bcl-2 in endometrial glandular epithelial cells throughout the menstrual cycle. P1 = early proliferative phase; P2 = mid-proliferative phase; P3 = late proliferative phase; S1 = early secretory phase; S2 = mid-secretory phase; S3 = late secretory phase. Staining score was calculated as described in the text. Error bar ± SD. Kruskal–Wallis test, P = 0.0047. P values of Scheffe’s F-test are indicated above.

Preamplification System (Superscript™ II RT; 200 IU; Gibco BRL, Grand Island, NY, USA). Control RNA is included in this kit to verify system performance. One µl (50 ng) of control RNA was used as a template for first strand cDNA synthesis. Following treatment with RNase H, cDNA samples were stored at –20°C.

Amplification of the target cDNA
Amplification of cDNA was performed with an Ambion Gene Specific Human Survivin Relative RT–PCR Kit (Ambion Inc, Austin, TX, USA). Primer sequences for human survivin used in PCR amplification were: 5′ GTGAATTTTTGAAACTGGACAG, 3′ CCTTTCCTAAGACATTGCTAAG (Genbank Accession no. U75285). PCR was carried out in a reaction volume of 25 µl for 5 min at 94°C for initial denaturing, followed by 35 cycles at 94°C for 1 min, 57°C for 1 min, and 72°C for 1.5 min, on a TaKaRa PCR Thermal Cycler MP (TaKaRa, Tokyo, Japan). Primers were confirmed in multiplex RT–PCR on mouse and human RNA samples. Primers yield a gene-specific product of the correct size and multiplex efficiently with the supplied 18S primers. Where possible, primers were designed to flank intron sequences. PCR products were analysed on a 2% agarose gel and visualised by staining with ethidium bromide.

Results
Results of immunohistochemistry are summarized in Table I and Figures 1 and 2.

Immunohistochemistry for survivin
Proliferative phase
The glandular epithelium and stroma were negative or very weakly positive for survivin immunostaining (Figure 3A). The surface epithelium (Figure 3B) and basal layer cells stained weakly and heterogeneously in proliferative phase samples. Staining was localized to the cytoplasm.

Secretory phase
Immunolocalization of survivin was confined to the glandular epithelial cells. The staining pattern was different between the basal layer and the functional layer. Immunostaining was nuclear in pattern within the glandular cells of the functional layer (Figure 3C), while cytoplasmic in pattern in the basal layer cells (Figure 3D). The intensity was strongest in the lower portion of the functional layer, and peaked during the late secretory phase. Survivin immunostaining was weak in the basal layer. Stromal cells were negative for survivin.

Immunohistochemistry for Bcl-2
Proliferative phase
The staining pattern was uniformly intense in the cytoplasm of glandular epithelial cells (Figure 3E). Stromal cells were negative for Bcl-2, whereas rare positive-staining cells seemed to be lymphocytes. Surface epithelial cells were mostly negative or weakly positive for immunoreactive Bcl-2 protein.

Secretory phase
Immunostaining for Bcl-2 disappeared from glandular epithelial cells as soon as secretory phase characteristics appeared (Figure 3F). In the premenstrual phase, the basal layer of the glandular cells was positive for Bcl-2 immunoreactive protein, as was found in the proliferative phase. Immunopositive stromal cells increased during the secretory phase, and peaked in the late secretory phase. Of the predecidualized stromal cells, some stromal granular lymphocytes were positive for Bcl-2, but predecidual cells were negative. The surface epithelium stained heterogenously and weakly for Bcl-2 protein.

Relationship between survivin and Bcl-2
Immunohistochemistry data on survivin and Bcl-2 staining in the normal endometrium during the menstrual cycle are shown in Figures 1 and 2. The cyclic changes of survivin and Bcl-2 protein expression demonstrated an inverse relationship.
between the proliferative phase and the secretory phase (Figure 4). In the secretory phase, immunostaining was positive for survivin but negative for Bcl-2, while in the proliferative phase, it was negative for survivin but positive for Bcl-2.

**RT–PCR of survivin**

In the normal endometrium, *survivin* mRNA expression was detected using RT–PCR in four out of seven endometrial samples obtained during the proliferative phase, in all of nine samples from secretory phase endometria, and in none out of three inactive endometria (Figure 5).

**Discussion**

It has been reported that *survivin* is expressed during embryonic and fetal development. However, its expression has been observed to be down-regulated and/or undetectable in many normal adult tissues (Ambrosini *et al*., 1997), but present in the thymus and placenta. The expression of *survivin* has not, however, been investigated in normal human endometrium.

This is the first study investigating *survivin* expression in the normal endometrium as it relates to hormonal fluctuations during the menstrual cycle. Prior to obtaining our results, we expected that *survivin* might be expressed during the proliferative phase, as was observed for *Bcl-2* expression in previous reports (Gompel *et al*., 1994; Otsuki 1994; Saitoh *et al*., 1999). In fact, the results from our experiments by immunohistochemistry and RT–PCR clearly show that *survivin* is expressed during the secretory phase, and is rare or absent in the proliferative phase.

Expression of *Bcl-2* has been demonstrated in normal...
Survivin and Bcl-2 expression in normal human endometrium during the menstrual cycle, being associated with oestrogen and progesterone status. Our finding that Bcl-2 immunohistochemical staining predominated in glandular cells, peaking during the proliferative phase and disappearing at the onset of the secretory phase, coincides with previous studies (Gompel et al., 1994; Otsuki et al., 1994; Koh et al., 1995).

Among the regulators of programmed cell death (apoptosis), IAP have recently attracted considerable attention for their ability to suppress an evolutionarily conserved step in apoptosis (Clem and Duckett, 1997), potentially involving direct caspase inhibition (Deveraux et al., 1997). It has been reported that survivin binds specifically to terminal effector cell death proteases, caspase-3 and -7 in vitro and inhibits caspase activity (Deveraux et al., 1997). Moreover, survivin is expressed in the G2/M phase of the cell cycle in a cell-regulated manner. At the beginning of mitosis, survivin associates with microtubules of the mitotic spindle in a specific and saturable reaction that is regulated by microtubule dynamics. Disruption of survivin-microtubule interactions results in the loss of survivin’s anti-apoptosis function and increased caspase-3 activity, a mechanism involved in cell death, during mitosis. These results suggest that survivin may counteract a default induction of apoptosis in the G2/M phase of the cell cycle (Fengzhi et al., 1998). The above information is seen to be of value considering the role of survivin in the mechanism of apoptosis in normally cycling endometrium.

We pointed out in the present study that the immunostaining pattern for survivin is different between glandular epithelial cells during the secretory phase and superficial cells during the proliferative phase. Nuclear staining was observed in glandular epithelial cells of the functional layer during the secretory phase. On the other hand, immunostaining was cytoplasmic in the proliferative phase, similar to that of the basal layer during the secretory phase. Furthermore, in previous reports concerning cancer tissues, immunohistochemical staining was observed in the cytoplasm and not in nuclei (Ambrosini et al., 1997; Lu et al., 1998). It is probable that the difference of immunolocalization for survivin is due to survivin–microtubule interactions. Since there is insufficient data to state a clear relationship between the staining patterns and any G2/M checkpoints, further study is necessary.

In addition, we found that the expression of caspase-3 was demonstrated immunohistochemically in endometrial glandular cells during the secretory phase, and not in the proliferative phase (R.Konno, unpublished data). It is likely that survivin may be expressed antagonistically to caspase-3 expression in order to keep the balance between inhibition and induction of apoptosis in the secretory phase. It is reasonable to suggest that the up-regulation of survivin expression by progesterone could play a role in the physiological homeostasis in normal endometrium.

In the present study, we found that immunohistochemical staining of survivin was restricted to endometrial glandular epithelium and was negative in endometrial stromal cells, while stromal cells were positive for Bcl-2. Previous studies (Koh et al., 1995; Jones et al., 1998; Konno et al., 1999) have demonstrated that most of the endometrial stromal granulocytes during the secretory phase were CD56-positive natural killer cells, containing perforin and granzyme B, which are cytotoxic granules inducing apoptosis (Konno et al., 1999), and revealed that endometrial stromal granulocytes were also expressing caspase-3 (R.Konno, unpublished data). Moreover, it has been suggested that endometrial stromal granulocytes expressing Bcl-2 and Ki67 simultaneously do not undergo apoptosis (Jones et al., 1998). We speculate that endometrial stromal cells during the secretory phase may antagonize caspase-3-mediated apoptosis via the Bcl-2 pathway and/or other cell proliferative mechanism, but not using survivin.

![Figure 4. Correlation between survivin and Bcl-2 staining scores on a per sample basis (r = -0.790, P < 0.0001). Data points are overlapped between 2 (#), 3 ($), and 4 (*) samples.](image)

![Figure 5. Survivin expression in 16 menstruating women and three menopausal women, detected by reverse transcription–polymerase chain reaction (RT–PCR). M = molecular weight marker; P = positive control; survivin mRNA is observed as a 243 bp signal; 18S served as an internal control (495 bp).](image)
In summary, survivin expression was demonstrated in the endometrium during the secretory phase in the human adult. It is suggested that survivin may play a role in the mechanism of endometrial menstruation independent of Bcl-2 expression.

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References


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