Involvement of the Fas Ligand and Fas System in Apoptosis Induction of Mouse Uterine Natural Killer Cells

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Abstract. Uterine natural killer (uNK) cells in the pregnant uterus are known to be associated with the normal development of the placenta. In the mouse pregnant uterus, many uNK cells exist during mid pregnancy, although they show a sudden decrease during late pregnancy and almost disappear before delivery. Our previous study indicated that uNK cells showed clear apoptotic morphology during late pregnancy. Therefore, the present study was carried out to define the involvement of Fas ligand (FasL) and Fas in apoptosis induction of uNK cells. Immunohistochemical analyses revealed that uNK cells expressed FasL in the cytoplasmic granules and Fas on the cell membrane during late pregnancy. In lpr/lpr mice, which genetically lack Fas, many uNK cells were clearly observed during late pregnancy compared with wild-type mice, and moreover uNK cells still existed at day-18 of pregnancy, although there were few in wild-type mice during the same period. In the experiment of in vitro culture, uNK cells derived from wild-type placenta showed chromatin condensation and DNA fragmentation frequently following the anti-Fas antibody treatment, as compared with the control. From these results, it is suggested that FasL and Fas-dependent apoptosis regulates cell appearance of uNK cells in the mouse pregnant uterus.

Key words: Apoptosis, Fas, Fas ligand, lpr/lpr mice, Uterine natural killer (uNK) cell

The apoptosis induction system that depends on Fas ligand (FasL) and Fas has an essential role in the process of negative selection against the autoreactive T cells [1, 2]. MRL-lpr/lpr mice, a model of human systemic lupus erythematosus (SLE) and genetically lacking Fas, show symptoms of autoimmune disease caused by a deficiency of the exclusion system for autoreactive T cells [3]. Endogenous expressions of FasL and Fas were detected in normal mouse uteri and their apoptosis effects were suggested to contribute to physiological cell turnover and immune privilege [4]. In human placenta, FasL production from the syncytiotrophoblast is considered to induce apoptosis in maternal aggressive lymphocytes [5]. In murine placenta, large granular lymphocytes exist as a major population in the metrial gland and decidua basalis regions [6]. The metrial gland region is formed within the mesometrial triangle after destruction of the circular smooth muscle of the uterine wall, and develops during mid-late pregnancy with the prominent appearance of large granular lymphocytes [6]. The phenotypes of the surface antigens on the large granular lymphocytes are the Ly49G2, NK1.1 and asialo-GM1 that belong on natural killer (NK) cells, and the cytoplasmic granules contain pore forming protein (perforin) and granzyme B [7]. Usually, uterine NK (uNK) cells appear in the decidua basalis of mouse

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placenta at days 7–11 of pregnancy, and the dominant localization sites move to the metrial gland at days 11–15 [6]. An abnormality in cell appearance of uNK cells can induce low fertility in pregnant mice [8]. We previously reported that the number of uNK cells suddenly decreases during late pregnancy period, and that the decrease is dependent on apoptotic cell death [9]. Furthermore, in pregnant lpr/lpr mice, retardation of the degenerative change in uNK cells was observed, as compared with wild-type mice [10]. The peripheral NK cells are known to express FasL following the activation of their Fcγ receptor [11]; however, FasL expression has not been examined in uNK cells. We speculated that the FasL and Fas system may act in mouse placenta to regulate the apoptosis of uNK cells. The aim of the present study was to define a relevance of the FasL and Fas system to apoptosis induction in uNK cells, and to provide a suggestion related to reproductive physiology for the apoptosis of uNK cells.

Materials and Methods

Mice

C57BL/6J (wild-type) and C57BL/6J-lpr/lpr mice were purchased from Japan SLC (Hamamatsu, Japan) and used at 8–10 weeks of age for the study. Both types of mice were bred in a specific pathogen free (SPF) facility, and the lpr/lpr mice were used before developing SLE symptoms. Female mice were paired to male mice of the same strain, and the detection of a vaginal plug at the next morning was designed as day 0 of pregnancy. Pregnant mice were euthanized by diethyl ether anesthesia and cervical dislocation from day-2 to day-20 of pregnancy. During pre-implantation periods, both of the uterine horns were collected (the oviduct was removed), and after implantation periods, the implantation sites were collected and divided individually. Some of the material was immediately frozen at –80°C for molecular experiments, and the rest was embedded in O.C.T. compound (Sakura Finetechnical, Tokyo, Japan) or fixed with 10% (v/v) neutral-buffered formalin for histological and immunohistological analyses. All animals received humane care as outlined in the Guide for the Care and Use of Laboratory Animals at Osaka Medical College.

Morphometry of the cell density of uNK cells

Paraffin sections were prepared from formalin-fixed tissue at a transverse section of the implantation sites, and were stained with periodic acid-Schiff's (PAS) reagent, with and without previous diastase digestion. Since large cells containing PAS-stained cytoplasmic granules that resist diastase digestion are identified as uNK cells [7], the cell density of uNK cells existing in the metrial gland region was measured in the PAS-stained sections using a Cosmose-15B morphometric system (Nikon, Tokyo, Japan). Measurement was conducted at ×200 magnification using 5 sections, prepared from 4 or more mice, during each day of pregnancy.

Immunohistochemistry

Paraffin sections were also used for immunohistochemical analysis for FasL, and cryostat sections, prepared from the O.C.T. compound-embedded tissue, were used for Fas analysis. For the primary antibody, rabbit polyclonal anti-FasL antibody (N-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit polyclonal anti-Fas antibody (M-20, Santa Cruz Biotechnology) were used. Both antibodies were diluted to 1/50 with phosphate buffered saline (PBS), and reacted for 2 h at room temperature (20–25°C). As the secondary antibody, fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antibody (Santa Cruz Biotechnology) was used for Fas detection in a 1/100 dilution with PBS, and reacted for 30 min at room temperature. To detect FasL, a Vectastain ABC kit (Vector Lab Inc., Burlingame, CA, USA) was used, and visualization of the peroxidase activity was conducted using a diaminobenzidine (DAB; Vector Lab Inc.) reaction. Following the DAB reactions, the sections were briefly counterstained with hematoxylin and mounted. Thymus sections prepared from adult mice were used as positive controls. Normal rabbit serum was reacted instead of the primary antibody as negative controls.

Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR analysis was conducted to detect mRNA expression of FasL and Fas in the pregnant uterus. Total RNA from the frozen tissue was isolated with TRIzol LS reagent (Invitrogen, Carlsbad, CA, USA) and reacted with DNase I (Invitrogen) for the
digestion of contaminated DNA. Synthesis of cDNA from total RNA was carried out by RT reaction with MMLV RNase (H–) point mutant (Promega, Madison, WI, USA) and dNTP (Takara, Kyoto, Japan) at 42 C for 1 h, 90 C for 5 min, and stopped at 4 C. The cDNA was amplified by PCR reaction using a Gene Amp PCR System 2400 (Perkin-Elmer, Foster City, CA, USA) at 95 C for 5 min; 30 cycles of 95 C for 15 sec, 56 C for 30 sec and 72 C for 90 sec; and finally 72 C for 7 min. PCR products were fractioned by 1.0 % (w/v) agarose gel electrophoresis, and stained with ethidium bromide. Primer sequences were designed as 5'-ATG AAT TAC CCA TGT CCC CA-3' and 5'-TGC AGG CAT TAA GGA CCA CT-3' for FasL [1, 2], and 5'-TAA TAG CAT CTC CGA TT-3' and 5'-CTC CAG ACA TTG TCC TTC AT-3' for Fas [3]. Detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was conducted for the internal control, and the primer sequences were 5'-TGT TCC TAC CCC CAA TGT GT-3' and 5'-TGG GTG CAG CGA ACT TTA TT-3' (GenBank, Accession Number: XM_136109). All primers were purchased from Invitrogen Japan (Tokyo, Japan).

Isolation of uNK cells

For in vitro study, uNK cells were isolated from the mouse placenta by the established method of explant culture [12]. Briefly, tissue samples of metrial gland were collected from the implantation sites of wild-type mice at day-14 of pregnancy. Tissues were minced into 1 mm cubes and hooked into a 60 mm plastic dish with a scalpel blade, and covered with 10% (v/v) fetal calf serum contained Inra Menozo B2 medium (Biomérieux SA, Marcy-l’Étoile, France). Then, the explants were treated with 100 IU mouse recombinant interleukin (IL)-2 (Genzyme/Techne, Minneapolis, MN, USA) and incubated for 4 h at 37 C and 5% CO2. After incubation, the medium containing the migratory uNK cells was collected and washed 3 times by centrifugation at 800 rpm in 4 C for 10 min. Cell number and viability of the isolated uNK cells were assessed by trypan blue dye exclusion.

The isolated uNK cells were treated with 5 µg/ml of rat anti-mouse Fas antibody (clone RMF2; MBL, Nagoya, Japan), which has been confirmed to induce apoptosis in mouse T cell lymphoma WR19L cells [13], at 37 C and 5% CO2 for 3 h. After treatment, apoptotic changes in the uNK cells were examined by morphological observation with Giemsa stain, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-FITC nick end labeling (TUNEL) staining and genomic DNA electrophoresis. As control treatment, normal rat serum was applied instead of anti-Fas antibody.

TUNEL stain

To detect apoptotic cells, TUNEL stain was applied using an apoptosis in situ detection kit (Wako). Procedures were according to the manufacturer’s instructions. Briefly, suspended cells were fixed on a glass slide using cytopsin, and chemically fixed with 4% (v/v) neutral-buffered formalin. After washing with PBS, 0.1% (v/v) Triton X-100 [in 0.1% (w/v) sodium citrate] was treated on the slide for enhancement of penetration. These slides were reacted with TdT/FITC-dUTP solution for 1 h at 37 C. After washing, the slides were incubated with peroxidase-conjugated anti-FITC antibody for 30 min at 37 C. Peroxidase activity was detected by DAB reaction, and counterstain was conducted with methyl green.

DNA electrophoresis

DNA electrophoresis was conducted to detect the fragmentation of genomic DNA accompanying apoptotic cell death. Cultured uNK cells were suspended with 10 mg/ml proteinase K (Sigma-Aldrich Co., St. Louis, MO, USA), 10 mg/ml RNase (Sigma-Aldrich Co.) and 10% SDS for 30 min at 37 C, and then reacted with NaI solution [6 M NaI, 13 mM ethylenediaminetetraacetic acid (EDTA), 0.5% sodium-N-lauroylsarcosinate, 10 mg/ml glycogen and 26 mM Tris-HCl, pH 8.0] for 15 min at 60 C. The extracted DNA was washed 3 times by absolute and 50% (v/v) isopropyl alcohol and centrifuged at 20,000 × g for 15 min. The DNA pellets were air-dried and dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0). The extracted DNA was quantified by spectrophotometer (U-2000A; Hitachi, Tokyo, Japan) using an extinction coefficient of OD260, and fractionated by electrophoresis with 1.5% (w/v) agarose gel. Subsequently, the agarose gel was stained with 0.5 µg/ml ethidium bromide and observed using a UV illuminator (Vilber Lourmat, Marne-La-Vallée, France). As controls for DNA electrophoresis, splenocytes derived from wild-type mice were used and reacted with dexamethasone (Wako, Osaka, Japan) to induce apoptosis.
**Statistical analysis**

The significance of differences among the groups in cell density, number of apoptotic cells and percentage of TUNEL-positive cells was statistically evaluated by two-sided Student’s t test via the method of Welch. Significant and most significant differences were determined if the value was \( P < 0.05 \) and \( P < 0.01 \), respectively.

**Results**

*FasL and Fas expression in the mouse placenta*

The uNK cells in wild-type mice (WT) could be identified by the PAS-stained cytoplasmic granules and observed in large numbers in the metrial gland region (Fig. 1A). Immunohistochemical analysis revealed that uNK cells had a clearly positive reaction for FasL in the cytoplasmic granules at day-16 of pregnancy (Fig. 1B). With regard to Fas, immunohistochemical signals of Fas were detected on the cell membrane of uNK cells at day-16 of pregnancy (Fig. 1C). RT-PCR analysis showed expression patterns of FasL and Fas in the uterus of pregnant wild-type mice (Fig. 1D). FasL expression could be detected strongly at day-10, and continuously observed until day-18. Fas expression was undetectable or at a very low level before day-10, although its level clearly increased at day-14 and -18.

*Influence of Fas deficiency on the cell appearance and apoptosis in uNK cells*

Interestingly, alteration of uNK cell appearance in the metrial gland region was observed in the Fas lacking *lpr/lpr* mice compared with WT mice (Fig. 2). Recognition of uNK cells was difficult before day-10 of pregnancy in both mice due to the underdevelopment of PAS-stained granules. In WT mice, uNK cells increased during day-10 to 12 and reached a maximum cell-density at day-12. Subsequently, the uNK cells in WT mice decreased in number after day-12, and most of them had disappeared by day-18. In *lpr/lpr* mice, larger numbers of uNK cells were observed than in WT mice at day-14 and -16, and significant numbers of

![Fig. 1](image_url). Histological study of wild-type mice (A-C). Mature uNK cells exist in the metrial gland at day-14 of pregnancy, containing PAS-stained cytoplasmic granules (arrows in A). In the immunohistochemistry of FasL, positive reactions are observed in the granules of uNK cells at day-16 (arrows in B). Immunohistochemistry of Fas shows positive reactions on the surface of uNK cells at day-16 (arrows in C). A-C: ×400. RT-PCR analysis of Fas and FasL in the uterus of pregnant wild-type mice (D). Each expression can be seen starting from day-6 in FasL and from day-10 in Fas, and both expressions continue until day-18.
uNK cells remained even at day-18. Finally, the uNK cells in lpr/lpr mice had almost disappeared at day-20.

**Discussion**

The present study demonstrated that the FasL and Fas system was closely related to the induction of apoptosis in uNK cells. Our previous study proposed that the apoptotic cell death of uNK cells contributes to their decrease during late pregnancy and disappearance from the implantation site before parturition [9]. It is possible that the FasL and Fas system has an important role in the process that decreases uNK cells.

In the peripheral blood, activated NK cells are Fas positive, and sensitive to Fas-mediated apoptosis [14]. The experiment with peripheral NK cells showed that both the expression of FasL and Fas was enhanced following treatment with IL-2, which can strongly activate the cytotoxicity of NK cells [15]. In normal mouse placenta, the expression of the IL-2 receptor β-chain, which is an essential subunit for the signal transduction of IL-2, was clearly detected, especially during late pregnancy [16]. IL-2, as well as IL-15, which shares the IL-2 receptor β-chain and has a similar effect to IL-2, may have an important role for the regulation of Fas expression in uNK cells. Furthermore, peripheral NK cells are induced to express FasL following the stimulation of the Fc γ receptor, which initiates the NK cell-killing activity that is referred to as antibody-dependent cell-mediated cytotoxicity (ADCC) [11], and it is suggested that FasL down-regulates the cytotoxicity of NK cells through the cell death of NK cells themselves [17]. In the process of negative selection for autoreactive immature T cells, both FasL and Fas gene expression were found after stimulation of the T cell receptor (TCR) with self tissue antigen, and it was suggested that autoreactive and activated T cells were killed by FasL-presented T cells. [18]. Resembling the apoptosis in T cell-negative selection, apoptotic cell death may be induced in activated uNK cells specifically by the interactions among the uNK cells.

The present study indicated both expressions of FasL and Fas in uNK cells, and the possibility of apoptosis induction among uNK cells. Although FasL could not be detected on the cell surface of uNK cells extracted from Fas-treated uNK cells showed a typical ladder-figure, although it was undetected in uNK cells with the control treatment (Fig. 3C).
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uNK cells, it may be possible to explain that FasL is released in a soluble form from the stimulated uNK cells, as referred to in the report that human NK cells are enhanced to release FasL after recognition of human leukocyte antigen (HLA)-E and F [19]. Interestingly, HLA-E is known to express specifically on the trophoblast in human placenta and to suppress cytotoxicity of NK cells [20]. Placental morphogenesis accompanied with trophoblast development may be associated with the apoptosis of uNK cells through the regulation of FasL release.

In insulin-like growth factor (IGF)-1 overexpressing mice, apoptosis of uNK cells is clearly inhibited, and moreover, fetal loss is frequently occurred following the progress of pregnancy [21]. This report estimated a functional necessity of uNK cells in the normal process of successful pregnancy. In normal pregnant mice, a hormonal effect is confirmed to disturb the appearance of uNK cells, especially in regard to progesterone [22]. Progesterone-treated pregnant mice showing abnormal prolongation of

Table 1. Apoptosis detection in cultured uNK cells following treatment with anti-Fas antibody

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Control</th>
<th>Anti-Fas antibody</th>
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<tr>
<td>Apoptotic morphology (%)</td>
<td>18.0 ± 1.65</td>
<td>33.8 ± 1.38**</td>
</tr>
<tr>
<td>TUNEL-positive cells (%)</td>
<td>42.4 ± 11.2</td>
<td>80.2 ± 15.5**</td>
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**: P<0.01, control vs. anti-Fas antibody.

Fig. 3. Apoptotic analysis of cultured uNK cells isolated from metrial gland tissue at day-14 of pregnancy following treatment with anti-Fas antibody. Histological analysis with Giemsa stain shows that normal uNK cells have large cytoplasm and granules (arrow in A). Apoptotic uNK cells that are characterized by condensed and fragmented nuclei, budding cytoplasm and reduced size are seen (arrowheads in A). Many uNK cells show positive nuclei for TUNEL stain after treatment with anti-Fas antibody (arrows in B). A and B: × 500. Electrophoresis of genomic DNA extracted from cultured uNK cells following the control (lane 1) and anti-Fas antibody treatments (lane 2), and intact (lane 3) and dexamethasone-treated splenocytes (lane 4) are shown in (C). Ladder formation can be seen in lanes 2 and 4, indicating typical apoptosis-dependent genomic DNA fragmentation.
parturition also show the abnormal remains of uNK cells similar to our present results in lpr/lpr mice (unpublished data, Kiso et al.). It is suggested that there is a correlation between parturition onset and the functional activity of uNK cells. It has been established that uNK cells have an essential role for the vascular modification of developing mouse placenta, mediating by the effect of interferon (IFN)-γ [23]. In the late pregnancy placenta, weakness of uNK cell function accompanied by apoptotic cell death may be involved in the arrest of vascular modification and in the preparation for placental expulsion at parturition period.

If the recognition of target cells was an essential step for FasL and Fas-mediated apoptosis, migration and binding would be necessary for effector uNK cells. It has been studied that uNK cells respond to several extracellular matrix (ECM) proteins in vitro [24], and that they express ECM receptors of the VLA-integrin family in vivo [25]. The expression of VLA-integrin family receptors is altered due to the stage of uNK cell differentiation, and overproduction of type I and III collagens results in a disruption of the appearance of uNK cells [24]. Transition of VLA-integrin receptor expression may be associated with the functional activation of uNK cells relating migration and adhering in the apoptosis process. Otherwise, the existence of ECM receptors may reflect a possibility of uNK cells escaping from the pregnant uterus. In the present study, since uNK cells in lpr/lpr mice eventually disappeared from the pregnant uterus at day-20, apoptosis induced by the FasL and Fas system could not fully explain the decrease of uNK cells, especially during the preterm period.

Previous study shows the frequent existence of many uNK cells in the blood vessels [6], and migrating uNK cells through blood vessel walls are often observed (unpublished data, Kusakabe et al.). Alteration of ECM properties in the pregnant uterus may influence the escape of uNK cells into circulating blood from preterm uterus.

Analogous-types of cells to uNK cells are commonly found in many kinds of viviparous animals, including domestic animals and human, and their contribution to normal pregnancy has been confirmed [8, 24]. It is possible that uNK cells have a crucial reproductive role commonly in many mammals, and that they may be involved in reproductive disturbance or infertility. Further elucidation of the uNK cell properties will be useful for the development of new aspects in reproductive study.

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