Involvement of Granule-Mediated Apoptosis in the Cyclic Changes of the Normal Human Endometrium

TSUKASA IGARASHI, RYO KONNO, SATOSHI OKAMOTO, TAKUYA MORIYA, Shinji SATOH and AKIRA YAJIMA

Department of Obstetrics and Gynecology, and
Department of Pathology, Tohoku University School of Medicine, Sendai 980-8574

IGARASHI, T., KONNO, R., OKAMOTO, S., MORIYA, T., SATOH, S. and YAJIMA, A. Involvement of Granule-Mediated Apoptosis in the Cyclic Changes of the Normal Human Endometrium. Tohoku J. Exp. Med., 2001, 193 (1), 13-25 —— Our objective is to investigate the involvement of granule-mediated apoptosis in the cyclic changes of the endometrium. We demonstrated the localization of CD56, perforin, granzyme B and caspase-3 in the endometrium by immunohistochemistry. We also confirmed the localization of perforin by immuno-electron microscopy, and demonstrated apoptosis in endometrial glandular cells by TdT-mediated dUTP-biotin nick end labeling (TUNEL) and electron microscopy. Uterine CD56-positive natural killer (NK) cells expressed perforin and granzyme B in its cytoplasm. Uterine NK cells increased significantly in the endometrial stroma during the secretory phase, and peaked during the late secretory phase. These cells started decreasing in number during the menstrual period. In endometrial glandular cells, caspase-3 and TUNEL-positive cells increased significantly from the late secretory phase, with apoptosis reaching a peak during the menstrual period. Using electron microscopy, we observed uterine NK cells with chromatin rich, segmented nuclei and intracytoplasmic granules in the stroma obtained from late secretory phase endometria. These cells extended projections to the lining of endometrial glandular cells and attached to form a cell-to-cell contact. In addition, nuclear chromatin was observed to have already cohered and small cytoplasmic organelles were beginning to disappear, suggesting that these endometrial glandular cells were undergoing apoptosis. Utilizing immuno-electron microscopy, intracytoplasmic granules in uterine NK cells were stained with anti-perforin antibody. The findings of this study suggest that granule-mediated apoptosis in endometrial glandular cells induced by NK cells expressing perforin and granzyme B may be associated with the onset of menstruation. —— apoptosis; endometrium; NK cell; perforin; granzyme B

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Address for reprints: Tsukasa Igarashi, M.D., Department of Obstetrics and Gynecology, Tohoku University School of Medicine, 1-1 Seiryoumachi, Aoba-ku, Sendai 980-8574, Japan.
e-mail: igarashi@ob-gy.med.tohoku.ac.jp
Menstruation has long been recognized as an ischemic necrosis of the endometrium caused by contraction of spiral arteries due to a decrease in sex steroid hormones (Markee 1940; Bartelmez 1957; Speroff and Vandey 1971). Since Hopwood and Levison (1976) reported the presence of apoptotic bodies in the human endometrium in 1976, reports suggesting the involvement of apoptosis in the endometrium during the menstrual cycle have appeared (Tabibzadeh 1995; Kokawa et al. 1996). However, the mechanism of apoptosis in the endometrium has yet to be clarified.

Decidual cells and endometrial granulated lymphocytes (eGLs) with chromatin-rich and segmented nuclei appear in the endometrium during the late secretory phase of the menstrual cycle. Dallenbach-Hellweg (1987) described that eGLs are differentiated from undifferentiated endometrial stromal cells, as well as decidual cells. In recent years, however, it has been recognized that more than half of all endometrial stromal cells are in fact natural killer (NK) cells. NK cells are characterized by several subtypes including CD56 dim, CD16+ NK cells, and CD56 bright, CD16- NK cells. CD56 dim, CD16+ NK cells have CD16 antigen and constitute NK cells found in the peripheral blood. Special type CD56 bright, CD16- NK cells have no CD16 antigen and strongly express CD56. Moreover, CD56 bright, CD16- NK cells have been shown to be expressed in the human endometrium (King et al. 1989; Bulmer et al. 1991; Klentzeris et al. 1992). During the menstrual cycle, CD56 bright, CD16- NK cells have been reported to increase in number from the proliferative phase to the secretory phase, and account for 60% of leukocytes in the endometrial stroma during the late secretory phase, and reach more than 70% during the first trimester of pregnancy (Bulmer et al. 1991). There have been reports suggesting that these NK cells play an important role in implantation and in the early formation of the placenta (King and Loke 1991; Haller et al. 1993; Tian et al. 1998). However, details of their role in the endometrium have yet to be clarified.

NK cells circulating in the peripheral blood possess both perforin and granzyme B in the granules of its cytoplasm (Young and Liu 1988; Peters et al. 1989; Cohen et al. 1992). Perforin released by exocytosis polymerizes itself onto the cell membrane of target cells in the presence of Ca2+ to form pores, thereby making it possible for granzyme B to enter the cytoplasm of these target cells (Berke 1995). Granzyme B, which is a serinprotease in the cytoplasm of target cells, activates caspase-3 to induce apoptosis (Darmon et al. 1995). This apoptotic pathway has been identified as a granule-mediated apoptosis. The function of NK cells as a factor regulating the cyclic changes of the endometrium in relation to apoptosis is very interesting, however much remains obscure and our previous report is the only study that has studied this function to date (Konno et al. 1999).

In our present study, therefore, we attempted to demonstrate, by immunohistochemistry and electron-microscopy, the involvement of granule-mediated apoptosis in the endometrium by NK cells via perforin and granzyme B, and investigated its association with the cyclic changes in the normal human endometrium.

**Materials and Methods**

*Immunohistochemistry and TdT-mediated dUTP-biotin nick end labeling method*

Normal endometrial specimens without lesions were collected from 99 patients after obtaining informed consent at Tohoku University Hospital, Sendai, Japan. The phase of the endometrial specimens were dated as: early proliferative phase \((n=15)\), mid-proliferative phase \((n=19)\), late proliferative phase \((n=7)\), early secretory phase \((n=13)\), mid-secretory phase \((n=19)\), late secretory phase \((n=18)\), and menstrual period \((n=8)\). Specimens obtained
from patients being treated with hormones and Gn-RH analogue were excluded from this study.

For immunohistochemistry analysis, the above mentioned 99 endometrial specimens were fixed in formalin, paraffin-embedded and thin sectioned and stained by the streptavidin-biotin-peroxidase complex method (SABC) using the HISTOFINE immunohistochemical staining system (Nichirei, Co., Tokyo) with antigen retrieval. Following deparaffinization, sections were treated with methanol/3% hydrogen peroxide for 5 minutes 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 minutes at 121°C. The slides were incubated for 60 minutes with primary antibody, followed by a 30-minute incubation with a biotinylated secondary antibody, and then with peroxidase-labeled streptavidin for 30 minutes. Negative control slides in the absence of primary antibody were included for each staining. Finally, 3,3'-diaminobenzidine was used for color development, and hematoxylin was used for counterstaining. We then immuno-localized CD56, perforin, granzyme B and caspase-3 in the normal human endometrium during the menstrual cycle. Primary antibodies utilized in this study are summarized in Table 1. Some of the endometrial specimens obtained from the late secretory phase were subjected to double immunohistochemical staining for CD56 and perforin. Apoptosis was detected by TdT-mediated dUTP-biotin nick end labeling (TUNEL) method using an Apop Tag In Situ Detection Kit (ONCOR Co., Gaithersburg, MD, USA), as previously described (Konno et al. 1999).

Electron microscopy and immuno-electron microscopy

In addition to the 99 endometrial specimens fixed and stained for standard immunohistochemistry, two fresh surgical specimens from a late secretory phase endometrium were fixed in 1/2 Karnovsky (2.5% glutaraldehyde + 2% paraformaldehyde), and epon resin blocks were prepared. From these blocks, 85 nm-thick sections for electron microscopy were prepared and subjected to double-staining (uranyl acetate staining and lead salt staining), and apoptosis in endometrial glandular cells and localization of perforin in the cytoplasm of NK cells were observed. Simultaneously, 1 μm-thick semi-ultrathin sections were prepared from epon resin blocks. For these sections, double staining with methylene blue-basic fuchsin and toluidine blue (BF/TF staining) were performed (Bennett et al. 1976; Mochizuki et al. 1996), and the region corresponding to that observed under the electron microscope was observed. Furthermore, 4% periodate, lysin, paraformaldehyde solution-fixed and frozen samples of the same specimens were subjected to preembedding immuno-electron microscopy by the enzyme-labeled antibody method, and localization of perforin in NK cells was confirmed. This staining was performed using the HISTOFINE immunohistochemical staining system (Nichirei, Co., Tokyo) by the SABC method. The primary antibody utilized in immuno-electron microscopy is described in the perforin column in Table 1.
Evaluation

Three fields of the endometrial functional layer at each phase of the menstrual cycle were observed by a light microscope in a high power field (×400). The mean number of CD56, perforin, and granzyme B immuno-positive cells in endometrial stromal tissue in these three ×400 fields were calculated and referred to as the Labeling Index (CD56 L.I., p L.I. and gr B L.I.). To investigate the relationship between apoptosis and the expression of caspase-3 in endometrial glandular cells, endometrial glands at each phase of the menstrual cycle were observed by a light microscope in a high power field (×400), and the numbers of immuno-positive cells among the total number of glandular cells in five glands were measured. The apoptotic index (A.I.) and caspase-3 labeling index (caspase-3 L.I.) were calculated as follows:

A.I. and caspase-3 L.I. = (number of staining-positive cells/total number of glandular cells in five endometrial glands) multiplied by 1000.

The Kruskal-Wallis test and Scheffe’s F test were used to statistically analyze A.I. and L.I. for each antibody. A p-value < 0.05 was considered significant. Furthermore, regression analysis was performed and the correlations between CD56 and perforin, CD56 and granzyme B, perforin and granzyme B, CD56 and apoptosis, perforin and apoptosis, granzyme B and apoptosis, and between caspase-3 and apoptosis, respectively, were examined in the endometrial functional layer.

RESULTS

CD56, perforin and granzyme B immuno-positive cells in the stroma of the endometrial functional layer hardly appeared during the proliferative phase, and then increased during the secretory phase, and reached peak levels during the late secretory phase. Furthermore,
these cells decreased in the number of positive staining cells during the menstrual period (Fig. 1). CD56 immuno-staining was localized to the cell membrane, while perforin and granzyme B immunoreactive proteins were identified in intracytoplasmic granules (Figs. 2A and 2B). Light microscopic findings of NK cells in the endometrial stroma by BFTB staining was obtained (Fig. 3A) and the localization of intracytoplasmic granules in NK cells by electron microscopy (Figs. 3B and 3C) was noted. In addition, the localization of perforin in the cytoplasm of NK cells was observed under an immuno-electron microscope (Fig. 4). In endometrial glandular cells, immuno-reactive caspase-3 was noted in the cytoplasm. Moreover, the number of these caspase-3 expression-positive cells increased significantly during the late secretory phase ($p < 0.0001$), reaching peak levels during the menstrual period, and decrease-
Fig. 3. (A) BFTB staining in the human normal endometrium during the late secretory phase \((\times 400)\). (B) Electron microscope photograph of a NK cell in the endometrial stroma during the late secretory phase \((\times 18,000)\). This endometrial NK cell shown here contains a lot of granules and a kidney-shaped nucleus. (C) Two NK cells with segmented nuclei and intracytoplasmic granules on the basal surface of endometrial glandular cells are seen \((\times 5000)\). These cells are indicated with an arrow in the BFTB staining (Fig. 3A).
Fig. 4. Immuno-electron micrograph for perforin in a NK cell during the late secretory phase \((\times 6500)\). Cytoplasmic granules are stained with anti-perforin antibody (arrows).

Fig. 5. Apoptotic Index (A.I.) of endometrial glandular cells during each phase of the menstrual cycle. TUNEL-positive apoptotic endometrial glandular cells increase significantly from the mid-secretory phase and reaching peak level during the menstrual period. EP, early proliferative phase; MP, mid-proliferative phase; LP, late proliferative phase; ES, early secretory phase; MS, mid-secretory phase; LS, late secretory phase; M, menstrual period. Scheffe’s F test: \(^*p<0.0001\).
Scheffe’s F test: \(^*p=0.0006\).

...ing significantly in the early proliferative phase \((p=0.0083)\). TUNEL-positive cells in endometrial glandular cells increased significantly from the mid-secretory phase and reaching peak level during the menstrual period (Figs. 5 and 6). Using electron microscopy, we observed uterine NK cells with chromatin rich, segmental nuclei and intracytoplasmic granules in the stroma obtained from late secretory phase endometria. These cells extended projections to the lining of endometrial glandular cells and attached to form a cell-to-cell contact (Fig. 7A). In these endometrial glandular cells, nuclear chromatin was observed to have already cohered...
and small cytoplasmic organelles were beginning to disappear, suggesting that these cells were undergoing apoptosis. Moreover, cell-to-cell contacts were lost between apoptotic cells and adjoining endometrial glandular cells (Fig. 7B).

A high positive correlation was found between the number of CD56-positive cells and the number of perforin-positive cells \((p < 0.0001, r = 0.900)\), and between the number of CD56-positive cells and the number of granzyme B-positive cells \((p < 0.0001, r = 0.860)\), respectively. Furthermore, a high positive correlation was noted between perforin and granzyme B immunoreactive expression \((p < 0.0001, r = 0.897)\). These cells were shown to be the same cells by double immunohistochemical staining for CD56 and perforin (Fig. 8). According to the regression line for CD56 and apoptosis, a group demonstrating very high A.I. was conspicuous despite a low CD56 L.I. (Fig. 9). The expression pattern in this group was consistent with that observed during the menstrual Period. When eight samples of endometria, which were obtained from the menstrual period, were included in the correlation analysis, the coefficient of correlation was lower than the analysis excluding these samples. Similar tendencies were also observed between apoptosis and perforin, and between apoptosis and granzyme B, respectively. The results of the coefficient of correlation are shown in Table 2. During the menstrual period, groups consisting of many apoptotic stromal cells were found to be negative for the expression of CD56, perforin and granzyme B. These findings suggest that apoptotic cells in the endometrial stroma during the menstrual period are not NK cells. In addition, a high positive correlation was noted between caspase-3 and apoptosis in endometrial glandular cells (Fig. 10).

**DISCUSSION**

As shown by the results of the present study, the appearance rate of cells positive for each of CD56, perforin and granzyme B increased sharply from the mid to late secretory phase, whereas the appearance of these cells was only partly observed during the proliferative phase. Positive correlations were found between CD56 and perforin, CD56 and granzyme B, and between perforin and granzyme B, respectively, in the number of cells positive for each of these proteins. From our results of double immuno staining for CD56 and perforin,
Fig. 7. (A) Electron microscope photograph of an endometrial NK cell (single arrow) in contact with an apoptotic endometrial glandular cell (double arrows) ($\times 6500$). This NK cell extends projections to the lining of an endometrial glandular cell and attached to form a cell-to-cell contact.

(B) Electron microscope photograph of an apoptotic endometrial glandular cell. In this endometrial glandular cell, nuclear chromatin is observed to have already cohered, and small cytoplasmic organelles are beginning to disappear, suggesting that this cell is undergoing apoptosis. This photo also shows that cell-to-cell contact has been lost between the apoptotic cell and adjoining endometrial glandular cells ($\times 12000$).
CD56 positive endometrial stromal cells were found to be positive for perforin. From the late secretory phase, caspase-3 immuno-positive cells and apoptotic cells in endometrial glandular cells increased significantly and the number of apoptotic cells reached a peak during the menstrual period.

These findings suggest that apoptosis of endometrial glandular cells via caspase-3 is associated with the onset of menstruation and that this apoptosis is granule-mediated apoptosis induced by CD56 positive NK cells.
Table 2. Coefficient of correlation between apoptotic index (A.I.) and CD56 labeling index (L.I.), between A.I. and perforin L.I., and between A.I. and granzyme B L.I.

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<th>Included in the menstrual phase</th>
<th>Excluded in the menstrual phase</th>
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<td>A.I. vs. CD56</td>
<td>0.534&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.887&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>A.I. vs. perforin</td>
<td>0.623&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.880&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>A.I. vs. granzyme B</td>
<td>0.561&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.812&lt;sup&gt;a&lt;/sup&gt;</td>
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<sup>a</sup><i>p</i> < 0.001.

Fig. 10. Correlation between Labeling Index for caspase-3 (caspase-3 L.I.) and A.I. in endometrial glandular cells.

\[ Y = 2.674 + 0.555 \times X; \ r^2 = 0.509. \]
\[ r = 0.713, \ p < 0.0001. \]

There have been several reports suggesting that apoptosis in the endometrium increases significantly from the late secretory phase to the menstrual period (Hoopwood and Levison 1976; Tabibzadeh 1995; Kokawa et al. 1996). It has also been reported that NK cells increase during this period (King et al. 1989; Bulmer et al. 1991). However, the pathway by which apoptosis is induced in the endometrium has yet to be elucidated. In a previous study, we demonstrated that the granule-mediated apoptosis pathway may be involved in human endometrial apoptosis (Konno et al. 1999). In the present study, we have substantially increased the number of samples (n = 99). In addition, we analyzed apoptosis in endometrial glandular cells and the expression of apoptosis-related compounds such as perforin, granzyme B and caspase-3 in the normal human endometrium, and then performed regression analysis among these parameters. Furthermore, we ultrastructurally observed the endometrial functional layer in the late secretory phase, so that we could firmly suggest the association between NK cells and the induction of granule-mediated apoptosis observed in endometrial glandular cells during the menstrual period. However, the possibility that apoptosis of endometrial glandular cells via Fas/Fas ligand being present has also been suggested (Yamashita et al. 1999). Further studies are required to investigate the relationship of other apoptotic pathways in the normal human endometrium.

Various investigators have suggested that apoptosis is present in endometrial glandular cells from the late secretory phase to the men-
strual period. However, Kokawa et al. (1996) have stated that apoptosis is present in the endometrial stroma at the beginning of the late secretory phase and spreads gradually to every region of the functional layer. Results of our present study have demonstrated that the apoptosis of NK cells was hardly observed in the stroma at any phase of the menstrual cycle. Rebecca and colleagues (1998) found that bcl-2 immuno-positive cells increased in the endometrial stroma from the proliferative phase to the premenstrual period, many of which were positive for double immuno staining with CD56. In their report, they maintained that CD56-positive cells expressed immuno-reactive bcl-2 and Ki67 even during the late menstrual period, although no apoptosis was observed in these cells. These findings suggest that many NK cells in the endometrial stroma do not go into apoptosis even during the menstrual period and are therefore decreasing owing to other mechanisms. While the exact role of these NK cells has not been fully elucidated, it appears that apoptosis in endometrial glandular cells is closely associated with the onset of menstruation.

The findings described above suggest that the cyclic changes of the endometrium are regulated delicately by the cyclic appearance-disappearance of CD56 positive NK cells. Tian et al. (1998) reported that perforin-positive cells are found to be located in the endometrial stroma and express CD56, and these cells demonstrate an increase in number from proliferative phase to secretory phase through first-trimester decidua. They found that these cells are absent in the postmenopausal endometrium. It has also been reported that perforin-positive lymphocytes increase sharply in the endometrial stroma with the administration of progesterone (Hameed et al. 1995). Our results also appear to support these two reported findings, in that the number of uterine NK cells increased following the ovulatory phase. Therefore, it is possible to speculate that apoptosis in the endometrium may be controlled by apoptotic pathways which involve NK cells. Then by what mechanism do sex steroid hormones control NK cells? It has been reported that neither estrogen nor progesterone receptors are present in CD56-positive cells of the endometrial stroma in any phase of the menstrual cycle (King et al. 1996; Stewart et al. 1998). Thus, the indirect actions of mediators, such as cytokines, existing in the stroma and epithelium rather than the direct action of steroid hormones on CD56-positive cells may be involved (Stewart et al. 1998).

Results from our present study suggest that the cyclic changes of the endometrium are regulated by the cyclic appearance-disappearance of NK cells in the endometrial stroma. In addition, granule-mediated apoptosis of these glandular cells via perforin, granzyme B and caspase-3 may be associated with the onset of menstruation. The cyclic expression pattern of these important mediators of apoptosis in the normal human endometrium suggests that sex steroid hormones are likely regulators of these compounds. However, the role, mechanism of appearance-disappearance of NK cells in the endometrial stroma and the detailed effects of sex steroid hormones on NK cells remain obscure. These mechanisms will be the subject of future studies.

References


Granule-Mediated Apoptosis in the Endometrium


