Cell Death of Uterine Natural Killer Cells in Murine Placenta during Placentation and Preterm Periods

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ABSTRACT. In the murine uterus granulated metrial gland (GMG) cells appear only during normal pregnancy. GMG cells belong to a member of natural killer (NK) cells and play an important role in fetus survival and placental growth. Our previous study revealed that mouse GMG/uterine NK (uNK) cells in the late pregnancy rapidly disappear from the uterus, due to the degenerative change classified as necrosis. But there are few reports regarding appearance and morphology of uNK cells during late pregnancy. We examined histologically and histochemically how and when uNK cells undergo cell death. The uNK cells in the metrial gland increased in number and reached maximum until day 12 of pregnancy. Sudden disappearance, however, occurred after day 15 and the granules reduced in both number and size. In situ DNA fragmentation detection revealed that DNA fragmented uNK cells increased in number during days 13 to 15 and reached 70.2% at day 15 of pregnancy. From days 13 to 17, uNK cells were positive against anti-perforin antibody. Ultrastructurally, uNK cells at day 15 showed poor organelles and unusual granules in structure. In uNK cells at day 17, condensation of nucleus chromatin, reduction in size and phagocytosis into other uNK cells were observed. These results suggested that uNK cells undergo at least two types of cell death, classified as necrosis and apoptosis, at the different stages of pregnancy, and that perforin is not a mediator for cell death.—KEY WORDS: apoptosis, GMG cell, metrial gland, necrosis, uterine NK cell.

Granulated metrial gland (GMG) cells exist in the murine pregnant uterus as a major cell population [16]. Since GMG cells have activities of perforin and serine esterase in the granules and express phenotypes belonging to a member of natural killer (NK) cell lineage [2], they are called as uterine NK (uNK) cells. Recent studies of the TgE26 transgenic mouse, genetically deficient in NK and T cells, showed that the placenta was poorly developed, that uNK cells were rare, and that a sudden onset of fetal loss occurred at day 10 of gestation through pathological degeneration in placental vessels [6, 8]. Furthermore, after reconstitution of uNK cells in TgE26 mouse, placental development, vascularization and fetal growth were recovered [7]. Thus, uNK cells were suggested to have vital roles for the fetus growth, placental formation and pregnancy success.

The uNK cell precursors reside in the non-pregnant uterus [13]. In the pregnant uterus, maturation of uNK cells begins during days 6 to 10 of pregnancy with proliferation and granule development. The differentiation of uNK cell is influenced in early pregnancy by mesenchyme [12], but not by the microbiological environment, T/B lymphocytes and macrophage lineage [11]. At days 12 to 14 of pregnancy, most of the uNK cells are localized in the metrial gland (MG). Cell number of uNK cells in the MG is most frequent in mid-pregnancy. During late pregnancy to parturition, uNK cells decrease dramatically in number and disappear from the MG [15].

Peel reported that degenerative changes are observed in uNK cells at day 17 to parturition and that they include pycnotic nucleus like in an apoptotic change [15]. Our previous study using MRL-lpr/lpr mice, genetically lacking Fas, established that necrotic cell death occurred in uNK cells at late pregnancy [4]. Necrosis involves unrepaired damage to the cell membrane and is associated with stimuli such as hypoxia, complement injury, and lytic viral infection [17]. On the contrary, apoptosis occur in the physiological circumstances during development, in response to hormones and other mediators [17]. Logically, apoptosis can be induced in the pregnant uterus, because physiological valances in the placenta, especially hormones and cytokines that can be promoters for apoptosis, change with pregnant periods. Indeed, dexamethasone elevation can regulate apoptosis for thymocytes [18]. In the term period of pregnancy, apoptosis was seen in the trophoblast in the mouse placenta, while experimental preterm delivery was induced by lipopolysaccharide [10]. DNA fragmented trophoblasts increased in number until delivery, due to interleukin (IL)-1α and tumor necrosis factor (TNF)-α. We previously reported that uNK cells in vitro showed apoptotic change after 4 hr of culture, in spite of the supplement of the medium with estrogen, IL-2, various extracellular matrix proteins and decidual cells [3]. Apoptosis of uNK cells remains to be fully understood, because there are few reports regarding appearance and morphology of uNK cells during late pregnancy. Besides, perforin activity has not been examined at late pregnancy, although perforin is capable to cause apoptosis [17]. We studied cell behavior, detailed structures and perforin of uNK cells to establish cell death.
process of uNK cells, as an important event in successful pregnancy.

MATERIALS AND METHODS

Animals: ICR mice, purchased from Japan Clea (Osaka, Japan) at 10–12 weeks of age, were used in this study. They were bred in a conventional facility. Females were paired to males and the detection of vaginal plug at the next morning was designated as day 0 of pregnancy. Females were sacrificed at 9, 11, 13, 15 and 17 day of pregnancy and the implantation sites of the pregnant uterus were sampled. All animals received humane care as outlined in the Guide for the Care and Use of Laboratory Animals from the Institute of Laboratory Animal Resources (ILAR) in 1996.

Light microscopy: For light microscopy, uterine samples were fixed in 10% neutral buffered formalin for embedding in paraffin. Sections were made transversely through the central region of the implantation site and stained with periodic acid-Schiff (PAS) reagent, with or without previous diastase digestion. The uNK cells with PAS-positive granules were studied morphologically at each day. Tissue samples were collected from at least 2 implantation sites per female. More than 2 females were sacrificed at days 9, 11, 12, 13, 15 and 17 of pregnancy. To search the absolute number of uNK cells in whole area of the MG in a cross section, the cell density and the cross area of the MG were measured and multiplied with each other. For this measurement, 2 randomly selected fields in each 4 sections from 1 implantation site were analyzed using an automatic morphometry system (Cosmozone-1SB; Nikon, Tokyo, Japan).

Statistical analysis: The ANOVA analysis with a Fisher’s protected least significant difference test in morphometric data was carried out with Stat View 4.0 program using a Macintosh computer. Differences at a probability of p<0.01 were considered significant.

Electron microscopy: The MGs were carefully sampled from the uterus at days 13, 15 and 17 of pregnancy and were cut into small pieces. They were pre-fixed with 2.5% glutaraldehyde-2% paraformaldehyde in PBS (pH 7.4), post-fixed with 1% osmium in PBS and then embedded in epoxy resin. The thin sections were made from 3 blocks per each day using ultramicrotome, stained with uranyl acetate and lead citrate and observed with an electron microscope (H-1200; Hitachi, Tokyo, Japan).

In situ detection of fragmented DNA: Apoptotic cells were displayed histochemically by the TUNEL (terminal deoxynucleotidyl transferase (TdT) mediated dUTP-biotin nick end-labeling) method using a commercial kit (ApopTag™ Plus; Oncor, Gaithersburg, MD, U.S.A.) according to the manufacturer’s protocol. For this experiment, paraffin sections were used from the implantation site at days 11, 13, 15 and 17 of pregnancy. To recognize uNK cells, dual stains of TUNEL and PAS were conducted. The positive controls were made from the mammary gland immediately after weaning. For the negative controls, distilled water was applied instead of TdT. All uNK cells and positive uNK cells in the MG were counted in the 2 sections from 1 implantation site of at least 3 females per each day for calculation of positive proportion.

Immunohistochemistry: To detect whether uNK cells have perforin in the late pregnancy, paraffin sections were prepared from the MGs at days 13, 15 and 17 of pregnancy. Rabbit anti-mouse perforin antibody generously provided by Dr. L. M. Zheng (Rockefeller University, New York, NY, U.S.A.) was used as primary antibody. Sections were incubated at 1/50 dilution of the antibody, at room temperature (RT) and overnight. Hist Scan™ Kit (BioMeda, Foster City, CA, U.S.A.) was used for the treatment of secondary antibody, avidin-biotin and color development. Samples of spleen from ICR mouse bred in the same facility were used as positive controls. For the negative controls, rabbit non-immune serum was used instead of the primary antibody.

RESULTS

In ICR mice, at day 9 of pregnancy, uNK cells had several, PAS-positive, small granules (Fig. 1A). Three to 8 percentages of them were large in shape, but most of them appeared small. Large uNK cells were frequently localized in the decidua basalalis (DB). The MG was not well-developed. At day 11, uNK cells increased in number, becoming a major population in the pregnant uterus. At day 13 most of uNK cells were swollen and included huge granules in the cytoplasm (Fig. 1B). The uNK cells was localized mainly in the MG that was well-developed at day 13. At day 15, granules seemed to reduce their size and number (Fig. 1C). In the aggregation of uNK cells, agranular cells were observed, showing large cytoplasm and the same size as uNK cells (Fig. 1C, arrows). At day 17, there were a few uNK cells in the pregnant uterus (Fig. 1D) and the MG could not be observed as a definitive structure. The uNK cells at day 17 had many, very small granules (Fig. 1E), and included both fragmented nuclei and PAS-positive granules (Fig. 1F).

The MG occupied a very narrow area in the placenta until day 11 of pregnancy (under 0.70 mm²) (Table 1). After day 12, the MG rapidly developed (up to 3.24 mm²) and at day 12 the MG area reached maximum. However, during days 12 to 15, 28.4% of the area was slowly reduced. From days 15 to 17, further 59.8% of area was sharply reduced and the MG disappeared at day 17. Absolute number of uNK cells in the MG was also summarized in Table 1. During days 9 to 12, the number of uNK cells sharply increased in the MG and reached maximum at day 12. During days 12 to 15, 49.2% of uNK cells gradually disappeared from the MG. From days 15 to 17, 48.5% of uNK cells were further reduced. At day 17, only 13.9 cells per the MG area remained.

In situ DNA fragmentation detection revealed that TUNEL-positive reactions were hardly seen in the nuclei of
uNK cells at day 11 (Fig. 2A). The positive proportions were only 10.9%. At day 13, the population of uNK cells with low positive reaction (under 20% nuclei) was observed at the center region of the MG (Fig. 2B), while the cell proportion with highly positive reaction (up to 70% nuclei) was at the peripheral region close to myometrium (Fig. 2C). Total proportion at day 13 was 49.9%. At day 15, positive uNK cells were randomly seen in high proportion (70.2%, Fig. 2D). At day 17, 36.4% of uNK cells were positive, although they existed in small number.

Ultrastructural analysis showed that uNK cells at day 13 of pregnancy were 15–20 µm in diameter and had many granules, well-developed rERs and Golgi complexes, and 1 or 2 nuclei containing large nucleoli (Fig. 3A). The granules were approximately 2.0 µm in diameter, and displayed the homogeneous electron-dense core in the central region and...
the 'cap' membrane structure in the peripheral region (Fig. 3B). At day 15, uNK cells became approximately 25 µm in diameter and the cytoplasm was very low in electron density due to poor organelles and granules (Fig. 4A). Nuclei showed clumpy and coarsely aggregated chromatin. Granules were irregular in shape, showing the different electron density in the central region (Fig. 4B). Various changes were observed in granule structures, including formation of 1 or 2 highly dense compositions (Fig. 4B), components of digested-like structure (Fig. 4C), myelin-like bodies (Fig. 4D) and very huge body (6 µm in diameter) that is up to 3 times larger than normal granules of day 13 (Fig. 4E). At day 17, uNK cells included many, but smaller granules (approximately 1.2 µm in diameter) than the normal (Fig. 5A). The uNK cells at day 17 tightly attached with neighboring uNK cells and both cytoplasmic membrane in attached area disappeared, i.e., both cells fused (Fig. 5B). Chromatin-condensed, shrunk cells (approximately to 6 µm in diameter) contained uNK-like granules. Shrunk uNK-like cells were incorporated into the cell that had many granules and extended cytoplasmic projections (Fig. 5C). Granular cells included several huge bodies that were composed of granule-like structures (Fig. 5D).

Immunostaining analysis revealed that uNK cells were positive against anti-perforin antibody at all stages tested, although uNK cells at days 15 and 17 showed weak reaction (Fig. 6A-C).

**DISCUSSION**

The present study established morphological degeneration of uNK cells during placentation and preterm periods, suggesting that different types of cell death occur in uNK cells at different stages of pregnancy. From days 13 to 15, both granule size and number were reduced and few granules were ultrastructurally observed in uNK cells. Besides, granules at day 15 included myelin-like figures, vacuole formation and uneven density, resembling the secondary lysosome. Secondary lysosomes are formed by fusion of primary lysosomes with the uptakes through pinocytosis or

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**Fig. 2. In situ DNA fragmentation detection of uNK cells in the MG at day 11, 13 and 15 of pregnancy.** (A): At day 11, few uNK cells show TUNEL-positive reaction in the nucleus. (B), (C): At day 13, cell population of low positive uNK cells is localized in the central region of the MG (B), and that of highly positive uNK cells (arrowheads) are observed in the peripheral region close to myometrium (C). (D): At day 15, many uNK cells show strongly positive reaction in the nucleus (arrowheads). (A)-(C): × 300, (D): × 500.
autophagy of degenerative organelles. Actually, uNK cells at day 15 had very poor organelles. In granules, uNK cells have lysosome activities such as β-glucuronidase, acid phosphatase, aryl sulphatase, aminopeptidase and serine esterase [15]. In these periods, lysosome activity would be elevated for autophagy against degenerative organelles and granules. On the other hand, at day 17, few granules showed deformation, although uNK cells were observed as apoptotic bodies. Abnormally huge bodies in uNK cell resulted from the aggregation of granules. These findings suggested that uNK cells during days 15 to 17 undergo apoptotic cell change, remaining the granule structure. Apoptotic uNK cells would adhere to neighboring uNK cells and be incorporated for phagocytotic digestion. This phagocytosis may also be due to macrophage, expressing scavenger receptor in mice uterus [15]. Fukasawa et al. proposed that in the rat uNK cell, protein synthesis for apoptosis differs according to the stage of pregnancy and at least two different
subtypes exist [5]. Immunohistochemically, perforin was detected in uNK cells at all stages from days 11 to 17 of pregnancy, suggesting that perforin is not related to uNK cell death. Cell death process may depend on the subtypes of uNK cells under condition of different mediator and sensitivity for each type of cell death.

Clumpy chromatin and degenerative cytoplasm appeared in uNK cells of day 15 were classified as necrotic change. Delgado et al. indicated that in murine uNK cells the onset of necrosis was prospected between day 11 and 12 of pregnancy [4]. This study showed that at day 13, highly positive TUNEL-reaction, indicating DNA fragmentation, was observed in uNK cell population localized in the MG close to myometrium. TUNEL-reaction was detectable not only in apoptotic but also necrotic cells [1]. Thus, onset of necrosis is suggested to begin between day 12 and 13 from the mesometrial side of the MG. Proportion of positive uNK cells for TUNEL was 70.2% at day 15, although 98.6% of uNK cells disappeared from the MG during days 15 to 17, indicating that more than 70% of uNK cells disappeared from the endometrium due to cell death during these period.

The present study also showed that changes in the developing and degenerating MG area affected the uNK cell population. During days 12 to 15, approximately 30% of the MG area decreased and then, approximately 60% during days 15 to 17. The MG of day 13 included large maternal vessels, especially veins that were from 40 µm to more than 200 µm in diameter (data not shown). After day 15, there were only small vessels, smooth muscle fibers extending from the mesometrium and stromal cells within

![Ultrastructure of uNK cells at day 17 of pregnancy.](image-url)
the MG, suggesting remodeling of the MG. Hunt et al. reported that uNK cells expressed the gene for inducible nitric oxide synthase (iNOS) [9]. The iNOS expression accounts for the relaxation of vascular smooth muscle in the placenta. Besides, we defined the production and release of epidermal growth factor (EGF) from uNK cells and the expression of EGF receptor mainly localized in the MG and placental labyrinth [14], indicating the importance of uNK cells for placental growth. Apoptosis of uNK cells during days 15 to 17 would prevent the development of the MG, due to end of iNOS and EGF production, and would contribute to the alternation of placental structure before parturition.

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