

Apoptosis of Human Endometrium Mediated by Perforin and Granzyme B of NK Cells and Cytotoxic T Lymphocytes

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KONNO, R., IGARASHI, T., OKAMOTO, S., SATO, S., MORIYA, T., SASANO, H. and YAJIMA, A. *Apoptosis of Human Endometrium Mediated by Perforin and Granzyme B of NK Cells and Cytotoxic T Lymphocytes*. Tohoku J. Exp. Med., 1999, 187 (2), 149–155 — Endometrial stromal granulocytes (EGs) were found to be a major component of human endometrial stroma in the late secretory phase. However, the role of EGs in the mechanism of human endometrial menstruation has not been clarified. Immunohistochemistry of CD56, perforin, granzyme B, and vimentin, in situ detection of apoptosis by TUNEL (TdT-mediated dUTP-biotin nick and labeling) and electron microscopy were performed in endometrial tissue samples with normal menstrual cycles. We analyzed the number of immunostained cells in the functional layer of stroma and the number of apoptotic cells detected by TUNEL in the endometrial glandular cells. Double-staining revealed that CD56-positive endometrial stromal cells were simultaneously positive for both perforin and granzyme B, and negative for vimentin, which recognized stromal tissue. Vimentin was positive for the predecidual cells and negative for EGs. CD56-positive EGs involving perforin and granzyme B were progressively recruited during the secretory phases before menstruation. Apoptosis in endometrial glandular cells increased from the late secretory phase, which maximized at the menstrual period. This finding suggests that the cytotoxic granules released from EGs, which are derived from cytotoxic T lymphocytes and natural killer cells, are initiators of the apoptotic pathway that induces endometrial menstruation. — endometrium; menstruation; apoptosis; perforin; granzyme B © 1999 Tohoku University Medical Press

Menstruation has been regarded as ischemic necrosis of a functional layer caused by contraction of spiral arteries, and is dependent on sex hormone concentrations (Speroff and Vande Wiele 1971). Endometrial stromal cells in the late

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secretory phase develop into two kinds of cells, predecidual cells and endometrial stromal granulocytes (EGs), also called Kernchenzellen or "K" cells (Mazur and Kurman 1995). EGs were considered to be derived from undifferentiated endometrial cells, as well as predecidual cells (Dallenbach-Hellweg 1987).

However, recent immunohistochemical studies revealed that most of these cells are T lymphocytes (Bulmer et al. 1987; King et al. 1989). Other studies have reported that perforin-positive endometrial lymphocytes increase in number in the middle until the late secretory phase and 80% of the endometrial lymphocytes are CD3⁻, CD56⁺ and 20% are CD3⁺, CD8⁺ (Hameed et al. 1995). Granzyme B is also one of the important cytolytic granules in NK cells and CTLs, as well as perforin. Perforin facilitates the entry of granzyme B into the target cell (Berke et al. 1995). Granzyme B cleaves and activates Caspase-3/CPP32 and induces DNA fragmentation and apoptosis (Darmon et al. 1995).

Little is known regarding the role and character of EGs in human endometrium. We immunohistochemically demonstrated that EGs are NK cells and CTLs with perforin and granzyme B, involving apoptosis and are not derived from the undifferentiated stromal cells.

MATERIALS AND METHODS

Tissue samples of endometria were obtained from 27 women with normal menstrual cycles (5 in the early proliferative [EP] phase, 4 in the mid-proliferative [MP] phase, 3 in the late proliferative [LP] phase, 4 in the early secretory [ES] phase, 2 in the mid-secretory [MS] phase, 6 in the late secretory [LS] phase, and 3 during the menstrual [M] period) after informed consent was obtained. No pathological findings related to the endometrium were detected. Formalin-fixed and paraffin-embedded samples were prepared for histological diagnosis, immunohistochemical analysis, in situ detection of apoptosis, and electron microscopy. Endometrial dating was reconfirmed by light microscopy according to the standard criteria (Noyes et al. 1950).

Immunohistochemistry was performed using a streptavidin biotin method with antigen retrieval using an autoclave. The primary antibodies are summarized in Table 1. Moreover, to distinguish EGs from other stromal cells, the double-immunohistochemical staining technique was used. The number of immunostained cells in the functional layer of endometrial stroma was counted by conventional light microscopy and the average number of cells stained in each of the 3 microscopic fields was calculated (magnification $\times 400$). Apoptotic cells in the endometrial gland were detected by TUNEL (TdT-mediated dUTP-biotin nick and labeling: Apoptag, Oncor Inc., Geithersberg, MD, USA). The number of apoptotic cells in the endometrial gland was counted by conventional light microscopy. The apoptotic index (A.I.) was obtained by the following formula;

$$\text{A.I.} = (\text{positive cells/total cells of 5 endometrial glands}) \times 1000.$$

Statistical analysis was performed using Kruskal-Wallis test and Scheffe's F

TABLE 1. *Antibodies and specification*

Antibodies	Manufactures	Clone	Antigens/cells stained
CD56	Novocastra Lab., Newcastle upon Tyne, UK	1B6	NK cells, CTLs
Perforin	Kyowa Medex, Tokyo, Japan	KM585	NK cells, activated CTLs
Granzyme B	Kamiya Biomedical Company, Seattle, WA, USA	GrB-7	NK cells, activated CTLs
Vimentin	Novocastra Lab., Newcastle upon Tyne, UK	V9	Cells with mesenchymal origin

NK cells, natural killer cells; CTLs, cytotoxic T lymphocytes.

test. $p < 0.05$ was selected as the significant level.

RESULTS

The number of CD56-, perforin- and granzyme B-positive cells in endometrial stroma increased progressively and became confluent at the end of the late secretory phase (Kruskal-Wallis test, $p < 0.001$; Scheffe's F test, EP, MP, LP, and ES vs. LS, $p < 0.001$) (Table 2 and Fig. 1). Immunohistochemistry demonstrated that the anti CD56 antibody was positive for EG surface membranes, which showed small round or kidney-shaped and dense nuclei by H&E staining. The localization and frequency of the positive cells for CD56, perforin, and granzyme B were similar in the serial sections (Table 2 and Fig. 2). Some endometrial glandular cells were positive for granzyme B, but negative for perforin.

Double-staining revealed that CD56-positive endometrial stromal cells were simultaneously positive for both perforin (Fig. 2) and granzyme B, and negative for vimentin, which recognized stromal tissue. Vimentin was positive for the

TABLE 2. *Immunostained cells in the endometrial stroma and apoptotic index in the endometrial glandular cells in high power fields*

	EP	MP	LP	ES	MS	LS	M
CD56 ^a	5.1	2.1	3.2	79.4	190.7	373.3 ^b	82.5
Perforin ^a	31.2	21.0	10.7	90.5	179.5	325.7 ^b	167.3
Granzyme B ^a	12.3	18.3	12.9	102.3	166.3	289.3 ^b	117.6
Apoptotic index ^a	11.7	9.1	11.6	9.7	14.5	65.3	93.7 ^c

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.

^a Kruskal-Wallis test, $p < 0.001$.

^b Scheffe's F test, EP, MP, LP, and ES vs. LS, $p < 0.001$.

^c Scheffe's F test, EP, MP, LP, ES, and MS vs. M, $p < 0.001$.

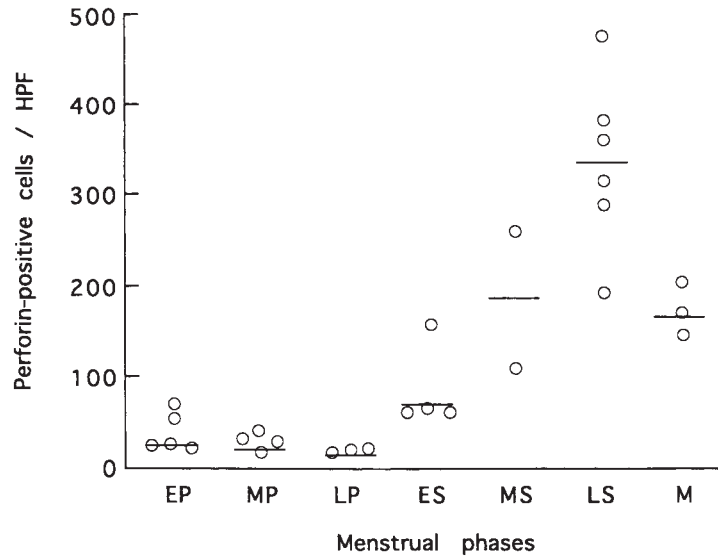


Fig. 1. Quantitative analysis of immunohistochemical perforin-positive cells in endometrial stroma. EP: early proliferative phase ($n=5$), MP: mid-proliferative phase ($n=4$), LP: late proliferative phase ($n=3$), ES: early secretory phase ($n=4$), MS: mid-secretory phase ($n=2$), LS: late secretory phase ($n=6$), M: menstruation period ($n=3$).

Perforin-positive cells in endometrial stroma progressively increase and become confluent at the late secretory phase and decrease at the menstruation period. Significant differences were obtained by the Kruskal-Wallis test ($p < 0.001$) and in EP, MP, LP, and ES vs. LS by the Scheffe's F test ($p < 0.001$).

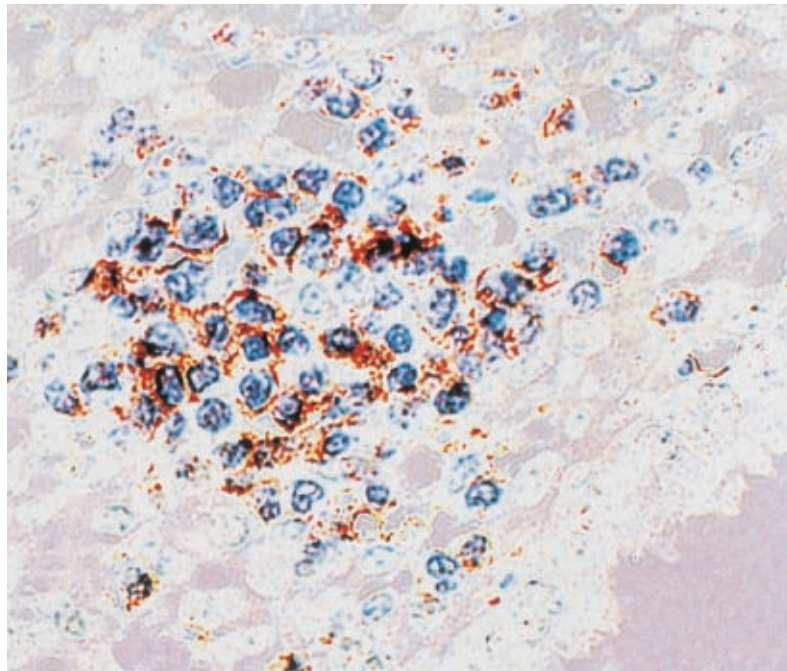


Fig. 2. Double-immunohistochemical staining in the late secretory endometrium. CD56-positive (brown) endometrial stromal granulocytes (EGs) are simultaneously positive for perforin (blue), and predecidual cells are negative for those antibodies. Staining of CD56 is positive for the cell membrane and staining of perforin is positive for cytoplasm (Original magnification was $\times 400$).

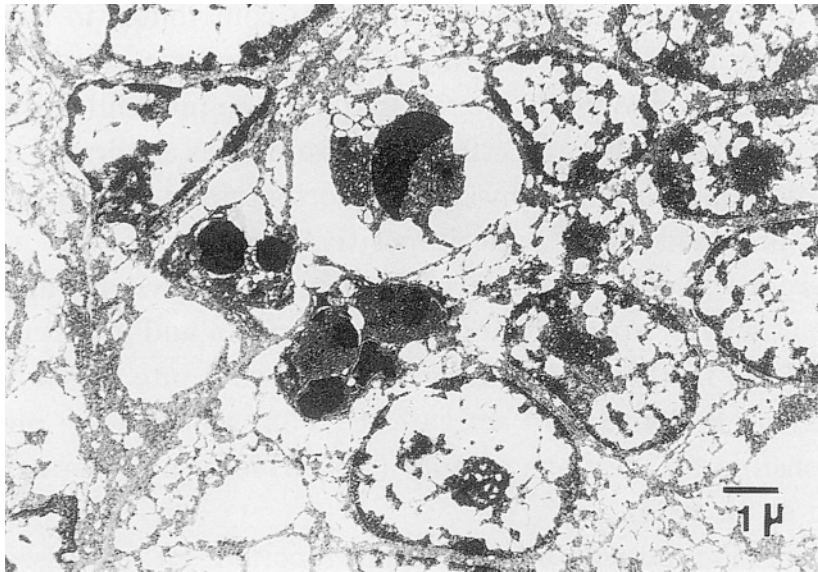


Fig. 3. Apoptosis of the endometrial glandular cells. Apoptotic bodies are shown in the endometrial glandular cells by electron microscopy.

predecidual cells, which showed large, round, clear nuclei and abundant cytoplasm by H&E staining.

Apoptosis in endometrial glandular cells increased from the late secretory phase, which maximized at the menstrual period (Table 2). Apoptosis of endometrial glandular cells in the late secretory endometrium was detected by TUNEL and reconfirmed using electron microscopy (Fig. 3). EGs likely surrounded the endometrial glandular cells to induce apoptosis by releasing perforin and granzyme. Rare apoptotic cells were observed in the endometrial stroma in the secretory phase.

DISCUSSION

The mechanism of endometrial stromal differentiation was proposed to be that both decidual cells and endometrial granulocytes are derived from poorly differentiated stromal cells (Dallenbach-Hellweg 1987). At the late secretory phase, dissociation of the stromal cells becomes apparent in the compact layer due to the beginning of reticulin fiber dissolution. This is caused by secretion of relaxin from EGs, which begins immediately before menstruation (Dallenbach-Hellweg and Poulsen 1996). However, we immunohistochemically demonstrated localization of perforin- and granzyme B-positive granules in EGs, which are also simultaneously positive for CD56 and negative for vimentin. Functionally active cytotoxic cells with positive staining for perforin and granzyme B are progressively recruited at the secretory phases before menstruation. We proposed that EGs are NK cells or CTLs in the endometrium, and they are not derived from undifferentiated endometrial stromal cells, but from bone marrow (NK cells) or the thymus (CTLs).

CTL-mediated cytotoxicity with perforin represents the body's major defense

against virus-infected and tumorigenic cells, and contributes to transplant rejections and autoimmune disease. During killing, cytotoxic granules are removed via exocytosis, and their contents are released into the intracellular space between the target cell and the effector. Perforin expression was confirmed in endometrial lymphocytes by immunohistochemistry and Northern blot analysis (Hameed et al. 1995). They proposed that perforin-positive cytotoxic cells with NK-like cytotoxicity are involved in endometrial stromal breakdown during the menstrual cycle. The pathway of apoptosis induced by perforin and granzyme B was later revealed. Perforin facilitates the entry of granzyme B into the target cell (Berke 1995). Granzyme B cleaves and activates CPP32/caspase3, the precursor of the protease responsible for cleavage of poly (ADP-ribose) polymerase in the target cells (Darmon et al. 1995).

A study of the human endometrium reported an increased number of apoptotic bodies during the late secretory and menstrual phases (Hopwood and Levison 1975), and disappearance of bcl-2 expression in glandular cells at the late secretory phase was consistent with the appearance of apoptotic cells (Otsuki et al. 1994). Furthermore involvement of apoptosis was recognized as a possible mechanism of menstruation in the endometrium. Although, the exact pathway that induces apoptosis has not been clarified, the mechanism of menstruation may be apoptosis of endometrial glandular cells mediated by perforin and granzyme B released from EGs.

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